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Novel Natural Products: Therapeutic Effects in Pain, Arthritis and Gastro-intestinal Diseases

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Preface

In recent years there have been a number of significant developments of natural products for the treatment of rheumatic diseases, pain and gastro-intestinal ulcers and inflammation. It is proposed to cover chapters on some of these novel developments of natural products which are of current and future interest as therapies for the above-mentioned conditions.

The current volume could be considered to be a follow-on from the last two PDR volumes on "Natural Compounds as Drugs", Volumes I and II (Frank Petersen and Rene Amstutz, editors, 2008). These volumes covered a wide range of biological and technological aspects of natural products and their discovery, some involving synthesis and properties of chemical compounds. The difference in this volume is that the natural products have a focus of their therapeutic effects on pain, arthritic and gastro-intestinal diseases.

Some of the natural products covered in the current volume can be considered novel with respect to their anti-inflammatory, gastro-intestinal and anti-microbial activities. They are either at the experimental stage of development while some others are well-established clinical used products. Each has its own unique place in therapy.

This volume has mostly arisen from on long-standing research interests and scientific contacts by the editors. To some extent the link between anti-inflammatory and gastro-intestinal diseases is part of an evolving trend. Issues concerning the fact that many anti-inflammatory agents exhibit gastro-intestinal (GI) adverse reactions as part of their pharmacological actions is considered as a basis for developing new agents that are either devoid of the GI effects or that the gut microbes may be therapeutically manipulated to influence some of the systemic manifestations of inflammatory diseases. Traditional or plant sources continue to serve not only as valuable sources of therapies or as basic leads for developing specific anti-inflammatory therapeutics with lesser GI effects than seen with conventional drugs.

May 2015

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Contents

Gastrointestinal Tract Commensal Bacteria and Probiotics: Influence on End-Organ Physiology 1
Luis Vitetta, Talia Palacios, Sean Hall and Samantha Coulson

Therapeutic Effects of Ribunucleinate (Ribonucleotides) in Immuno-Inflammatory and Arthritic Diseases 35
G. Stommel, S. Schuehlein, K.-H. Schuehlein and K.D. Rainsford

***Perna canaliculus* (Green-Lipped Mussel): Bioactive Components and Therapeutic Evaluation for Chronic Health Conditions** 91
Samantha Coulson, Talia Palacios and Luis Vitetta

Celery Seed and Related Extracts with Antiarthritic, Antiulcer, and Antimicrobial Activities 133
Michael C. Powanda, Michael W. Whitehouse and K.D. Rainsford

Traditional Medicinal Oils Sourced from Birds: Anti-inflammatories and Potential Immunoregulants 155
Athol Turner, Gerald Hancock, John Wells and Michael Whitehouse

The Genus Aloe: Phytochemistry and Therapeutic Uses Including Treatments for Gastrointestinal Conditions and Chronic Inflammation 179
I.E. Cock

Silver Pharmacology: Past, Present and Questions for the Future 237
Michael W. Whitehouse

Index 275

Gastrointestinal Tract Commensal Bacteria and Probiotics: Influence on End-Organ Physiology

Luis Vitetta, Talia Palacios, Sean Hall and Samantha Coulson

Abstract Bacteria represent the earliest form of independent life on this planet. Bacterial development has included cooperative symbiosis with plants (e.g., *Leguminosae* family and nitrogen fixing bacteria in soil) and animals (e.g., the gut microbiome). It is generally agreed upon that the fusion of two prokaryotes evolutionarily gave rise to the eukaryotic cell in which mitochondria may be envisaged as a genetically functional mosaic, a relic from one of the prokaryotes. This is expressed by the appearance of mitochondria in eukaryotic cells (an *alpha-proteobacteria* input), a significant endosymbiotic evolutionary event. As such, the evolution of human life has been complexly connected to bacterial activities. Hence, microbial colonization of mammals has been a progressively driven process. The interactions between the human host and the microbiome inhabiting the gastrointestinal tract (GIT) for example, afford the human host the necessary cues for the development of regulated signals that in part are induced by reactive oxygen species (ROS). This regulated activity then promotes immunological tolerance and metabolic regulation and stability, which then helps establish control of local and extraintestinal end-organ (e.g., kidneys) physiology. Pharmacobiotics, the targeted administration of live probiotic cultures, is an advancing area of potential therapeutics, either directly or as adjuvants. Hence the continued scientific understanding of the human microbiome in health and disease may further lead to fine tuning the targeted delivery of probiotics for a therapeutic gain.

Keywords Probiotics · Gastrointestinal tract · Inflammatory diseases · Arthritis · Dysbiosis · Pharmacobiotics · Mitochondria · Endosymbiotic · Microbiome

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1 Introduction

The evolution of a primordial prokaryote that led to the development of the eukaryotic cell (i.e., acquisition of mitochondria) has been hypothesized as arising from an oxygen consuming bacterial ancestor (Dolan and Margulis 2007; Shih and Matzkeb 2013) by endosymbiosis. This has been reported as an event that highlights a significant evolutionary step that is obligatory for the continued symbiotic existence between bacteria, plants and animals, which is vital for life and survival on this planet.

During the twentieth century, there was an increasing dependency on antimicrobial compounds as mainstream therapy for bacterial infections. It is only somewhat recent that the focus of basic scientific and clinical research has moved beyond the premise that all bacteria are harmful to now clarifying the critical interrelationships that exist between the human host and its microbiome in regards to health/wellness and disease. This new understanding has redefined the interactions between gut microbes and vertebrates, now recognizing that the microbial active cohort and its mammalian host have shared coevolutionary metabolic interactions that span millennia.

At the time of birth, humans experience an induced proinflammatory beneficial event. The mediators of this encouraged activity is a fleet of bacteria that assault all mucosal and extramucosal tissue sites that include the mouth, hair, nose, ears, eyes, urogenital tract, lungs, GIT, and skin which express their own unique microbiomes (Dominguez-Bello et al. 2010) thus initiating effects that eventually provide the infant with immune tissue maturation. These effects occur beneath an emergent immune system surveillance and antigenic tolerance capability radar. Over time, continuous and regulated interactions with environmental, as well as commensal microbial, viral, and other antigens lead to an adapted and maintained symbiotic state of tolerance, especially in the GIT, the organ site of the largest microbial biomass.

The GIT is reported to harbor thousands of bacterial species while being the site of the most dense and diverse microbiome cohort known on the planet with an average of 10^{12-14} bacterial cells (predominantly in the large bowel) per wet weight of luminal content. Notwithstanding, recent reports suggest that the skin microbiome may be just as dense and complex (Grice and Segre 2011). The number of bacterial cells residing within and on the human body of the average healthy adult is estimated to outnumber human cells by a factor of 10 to 1.

Bacteria that colonize the GIT metabolically outperform somatic cells with enzymatic/metabolic activity, that include (1) triggering and regulating the normal development and function of the mucosal barrier (Xu and Gordon 2003); (2) assisting with the maturation of immunological tissues, which in turn promote immunological tolerance to antigens from the external environment/foods or potentially pathogenic organisms that are ingested or reside within the GIT (Berg and Savage 1975); (3) the control of nutrient metabolism and assimilation

(Mazmanian et al. 2005; Rakoff–Nahoum et al. 2004), and (4) the prevention of the proliferation of pathogenic bacteria (Tappenden and Deutsch 2007). Early reports have hypothesized that changes in the profile of the resident GIT microbiota may reduce beneficial functions and affect the regulation of GIT immune and inflammatory responses (Pickering 1950). Hence, in addition to its inherited constitution of genes, the GIT microbiota together with environmental influence may comprise critical factors in disease causation (Pickering 1950).

2 Immunological Tolerance and the Control of Inflammation Originating from the GIT

Prebirth mammalian young were previously thought to be sterile in utero. However, a recent review has documented that neonatal colonization actually begins in utero (Rautava et al. 2012). Hence establishing clearly that mammals on this planet engage with bacterial species throughout a lifetime (Cert–Bensussan and Gaboriau–Routhiau 2010). In vivo studies with germ-free animals have confirmed the posit that antigenic tolerance has a bacterial triggered requisite. Comparative studies between mice that were raised in conventional versus germ-free environments highlight the importance of the intestinal microbiota for the development of the peripheral immune system in immunocompetent hosts (Hooper and MacPherson 2010). Most notably, the spleens of germ-free mice contain fewer and smaller germinal centers (Bauer et al. 1963) and decreased numbers of memory $CD4^{+}$ T cells whose cytokine production demonstrates a Th2-type profile (Mazmanian et al. 2005). Moreover, a balanced microbiome prevents the growth of disease-causing bacteria within the intestine via a number of mechanisms. The GIT microbiota produce vitamins (e.g., vitamin K and B12) and are also important for maintaining the muscular activity of the small intestine. Specifically, studies reveal that the functions of the human GIT immune system are only partially encoded in the host's genes and that cues are required from the symbiotic microbial cohort for its full development (Hooper 2004). The bacteria that colonize the adult human GIT hence function collectively as a metabolic organ (Backhead et al. 2007) and within this evolutionary paradigm, the development of an immune-metabolic-competent host may be a necessary response for survival. The microbiota that then colonize the human GIT exhibit a high phylogenetic diversity reflecting their immense metabolic potential. The mechanism by which bacteria colonize the GIT alludes to the cues required by the GIT to develop a regulated immuno-metabolic-competent profile. This basic scientific understanding reinforces the idea that bacteria can drive the epigenetic control of the host genome and hence host survival.

Upregulated immune responses in an individual are necessary to clear the GIT of pathogenic cells. The immune system achieves this by initiating a proinflammatory response. The microbiota act partly in an immune-surveillance role by detecting an overgrowth of pathogenic bacteria, stimulating the immune system and

subsequently initiating an appropriate eradicative inflammatory response (Eckmann 2006). Once the pathogenic cells are reduced/cleared, anti-inflammatory signals are switched on to restore the pro-inflammatory response back to a normal level. Accordingly, the healthy gut is in a constant state of *regulated inflammation*. The role that the GIT microbiota play in triggering the anti-inflammatory response is still unclear. Accumulating evidence indicates that the balance of commensal bacteria within the GIT may be associated with the development of some GIT disorders (Swidsinski et al. 2002). Patients diagnosed with inflammatory bowel disease (IBD) or irritable bowel syndrome (IBS) have been reported to present with increased proinflammatory or potentially pathogenic bacterial species such as *Escherichia coli* (Mylonaki et al. 2005; Martin et al. 2004), members of the genus *Bacteroides* (Swidsinski et al. 2002) and *Enterococci*; and decreased *Bifidobacteria* and *Lactobacilli* species (Giaffer et al. 1991; Van de Merwe et al. 1988). For example, the etiology of IBD is not fully understood, but is considered to be a T-cell-driven inflammatory response resulting from a persistent preponderance of pro-over anti-inflammatory cytokine production (Hvas et al. 2007), whereas, Crohn's disease (CD) is reported to be driven by an T-helper 1 (Th1) immune response (Matsuoka et al. 2004; Fuss et al. 1996) that can affect any part of the GIT, i.e., from the mouth to the anus. By contrast, ulcerative colitis (UC) is a T-helper 2 (Th2) driven response, and is restricted to the mucosa of the colon and rectum (Heller et al. 2005; Fuss et al. 1996). Figure 1 illustrates a diagrammatic view of the control of proinflammatory activity that is attenuated by probiotic live cultures.

3 The Hygiene Hypothesis

The past 60 years has seen a significant increase in the prevalence of autoimmune diseases (Mackay et al. 2001; Sironi and Clerici 2010). This trend has triggered the formulation of the hygiene hypothesis that has been defined as a lack of early childhood exposure to infectious agents, symbiotic microorganisms (e.g., gut microbes or probiotic bacteria) and parasites that increases an individual's susceptibility to allergic diseases by suppressing the natural development of tolerance by the immune system.

Over the past two to three decades, the hygiene theory has been tested and tweaked, expanded and extended (Sironi and Clerici 2010). This hypothesis provides a biologically plausible explanation for the trend that implicates diminished exposure in early childhood to those *commensal* infections that boost immune defenses while promoting tolerance. This deficit subsequently enhancing the risk for later life GIT proinflammatory shifts that disrupt normal regulated GIT inflammatory responses increasing the susceptibility to developing autoimmune diseases (Bach 2002).

This proposed postulation of reduced early childhood exposure to infections is possibly linked to diminished family size and better personal hygiene, which may contribute to decreased antigenic tolerance and a concomitant increase in the risk of

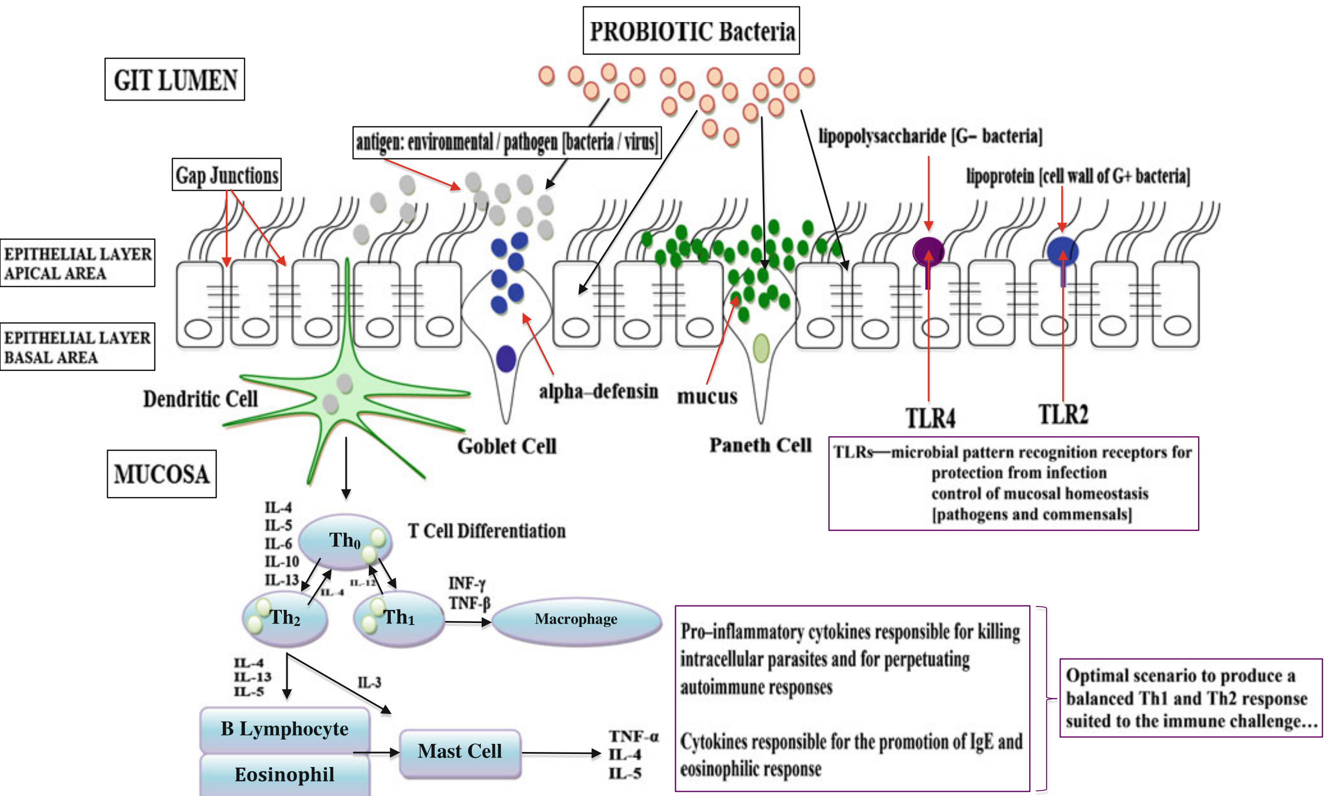

Fig. 1 Diagrammatic representation of GIT epithelial and mucosal homeostasis. Probiotic bacterial actions include those on epithelial cells, mucus producing cells, gap junctions between epithelial cells and integrity maintenance and the control of pathogenic bacteria. Multiple microbial pattern recognition receptors (e.g., TLR4 and TLR2) are known and are involved in supporting homeostasis by recognition and protection from opportunistic pathogenic infections and commensal bacterial tolerance. * *G*−Gram-negative *G*+ Gram positive

developing allergic disease (Bach 2002). The interface of the microbial environment with the Innate immune system can be significantly modulated so that its ability to impart instructions to adaptive/regulatory immune/inflammatory responses are adversely affected, particularly when such interactions occur in utero and or are presaged in early life. Bach (2002) documented this trend highlighting that an epidemic of both GIT autoimmune diseases in which the immune response was dominated by Th1 cells (such as type 1 diabetes mellitus, CD, multiple sclerosis) or allergic diseases in which the immune response was dominated by Th2 cells (such as asthma, allergic rhinitis, and atopic dermatitis) were becoming increasingly prevalent in Western communities.

Evolution has naturally endowed the human species with immune/inflammatory regulatory mechanisms activated by the interactions with both the external and internal microbial environments. These then serve to fine-tune both Th1 and Th2 antigen-driven effector responses (Wills–Karp et al. 2001). The innate immune system samples the environment and accordingly modulates the T regulatory arm, the ultimate keeper of the balance between antigen tolerance and responsiveness. The efficiency of the regulatory interface in its current state would paradoxically be jeopardized by a decrease in the microbial burden that the immune system has coevolved with (Wills–Karp et al. 2001).

Studies exploring the molecular mechanisms that might underpin the hygiene hypothesis have focused mostly on the interactions between bacterial products and Toll-like receptors (TLRs)—the main transducers of microbial signals to the innate immune system and critical regulators of $CD4^{+}$ T-cell activation and regulation (O'Neill 2006; Pasare and Medzhitov 2004) (Fig. 1). Therapeutically, a recent review has highlighted how individuals with chronic helminth infections often have a reduced prevalence of inflammatory disorders, including allergic diseases (Hussaarts et al. 2011). Mechanistically, it is purported that inducing or expanding regulatory B cells with helminth infection, novel avenues for the treatment of inflammatory diseases such as allergic asthma are revealed (Hussaarts et al. 2011).

4 The Influence of Probiotic Therapies

The World Health Organization and others have defined probiotics as live bacterial cultures that when consumed in foods (e.g., yogurts) and dietary supplements can improve the health of the host beyond their inherent basic nutritional content (Fuller 1989; Morelli and Capurso 2012).

Perhaps the most studied site for investigating probiotic efficacy is the GIT and inflammatory conditions such as CD, UC, and IBS. It has been reported that probiotic bacteria may operate on three levels of host functionality that enhances GIT and extraintestinal activity (Fig. 1) namely by (a) interfering with the growth of pathogenic bacteria in the lumen of the GIT; (b) strengthening gut epithelial barrier function and mucosal immunity as well as mucus production; and (c) beyond the gut influencing both the systemic immune and organ systems such as the liver,

brain, and heart. A series of clinical trials (reviewed elsewhere c.f. Vitetta et al. 2014a, b) that implemented various combinations of probiotic species frequently demonstrated efficacy in treating GIT conditions/diseases and various other end-organ tissues (Vitetta et al. 2014a, b). The core notion emanating from the reviews was the administration of multistrain probiotic formulations could, in addition to improving GIT function, influence numerous end-organ tissues beneficially. Furthermore, clinical studies indicate that administration of probiotic bacterial species provides efficacious results in restoring the GIT microbiome to a more balanced metabolic state. This possibly achieved in part by reducing pathogenic bacterial overgrowth and the resulting adverse localized effects that in turn affect end-organ physiology.

It is postulated that a dysbiotic GIT, induced by a microbiome drift toward an overgrowth of pathogenic bacteria, may play a significant role in the induction of pro-inflammatory mediators that begin in the GIT and then may affect different end-organs as shown in Fig. 2. For example, disruption of the GIT epithelial barrier that accompanies chronic anti-inflammatory/analgesic (e.g., NSAIDs) medication use or over prescription of others (e.g., antibiotics) exacerbates local

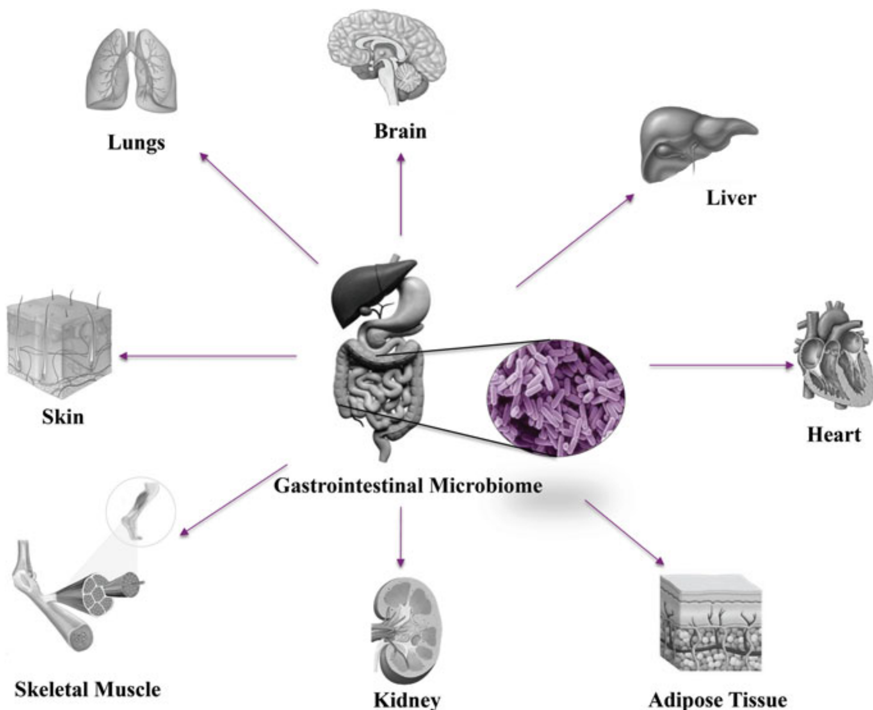

Fig. 2 The GIT microbiome can influence numerous end-organ tissue physiology beneficially and adversely through mirobial metabolic activity and host immune cell and hormonal molecular actions

proinflammatory responses induced by the pathogenic commensal cohort. This activity can further disturb GIT physiological and epithelial barrier function leading to disruption of controlled pro-inflammatory actions.

The rescue of an adversely affected GIT microbiome may depend on the introduction of a multistrain probiotic rather than that of single strains. Given the extensive array of microorganisms that inhabit the GIT, probiotic combinations may be a more biologically plausible therapeutic option in rescuing GIT microbiome aberrant functionality. Recently, it was reported that multistrain probiotics may appear to be more effective against a wide range of end points (Chapman et al. 2011). Chapman et al. (2011) also reported that based on a limited number of studies, multistrain probiotics may show greater efficacy than single strains, including strains that are components of the mixtures themselves demonstrating an additive effect.

A problem with probiotic therapy is ascertaining and correctly administering the optimal therapeutic dose for the condition being treated. As each probiotic species/strain has been clinically trialed using variable dose concentrations for varying disease etiologies, defining the *ideal* dose is complex. To assess the efficacy of probiotics, improvements in disease outcome measures have been assessed. Whorwell and colleagues (2006) investigated the effects of three different doses of *Bifidobacterium infantis* 35624 (1×10^6, 1×10^8 and 1×10^{10}, CFU/mL) in treating primary-care IBS patients. The dose of 1×10^8 CFU/mL proved superior in relieving abdominal pain compared with the placebo and other doses. Further investigation of the highest dosage demonstrated that the probiotics *coagulated* into a firm glue-like mass making them resistant to acid and agitation. The lowest dose of probiotics may not have been effective because of the duration of the study, or insufficient biological activity. These findings highlight the potential importance of how probiotics are dosed and administered in order to maximize bioavailability within the GIT.

Probiotics have been shown to reduce abdominal pain, discomfort, and symptom scores in patients with IBS when administered *Lactobacillus acidophilus* (Sinn et al. 2008), *Lactobacillus plantarum* 299 V (Niedzielin et al. 2001), or ProSymbioflor (a combination of *E. coli* DSM 17252 and *Enterococcus faecalis* DSM 16640) (Enck et al. 2008) compared with a placebo. In contrast, Drouault-Holowacz and colleagues (2008) found that *Bifidobacterium longum* LA 101, *L. acidophilus* LA 102, *Lactobacillus lactis* LA 103 and *Streptococcus thermophilus* LA 104 were not superior to the placebo treatment for relieving disease symptoms except that of abdominal pain, due to a strong placebo[1] effect. Further analysis of the IBS subgroups revealed that patients with changing bowel habits (alternating between constipation and diarrhea and those with short durations of symptom exacerbation and remission) reported significantly less abdominal pain with constipation-predominant IBS patients reporting improved bowel motions. These results indicate that different disease etiologies may exist between IBS subgroups and that some probiotics may be more efficient than others for treating

[1]A placebo is a substance containing no medication benefit and prescribed to reinforce a patient's expectation of possibly attaining a beneficial effect.

symptoms within these subgroups. These findings also point to the need to further classify patients into relevant sub groups whenever possible for assessing the efficacy of a probiotic.

A number of studies investigating the effects of probiotics within specific subgroups of IBS have shown the beneficial effects of probiotic supplements. Therefore, Zeng and colleagues (2008) first separated patients with IBS into sub groups (those with increased small bowel permeability and those with increased colonic permeability), treating diarrhea-predominant IBS patients with *S. thermophilus and Lactobacillus bulgaricus*, *Lactobacillus bulgaricus* and *B. longum*. The proportion of patients with increased small bowel permeability (lactulose/mannitol ratio >0.025) decreased significantly ($p < 0.023$) after treatment. These patients also demonstrated improved IBS scores with diminished abdominal pain and flatulence. Similarly, symptoms improved after treatment with the probiotic VSL#3 in subjects with either diarrhea-predominant IBS (Kim et al. 2003) or IBS with bloating (Kim et al. 2005). In subjects complaining of IBS with bloating, the VSL#3 reduced flatulence scores and retarded colonic transit time, without altering bowel function. In patients with diarrhea-predominant IBS, VSL#3 only relieved abdominal bloating, having no effect on mean transit measures, bowel function scores or satisfactory relief of symptoms. VSL#3 has also been shown to be superior to a placebo in children with IBS. VSL#3 supplementation improved overall IBS symptoms as assessed by abdominal pain/discomfort, abdominal bloating/gassiness, and on family life disruption.

To date, monitoring disease symptoms is usually employed to assess the efficacy of probiotics in conditions such as IBS and IBD. These subjective measures, usually self-assessed by the patients, have provided slight indications of the underlying mechanisms of probiotics or the disease etiology. In an attempt to understand the physiological mechanisms of probiotics, Kajander and colleagues (2005, 2008) administered patients with IBS a mixture of probiotic species/strains containing *Lactobacillus rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* Bb99 and *Propionibacterium freudenreichii* ssp. *shermanii* JS (Kajander et al. 2005) or *L. rhamnosus* GG, *L. rhamnosus* Lc705 (DSM 7061), *P. freudenreichii* ssp. *Shermanii JS* (DSM 7067), and *Bifidobacterium animalis* ssp. *lactis* Bb12 (Kajander et al. 2008). Serum C-reactive protein and pro- and anti-inflammatory cytokines (IFN-γ, TNF-α, IL-2, IL-4, IL-6 and IL-10) concentrations were generally below the limit of detection, and therefore did not indicate any differences between the treatment groups (Kajander et al. 2008). Both studies did, however, report an improvement in the IBS scores from baseline, particularly for distention and abdominal pain. The IBS score had at 5 months decreased by 14 points (−19 to −9) with the multispecies probiotic versus 3 points (−8 to 1) with placebo ($P = 0.0083$). Moreover, the study also reported that there was a stabilization of the GIT microbiota as the microbiota similarity index increased with the probiotic supplementation (1.9 ± 3.1), while it decreased with placebo (−2.9 ± 1.7).

In contrast to the benefits of probiotics in relieving IBS symptoms, other studies report few or no beneficial effects of probiotics. O'Mahony and colleagues (2005) found disparate effects when providing *L. salivarius* UCC4331, *B. infantis* 35624, or a placebo to subjects with IBS and to healthy volunteers. Following supplementation, the composite score (weeks 1–8), pain/discomfort (at weeks 1, 2, 4, 5, and 7), bloating/distention (at weeks 2, 5, and 6), and difficulty with bowel movements (at weeks 2, 3, 5, and 6) were generally lower in the *B. infantis* group than in the placebo (malted milk drink) group. Composite score was only lower in the *L. salivarius* group than the control group in the second week of supplementation, indicating that the effects of *L. salivarius* were short-lived and intermittent. In vitro production of IL-10 and IL-12 by isolated mononuclear cells (peripheral blood mononuclear cells [PBMC]) from whole blood was a proinflammatory profile at baseline in patients with IBS. Patients with IBS had low levels of IL-10 and high levels of IL-12 synthesis compared with healthy volunteers. Notably, however, supplementation with *B. infantis* restored IL-10 and IL-12 syntheses to levels similar to those observed in healthy volunteers.

To understand the apparent lack of effect of probiotics in some clinical studies, it is essential that the placebo effect or natural healing cycle needs to be critically considered. Niv and colleagues (2005) provided *Lactobacillus reuteri* ATCC 55730 or a placebo to subjects with IBS. Following supplementation, an improvement in IBS symptoms was reported. However, a similar response occurred in the placebo group (treatment versus placebo $P = 0.0714$ and $P = 0.0971$, respectively). This may demonstrate a strong placebo effect or stimulation of the natural healing cycle of the disease allowing some IBD and IBS patients to more frequently enter periods of remission. Notwithstanding, the overall therapeutic effect of probiotics in humans supports beneficial findings. While some results were inconsistent, probiotic treatment generally reduced symptoms of IBS, particularly abdominal pain, and restored the balance of pro- and anti-inflammatory cytokines.

5 The GIT, Probiotics and End-Organ Physiology

Table 1 presents an overview of clinical studies that have administered a probiotic strains (formulations) and have reported improvements on numerous conditions and symptoms in various organ systems (c.f. Vitetta et al. 2014b).

5.1 Probiotics and the Liver

It has recently been reported that there exists a gut–liver axis that suggests the GIT microbiota may significantly affect liver physiology and act as a cofactor in the etiology of chronic liver disease (Loguercio et al. 2002). This hypothesis has

Table 1 A summary of clinical trials that administered different probiotic bacteria and reported improvements for specific conditions and symptoms for different end-organs

Genera/species		[a]*L. gasseri*	*L. casei*	*L. helveticus*	*L. salivarius*	*L. johnsonii*	*L. acidophilus*	*L. plantarum*	*L. paracasei*	*L. fermentum*	*L. rhamnosus*
System	Improvement condition/symptom										
Nervous	Anxiety		X	X							
	Depression		X	X							
Respiratory	Asthma symptoms	X									X
	Allergic rhinitis symptoms		X								X
	Antigen–induced cytokines	X	X	X					X		
Obesity	Body weight	X									
	Total cholesterol						X				X
	Ldl						X				X
	Triglycerides						X				X
	Abdominal visceral fat	X									
	Improved insulin sensitivity										
Liver non–alcoholic fatty liver disease	Inflammatory cytokines				X		X	X			
	Blood ammonia						X	X	X		
	Liver enzymes							X			
	Ascitic fluid						X				
Gastrointestinal tract	Irritable bowel syndrome										
	Pain						X	X	X		
	Altered bowel habits				X		X				

(continued)

Table 1 (continued)

Genera/species		[a]*L. gasseri*	*L. casei*	*L. helveticus*	*L. salivarius*	*L. johnsonii*	*L. acidophilus*	*L. plantarum*	*L. paracasei*	*L. fermentum*	*L. rhamnosus*
	Bloating						X	X	X		
	Antibiotic diarrhoea				X		X	X	X		
	Infectious diarrhoea	X							X		
	Helicobacter pylori						X				
	Pouchitis						X	X	X		
	Inflammatory bowel disease										
	Crohn's disease										
	Ulcerative colitis						X	X	X		
Chronic kidney disease	Uric acid						X				
	Blood urea nitrogen						X				
	Serum p–cresol				X						
Skin	Uv–induced damage					X					
	Eczema						X		X		X
	Atopic dermatitis						X			X	X

L. delbrueckii ssp. *bulgaricus*	*L. reuteri*	*B. longum*	*B. breve*	*B. infantis*	*B. animalis* ssp. *B. lactis*	*B. bifidum*	*Bacillus bifidus*	*Streptococcus thermophiles*	*Saccharomyces boulardii*
		X							
		X							
	X		X						
					X				
X		X	X					X	
X		X	X					X	
X		X	X					X	

(continued)

Table 1 (continued)

L. delbrueckii ssp. *bulgaricus*	*L. reuteri*	*B. longum*	*B. breve*	*B. infantis*	*B. animalis* ssp. *B. lactis*	*B. bifidum*	*Bacillus bifidus*	*Streptococcus thermophiles*	*Saccharomyces boulardii*
					X				
					X				
X		X	X	X				X	
		X							
X		X				X		X	
X							X	X	
X	X	X	X	X	X	X		X	
		X		X	X	X		X	
X		X	X	X	X	X		X	
X		X	X	X	X			X	X
	X	X			X				
	X			X					
X		X	X	X				X	
									X
X		X	X	X		X		X	
		X						X	
		X						X	
			X						
	X	X			X	X			
			X		X				

[a]*L. Lactobacillus B. Bifidobacterium*

This table presents in summary form clinical trial data that administered different probiotic bacteria (genera/species) testing efficacy for different end-organ conditions and symptoms. The data presented in this table is a synthesis from Vitetta et al. (2012, 2013, 2014a, b)

stemmed largely from the long-standing practice of using lactulose in the treatment of hepatic encephalopathy (Polson and Lee 2005). This suggests that the gut microbiota is intimately involved in the management of chronic liver disease. A GIT microbiota that sustains a persistent low-level proinflammatory pathogenic profile could modulate liver damage caused by ethanol and other toxic compounds such as acetaldehyde, phenols and endotoxins. We have reviewed the clinical evidence of studies that have employed probiotics in the treatment of chronic liver diseases reporting significant improvements (c.f. Vitetta et al. 2014b). Clinical studies that demonstrated efficacy were related to improving endotoxaemia that in turn improved liver functionality. It would seem that the probiotic actions most relevant to chronic liver diseases were modification of intestinal barrier function and the prevention of bacterial/toxin translocations. Increased GIT overloads with Gram-negative bacteria, increased permeability and impaired immunity may all contribute to increased bacterial/toxin translocations. Furthermore, a strong correlation between the rate of bacterial/toxin overload and the severity of cirrhosis has been demonstrated. Hence, the combined clinical studies seem to suggest that multistrain probiotics may alter gut microbiota and rescue the GIT microbiome toward a protective commensal profile with a concomitant increase in GIT epithelial barrier function.

5.2 Probiotics and Obesity

In vitro screening experiments with bacteria from the genus *Lactobacillus* and *Bifidobacteria* isolated from the human GIT have demonstrated significant cholesterol-lowering actions (Pereira and Gibson 2002). Recent research findings suggest that a high-fat diet and the GIT bacterial cohort interact to promote early inflammatory changes in the gut that contribute to the development of obesity and insulin resistance (Ding and Lund 2011).

Clinical studies investigating probiotic preparations in obesity (c.f. Vitetta et al. 2014b) have reported an overall trend that demonstrates probiotic preparations could positively influence weight reduction. Particularly, in a study with healthy infants (Chorell et al. 2013) it was demonstrated that probiotic administration significantly lowered levels of palmitoleic acid and significantly increased levels of putrescine. The data suggested that palmitoleic acid a major monounsaturated fatty acid that has strongly been linked to visceral obesity was reduced with probiotic supplementation whereas putrescine an important polyamine with importance for gut integrity was beneficially increased. Probiotic supplementation in adulthood (Kadooka et al. 2010) and during childhood (from birth to 10 years) (Luoto et al. 2010) demonstrated that probiotics in part assisted with the metabolic control of abdominal visceral and subcutaneous fat. In an additional study, administration of a multispecies probiotic supplement provided a synergistic effect on overweight and obese individuals when provided with a weight loss diet (Zarrati et al. 2013). In a further study with overweight children, a multispecies probiotic and prebiotic

(synbiotic) formulation demonstrated a significant decrease in blood lipid profiles (Safavi et al. 2013).

Mechanistically, Tien et al. (2006) have reported that the anti-inflammatory effects of *Lactobacillus casei* were aLactobacillus caseisociation with NF-κB activation. Therefore, suggesting that the health properties of probiotics could be related to peroxisome proliferator-activated receptor gamma (PPAR-g) activation, which then blocks the activity of NF-κB (Amaral et al. 2008; Nakamura and Omaye 2012). Interestingly the overconsumption of food triggers GIT proinflammatory bacterial activity; this then may induce GIT metabolic dysfunction increasing the risk of metabolic diseases. Whereas, a balanced diet with an optimally balanced GIT microbiota that promotes regulated/controlled PPAR-g activation could alleviate or suppress the risk of developing metabolic diseases such as T2DM.

5.3 Probiotics and the Brain

There is an increasing body of preclinical evidence supporting the important role that the gut microbiota plays in influencing emotional behavior as well as monitoring underlying brain mechanisms (c.f. Vitetta et al. 2014b). Studies with germ-free mice have demonstrated the important role of the gut microbiota in brain development and resultant adult pain responses and emotional behaviors, as well as on adult hypothalamic–pituitary axis responsiveness. Of the scant clinical trials that have investigated probiotics and brain function, the results have shown significant improvement in behavior with probiotic administration (c.f. Vitetta et al. 2014b). In one study, assessing patients with traumatic brain injury, probiotic supplementation improved the anti-inflammatory clinical picture (Tan et al. 2011).

5.4 Probiotics and Joint Disease

Patients diagnosed with joint diseases have been reported as being predisposed to GIT disturbances (Lee et al. 2010). There are a small number of human clinical trials that have assessed the therapeutic efficacy of administering probiotics to patients with autoimmune arthritic diseases (c.f. Vitetta et al. 2014b). However, there are no clinical studies that have investigated the role of probiotics in reducing the symptoms of osteoarthritis. A recent animal study however has provided plausible data that the probiotic species *L. casei* could act as a potent nutraceutical modulator for the treatment of osteoarthritis. Pain was reduced, as were inflammatory responses and articular cartilage degradation (So et al. 2011).

5.5 Probiotics and Respiratory Diseases

Respiratory allergies include allergic rhinitis, sinusitis and asthma. As previously presented herein, the advent of the hygiene hypothesis has proposed that the increase in allergic diseases reflects a decrease in infections during childhood. Clinical trials have also suggested that the exposure to microbes through the GIT robustly shapes immune function (Dominguez-Bello et al. 2010). Probiotics have been reported to exert a beneficial effect in the prevention as well as the treatment of allergic diseases through modification of the immune system of the host via the GIT ecosystem. This has prompted studies of feeding probiotics in the prevention as well as the treatment of respiratory allergies (c.f. Vitetta et al. 2014b). The clinical data presents a contentious profile of probiotic efficacy. In a recent controlled study, it was reported that long-term consumption of fermented milk containing *L. casei* may improve the health status of children with allergic rhinitis, however no effect was found in asthmatic children (Giovannini et al. 2007).

5.6 Probiotics and Skin Conditions

Lactobacillus rhamnosus GG has been reported to reduce clinical symptoms, intestinal inflammation and mucosal barrier permeability in infants with allergic dermatitis (MacDonald and Sabatino 2006).

Allergic conditions are caused by abnormal or exaggerated immune reactions of the skin. A range of symptoms can be expressed however the most common chronic allergic conditions of the skin are atopic dermatitis/eczema. Probiotics are reported to exert some benefit in such conditions, which is thought to be due to the immune modulating effects of the bacteria. Studies demonstrate that probiotics contribute to relief of symptoms and also prevention of atopic conditions in infants (c.f. Vitetta et al. 2014b). In one study, a probiotic preparation induced the repair of ultra violet damaged skin (Bouilly-Gardner et al. 2010).

5.7 Probiotics and Chronic Kidney Disease (CKD) [See Further Sect. 6]

Dysfunction of the kidneys leads to disturbed renal metabolism impaired glomerular filtration and tubular secretion/reabsorption problems. This results in the retention of toxic solutes, which affect all organs of the body. It has been posited that toxins generated by gastrointestinal dysbiosis, and introduced into the body via the small and large bowel, may all contribute to CKD. They comprise advanced glycation end products, phenols, and indoles (Vitetta and Gobe 2013). Moreover, recent reports suggest that the bacterial load and the adverse products of the

intestinal microbiota might influence chronic disease pathogenesis (Wu et al. 2005; Arumugam et al. 2011). This is particularly relevant to the development of CKD, a disease of increasing prevalence in many Western societies. It has also been recently reported that the pharmacobiotic potential of the GIT micro-biometabolome may provide a plausible therapeutic role with the administration of live multistrain probiotic cultures (Vitetta and Alford 2013).

Although current evidence of probiotic efficacy in reducing uremic toxins is limited, clinical evidence does demonstrate that specific strains in a multiple species matrix configuration, in combination with prebiotics, may be most beneficial in reducing gut-derived uremic toxins (c.f. Vitetta et al. 2014b). In addition, selecting and administering probiotic species with known metabolic functions, such as *Streptococcus thermophilus*, for metabolizing urea as a nitrogen growth source, could contribute to reducing uremia.

5.8 Prebiotics

The introduction of prebiotics in Japan and Europe as food additives point to the need for further controlled clinical studies before prebiotics can be unequivocally recommended as a food additive for infant formulas and yogurts or as dietary supplements that should be consumed on a daily basis. No human studies have been conducted to confirm the suggested in vitro and animal study effects of prebiotics on carcinogenesis. Long-term trials with prebiotics, perhaps among patients with chronic digestive diseases such as colon cancer-prone patients, would certainly be useful (de Vrese and Schrezenmeir 2008).

The symbiotic effect of combining prebiotics with probiotics needs to be further evaluated and quantified. Such studies could include investigations as to whether there is altered bacterial colonization in the gut following the ingestion of both prebiotics and probiotics. For example, by determining the natural adaptation of the gut to recolonization with commensal bacteria and their growth-promoting nutrients (after pre/probiotic treatment) may establish how dietetic influences could influence the pathogenesis of inflammatory diseases of the digestive system.

The FAO/WHO Expert Consultation and Working Group on probiotics presented their recommendations to Codex (Pineiro et al. 2007) with the hope that these will be used for a science-based risk assessment process for managerial decisions concerning probiotics.

The resolution of some human diseases does not reside solely within the host but rather could involve the host's interface with the microbial environment. Manipulating the gut microbial cohort is a realistic therapeutic and prophylactic strategy for many infectious, inflammatory and neoplastic diseases within the gut. But the promise of *pharmacobiotics* (therapeutic exploitation of the commensal cohort) is only likely to be fulfilled following greater attention to the endogenous enteric microbiome. The GIT microbiome is certainly an affluent repository of metabolites that can be exploited for therapeutic benefit. Elucidating the molecular

details of host–gut microbiome interactions is therefore, a prerequisite for a bacterially derived metabolite program of discovery and control of GIT inflammation. Reports that have demonstrated dietary neosugar, a fructooligosaccharides non-nutritive sweetener can significantly influence the feacal microbiome and activities of reductive enzymes (Buddington et al. 1996) certainly warrant further study.

5.9 Synbiotics

Synbiotics are products that contain both a probiotic and prebiotic component (Bengmark and Martindale 2005; Scholz–Ahrens et al. 2007). The rationale for such combined formulations is to enhance the survival of probiotic bacteria in transit through the proximal GIT; improve colonization of the probiotics in the large bowel and to induce a stimulating effect on the growth of the endogenous microbiome as well (Bengmark and Martindale 2005). This effect may rescue the GIT from a dysregulated inflammatory response that may increase risk of disease.

6 Commensal GIT Bacteria/Probiotics Cellular Signaling and Macromolecular Redox Changes—Chronic Kidney Disease as an Exemplar

Over the last decade, oxidative stress has been proposed to play a major role in the development of co-morbid conditions such as cardiovascular disease (CVD) among, for example, renal failure patients (Himmelfarb et al. 2002; Terawaki et al. 2004), advocating that antioxidant strategies should become part of the treatment for pre-dialysis renal failure (Rutkowski et al. 2007). Additionally, it has been proposed uremic toxins and oxidative stress play significant roles in the development of uremia and its complications (Vanholder et al. 2003). Thus, the concept of oxidative stress being a major deleterious player in all manner of situations has been massively supported by expansive literature (Rutkowski et al. 2007; Chiang et al. 2012; Dounousi et al. 2006). We assert this is incorrect. Reactive oxygen species (ROS) are known to play a major role in maintaining normal physiological function (Linnane et al. 2007). The investigations on protein albumin thiol oxidations and serum protein carbonyl formations indicate that these biochemical events progressively increase with advancing stages of CKD (Dounousi et al. 2006), leading to the conclusion that there exists a close association between oxidative stress and carbonyl formation and that there is a correlation with carbonyl formation and renal dysfunction among pre-dialysis CKD patients (Dounousi et al. 2006). This inference further nurtures support for the administration of antioxidant therapies. However, there are no reported clinical trials that support this conclusion, indeed

studies on the protective antioxidant role of administered alpha lipoic acid for the prevention of contrast-induced nephropathy in diabetic patients demonstrated no benefit (Cicek et al. 2013). A recent systematic review and commentary reported that antioxidant therapy with vitamin C does not reduce the risk of death or cardiovascular events overall in CKD, but that possibly may benefit people with more advanced kidney failure (Jun 2013). It has been (LV, AWL c.f. extensive review) (Linnane et al. 2007) previously considered and challenged that the commonly held view that proteins are randomly oxidized in an uncontrolled process by superoxide anion, hydrogen peroxide, nitric oxide, and peroxynitrite, thereby contributing directly to the development of chronic diseases and the aging process is a simplistic view of complex physiological processes. It was concluded that this concept is not tenable and it is in error, misrepresenting stringently regulated cellular redox metabolism.

The oxidation of protein amino acid residues, since their discovery some decades ago, has been almost universally reported as leading to protein inactivation and requiring mandatory proteolysis to prevent their deleterious cellular accumulation. It is clear that oxidatively modified proteins do not simply arise as the result of random oxidative damage (hydroxylations of various amino acid residues, sulphoxidation of methionines, nitrosylations of sulphydryl groups, and so on). There is an increasing number of situations where free radical protein modifications can be shown to be part of normal cellular regulatory signaling activity. To support these conclusions, some examples follow.

6.1 Specific Protein Oxidations

i. One of the most sensitive amino acids to oxidation is methionine, being converted to methionine sulfoxide. This phenomenon is commonly cited as an example of random oxidative damage to proteins. The following example would bring such an overriding conclusion into serious question. Calmodulin function and its regulation by superoxide anion/hydrogen peroxide oxidation of specific methionine residues is well-documented (Yin et al. 1999). The oxidation of only two of the seven specific methionine residues (144 and 145) of calmodulin is involved in the process of down-regulating plasma membrane-Ca^{++}ATPase. Using genetically engineered calmodulin in which the two methionines (144, 145) were replaced by glutamines, it was shown that oxidation of the remaining methionines did not significantly downregulate calmodulin-plasma membrane-Ca^{++}ATPase activation (Yin et al. 2000). It has also been reported from the same laboratory (Sun et al. 1999) that methionine sulfoxide reductase can act reductively to restore the ability of oxidized calmodulin to regulate plasma membrane-Ca^{++}ATPase. These results showed that superoxide anion and/or hydrogen peroxide are functioning as part of the controlled regulation of the calmodulin-plasma membrane-Ca^{++}ATPase complex. Further that proteasomal degradation of oxidized calmodulin, when

and where it occurs, is part of the normal process of regulated protein turnover. Protein turnover is rigidly controlled, some proteins turnover in minutes, others in hours and longer, but all these proteins are part of a system tightly regulated by the ubiquitin/proteasome system.

ii. The turnover of the hypoxia-inducible factor-alpha (HIF**α**) and its proteasome degradation is clearly regulated by hydroxylation of its prolyl residues (Stolze et al. 2006). This is an ordered process involving signaling by the free radical system comprised of superoxide anion, nitric oxide and peroxynitrite.

iii. Bota et al. (Bota et al. 2002, 2005) have reported that mitochondrial aconitase is preferentially oxidatively modified and inactivated, and that the ATP activation of the mitochondrial Lon protease specifically acts to degrade the oxidized inactivated enzyme. The authors interpret their results as demonstrating the toxicity of ROS. We believe that this conclusion is in error; rather their results demonstrate how tightly regulated is the formation of ROS and its directed activity in regulating the metabolome. The controlled specific degradation of aconitase (among the hundreds of mitochondrial proteins) to regulate citric acid cycle activity is an excellent example of the regulatory role that ROS play in the modulation or control of the metabolome, and that ROS do not randomly contribute to the damage or degradation of cellular metabolic processes.

iv. Consider the nitrosylation of sulphydryl groups, proposed as a damaging phenomenon. We have previously referred to and sited the hemoglobin system as a remarkably regulated machine, finely tuned allosterically for the carriage of the daily massive amounts of inhaled oxygen from lungs to cells (Linnane et al. 2007). It is now recognized that hemoglobin undergoes subtle but critical changes as result of sequential reactions with dioxygen, protons and CO_2 to regulate the delivery of oxygen to the tissues. As part of this process, it is relatively recently recognized that NO• participates in the regulation (Singel et al. 2005). In the cyclic oxygen carriage by hemoglobin, NO• reacts with the b subunit ferrous ions. Subsequently on the b subunit binding of dioxygen, the NO• is displaced to nitrosylate the cys 93 thiol group of the hemoglobin b subunit. These changes are accompanied by an allosteric change from the T (tense) to the R (relaxed) form. The various allosteric changes which hemoglobin undergoes are now recognized as of the utmost importance to hemoglobin function. They are the outcome of over 80-years study (that includes the Bohr effect/Perutz X ray structural studies/the detailed effector allosteric inducers, namely H^+, CO_2, O_2, NO•, 2;3-bisphosphoglycerate/and the allosteric positive and negative cooperative changes that occur) (Linnane et al. 2007). Suffice for here, is that the recently recognized NO• nitrosylation of hemoglobin is part of the normal physiological transport of oxygen delivery to tissues; nitrosylation of proteins is not conditionally deleterious. Parenthetically, it may be added that superoxide anion continually formed in small amounts during the process oxidizes hemoglobin to meet hemoglobin in the order of a steady-state amount of 1–3 %. The met hemoglobin formed is itself continually reduced back to hemoglobin by erythrocyte met hemoglobin reductase to maintain regulated oxygen homeostasis.

v. Farout and Friguet (Farout and Friguet 2006) have considered that there is an age-related deleterious accumulation of oxidized proteins resulting from impaired redox homeostasis and proteolysis. Further, they consider that changes in proteasome structure with increasing age and dysfunction of the proteasome leads to an exacerbated accumulation of oxidatively modified proteins due to their impaired proteolysis. Somewhat contrary to this interpretation, it has been reported when cellular proteasome activity is inhibited, the resultant decrease in its activity leads to a concerted increase in cellular synthesis of the proteasome (the phenomenon of hormesis) (Meiners et al. 2003). Husom et al. (2004) have reported an increase in the 20S proteasome in aged rat skeletal muscle, albeit with some change in function.

It is suggested that the proteasome activity and changes in structure with increasing age and organ dysfunction (for example, in the kidneys and the gut epithelium) be viewed from a different perspective. The proteasome system makes a major demand on the available cellular ATP and will become increasingly dysfunctional in the absence of sufficient ATP substrate. Central to any consideration of aging and its outworking is the universally recognized decline in bioenergy capacity with age from increasingly dysfunctional mitochondria (DNA mutations and deletions). Arising from this consideration, declining ATP availability leads to declining proteasome function which contributes to the multisystem aging process, that includes single organ dysfunction, albeit not as a primary effector and not as a direct result of oxygen radical damage to proteins. Furthermore, with the increasing understanding of the upstream regulation of the superoxide anion/hydrogen peroxide second messenger system, it is becoming increasingly apparent that they play a major role in the ordered regulation of proteolysis and protein homeostasis; and that the damage process is far from random.

6.2 *The Antioxidant Effect*

Vitamin C has long been promoted as an outstanding antioxidant and of benefit in the prevention or amelioration of age-associated diseases proposed arising from oxygen radical damage. There is no doubt that vitamin C is an essential nutritional supplement required for normal mammalian function but it has yet to be demonstrated by clinical trial that it has any role as a meaningful therapeutic antioxidant.

Ascorbic acid plays an essential co-enzyme oxidoreductase role in the hydroxylations of pro-collagen (pro-collagen trimer formation and release from the endoplasmic reticulum), dopamine (to give rise to norepinephrine), and HIF (regulation). Ascorbate occurs in high concentration in the adrenal and pituitary glands but it is not evenly distributed throughout mammalian tissues. Its occurrence is low in tissues such as skeletal muscle, testes, thyroid and lung (Hornig et al. 1975), so that it would not constitute a general endogenous tissue antioxidant, if that were its proposed antioxidant role. Recently, it was reported that administered ascorbate acts

as a pro-oxidant during surgical ischemia-reperfusion (Bailey et al. 2006). On the contrary in large doses it may act as a pro-drug for the production and delivery of H_2O_2 to tissues, especially as has been reported for the treatment of some cancers (Chen et al. 2005). There is no convincing clinical evidence for ascorbic acid acting beneficially in mammals as an antioxidant. Furthermore and importantly, vitamin C has been demonstrated to directly inhibit bacterial *spreading factor* (Li et al. 2001). In that study vitamin C was reported to competitively inhibit hyaluronan degradation by *Streptococcus pneumoniae* hyaluronate lyase, highlighting a compound capable of inhibiting a pathogenic bacterial enzyme in order to maintain homeostasis.

6.3 Commensal/Probiotic Bacteria and Mechanism of Action

CKD has been recently linked to severe disruption of the gut epithelial tight junction barrier (Vaziri et al. 2012; Vinik et al. 2011) with oxidative stress the reported primary factor that drives metabolic abnormalities that contribute to CKD development (Vinik et al. 2011).

It is extensively reported that probiotics can temper a range of GIT physiological functions, including control over immune responses, epithelial barrier function, and cellular proliferation (Vitetta et al. 2012). A recent study has demonstrated that some genera of human GIT bacteria can induce a rapid increase of ROS, eliciting a physiological response through the activation of epithelial NADPH oxidase-1 (Nox1) (Bermudez-Brito et al. 2012; Neish 2013). In addition, reports cite in vitro experiments with epithelial cells that, when co-cultured with specific probiotic bacteria, show an increased and rapid oxidation reaction of soluble redox sinks, namely glutathione and thioredoxin (Bermudez-Brito et al. 2012; Neish 2013) that indicate the presence of a regulated process. This effect was demonstrated as an increase in the oxidoreductase reaction of transcriptional factor activations such as NFκB, NrF2 and the antioxidant response element, reflecting a cellular response to increased ROS production that is regulated (Bermudez-Brito et al. 2012; Neish 2013). This effect must be decisive in order to elicit a restrained anti-infective response with a minimal chance of proinflammatory damage to the tissue. These reactions define potent regulatory effects on host physiological functions that include immune function and intracellular signaling.

The reported mechanisms of action of probiotics are similarly aligned acting to enhance the epithelial barrier, increased bacterial adhesion to the intestinal mucosa, with an attendant inhibition of pathogen adhesion to the competitive exclusion of pathogenic microorganisms ((Bermudez-Brito et al. 2012; Neish 2013; Lin et al. 2009a; Lee 2008; Yan et al. 2007; Patel et al. 2012; Collier-Hyams et al. 2005). Furthermore, probiotic species have also been reported to generate a range of antimicrobial substances and to positively affect and modulate immune system function.

Lee (2008) has reported that the enteric commensal bacteria, by rapidly generating ROS, negotiate an acceptance by the GIT epithelia. Different species of commensal bacteria can elicit markedly different levels of ROS from contacted cells. *Lactobacilli* are especially potent inducers of ROS generation in cultured cells and in vivo, though all bacteria tested have some ability to alter the intracellular oxidoreductase environment (Lin et al. 2009b). Yan et al. (2007) has reported that there are soluble factors that are produced by species of *Lactobacilli* that are capable of mediating beneficial effects in in vivo inflammatory models. This result expands our understanding that there are ROS-stimulating bacteria that possess effective specific membrane components and or secreted factors that activate cellular ROS production to maintain homeostasis.

It has been reported that redox signaling by microbial ROS formation is in response to microbial signals via formyl peptide receptors and the gut epithelial NADPH oxidase 1 (Nox1) (Neish 2013). As previously documented (Linnane et al. 2007) ROS generated by Nox enzymes have been shown to function as essential second messengers in multiple signal transduction metabolic pathways through the rapid and transient oxidative inactivation of a distinct class of sensor proteins bearing oxidant-sensitive thiol groups. These redox-sensitive proteins include tyrosine phosphatases that attend as regulators of the MAP kinase pathways, focal adhesion kinase (Linnane et al. 2007; Neish 2013). These reports focus our understanding on the importance of second messenger functionality for the maintenance of homeostasis and brings into serious question of the annulment of ROS by antioxidant supplements for the amelioration of chronic diseases such as CKD. The established importance of recent investigations regarding probiotic/microbial-elicited ROS teaches that stimulated cellular proliferation and motility is strictly controlled and is a regulated signaling process for proper innate immunity and gut barrier functionality (Lin et al. 2009a, b; Patel et al. 2012; Collier-Hyams et al. 2005) The observations that the vertebrate epithelia of the intestinal tract supports a tolerable low-level inflammatory response toward the GIT microbiome, can be viewed as an adaptive activity that maintains homeostasis (Neish et al. 2000).

7 Fecal Microbiota Transplants

The accepted definition for fecal microbiota transplants (FMT) is a term that describes the *infusion of a fecal suspension from a healthy individual into the gastrointestinal tract of an individual with colonic disease* (Borody and Campbell 2012).

Recently, there has been strong interest in the use of FMT for the treatment of gastrointestinal and non-gastrointestinal diseases (Sha et al. 2014). A systematic review that reported on 844 patients who had undergone FMT was identified from 67 published clinical studies (Sha et al. 2014). The most common indications in this review were for refractory/relapsing *Clostridium difficile* infection (CDI) (76.3 %)

and inflammatory bowel disease (IBD) (13.2 %). Seven publications reported FMT use in pediatric patients with a total of 11 treated, 3 with chronic constipation and the remainder with recurrent CDI or ulcerative colitis (UC). Patients diagnosed with refractory/relapsing CDI had a 90.7 % cure rate and 78.4 % of patients with IBD were in remission after FMT. It was further reported that FMT therapy could also be effective in the treatment of some non-gastrointestinal disorders such as chronic fatigue syndrome. The only reported serious adverse event attributed to the therapy was a case of suspected peritonitis. Furthermore, the review reported that at the time of the review there had been only one placebo-controlled trial, reporting a successful treatment of 43 patients diagnosed with recurrent CDI (Van Nood et al. 2013).

We have previously reviewed and reported (Bella et al. 2014) that multistrain probiotic formulations may also be efficacious for the treatment and also prevention of proton pump inhibitor induced *Clostridium difficile* associated diarrhea (CDAD). In that review we reported that there are numerous probiotic species hat have been investigated as useful and these include *L. rhamnosus* GG, various *Lactobacillus* and *Bifidobacterium* species, and the yeast *Saccharomyces boulardii* (Hickson 2011). The groups of probiotics that have been investigated varied from single species (*Saccharomyces boulardii, L. rhamnosus* GG, *Bacillus clausii*, *B. longum*, *Clostridium butyricum miyairir*, *L. acidophilus*, *Enterococcus faecium SF68*), to mixtures of two types of probiotics and to a synbiotic (a probiotic combined with a prebiotic substance) (Hickson 2011). A meta-analysis reporting on probiotics for the prevention of CDAD efficacy was reported for *L. rhamnosus* GG [dose: 10^9–10^{10} CFU/day]; *L. acidophilus* [dose: 10^9–10^{10} CFU/day]; *S. boulardii* lyophilized [dose: 10^9 CFU/day]; *L. plantarum* [dose:10^9 CFU/day]; *L. acidophilus* and *L. casei* [dose:10^9 CFU/day], and VSL3# [dose:10^9 CFU/day]. The duration of follow-up varied from 2 weeks to 12 weeks and the risk of developing CDAD was 0–24 %. Hence the systematic review reported that 20 randomized trials testing the effect of probiotics in patients receiving antibiotics showed a large relative risk reduction in the incidence of CDAD of 0.34 (CI, 0.24–0.49).

8 Discussion

Inflammation is an essential physiological response by body tissues to injury, chemical irritation, or an assault by generally pathogenic bacteria (Mazmanian et al. 2008). Once the insult is neutralized, normal physiological function needs to be restored. In the GIT, an inflammatory response can be elicited to clear pathogenic bacteria with adaptive responses by commensal and probiotic bacteria that can then subsequently reduce the inflammatory event, thereby promoting a *regulated* pro-or anti-inflammatory state and assisting in reducing the symptoms of conditions such as IBS or IBD. Figure 1 illustrates diagrammatically the complexity exhibited by the GIT in the continuous regulation of inflammation that is required throughout a lifetime. Furthermore, research (Parassol et al. 2005; Zyrek et al. 2007) supports the

notion that increased intestinal permeability resulting from the disruption of the epithelial tight junction may initiate or promote dysregulated inflammation. Maintaining and protecting the tight junctions, preserves barrier function (Nunbhakdi–Craig et al. 2002; Schneeberger and Lynch 2004). It has been demonstrated in vitro that treatment of T84 and Caco-2 cells with probiotics restored or maintained tight junction complexes, thereby restoring the epithelial barrier function in enteropathogenic *E. coli* stimulated cells. Regardless of the timing, incubation with *E. coli* Nissle 1917 or *L. casei* following or during enteropathogenic *E. coli* infection restored the integrity of the epithelial cell barrier (Parassol et al. 2005; Zyrek et al. 2007).

The clinical evidence for the benefits of probiotics is sometimes contentious, however, the data presented herein indicates that probiotics provide both a prophylactic and therapeutic benefit in improving inflammatory conditions (e.g., in the GIT, skin) by regulating cytokine and cell signaling pathways (Mencarelli et al. 2011).

Commensal bacteria and vertebrate immune systems form a symbiotic relationship and have a coevolutionary profile. Such that proper immune development and function relies on colonization of the GIT by commensal bacteria and the maturation cues elicited by the bacterial cohort.

Modification of the gut bacterial cohort has strong therapeutic implications. The demonstration that commensal bacteria are not sequestered by the gut epithelium but are instead recognized by TLRs under normal steady-state conditions attests to this complexity. Indeed, the interaction of commensal bacterial products with host microbial pattern recognition receptors plays a crucial role in resistance to epithelial injury and promoting intestinal homeostasis (Rossi et al. 2013). Because mammalian TLRs recognize products of both pathogenic and commensal bacteria, they might have at least two distinct functions, namely: (i) protection from infection and (ii) control of mucosal homeostasis, both of which are dependent on the recognition of microorganisms (pathogens and commensals, respectively) (Fig. 1). This dual function might explain why some of the TLR-induced gene products, such as inflammatory cytokines and chemokines, are intricately involved in both host defense and tissue repair.

Previously reviewed published human studies of probiotics and of prebiotics (a nutritional supplement favoring the growth and increasing the lifespan of probiotic bacteria) demonstrate their effects on several clinical scenarios (Vitetta and Sali 2008; Vitetta et al. 2012, 2014a). Their beneficial effects can occur when the internal human environment is influenced by the commensal/probiotic environment throughout the digestive tract. Understanding both the bacteria—bacteria interactions and the bacteria—host interactions, especially in the distal GIT, will provide further opportunities for modulating the bacterial cohort of bacteria for therapeutic gain. Although many clinical studies indicate promising trends, the present consensus is that a number of larger controlled trials will be necessary before warranting the use of probiotic supplements as a routine medical treatment for numerous conditions.

There is considerable public and scientific interest in various *natural* products that include probiotics and prebiotics in modulating intestinal as well as end-organ

physiology. Probiotic bacteria demonstrate promise as a biotherapeutic modality as scientific evidence continues to accumulate on the properties, functionality and benefits reported for promoting human health. Manipulating the GIT, a most complex ecosystem, is particularly challenging for therapeutic interventions that aim to regulate the GIT microbiome for the effective treatment of diseases such as irritability and inflammation of the GIT and possibly also cancer of the large bowel. However, the promise is often admixed with the hype. It is untenable to think that one probiotic will cure all diseases, but rather that probiotics are certainly an integral part of the integrative approach to health.

9 Future Research

Future directions for research may involve exploring the optimal doses of probiotics, duration of treatment, their effects in different models (in vitro and animal) of inflammatory disease, and suitability as a prophylactic and or therapeutic treatment. Probiotics and prebiotics can be delivered to the GIT but the percentage of ingested viable bacteria that reach the intestinal tract is not well characterized. Studies aimed at calculating the quantity of viable probiotic bacteria that reaches the upper and lower GIT may be useful. There is still a clear lack of evidence about the effect probiotics have on patients with for example IBD in terms of restoring the GIT microbiome profile. While alleviating the symptoms of IBD and IBS is clinically relevant, future research may also benefit from collecting colon tissue from CD and UC subjects for analysis of gastrointestinal inflammation, bacterial adhesion to the mucus barrier and epithelial cells and colonic crypts histology. This would certainly provide a better understanding of the underlying mechanisms of each condition and possibly lead to better strategies for treatments. Ultimately, probiotic research may also need to examine the synergistic benefits associated with individual bacterial strains that are currently used to formulate commercially available probiotic mixtures.

Within the beneficial efficacy of FMT could probiotics have an adjuvant role? This is a plausible accessory that may further improve FMT efficacy. Patients who elect to undergo FMT could gain a further benefit with the prior administration of probiotics (i.e., days–weeks in advance). The effect may be translated to a further reduction in the risk of gastrointestinal disease relapse.

Mechanistically, the causal relationship between reactive oxygen species and the unbridled damage proposed to macromolecules has led to an over simplification of complex biological processes. It has previously been reported that the formation of superoxide anion/hydrogen peroxide and nitric oxide do not conditionally lead to random macromolecular damage as under normal physiological conditions their production is actually regulated consistent with their second messenger roles (Linnane et al. 2007). As for the GIT it would expected that it too would behave in a manner that sustains a redox regulated state. Intestinal cells that maintain a redox balance preserve the cellular and microbial environment that supports physiological processes and orchestrates networks of enzymatic/metabolic reactions whereby

inflammation remains regulated. Furthermore, the innate immune system presents a wide array of different receptors that can recognize specific bacterial molecular patterns. Hence, an enhanced understanding of the role played by individual probiotic molecular patterns becomes crucial in order to evolve the current complex area of live probiotic bacteria toward improved efficacious pharmacobiotic strategies (Caselli et al. 2011). This research area is complex and intellectually challenging.

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Therapeutic Effects of Ribunucleinate (Ribonucleotides) in Immuno-Inflammatory and Arthritic Diseases

G. Stommel, S. Schuehlein, K.-H. Schuehlein and K.D. Rainsford

Abstract Ribonucleic acids from different organs and from yeast have been used for the treatment of chronic and degenerative diseases in the context of naturopathic medicine in the last 60 years. This chapter provides general information about ribonucleinates as therapeutic agents. Past and present pharmacological and clinical investigations are discussed in the field of the central nervous system, sensory organs, cancer and degenerative diseases of joints and vertebra.

Keywords Osteoarthritis · Ribonucleotides · Cartilage · Bone · Synovia · Proteoglycans · Micro-RNA · Silencing RNA

1 Introduction

It is now well established that diseases affecting the nervous, immune and articular systems are among the most serious and debilitating conditions affecting human populations worldwide. The impact of these chronic conditions both on afflicted patients, the economies and health care systems is by far the most demanding of all human conditions. At present, the prevention and treatment of these conditions are based on a wide range of therapies and surgical procedures all of which are costly and in some cases only partially alleviate or ameliorate the various conditions. The possibility that a single group of therapeutic substances might be effective in treating such a wide range of conditions has some appeal. The reality is that this may not always prove to be the case in the long term.

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The concept that ribonucleic acids from different organs might regulate the regeneration of these organs was developed over 60 years ago by Professor Dr. Hanns Dyckerhoff and further commercialised by a company he formed (Dyckerhoff Pharma GmbH & Co. KG, Köln, Germany) (Becker et al. 1995). In essence, the concept he formulated was that ribonucleic acids (RNAs) are the key regulator of regeneration which is necessary to maintain healthy tissue and prevent disease (Becker et al. 1995). The thesis about RNA involvement in regeneration is that after the age of 40 the body lacks sufficient biologically active RNA. The RNA derived from specific organs is used therapeutically to enhance the production of proteins in the organs which should benefit from the treatment.

1.1 Development of RNA Regenerative Therapy

Decades ago, ribonucleic acid was expected to have modulating effects in the cellular metabolism especially the protein biosynthesis with high potential in regenerating cellular metabolism in degenerating tissues. RNA from different sources was tested in biological studies about the influence on the synthesis of RNA (Kanehisa et al. 1977; Grabowska et al. 1981; Liu et al. 1981; Novakova et al. 1979), the synthesis of DNA (Beljanski and Plawecki 1979; Plawecki and Belianski 1981; Lodemann et al. 1989) and the protein biosynthesis (Amos and Moore 1963; Malpoix 1964, 1967; Rollins et al. 1966; Bogdanovsky et al. 1973; Kelly et al. 1983).

1.1.1 Pharmacodynamics

Early research was done in the field of RNA effects in the brain showing improved brain function and learning tested in different animal species (Hydén and Pigon 1960; Hydén and Egyhazi 1963; Cook and Davidson 1963; Solyom et al. 1967; Guyette et al. 1980; Rosenzweig 1984; Davis and Squire 1984; Högger 1999).

A variety of modulating effects of RNA on the immune response were shown including interferon induction (Aksenov et al. 1970; Taborsky and Dolnik 1977; Wacker et al. 1981; Lacour et al. 1984; Sula and Nouza 1984; Lodemann et al. 1989), virus inhibition (Gifford 1965; Stebbing et al. 1977; Stebbing and Lindley 1980; Zemskov 1977; Iliescu et al. 1983; Repanovici et al. 1983; Nosik et al. 1984; Ignat'ev et al. 1988), stimulation of macrophages and propagation of plasma cells (Merritt and Johnson 1965; Engibarian 1977; Stebbing et al. 1980; Zemskov 1980; Razvorotnev et al. 1987; Ikeda et al. 1994), increase in colony stimulating cells of the hematopoietic system (Semina et al. 1976), increase in host versus graft immune-tolerance (Ashley et al. 1960; Jolley et al. 1961; Largiadèr et al. 1968; Groth et al. 1968), anti-Inflammatory effects (Davis et al. 1981, 1985) and the inhibition of cancer (DeCarvalho and Rand 1961; Esposito 1964; Matienko et al. 1971; Demin 1973; Beljaew et al. 1974).

Hormone-like effects of RNA preparations have been tested for RNA extracts including those from adrenal glands and testis (Villee 1967), thyroid gland and liver (Mu 1973), uteri, kidney, lungs, skeletal muscle, thymus and liver (Mansour 1968;

Fencl and Villee 1971) and from seminal vesicle, ovary, prostate and liver (Fujii and Villee 1969; Niu et al. 1973). In these studies, RNA preparations from different tissues showed organ-specific effects.

Regenerative effects were found in the healing of bones and wounds (Williamson and Guschlbauer 1961a, b, 1963; Belous and Pankow 1966, 1969; Babiichuk et al. 1969; Klyuewa et al. 1977; Semochkin et al. 1999, 2001; Bekman et al. 2001), in the regeneration of nerves (Batkin 1966; Razumova 1970; Vichikova 1982) and of heart, liver, pancreas and bone marrow (Wool et al. 1968; Chernukh et al. 1970, 1971; Breslavskii et al. 1978; Skuba and Levkova 1980; Beljanski et al. 1983). RNA extracts induce regeneration after radiation damages (Sugahara et al. 1966; Ebel et al. 1969; Vladimirov et al. 1985). There is an indication for RNA eliciting differentiation in embryonic myocardium cells (Deshpande et al. 1977; Deshpande and Siddiqui 1978; McLean et al. 1977).

1.1.2 Pharmacokinetics

Cellular uptake of oligonucleotides has been shown to occur by receptor-mediated endocytosis and by unspecific non-receptor-mediated mechanism (Bennet et al. 1988, 1991; Yakubov et al. 1989; Loke et al. 1989; Rieber et al. 1989; de Smidt et al. 1991; Barry et al. 1993; Geselowitz and Neckers 1992; Iversen et al. 1992; Krieg et al. 1991, 1993; Vlassov et al. 1993; Saijo et al. 1994; Wu-Pong et al. 1994; Zamecnik et al. 1994; Giles et al. 1995). Oligonucleotides can be found in the nucleus and in the cytoplasm of incubated cells (Yakubov et al. 1989; Chin et al. 1990; Hawley and Gibson 1992; Barry et al. 1993; Gao et al. 1993; Wu-Pong et al. 1994; Giles et al. 1995). After intravenous, intraperitoneal, intradermal, oral or mucosal administration of oligonucleotides, these are distributed throughout the body (Bazanova et al. 1991; Vlassov et al. 1993; Cossum et al. 1994; Crooke et al. 1994; Sands et al. 1994; Saijo et al. 1994; Agrawal et al. 1995; Zhang et al. 1995). Oligonucleotides are metabolised in cells and serum and excreted mainly in the urine (Crooke et al. 1994; Galbraith et al. 1994; Agrawal et al. 1995). Toxicological studies showed no evidence of acute or chronic toxic effect, teratogenetic effects or mutagenicity (Goossens and Gastpar 1960; Caujolle 1966; Lapik and Matienko 1970; Bormann and Reyher-Pauly 1972; Leuschner 1974a, b, c, d, 1975, 1984, 1988). No cancerogenic effect of oligonucleotides could be found in RNA extracts from healthy tissues (Niu et al. 1961; Esposito 1964; Demin 1973; Beljaew et al. 1974; Svirnovskii et al. 1974; McLean et al. 1977).

1.2 Modern Concepts of RNA Regenerative Therapies

1.2.1 Micro-RNA/Silencing RNA

Current research on micro-RNA/silencing RNA shows that there is highly specific effects of these small RNA chains in cardiovascular disease (Kataoka and Wang 2014), disorders of the immune system and inflammatory diseases

(Tomakova et al. 2011) and osteoarthritis (Stanczyk et al. 2008, 2011; Miyaki et al. 2009; Nakamachi et al. 2009; Tardif et al. 2009; Abouheif et al. 2010; Niimoto et al. 2010; Kawano and Nakamachi 2011; Kurowska-Stolarska et al. 2011; Li et al. 2011, 2012a, b; Nakasa et al. 2011; Tew et al. 2011; Yu et al. 2011; Dai et al. 2012; Díaz-Prado et al. 2012; Dong et al. 2012; Goldring and Marcu 2012; Liang et al. 2012; Martinez-Sanchez et al. 2012; Steck et al. 2012; Swingler et al. 2012; Ukai et al. 2012; Yamasaki et al. 2012; Akhtar and Haqqi 2012; Le et al. 2013; Matsukawa et al. 2013; Song et al. 2013, Trenkmann et al. 2013; Wang et al. 2013). Synthetic micro-RNA for medicinal use is of rapidly expanding relevance (Gibson 2014).

1.2.2 Natural RNA Extracts Containing Micro-RNA

RNA regenerating therapy is based on pharmacological data described above. This may be related to the actions of the components of Regeneresen® comprising the RNA extracts from different organs and from yeasts. Recent analysis of Regeneresen® RNA extracts shows the complex composition of these natural organ extracts. Thus, the chain length of nucleotides (nt) has been found to be below 500 nt when extracts were tested using an Agilent Bioanalyzer (Fig. 1). This analysis shows that the majority of small RNA chains comprise transfer-RNA or smaller RNA types. Micro-RNA was analysed by RNA hybridisation in Regeneresen® RNA extracts which showed the presence of more than 100 micro-RNA types compared with those found in known sequences from different mammalian species

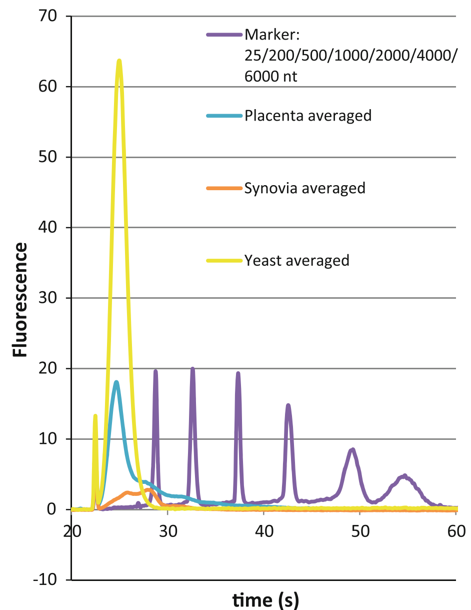

Fig. 1 BioanalyzerData (Exiqon, Denmark) of organ extracts from placenta (average of 4 batches), synovia (average of 3 batches) and from yeast extract (average of 2 batches). These extracts are the active ingredients of Regeneresen®. Standard nucleotides (nt) with chain length from 25 to 6000 nt were used as markers

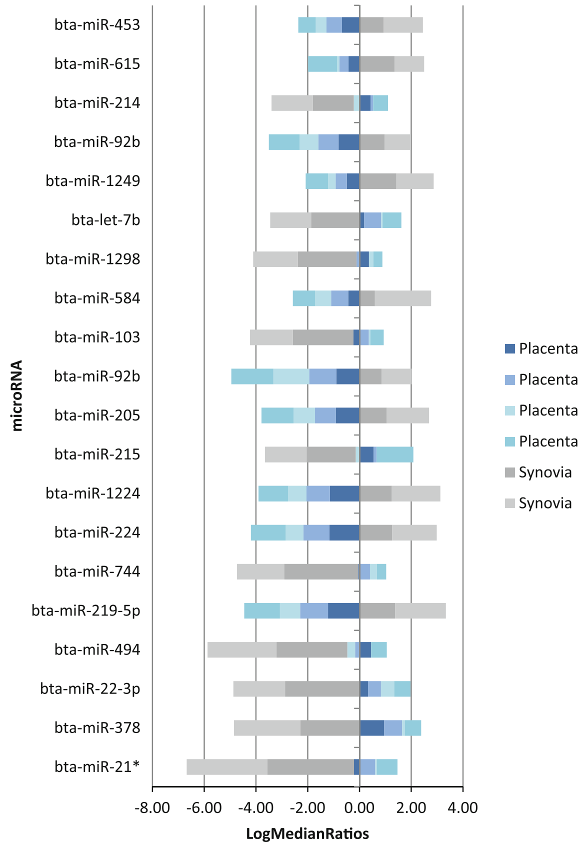

Fig. 2 Micro-RNA hybridisation analysis (miRCURY™ LNA Array, Exiqon, Denmark) of RNA extracts from synovia (2 batches) and from placenta (4 batches). 20 micro-RNAs have been sorted on basis of the standard deviation values with the highest differential expression on top. The numbers are log2(Hy3/Hy5) ratios

shown in standard databases. These hybridisation assays showed differences in the spectrum of micro-RNA for different organ RNA extracts (Fig. 2). Micro-RNA might contribute to organ-specific effects of natural RNA extracts found in pre-clinical tests.

1.3 Clinical Relevance of RNA Regenerative Therapies

There is evidence that RNA extracts of different sources and even synthetic "non-sense" RNA are of clinical relevance as well. RNA extracts from yeast or liver enhanced the recovery from hepatitis and fatty liver with long-term benefits (Levina et al. 1975; Frolov and Razenkova 1980; Rychnev et al. 1982; Hou et al. 1988). In patients with diabetes mellitus, the clinical chemistry of parameters of the pentose phosphate cycle including the activity of ATP-ase, 3-nucleotidase and transketolase increased, while the purine nucleotide concentrations were improved (Karabun and

Yefimov 1975). In patients with *Tapetoretinal dystrophia* or *Retinitis pigmentosa*, treatment with yeast RNA improved the visual field and acuity as well as the dark adaptation (Fuks et al. 1969, 1971; Shershevskaya et al. 1971; Shershevskaya and Levina 1978; Trutneva et al. 1972). Yeast RNA extract reduced dyspnoea and asthma in chronic obstructive lung disease (Zemskov et al. 1979; Silvestrov et al. 1981). Improved wound healing has been found after surgical intervention in *Otitis media epitympanic* after treatment with homologous bone RNA (Filatov et al. 1977). Phage double-stranded RNA accelerated the recovery of Herpes simplex infections and improved symptoms of *Herpes genitalis* or other virus-related dysplasia (Borecky et al. 1978; Gasparyan et al. 1991; Cheknev et al. 1994). Adjuvant treatment with synthetic "non-sense" polyadenylic–polyuridylic acid increased tumour-free intervals and the lifespan of patients with breast cancer in 8 years of observation (Lacour et al. 1980, 1984, 1988). A synthetic "non-sense" dsRNA Poly (I)-poly(CU) improved symptoms of lethargy and fatigue in patients with Chronic Fatigue Syndrome (Strayer et al. 1994). In patients with age-related dementia or memory loss due to other diseases of the central nervous system, yeast RNA improved memory and vigilance (Cameron and Solyom 1961 Cameron et al. 1963; Kral et al. 1967). Clinical experience and trials with Regeneresen® RNA Extracts are detailed below.

2 Regeneresen®

2.1 General Properties

Regeneresen® is a trade name for sodium salt-ribonucleic acid extracts (RNA) preparations from about 50 bovine organs and from yeast as mixtures of specific amounts of organ RNA and yeast RNA. One type of this is RN 13 Regeneresen which comprises a specific mixture of RNA components from adrenal cortex, cerebral cortex, heart, hypothalamus, kidney, liver, ovary, pituitary gland, placenta, spleen, testes, thalamus, vessel wall and yeast. RN13 has been employed for treating patients with geriatric conditions, age-related endocrine involution, general manifestations of ageing, immune deficiencies and for improving muscular strength. Another type of Regeneresen, known as AU4 Regeneresen comprises RNA derived from the auditory system, has been used in patients with presbyacusis, degenerative diseases or toxic injury to the internal ear, sudden deafness and tinnitus. Osteochondrin® is a mixture from connective tissues as described in the subsequent section.

Regeneresen®/AU 4/Osteochondrin® is administered by intramuscular injection, intravenous infusion, intraperitoneally, orally, or applied directly to the mucous membranes or the skin. While intramuscular injection has been the standard method of application over decades, topical application has been proven effective to some extend (Vlassov et al. 1993).

The dosage of Regeneresen® is 6 mg RNA per 5 ml ampoule with recommended daily injection of 2 ampoules. The weekly dosage is 4–12 ampoules with a total of 12–18 ampoules per treatment. There is also some clinical experience with intravenous infusion (Westphal 1997) as well. Since uric acid is a metabolite of RNA and phenylalanine was used as excipient patients with manifest gout, phenylketonuria could not be treated with Regeneresen®. Hypersensitivity reactions manifest in the form of itching, and exanthema occurs infrequently at a rate of less than 1:10,000 applications. In these very rare cases, the treatment was discontinued.

2.2 Clinical Observations

Clinical studies with Regeneresen® have been conducted in patients with diseases of the central nervous system, sensory organs, cerebral insufficiency and cancer.

Diseases of the auditory system have been the subject of several studies with adjuvant treatment with Regeneresen® compared to standard therapy alone or with placebo.

A randomised study with standard therapy and Regeneresen® containing RNA derived from auditory system (AU4 Regeneresen®), vessel wall, placenta and yeast compared with standard treatment alone (low-molecular dextran; naftidrofurylhydrogenoxalate; Vitamin B; saluretic medication) was performed in 50 patients in 4 study groups with Ménierè’s disease (2 × 10 patients), sudden deafness (onset recently 2 × 5 and onset formerly 2 × 5 patients) and old acoustic trauma (2 × 5 patients). Of these, 25 patients received standard therapy alone and 25 additional Regeneresen (120 mg RNA) injections over 3 weeks (Pilgramm and Schumann 1985). Relative changes in hearing derived from audiometric data averaged from 0.25/0.5/1/2/3/4/5/6 to 8 kHz showed no significant differences between standard treatment and adjuvant Regeneresen treatment. Dizziness was reported by 20 patients in the group with Menière’s disease before treatment. After treatment with standard therapy, 6 patients reported improvement of dizziness and 5 patients in the Regeneresen group. A complete relief of Tinnitus was found in the Menière’s disease group (standard treatment 33 % vs. Regeneresen 62 %), in the group with sudden deafness with recent onset (standard treatment 60 % vs. Regeneresen 100 %), in the group with sudden deafness and formerly onset (standard treatment 40 % vs. Regeneresen 80 %) and in the group with old acoustic trauma (standard treatment 0 % vs. Regeneresen 20 %) showing a benefit due to adjuvant Regeneresen treatment in all study groups. A total of 3 patients showed mild inflammation at the injection site of Regeneresen, and one patient interrupted the treatment after breaking out into sweat and sensation of heat.

AU 4 Regeneresen® containing RNA from the auditory system and yeast (36 mg RNA) was compared with placebo (glucose) in a controlled study with 20 patients after acoustic trauma (Pilgramm and Schumann 1986). All patients received standard treatment with dextran40. Hearing loss was evaluated by audiometry, and tinnitus shown a proportion of patients with full relief of symptoms. Hearing gain

was 24.6 % after 10 days and 25.2 % after 42 days in the Regeneresen® group and 24.1 % after 10 days and 24.2 % after 42 days in the placebo group. Tinnitus revealed in 60 % of patients after 10 days and in 70 % of patients after 42 days in the Regeneresen® group and in 70 % after 10 days and 60 % after 42 days in the placebo group. These changes were not significantly different between placebo and Regeneresen® group. The treatment was well tolerated.

RNAs derived from auditory system (AU4 Regeneresen®), vessel wall placenta and yeast were tested in a randomised double-blinded study with 2 × 20 patients with tinnitus after acoustic trauma or sudden deafness (Gottwik 1989). All patients received standard therapy with pentoxifylline and dextran in addition to Regeneresen® (120 mg RNA) or placebo (containing the excipients from Regeneresen® and low-dose riboflavine). Placebo and verum groups were divided each into 2 subgroups with recent onset of tinnitus or longer existing tinnitus. Hearing loss was evaluated by audiometry and rated (0 = no symptom; 1 < −20 dB, 2 < −40 dB, 3 < −50 dB). Tinnitus was rated by patients (full relief, improved, unchanged, worse). Improvement in tinnitus was seen after 3 weeks of treatment with Regeneresen® (Regeneresen: 50 % of patients improved, placebo 40 % of patients improved). Hearing gain (2 before treatment, 1.3 after 3 weeks; $p < 0.05$) was seen in the subgroup with recent onset of tinnitus (mean 0, 81 years). No significant hearing gain was seen in other subgroups. The treatment was well tolerated.

A placebo-controlled double-blinded study with RN 13 Regeneresen® was executed in 60 patients with cerebral insufficiency mainly due to degeneration (Held et al. 1989). RN 13 (RNA from adrenal cortex, cerebral cortex, heart, hypothalamus, kidney, liver, ovary, pituitary gland, placenta, spleen, testes, thalamus, vessel wall and yeast) and placebo (low-dose Vitamin B2) were injected intramuscular over 3 weeks. The dosage of RN 13 was 3 × 12 mg per week. There was no significant difference in effects of RN 13 (SCAG: −6.1 %) compared to placebo (SCAG: −5.2 %) in the Clinical Assessment Geriatric Scale (SCAG) as well as in the tolerance of the treatments. Mild local inflammation at the injection site was seen in 6 of 30 patients in the placebo group and in 7 of 30 patients in the RN 13 group.

However, a subgroup of 24 patients (14 RN 13, 10 placebo) with severe clinical signs (SCAG at least 75) showed significant effects for RN 13 in SCAG (RN 13: −10.9 %, placebo: −3.5 %; $p < 0.039$) after 3 weeks. There was some evidence of effects after week 1 (RN 13: −8.1 %, placebo: −2.2 %; $p < 0.055$), but this tapered off after injections ceased; after week 5 (RN 13: −11.0 %, placebo: −4.3 %; $p < 0.093$) and week 8 (RN 13: −10.4 %, placebo: −6.6 %; $p < 0.68$), a trend to better results with RN 13 was found.

A total of 13 patients with Parkinson's disease were treated with 132 mg RNA from cerebral tissues as adjuvant treatment over a period of 12 days (Fornadi 1993). In this pilot study, all patients received L-Dopa and at least one further medication for this disease and physical therapy. Mini-Mental-State Scale, ZUNG Depression Scale and CURS (Columbia University Rating Scale) were evaluated.

After 5 weeks, the CURS rating was reduced from 19.3 to 11.4. The other parameters were unchanged. The treatment was well tolerated.

The adjuvant treatment with RN 13 Regeneresen and RNA from bone marrow was tested in an open-controlled randomised study with 45 patients with breast cancer (Tchaika et al. 1999). All patients underwent surgery and chemotherapy (cyclophosphamide, methotrexate, fluorouracil; 4 cycles with 3 months time interrupt). Regeneresen (72 mg RNA) was tested versus standard adjuvant treatment (Hämodes, Aerosyl, Cerucal, Navoban). Patients were divided into three groups with 15 patients each receiving standard, adjuvant treatment, RN 13 Regeneresen® and Regeneresen® bone marrow. Primary endpoints were leukocyte and thrombocyte count. In patients treated with Regeneresen®, bone marrow leukocytes recovered in mean from 3.3 Gpt/l before to 4.7 Gpt/l after 10 days of treatment. Thrombocytes recovered in mean from 145.4 Gpt/l before to 220.7 Gpt/l after treatment. In patients treated with RNA, 13 leukocytes recovered in mean from 3.1 Gpt/l before to 4.7 Gpt/l after 10 days of treatment. Thrombocytes recovered in mean from 168.1 Gpt/l before to 233.4 Gpt/l after treatment. In patients treated with standard therapy, leukocytes recovered in mean from 3.1 Gpt/l before to 4.2 Gpt/l after 10 days of treatment. Thrombocytes recovered in mean from 159.8 Gpt/l before to 202.4 Gpt/l after treatment differences were not statistically significant. One patient treated with Regeneresen bone marrow showed allergic exanthema after one injection. The consecutive treatment was well tolerated, and the patient finished the treatment accordingly.

3 Osteochondrin®

3.1 General Properties

Osteochondrin® is a trade name for a special mixture of Regeneresen® containing sodium salt-ribonucleic acid extracts (RNA) preparations from cartilage, intervertebral disc, synovia, placenta and yeast. It has been used for treatment of patients with osteochondrosis, osteoporosis, osteoarthritis, spondylosis and brachialgia.

3.1.1 Current Therapies for Osteoarthritis

Osteoarthritis (OA) affects over 90 % of the western population with increase in frequency and severity with age (Buchanan et al. 2003). Recent studies have given much information on the type and pattern of inflammatory changes that accompany this disease (Pelletier et al. 2001). The treatment of OA is conventionally symptomatic and relies on drugs and/or physical therapies to relieve the symptoms of pain, swelling, stiffness and immobility (Altmann 1991; Buchanan and Kean 2002a). Joint pain is the most significant clinical parameter for the patient

(Buchanan and Kean 2002a), but the precise origins of this are not clear (Creamer et al. 1998).

There are no therapies currently available that can either arrest or reverse the disease (Wieland et al. 2005) although there are some new developments which are encouraging (Wu and Kalunian 2005). In severe OA, surgery is the only realistic option where there is unremitting pain, immobility or instability of the affected joint (Brandt and Flusser 1991; Buchanan and Kean 2002a, b, c; Buchanan et al. 2003). While considerable advances have been made in surgical techniques since the development of the “Charnley” prosthetic hip nearly half a century ago and with advances in biomaterials, conventional surgery is still a procedure of last resort, which has considerable costs, some risks of failure and in some cases limited benefit within the scope of the lifespan of the individual (Brandt and Flusser 1991; Buchanan and Kean 2002a, b, c; Buchanan et al. 2003). Stem cell therapy, while attractive as a means of reversing joint damage, is still only experimental (Baker and Ferguson 2005). Most patients with OA will rely on therapy (self- or doctor-prescribed) with drugs comprising non-steroidal anti-inflammatory drugs (NSAIDs), non-narcotics (e.g. paracetamol, dipyrone), narcotics and a range of herbal or natural products to relieve symptoms of pain and joint inflammation (Buchanan and Kean 2002a). While having some benefit, these natural therapies have variable responses and the risks of developing adverse reactions, despite relative safety, are sometimes limitations to their applications. More significant is that despite claims for “chondro”- or cartilage protection with some NSAIDs (Rainsford 1996, 1999), and these claims are clinically unproven. With a few NSAIDs (e.g. aspirin, indomethacin), there is evidence that they may even accelerate cartilage destruction either as a consequence of over-use from analgesia or biochemical effects (e.g. impaired connective tissue metabolism).

3.1.2 Natural Products and Derivatives

In the past decades, much interest has been shown in therapy with oral glucosamine sulphate and/or chondroitin sulphate (Pavelka et al. 2002; Brenner et al. 2004; Bruyere et al. 2004; Reginster et al. 2001; Richy et al. 2003), and intra-articular hyaluronic acid (Hyal®, hyaluronan) (Leopold et al. 2003; Caborn et al. 2004; Kotevoglu et al. 2006; Neustadt et al. 2005; Ozturk et al. 2006; Raynauld et al. 2002, 2005) and intramuscular injection of galactosamino–glycuronylglycan sulphate (Rovetta 1991; Chevallard et al. 1993; Baker and Ferguson 2005; Moskowitz and Hooper 2005; Goldberg and Buckwalter 2005) as treatments for control of joint destruction as well as achieving relief of pain and inflammation. The biochemical rationale for the case of glucosamine/chondroitin sulphate, while not proven, rests on claims for inhibiting pro-inflammatory cytokine-mediated cartilage destruction as well as stimulating proteoglycan synthesis (by mechanisms that are not clear but could depend on provision of substrates). There are claims for analgesic and anti-inflammatory efficacies, and these effects are mild to moderate. There is also little or no substantial clinical benefit proven with chondroitin/glucosamine sulphate

preparations in *reversing joint damage per se*, although there may be some protection of cartilage. Similar conclusions can be drawn with the use of hyaluronic acid preparations (Buchanan and Kean 2002a; Brandt and Mazzuca 2005). Here, the utility and applications, like those of intra-articular corticosteroids (Bellamy et al. 2005a, b; Raynauld et al. 2005), are limited because of the need for injection into joints with associated risks of operative injury or side effects from the therapy.

Antioxidants have been proposed as another means for preventing or controlling cartilage destruction in OA. For some preparations, there is evidence for these to inhibit pro-inflammatory cytokine-mediated connective tissue degradation, but there is little clinical evidence that this is prevented with these agents Baker and Ferguson 2005; Moskowitz and Hooper 2005).

The central issue with the applications of all these agents is that there is no proven reversal of damage to both bone (notably subchondral bone) as well as cartilage in the joints of patients with OA. Here, some natural product extracts derived from connective tissues (e.g. Rumalon®, a cartilage-bone marrow-placental extract; Katona 1987; Pavelka et al. 2000) or polysulphated derivatives of glucosamine (e.g. Arteperon®; Arck 1982; Ghosh et al. 1992; Pavelka et al. 2000) have been tried, based on some experimental evidence for their effects in "controlling" joint destruction in animal models of joint injury. Concerns about unspecific immune reactions with Rumalon® (Ghosh et al. 1992) and liver toxicity from Arteperon® lead to their withdrawal or reduced interest (Rainsford 1996). Sterile abscesses in joints, and other adverse reactions have been reported in patients who have received these preparations (Schadelin et al. 1981; Berg et al. 1992) raising issues about the safety of these glycosaminoglycan products.

3.1.3 Osteochondrin

Osteochondrin is unique natural product derived from connective tissue sources but it is different from the above mentioned (which are glycoproteins or glycosaminoglycans; GAGs), where it is a ribonucleic acid/ribonucleotide extract product (RNP) derived from connective tissues and yeast (Schroeder et al. 1989; von Sulecki 1990; Rainsford 1996). The rationale for this (as well as a range of RNP's (including Regeneresen®) is that these stimulate regenerative processes, e.g. in bone and cartilage in an attempt to reverse or control the joint degenerative processes. There is evidence to support accelerated repair of experimentally induced fracture injury (Babayan et al. 1979; Bethge et al. 1979; Lodemann et al. 1989) and stimulation of joint GAGs and collagen production in regenerating bone of rats (Babiichuk et al. 1969) and other healing processes (Belous 1971). Osteochondrin/Regeneresen® products have been employed as medicinal products in some European and some other countries for several decades for relieving degenerative conditions in joints, the central nervous system (CNS) and other organs (Lodemann et al. 1989; Schroeder et al. 1989; von Sulecki 1990).

The basis for the actions of Osteochondrin in OA may be that it has combined actions on immuno-inflammatory reactions and connective tissue metabolism.

OA is accompanied by a variety of local immunological reactions with well-defined T-cell responses (Schlaak et al. 1995; Liossis and Tsokos 1998; Nakamura et al. 1999; Sakkas and Platsoucas 2002; Sakkas et al. 2004; Sturmer et al. 2004). Some of these immunological reactions are due to breakdown products of collagen, proteoglycan and bone connective tissues that are degraded in the osteoarthritic process (Liossis and Tsokas 1998; Schroeder et al. 1989).

Part of their actions may have an immunological basis since RNPs have been shown to act as immune stimulants (Wacker and Eichler 1981; Lacour et al. 1984; Zemskov et al. 1984; Berg et al. 1992; Ikenda et al. 1994) possibly in part by regulating T-cell functions (Rudolf et al. 1984; Kulkani et al. 1986; Bekman et all. 2001) so controlling chronic inflammatory conditions (Vladimirov et al. 1985; Beljanski 1991; Burmeister and Rainsford 1991) and the immune deficiencies from infection (Rudoff et al. 1984; Kulkarni et al. 1986). How these T-cell-mediated actions of RNPs affect degenerative processes in such diverse organs as the CNS and arthritic joints is not known. However, there is evidence from a wide range of immunological models for RNPs influencing abnormal immune functions and for the manipulation of T-cell functions to achieve reversal or control of chronic inflammatory-degenerative conditions.

Another action of RNPs may arise from the roles that (ribo)-nucleotides and RNAs have in regulating cell growth (Semochkin et al. 1999, 2001). Ribonucleotides and RNAs stimulate a number of metabolic reactions including mitochondrial oxidative metabolism (Germaniuk and Minchenko 1972, 1982; Germaniuk and Goidoash 1976; Germaniuk et al. 1976; Minchenko and Germaniuk 1976), and this may lead to increased metabolic reactions that lead to repair in connective tissues (Lodemann et al. 1989). Hitherto, difficulties that were envisaged in uptake of RNPs into cells so that they can stimulate growth processes have been overcome in well-characterised models. Moreover, the recent discovery of inhibiting RNAs (iRNA) that block specific translation of RNAs coding for proteins that in some cases may include pro-inflammatory cytokines, metalloproteinases and various inflammatory mediators (e.g. $PLA_{2,}$ COX-2) raises the tantalising prospect that some, albeit unspecific, iRNA-like activity may be present in RNPs to act as inhibitors of mRNAs coding for those cytokines and metalloproteinase enzymes (e.g. Jiang et al. 2005; Fukuyama et al. 2005) some of which are known to be central to the joint destructive processes in OA. Furthermore, ribonucleate sodium (Osteochondrin S) has been shown to inhibit cytokine-induced degradation of bones and cartilage (Rainsford et al. 2008) and modifies osteoclast activity reducing bone resorption in vitro (Cantley et al. 2010a, b). Osteochondrin® and its components showed a concentration-dependent inhibition of human osteoclast activity (Cantley et al. 2013). This inhibition by the RNA components of Osteochondrin® of the resorptive ability of osteoclasts is likely to occur at a late stage during osteoclast formation, downstream from the sites of action of NFATc1. Overall, the findings show that Osteochondrin S inhibition of osteoclast activity may be responsible for its beneficial effects on diseases of the bones such as osteoarthritis (Cantley at al. 2013).

Although these putative actions of RNPs have not yet been proven to have effects in control of regenerative or degenerative cell processes, let alone those

involved in joint destruction in OA, the need for an agent to attack these joint degenerative processes which is based on a non-protein, RNP product with some evidence for its clinical utility makes clinical studies with Osteochondrin®, a unique and novel strategy for treating OA.

3.2 Clinical Observations

Clinical studies have been conducted with Osteochondrin in patients with a variety of musculo-skeletal or arthritis condition. Among the most significant observations are significant improvements in osteoarthritis, lumbago and ischialgia.

A double-blinded randomised study with 57 patients with osteoarthritis of the ankle, knee or patella treated with Osteochondrin® S (27 patients, 120 mg RNA) or placebo (30 patients, low-dose vitamin B2) was conducted. All patients received standard physical therapy. Pain, stiffness and general function parameters were evaluated after 4, 8 and 12 weeks (Schröder et al. 1989). Osteochondrin S and placebo groups showed significant improvements of almost all parameters at week 12 compared to values before the treatment, and only the duration of pain was not significantly changed in the placebo group. Matched pair analysis with 18 patients each from placebo and verum groups could be applied considering age, severity and localisation of osteoarthritis showing superiority of verum in 61 %, equal results in 11 % and superiority of placebo in 28 % of the matched pairs suggesting that patients might benefit from an adjuvant treatment with Osteochondrin S. The treatment was well tolerated in both groups.

A total of 118 Patients with Lumbago or Ischialgia were included into a controlled study with Osteochondrin S (120 mg RNA) in two groups comparing efficacy and safety of paravertebral injections (58 patients) compared to intragluteal injections of Osteochondrin S (60 patients). Examination of the patients and injection of the patients were executed by two doctors independently achieving a blinding of the study. Superiority of paravertebral injection should be tested compared to intragluteal injection. Primary endpoints were the sum of the scores of pain, paraesthesia in the legs and reflexes after 2 weeks (118 patients) and after 3 months (100 patients). All patients received a standard physical therapy. The sum of the scores was 7.45 + 1.77 (mean + SD) before intragluteal injection and 7.64 + 1.97 (mean + SD) before paravertebral injection. The sum of scores (mean + SD) was reduced after 2 weeks by 1.90 + 1.79 with intragluteal injection and by 1.60 + 1.72 with paravertebral injection. After 3 months, the reduction was 2.66 + 1.93 (intragluteal) and 2.74 + 2.13 (paravertebral). Both groups were not significantly different. The treatment was well tolerated. Painful injections were reported in 28.3 % of the intramuscular treatments, and 47.5 % of paravertebral injection which was significantly more. Two patients finished the study after the first paravertebral injection one after a heat sensation in the back and the other after pain at the injection site with nausea and insomnia. These data suggest that both routes of administration might be effective but the paravertebral route causes more

adverse effects so the intragluteal injection might be recommended for Osteochondrin S as the standard application.

3.2.1 Multi-centre Clinical Trial

To establish the efficacy of Osteochondrin in patients, a randomised, parallel-group, multi-centre study was undertaken to investigate the effects of this RNP preparation in controlling pain and joint swelling in osteoarthritis of the knee

There were initially 20 centres recruited in this multi-centre study (under Chefarzt Dr. Med. Wolfgang Bolten, Aerztlicher Direktor, Klaus Miehlke Klinik, Wiesbaden as Principal Investigator). At the same time, there was a study at a single centre in Moscow. The study was designed and managed by the CRO, the Institut fuer Angewandte Statistik GmbH (IAS) (Bielefeld) under the Trial Manager, Dr. rer. Nat. Jörg Schnitker. Dipl. Math.

A total of 168 patients were enrolled in the 20 study centres in Germany and a further 48 at a centre of Moscow; the latter centre is excluded from further investigation in this report because of some protocol violations and inhomogeneity. Thus, the main report considered here is of the 168 patients initially enrolled in Germany. To these were applied standardised and appropriate Inclusion and Exclusion Criteria. Two patients withdrew consent, and thus 166 patients were randomised and considered for safety evaluation. After exclusion of two centres with substantial errors in records and other unacceptable deviations and patients with early termination without relation to efficacy, 145 patients constituted the Full Analysis Set (FAS). Subsequently, there were 12 major protocol deviations in the Osteochondrin Group and 13 in the placebo group treatment, and there were 60 patients in each group that comprised the Valid Case (VC) Set (Table 1).

The loss of patients due to deviations and violations was acceptable considering the circumstances in the trial which have been fully accounted for in the Report. From the statistical viewpoint, the number of patients in each of the treatment

Table 1 Demographics of patients enrolled for randomised treatment allocations

Patients	OST	PLA	All patients
Screening			**168**
Patients withdrew consent			**2**
Randomisation			166
Safety Analysis	**84 (100 %)**	**82 (100 %)**	**166 (100 %)**
Early termination not related to efficacy	4 (4.8 %)	1 (1.2 %)	5 (3.0 %)
Exclusion of centre No. 17[a]	4 (4.8 %)	4 (4.9 %)	8 (4.8 %)
Exclusion of centre No. 21[a]	4 (4.8 %)	4 (4.9 %)	8 (4.8 %)
Full Analysis Set (FAS)	**72 (85.7 %)**	**73 (89.0 %)**	**145 (87.3 %)**
Major protocol violations (with FAS)	12 (14.3 %)	13 (15.9 %)	25 (15.1 %)
Valid Case Set (FAS)	**60 (71.4 %)**	**60 (73.2 %)**	**120 (73.2 %)**

[a]These centres were excluded because of major protocol violations

groups of the FAS and VCS, respectively, is well balanced so that type II errors relating to loss of power (DiGiovanni and Hayes 2001) would be unlikely.

Measuring the Progression of OA and Responses to Therapy

OA (Osteoarthritis) is a complex disease which has variable clinical progression and outcomes (Altman 1991; Buchanan and Kean 2002b, c). In OA of the knee, long-term clinical and radiological studies have shown that although most deteriorate that a small proportion will actually improve and some will remain the same (Massardo et al. 1989). This variability in outcome presents considerable challenges to the investigator for measuring changes that occur in the knee and other joints in response to therapies (Dworkin et al. 2014). While routine X-ray and magnetic resonance imaging (MRI) may give a visual representation of changes in joints, application of these techniques to reliably quantify changes in bone and cartilage in OA has sometimes not been performed with standardised and fully validated techniques so that the assessment of changes and progression of joint injury or pathology may be highly variable (Buchanan and Kean 2002c). For the patients, the most significant clinical symptoms are pain and impairment of joint function (Buchanan and Kean 2002c). In assessing the treatment of OA, it will, therefore, be these parameters that will have greatest clinical significance (Buchanan and Kean 2002c). Quantifying changes in these clinical parameters as well as quality of life (QoL) assessments under standardised and validated procedures (Chassany et al. 2002) are the key components of assessing clinically relevant changes during therapy. In the present study, a standard clinometric approach was employed employing a health status instrument and assessment of QoL and global health status to determine the responses to Osteochondrin therapy.

Use of the WOMAC Instrument

In this study, the Western Ontario and McMaster Universities OsteoArthritis (WOMAC) Index was employed as the primary instrument for determining health status of the study patients.

WOMAC has been widely used as a health status instrument to determine outcomes from therapy with a wide variety of pharmacological agents, physical treatments and procedures used to treat osteoarthritis including that in the knee (Bellamy 2005; Lequesne and Maheu 2003; Salaffi et al. 2003, 2005).

Linguistic Forms of WOMAC and Applications

WOMAC has been evaluated in a number of different language forms, notably in German (Lesquesne 1994; Stucki et al. 1996; Kirschner et al. 2003). In the present study, the CRO has stated that the German version of WOMAC published by Lesquesne (1994) was employed.

Main Outcomes

Patients received treatments with intramuscular injection of 20 ampoules of Osteochondrin S with a dosage of 3 × 2 ampoules per week or placebo ampoules containing the excipients of Osteochondrin S and low-dose riboflavin (Fig. 3). Three subsequent treatment periods were conducted starting from 12th week after the onset of the previous cycle. Overall, the Primary Endpoint of the study in which the WOMAC total index showed response (≥20 % reduction of the baseline values) was achieved. This was shown in the form of statistically significant differences between treatment and control in the total index. Along with this were improvements in Osteochondrin c.f. placebo in the Pain, Stiffness and Physical Function Scales of WOMAC; these being Secondary Endpoints in the Valid Case Set using χ^2 test and logistic regression analysis and in the Full Analysis set using logistic regression following completion to series 2 and 3 of treatments (Rainsford et al. 2004; Stommel et al. 2008). In essence, this means that patients who received Osteochondrin showed significant improvement in total index, pain, stiffness and physical function over placebo.

Examination of the time-dependent changes reveals, overall, qualitative in the Valid Case (VC) population and there were, overall, striking improvements in the total WOMAC Index as well as the individual scales in both Osteochondrin and placebo groups (Fig. 4). The overall trend in the total WOMAC Index and all these component scales was a progressive reduction of about one-half in the VAS scores from the starting baseline (V1) over the three periods (V3, V5 and V7) of the VAS scores. While there was a slight reduction in some of the VAS scores between the period measurements at V5 (post series 2) and V7 (post series 3), the most striking

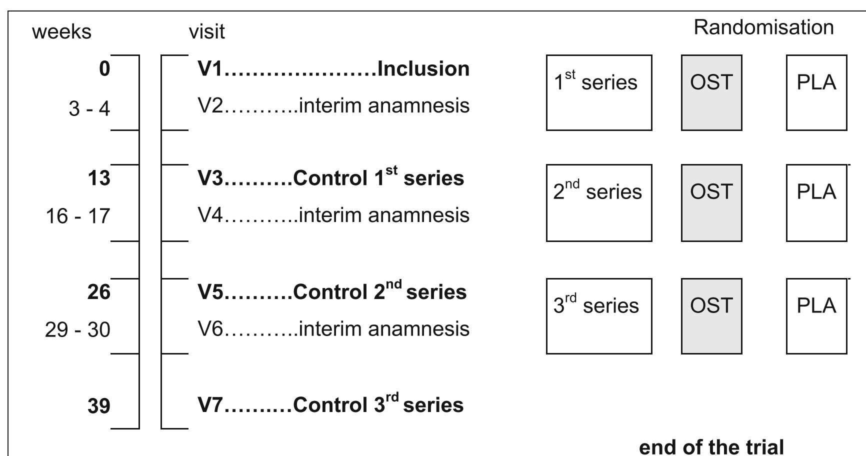

Fig. 3 Treatment and observation assessment. After randomisation patients were treated with 20 ampoules Osteochondrin S (OST) or Placebo (PLA) in three subsequent series with control of the parameters before treatment (V1), and 12 weeks after onset of each series of treatment (V3, V5, V7)

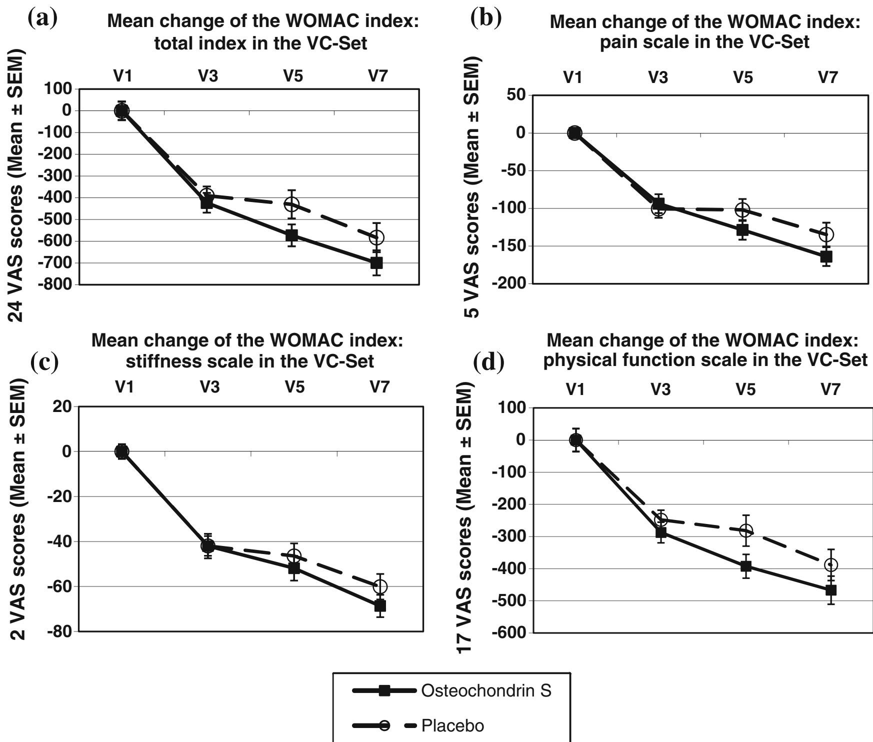

Fig. 4 Mean change of the WOMAC parameters (absolute values) in the Valid Case Set at study onset (V1) and in the course of treatment after the three series of treatment (V3, V5, V7). Differences in placebo and Osteochondrin groups are more obvious after shifting V1 mean values to 0. **a** WOMAC total index; V1 absolute mean values: 1332 in Osteochondrin S group and 1247 in placebo group. **b** WOMAC pain scale, V1 absolute mean values: 284 in Osteochondrin S group and 264 in placebo group. **c** WOMAC stiffness scale, V1 absolute mean values: 121 in Osteochondrin S group and 115 in placebo group. **d** WOMAC physical function scale, V1 absolute mean values: 928 in Osteochondrin S group and 865 in placebo group

changes in physical function, pain and total index seem to be evident at V5 (post series 2) compared with those at the other time points. These data suggest that there was a substantial influence of both treatments in the clinical outcomes shown by WOMAC measurements from the study.

Similarly, overall trends of both groups are evident in the VC population in the global health status (Fig. 5), as well as the OA status (Fig. 6). Again, the trend is towards a reduction of about one-half in the VAS scores over the entire period from the baseline in these global clinical parameters, with some trends being slightly greater than others. There is clearly a positive placebo effect evident, and since the trends towards improvements are apparent overall, this tends to disguise differences that are apparent with Osteochondrin treatment over placebo making the latter appear proportionately less.

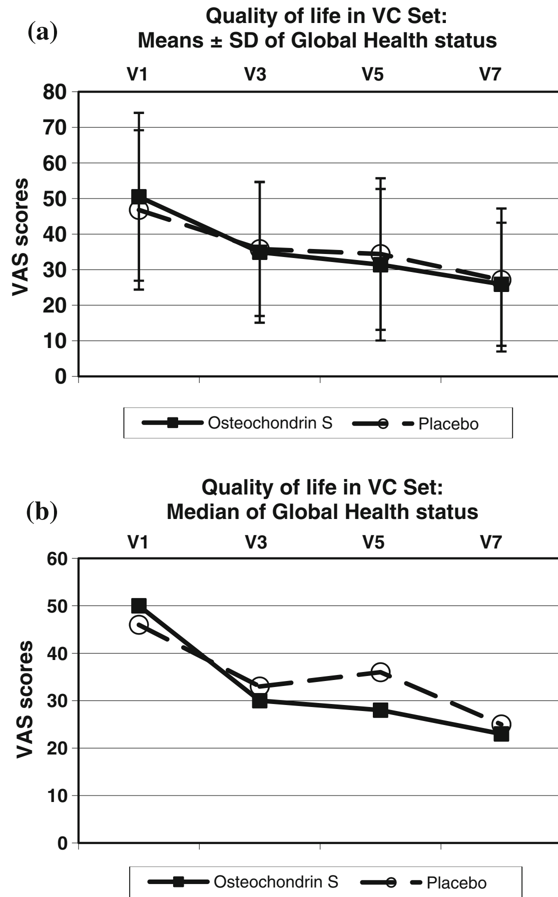

Fig. 5 Quality of life VAS scores in the Valid Case Set in the course of treatment after the three series of treatment (V3, V5, V7) with Osteochondrin S and placebo. Scales from 0 (very good) to 100 (very bad). **a** Means ± SEM of global health status. **b** Median values of global health status

Analgesic Consumption

As an indicator of pain status during the periods of the trial, the consumption of ibuprofen tablets (400 mg) did not reveal any differences in self-administration of this drug in the two treatments; whether the comparison of individual periods were considered (Table 2) or in the post-V7 period compared with V1 (1st injection) and there were no differences between the groups at baseline in both the VC or FAS groups. Overall, the consumption of ibuprofen was about 0.5–1.8 tablets per week which is relatively small and does not present a problem for developing gastro-intestinal or other side effects (Rainsford 1999). Although there was a trend to reduction in the intake of ibuprofen with both Osteochondrin and placebo groups, this difference did not achieve statistical significance in either the FAS or VC series populations. However, it was notable that patients in both groups were initially taking about 5 tablets of ibuprofen per week in both FAS and VC series so the relative reduction in rescue medication is small (since this was about 0.5–1.8 tablets/week).

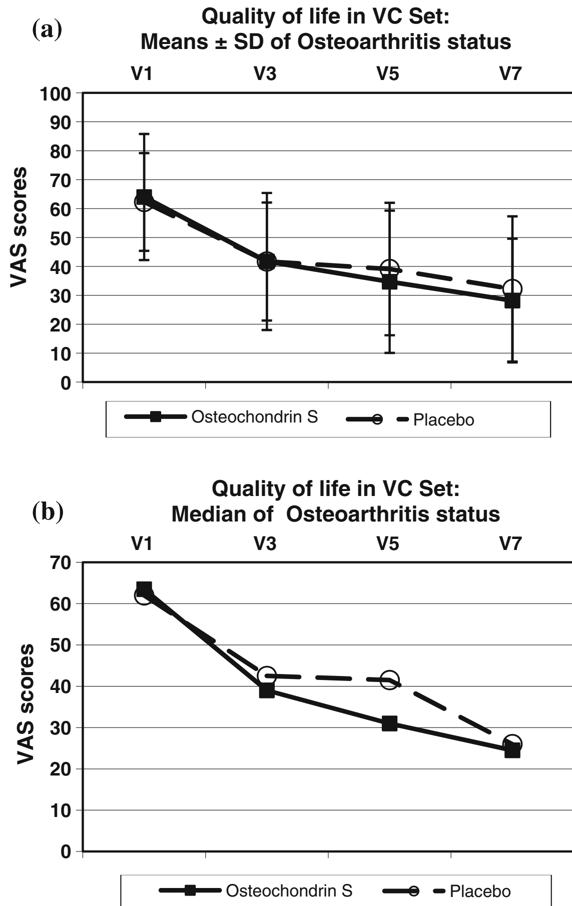

Fig. 6 Quality of life VAS scores in the Valid Case Set in the course of treatment after the 3 series of treatment (V3, V5, V7) with Osteochondrin S and placebo. Scales from 0 (very good) to 100 (very bad). **a** Means ± SEM of Osteoarthritis status. **b** Median values of Osteoarthritis status

Joint Parameters

The assessments of tenderness, mobility, circumference and swelling of knee joints were designated “Exploratory Target Criteria” in the measurements of efficacy in this study. These parameters are of considerable clinical significance and have advantages in being to some extent objective measures of joint inflammation and associated pain responsiveness to pressure application.

In both the FAS and VC series, the reduction in tenderness to application of pressure above the articular space of the affected knee (Table 3) was shown for the individually last series “post7”. This is a substantial reduction in pain responsiveness. Even though there were no statistically significant differences between the two treatments, this trend shows parallel with the WOMAC parameters of pain and physical function noted earlier. There was a reduction in the pain tenderness of contralateral knees in the FAS and VC series of both treatment groups as well but

Table 2 Rescue medication with Ibuprofen per week in the 1st, 2nd and 3rd series of treatment with Osteochondrin (OST) and placebo (PLA)

Rescue medication (FAS)	V1–V2		V2–V3		V3–V5		V5–V7		Post 7	
	OST	PLA	OST	PLA	OST	PLA	OST	PLA	OST	PLA
Mean	5.69	5.52	4.25	4.08	3.92	3.93	3.23	3.61	3.23	3.8
SD	7.25	6.16	4.88	4.70	5.55	5.02	5.46	5.13	5.36	5.45
Median	3.75	3.77	2.43	2.48	0.88	2.17	0.48	1.13	0.48	1.13
[a][FAS]										

Rescue medication (VC)	V1–V2		V2–V3		V3–V5		V5–V7	
	OST	PLA	OST	PLA	OST	PLA	OST	PLA
Mean	4.99	5.39	4.11	3.75	3.99	3.58	3.04	3.37
SD	5.7	5.8	4.8	3.93	5.28	4.33	4.85	4.70
Median	3.75	3.96	2.43	2.51	1.12	2.15	0.64	1.16
[b][VC-Set]								

[a]Findings in the time periods V1–V2, V2–V3, V3–V5, V5–V7 and in the individually last period (post7) compared to the first incection phase in Full Analysis Set
[b]Findings in the time periods V1–V2, V2–V3, V3–V5, V5–V7 in the Valid Case Set

again there were no differences between the treatment groups. The tenderness pain in the contralateral knees is about half that of the affected knees but is still quite pronounced. This is an interesting aspect and reflects the view that OA has systemic components.

The changes in tenderness pain had, to some extent, parallels with swelling of the knee joints (Table 4) which was reduced by about one-half, and the patients were symptom-free or improved over the period to the individually last series “post7” from baseline in about 60–70 % of individuals in both treatment groups. In the same way, the tenderness pain was symptom-free or improved in some 70–80 % of patients on both treatments (Table 3).

In values of the mobility of the affected joints, the values for the angles of stretch, bend and degree of mobility were improved in both treatment groups over the V1 to post V7 period by about 10 degree for the degree of mobility, and there were no changes in these parameters on the contralateral side (Table 5). Likewise, the values for the circumference of the knee joints of the affected side were reduced by about 10 mm in both treatment groups with a trend to get better results in the OST-group shown by the differences at the 1st, 2nd and 3rd series versus V1 at the affected side and contralaterally (Tables 6 and 7).

Overall, these parameters of joint inflammation and pain show that inflammatory pain is reduced in both treatment groups as well as indices of joint movement. There are no differences between the two treatment groups. The patient diary records show changes in pain in the knee by 40 % reduction in the OST-group and 30 % in the PLA-group for the last series compared to the first (Table 8), and this may relate to the changes in joint inflammatory/pain components for both treatments noted above with a clear trend to favour OST.

Table 3 Changes in tenderness on pressure from baseline to the individually last series 'post7' at

Changes from baseline	OST	PLA
Number of patients	72	73
Not affected	2	1
Symptom-free Improved Unchanged Worse	24 (34.3 %) 32 (45.7 %) 11 (15.7 %) 3 (4.3 %)	23 (31.9 %) 31 (43.1 %) 15 (20.8 %) 3 (4.2 %)
[a][χ^2 test: p = 0.891], affected side (FAS)		
Changes from baseline	OST	PLA
Number of patients	72	73
Not affected	37	31
Symptom-free Improved Unchanged Worse	18 (51.4 %) 4 (11.4 %) 11 (31.4 %) 2 (5.7 %)	23 (54.8 %) 5 (11.9 %) 12 (28.6 %) 2 (4.8 %)
[b][χ^2 test: p = 0.988], contralateral side (FAS)		
Changes from baseline	OST	PLA
Number of patients	60	60
Not affected	2	–
Symptom-free Improved Unchanged Worse	19 (32.8 %) 29 (50.0 %) 8 (13.8 %) 2 (3.5 %)	20 (33.3 %) 24 (40.0 %) 14 (23.3 %) 2 (3.3 %)
[c][χ^2 test: p = 0.552], affected side (VC)		
Changes from baseline	OST	PLA
Number of patients	60	60
Not affected	33	27
Symptom-free Improved Unchanged Worse	14 (51.9 %) 2 (7.4 %) 9 (33.3 %) 2 (7.4 %)	18 (54.6 %) 5 (15.2 %) 8 (24.2 %) 2 (6.1 %)
[d][χ^2 test: p = 0.739], contralateral side (VC)		

[a]The affected side [FAS]
[b]The contralateral side [FAS]
[c]The affected side [VC]
[d]The contralateral side [VC]

The measurements of walking time do not show any appreciable differences for both treatments, and there are no significant differences between the two treatments (Table 9).

The QoL Global Health State shows improvement in both groups from V1 to post 7 (Table 10) as well as the results in osteoarthritis status (Table 11) and global quality of life (Table 12) without significant differences between Osteochondrin and placebo.

The investigator's Global Assessment of Efficacy in relation to Clinical Global Impressions (CGI) showed an overall trend for improvement at the various periods

Table 4 Changes in swelling of the knee joint from baseline to the individually last series 'post7' at

Changes from baseline	OST	PLA
Number of patients	72	73
Not affected	13	5
Symptom-free Improved Unchanged Worse	28 (47.5 %) 12 (20.3 %) 18 (30.5 %) 1 (1.7 %)	33 (48.5 %) 13 (19.1 %) 19 (27.9 %) 3 (4.4 %)
[a][χ^2 test: p = 0.839], (FAS)		
Changes from baseline	OST	PLA
Number of patients	60	60
Not affected	9	4
Symptom-free Improved Unchanged Worse	25 (49.0 %) 12 (23.5 %) 14 (27.5 %) –	29 (51.8 %) 10 (17.9 %) 16 (28.6 %) 1 (1.8 %)
[b][χ^2 test: p = 0.710], (VC)		

[a]The affected side [FAS]
[b]The affected side [VC]

for both treatment groups (Table 13). When the values for CGI were correlated with changes in the WOMAC index in the FAS and VC groups that received Osteochondrin and placebo (Table 14), where there appeared to be no differences between the two treatments, there were overall improvements with both the two treatments. This is shown by the predominance of changes in improvements of the CGI of 1–3 ("very much improved" to "minimally improved") with relative changes from baseline in the WOMAC index >20 % (which are outlined in double-lined boxes in Table 14). Outliers have been listed in the comments in Table 14. However, the underlying assessment CGI and the components of the WOMAC Index would not be expected to have identical clinical responsiveness.

The Patient's Global Assessment of Efficacy (FAS and VC) showed a similar trend to that seen with the Investigator's assessment but with a trend to better results in favour of OST after the 2nd series of treatment (Table 15).

Adverse Events and Safety

About half the patients who received the treatments experienced adverse events (Table 16), and it was assumed that the treatments were possibly related in five patients who received Osteochondrin (6.0 %) and 6 who had placebo (7.3 %). The events reported (WHO Terms) were all minor, and there was no clear pattern of their occurrence (Table 17). It might be argued that the reports of pruritus (1 case) and erythematous rash (1 case) (both types of events have been reported earlier and are described in the SPC) might have resulted from the therapy because of vague possibility of some unspecified immunological reactions to the injection.

Table 5 Mobility of the Knee Joint (stretch, bend and degree of mobility) in the 1st, 2nd and 3rd series of treatment

OST					
Stat. estimate	V1	V3	V5	V7	post7
N	72	72	69	68	72
Mean	1.3	2.6	2.8	3.7	3.3
SD	5.9	5.2	5.7	5.7	5.9
Median	0.0	0.0	0.0	0.0	0.0
PLA					
N	73	73	71	71	73
Mean	1.8	2.0	2.7	2.6	2.6
SD	5.5	5.3	5.8	5.5	5.5
Median	0.0	0.0	0.0	0.0	0.0

[a]Stretching [°]of the affected knee (FAS)

OST					
Stat. estimate	V1	V3	V5	V7	post7
N	72	72	69	68	72
Mean	115.2	118.0	121.1	123.0	121.9
SD	15.8	16.8	15.4	15.4	16.5
Median	120.0	120.0	120.0	125.0	125.0
PLA					
N	73	73	71	71	73
Mean	112.8	117.0	119.5	121.3	120.7
SD	15.8	15.4	14.7	14.8	15.1
Median	120.0	120.0	120.0	120.0	120.0

[b]Bending [°] of the affected knee (FAS)

OST					
Stat. estimate	V1	V3	V5	V7	post7
N	72	72	69	68	72
Mean	116.5	120.6	123.9	126.8	125.2
SD	18.6	19.1	17.2	17.0	18.7
Median	120.0	121.5	125.0	130.0	130.0
PLA					
N	73	73	71	71	73
Mean	114.6	119.0	122.2	123.9	123.3
SD	17.8	17.3	16.8	16.8	17.1
Median	115.0	120.0	125.0	130.0	129.0

[c]Degree of mobility [°] at the affected knee (FAS)

OST				
Stat. estimate	V1	V3	V5	V7
N	60	60	60	60
Mean	1.9	3.0	2.7	3.5

(continued)

Table 5 (continued)

OST				
Stat. estimate	V1	V3	V5	V7
SD	5.7	5.0	5.8	5.5
Median	0.0	0.0	0.0	0.0
PLA				
N	60	60	60	60
Mean	2.1	2.4	3.3	3.0
SD	5.7	5.3	5.8	5.6
Median	0.0	0.0	2.5	0.0
[d]Stretching [°] of the affected knee (VC)				
OST				
Stat. estimate	V1	V3	V5	V7
N	60	60	60	60
Mean	114.5	117.9	120.3	121.8
SD	14.3	15.2	14.9	15.5
Median	117.5	120.0	120.0	125.0
PLA				
N	60	60	60	60
Mean	112.9	116.7	118.7	120.6
SD	15.6	15.2	14.5	14.5
Median	120.0	120.0	120.0	120.0
[e]Bending [°] of the affected knee (VC)				
OST				
Stat. estimate	V1	V3	V5	V7
N	60	60	60	60
Mean	116.4	120.9	123.1	125.2
SD	16.9	17.3	17.2	17.3
Median	120.0	121.5	124.5	130.0
PLA				
N	60	60	60	60
Mean	115.0	119.1	122.0	123.6
SD	17.2	16.6	16.7	16.5
Median	120.0	120.0	122.5	129.5

[f]Degree of mobility [°] at the affected knee (VC)					
OST					
Stat. estimate	V1	V3	V5	V7	post7
N	70	70	67	66	70
Mean	5.4	5.5	4.9	5.3	5.2
SD	5.1	5.0	5.3	5.1	5.1
Median	5.0	5.0	5.0	5.0	5.0

(continued)

Table 5 (continued)

OST					
Stat. estimate	V1	V3	V5	V7	post7
PLA					
N	72	72	70	70	72
Mean	5.1	4.5	4.7	4.7	4.6
SD	4.9	5.9	6.2	5.4	5.4
Median	5.0	5.0	5.0	5.0	5.0

[g]Stretching [°] of the contralateral knee (FAS)

OST					
Stat. estimate	V1	V3	V5	V7	post7
N	70	70	67	66	70
Mean	131.1	131.5	129.9	133.0	133.2
SD	12.3	12.8	18.0	17.3	18.1
Median	130.0	132.5	135.0	132.5	130.0
PLA					
N	72	72	70	70	72
Mean	130.0	130.7	129.1	128.7	128.5
SD	10.8	10.4	16.3	17.8	17.6
Median	130.0	130.0	130.0	130.0	130.0

[h]Bending [°] of the contralateral knee (FAS)

OST					
Stat. estimate	V1	V3	V5	V7	post7
N	70	70	67	66	70
Mean	136.5	137.0	134.8	138.3	138.4
SD	14.5	14.6	20.4	18.2	19.0
Median	140.0	140.0	140.0	140.0	140.0
PLA					
N	72	72	70	70	72
Mean	135.1	135.2	133.7	133.4	133.1
SD	13.2	13.2	18.1	18.8	18.6
Median	135.0	137.5	140.0	137.5	135.0

[i]Degree of mobility [°] at the contralateral knee (FAS)

OST				
Stat. estimate	V1	V3	V5	V7
N	58	58	58	58
Mean	5.9	5.7	5.0	5.4
SD	5.2	5.1	5.4	5.2
Median	9.0	9.0	5.0	7.0
PLA				
N	59	59	59	59
Mean	5.2	5.0	5.2	4.9

(continued)

Table 5 (continued)

OST				
Stat. estimate	V1	V3	V5	V7
SD	4.6	5.5	5.6	5.2
Median	5.0	5.0	5.0	5.0
[j]Stretching [°] of the contralateral knee (VC)				
OST				
Stat. estimate	V1	V3	V5	V7
N	58	58	58	58
Mean	130.7	131.6	129.8	131.0
SD	10.8	10.3	18.7	11.5
Median	130.0	132.5	132.5	130.0
PLA				
N	59	59	59	59
Mean	130.7	131.3	128.5	128.3
SD	10.0	9.6	16.9	18.4
Median	130.0	130.0	130.0	130.0
[k]Bending [°] of the contralateral knee (VC)				
OST				
Stat. estimate	V1	V3	V5	V7
N	58	58	58	58
Mean	136.6	137.3	134.8	136.4
SD	13.3	12.6	21.4	13.5
Median	140.0	140.0	140.0	140.0
PLA				
N	59	59	59	59
Mean	135.9	136.3	133.7	133.2
SD	11.4	11.1	17.8	18.8
Median	140.0	140.0	140.0	135.0
[l]Degree of mobility [°] at the contralateral knee (VC)				

Findings at V1, V3, V5, V7 in VC-Set (VC) and additionally for the initially last series “post7” in Full Analysis Set (FAS)
[a]Stretching of the affected side (FAS)
[b]Bending of the affected side (FAS)
[c]Degree of mobility at the affected side (FAS)
[d]Stretching of the affected side (VC)
[e]Bending of the affected side (VC)
[f]Degree of mobility at the affected side (VC)
[g]Stretching of the contralateral side (FAS)
[h]Bending of the contralateral side (FAS)
[i]Degree of mobility at the contralateral side (FAS)
[j]Stretching of the contralateral side (VC)
[k]Bending of the contralateral side (VC)
[l]Degree of mobility at the contralateral side (VC)

Table 6 Circumference of the Knee Joint (mm) in the 1st, 2nd and 3rd series of treatment

OST					
Stat. estimate	V1	V3	V5	V7	post7
N	71	71	68	67	71
Mean	422.8	418.4	414.7	413.0	411.8
SD	50.0	53.2	52.4	51.4	50.2
Median	410.0	400.0	400.0	400.0	400.0
PLA					
N	73	73	71	71	73
Mean	415.4	410.9	407.3	404.8	406.0
SD	40.6	38.4	37.9	39.3	39.5
Median	410.0	410.0	405.0	400.0	400.0

[a]Circumference [mm] of the affected knee (FAS)

OST				
Stat. estimate	V1	V3	V5	V7
N	59	59	59	59
Mean	428.0	423.7	418.2	415.8
SD	52.8	56.3	54.8	53.4
Median	429.0	416.0	417.0	410.0
PLA				
N	60	60	60	60
Mean	414.2	410.3	407.9	404.8
SD	42.1	40.2	39.8	40.7
Median	410.0	407.5	405.0	400.0

[b]Circumference [mm] of the affected knee (VC)

OST					
Stat. estimate	V1	V3	V5	V7	post7
N	67	67	64	63	67
Mean	410.9	409.8	409.6	409.8	407.6
SD	51.4	51.4	51.3	51.8	51.1
Median	400.0	400.0	408.0	400.0	400.0
PLA					
N	68	68	67	67	68
Mean	403.2	403.7	402.8	401.2	401.7
SD	42.2	40.8	40.5	38.8	38.7
Median	400.0	400.0	400.0	400.0	400.0

[c]Circumference [mm] of the contralateral knee (FAS)

OST				
Stat. estimate	V1	V3	V5	V7
N	55	55	55	55
Mean	416.3	415.3	413.3	412.6

(continued)

Table 6 (continued)

OST				
Stat. estimate	V1	V3	V5	V7
SD	54.4	54.4	53.9	54.3
Median	420.0	415.0	410.0	410.0
PLA				
N	57	57	57	57
Mean	402.4	403.3	403.2	401.8
SD	43.6	42.2	42.2	40.8
Median	400.0	400.0	400.0	400.0
[d]Circumference [mm] of the contralateral knee (VC)				

Findings at V1, V3, V5, V7 in Valid Case Set (VC) and additionally for the initially last series "post7" in Full Analysis Set (FAS)
[a]Circumference at the affected side (FAS)
[b]Circumference at the affected side (VC)
[c]Circumference at the contralateral side (FAS)
[d]Circumference at the contralateral side (VC)

Table 7 Differences in the Circumference of the Knee Joint [mm] versus V1 in the 1st, 2nd and 3rd series of treatment in the Valid Case Set [VC]

Affected side								
V3 versus V1			V5 versus V1			V7 versus V1		
Stat. estimate	OST	PLA	Stat. estimate	OST	PLA	Stat. estimate	OST	PLA
N	59	60	*N*	59	60	*N*	59	60
min. max. median mean standard dev.	–40 40 0.0 –4.2 12.5	–40 60 –0.5 –3.8 14.2	min. max. median mean standard dev.	–50 35 –10.0 –9.8 14.7	–50 50 –5.0 –6.3 13.9	min. max. median mean standard dev.	–60 30 –10.0 –12.1 17.2	–40 50 –10.0 –9.4 14.9
t test: p = 0.4348 U test: p = 0.6521			t test: p = 0.0938 U test: p = 0.1149			t test: p = 0.1730 U test: p = 0.3671		
Contralateral side								
N	55	57	*N*	55	57	*N*	55	57
min. max. median mean standard dev.	–25 15 0.0 –1.0 6.8	–28 40 0.0 0.8 8.7	min. max. median mean standard dev.	–30 15 0.0 –3.0 8.4	–37 65 0.0 0.8 12.0	min. max. median mean standard dev.	–55 40 0.0 –3.7 14.4	–36 65 0.0 –0.7 13.4
t test: p = 0.1135 U test: p = 0.1243			t test: p = 0.0282 U test: p = 0.0460			t test: p = 0.1302 U test: p = 0.3924		

[one-sided p values]

Table 8 Patient's diary in the 1st, 2nd and 3rd series of treatment for pain in the knee as an average of diary entries [0 = no, 1 = mild, 2 = severe]

OST					
Stat. estimate	V1–V2	V2–V3	V3–V5	V5–V7	post7
N	71	71	69	67	71
Mean	1.28	1.09	0.92	0.76	0.80
SD	0.36	0.43	0.49	0.47	0.49
Median	1.28	1.11	1.00	0.81	0.84
PLA					
N	71	71	70	69	71
Mean	1.23	1.05	0.95	0.85	0.87
SD	0.41	0.46	0.47	0.53	0.53
Median	1.22	1.06	1.00	0.99	1.00
[a](FAS)					

OST				
Stat. estimate	V1–V2	V2–V3	V3–V5	V5–V7
N	59	59	59	59
Mean	1.25	1.06	0.90	0.75
SD	0.36	0.43	0.49	0.48
Median	1.27	1.10	1.00	0.81
PLA				
N	58	58	58	58
Mean	1.24	1.06	0.96	0.87
SD	0.34	0.41	0.44	0.52
Median	1.22	1.06	1.00	0.98
[b](VC)				

[a]Findings in the time periods V1–V2, V2–V3, V3–V5, V5–V7 and in the individually last series in the FAS
[b]Findings in the time periods V1–V2, V2–V3, V3–V5, V5–V7 in the VC-Set

Table 9 Walking time [s] for 15 m in the 1st, 2nd and 3rd seriesof treatment in the VC-Set

OST				
Stat. estimate	V1	V3	V5	V7
N	60	60	60	60
Mean	23.2	21.9	21.4	20.6
SD	11.6	12.0	11.7	11.1
Median	19.5	18.5	18.0	17.0
PLA				
N	58	58	58	58
Mean	22.0	20.8	20.0	19.2
SD	11.3	11.0	10.4	9.5
Median	19.0	18.0	17.0	17.0

Table 10 Changes from baseline to the individually last series ‘post7’ in global health status in FAS and VC-Set

FAS		
Stat. estimate	OST	PLA
Number of patients	71	72
Median	–19.0	–17.5
Mean	–21.7	–19.3
Standard deviation	23.5	26.4
t test: p = 0.2838 one-sided; U test: p = 0.3292 one-sided		
VC		
Number of patients	59	59
Median	–21.0	–21.0
Mean	–24.6	–19.8
Standard deviation	23.9	26.6
t test: p = 0.1504 one-sided; U test: p = 0.2058 one-sided		

Table 11 Changes from baseline to the individually last series ‘post7’ in osteoarthritis status in FAS and VC-Set

FAS		
Stat. estimate	OST	PLA
Number of patients	72	73
median	–32.5	–32.0
mean	–32.7	–30.4
standard deviation	27.2	27.0
t test: p = 0.3019 one-sided; U test: p = 0.3154 one-sided		
VC		
Number of patients	60	60
Median	–35.0	–32.5
Mean	–35.8	–30.1
Standard deviation	26.0	26.5
t test: p = 0.1178 one-sided; U test: p = 0.1386 one-sided		

There were no statistically significant differences between the two treatment groups in the overall incidence of adverse events and most were minor general disorders, principally in the gastro-intestinal (GI), musculo-skeletal and the respiratory systems (Table 18).

Influenza-like symptoms (6 cases) in three patients who received Osteochondrin 3 with placebo could be coincidental even though these symptoms sometimes appear as a result of leucocyte reactions to immunological agents. Since there were no indications of leucocyte changes, there being only three patients who received Osteochondrin and five on placebo who exhibited abnormal leucocyte counts (Table 19). There was no evidence of fever in any of the patients given either of the

Table 12 Changes from baseline to the individually last series 'post7' in quality of life in FAS and VC-Set

FAS		
Stat. estimate	OST	PLA
Number of patients	72	72
Median	–16.0	–15.0
Mean	–18.7	–16.5
Standard deviation	24.5	24.0
t test: p = 0.2927 one-sided; U test: p = 0.3345 one-sided		
VC		
Number of patients	60	59
median	–17.5	–14.0
mean	–20.2	–16.8
standard deviation	24.2	23.8
t test: p = 0.2195 one-sided; U test: p = 0.2316 one-sided		

Table 13 Investigator's global assessment of efficacy as Responder rates (= incidences of improved physical status) from 1st, 2nd, 3rd series and the individually last series (post7) in FAS and from 1st, 2nd and 3rd series from VC-Set

FAS								
	V3		V5		V7		post7	
Stat. estimate	OST	PLA	OST	PLA	OST	PLA	OST	PLA
Responder rates	54/70 (77.1 %)	53/72 (73.6 %)	52/69 (75.4 %)	52/71 (73.2 %)	56/68 (82.4 %)	59/71 (83.1 %)	56/70 (80.0 %)	59/72 (81.9 %)
	p = 0.625		p = 0.774		p = 0.907		p = 0.768	
VC								
	V3		**V5**		**V7**			
Stat estimate	**OST**	**PLA**	**OST**	**PLA**	**OST**	**PLA**		
Responder rates	47/60 (78.3 %)	44/60 (73.3 %)	46/60 (76.7 %)	43/60 (71.7 %)	49/60 (81.7 %)	51/60 (85.0 %)		
	p = 0.522		p = 0.532		p = 0.624			

two treatments. Fever has been related to the use of polynucleotides designed for anti-viral therapy (Powanda et al. 1977).

Changes from baseline to endpoint of laboratory findings showed similar results for OST and PLA (Table 20). A difference of the p-value < 0.150 was obtained in HDL cholesterol (p = 0.068), Triglycerides (p = 0.086) and Total bilirubin (p = 0.036). These differences were due to different baseline values or within the normal range and clinically not significant.

Clinically relevant shifts (Table 21) occurred in two OST-treated patients (LDL, HDL and total cholesterol resp. triglycerides) and in one PLA-treated patient (γ-GT and alk. phosphatase). Laboratory findings were evaluated at V1 and at least at one visit after treatment in most cases at V7.

Table 14 Correlation between changes in WOMAC index and changes in patient's condition as compared to the start of therapy according to Clinical Global Impressions Item 2

OST								
Relative changes from baseline in WOMAC index	Changes in condition CGI Item 2							
	1	2	3	4	5	6	7	MV
≤–90 %	4	2	–	–	–	–	–	–
>–90 bis ≤–80 %	–	5	–	–	–	–	–	–
>–80 bis ≤–60 %	3	15	2	–	–	–	–	–
>–60 bis ≤–40 %	3	5	4	1	–	–	–	–
>–40 bis ≤–20 %	1	2	7	5	–	–	–	–
>–20 bis ≤+20 %	–	–	1	6	1	1	–	2
>+20 %	–	1	1	–	–	–	–	–
1 very much improved; *2* much improved; *3* minimally improved; *4* no change; *5* minimally worse; *6* much worse; *7* very much worse; *MV* missing value								
Comments: • 6 patients with improved WOMAC index ≥20 % were classified as unchanged or worse • 1/9 patient with marginal changes in WOMAC index within <20 % was classified asimproved								
PLA								
≤–90 %	5	5	1	–	–	–	–	–
>–90 bis ≤–80 %	–	4	–	–	–	–	–	–
>–80 bis ≤–60 %	6	11	1	–	–	–	–	–
>–60 bis ≤–40 %	–	9	1	–	–	–	–	–
>–40 bis ≤–20 %	–	2	4	1	1	–	–	–
>–20 bis ≤+20 %	–	3	7	7	2	1	–	1
>+20 %	–	–	–	–	1	–	–	–
1 very much improved; *2* much improved; *3* minimally improved; *4* no change; *5* minimally worse; *6* much worse; *7* very much worse; *MV* missing value								
Comments: • 2 patients with improved WOMAC index ≥20 % were classified as unchanged or worse • 10/20 patients with marginal changes in WOMAC index within <20 % were classified as improved								
[a](FAS)								

OST							
Relative changes from baseline in WOMAC index	Changes in condition CGI Item 2						
	1	2	3	4	5	6	7
≤–90 %	3	2	–	–	–	–	–
>–90 bis ≤–80 %	–	5	–	–	–	–	–
>–80 bis ≤–60 %	3	15	2	–	–	–	–
>–60 bis ≤–40 %	1	4	4	1	–	–	–
>–40 bis ≤–20 %	–	2	6	4	–	–	–

(continued)

Table 14 (continued)

OST							
Relative changes from baseline in WOMAC index	Changes in condition CGI Item 2						
	1	2	3	4	5	6	7
>–20 bis ≤+20 %	–	–	1	5	1	–	–
>+20 %	–	–	1	–	–	–	–
1 very much improved; *2* much improved; *3* minimally improved; *4* no change; *5* minimally worse; *6* much worse; *7* very much worse; *MV* missing value							
Comments: • 5 patients with improved WOMAC index ≥20 % were classified as unchanged • 1/7 patient with marginal changes in WOMAC index within <20 % was classified as improved							
PLA							
≤–90 %	3	4	1	–	–	–	–
>–90 bis ≤–80 %	–	4	–	–	–	–	–
>–80 bis ≤–60 %	4	10	1	–	–	–	–
>–60 bis ≤–40 %	–	8	1	–	–	–	–
>–40 bis ≤–20 %	–	2	4	–	1	–	–
>–20 bis ≤+20 %	–	3	6	5	2	1	–
>+20 %	–	–	–	–	–	–	–
1 very much improved; *2* much improved; *3* minimally improved; *4* no change; *5* minimally worse; *6* much worse; *7* very much worse; *MV* missing value							
Comments: • 1 patient with improved WOMAC index ≥20 % was classified as unchanged or worse • 9/17 patients with marginal changes in WOMAC index within <20 % were classified as improved							
[b](VC)							

[a]In the individually last series 'post7' FAS
[b]After series 3 in the VC-Set

Table 15 Patient's global assessment of efficacy as Responder rates (= incidences of improved physical status) from 1st, 2nd and 3rd series and the individually last series (post7) in FAS and from 1st, 2nd and 3rd series from VC-Set

FAS								
	V3		V5		V7		post7	
Stat. estimate	OST	PLA	OST	PLA	OST	PLA	OST	PLA
Responder rates	39/70 (55.7 %)	41/72 (56.9 %)	49/69 (71.0 %)	43/71 (60.6 %)	47/68 (69.1 %)	47/71 (66.2 %)	47/70 (67.1 %)	47/72 (65.3 %)
	$p = 0.883$		$p = 0.193$		$p = 0.713$		$p = 0.814$	
VC								
	V3		**V5**		**V7**			
Stat. estimate	**OST**	**PLA**	**OST**	**PLA**	**OST**	**PLA**		
Responder rates	35/60 (58.3 %)	35/60 (58.3 %)	43/60 (71.7 %)	35/60 (58.3 %)	42/60 (70.0 %)	40/60 (66.7 %)		
	$p = 1.000$		$p = 0.126$		$p = 0.695$			

Table 16 Adverse events

	Observed adverse events	
	OST	PLA
Number of patients	48 (57.1 %)	53 (64.6 %)
	X^2 test: $p = 0.323$	
	At least related as possible	
Number of patients	5 (6.0 %)	6 (7.3 %)

Table 17 Display of adverse events with at least possible relationship. Symptoms listed as WHO preferred terms from cases related to the study drug

OST	
WHO preferred terms	Action
Pruritus	No action taken
Circulatory failure	Study drug discontinued
Rash erythematous	Study drug discontinued
Sweating increased; Cramps legs	No action taken
Headache	No action taken
PLA	
Myalgia	Check for thrombosis
Arthralgia	Study drug discontinued
Paraesthesia	No action taken
Arthralgia; Hypoaesthesia; Fatigue and pain	No action taken
Dizziness; Fatigue	No action taken
Somnolence	Study drug discontinued

There do not appear to be any appreciable changes in the laboratory values over the treatment periods, and there appeared to be no differences in the occurrence of abnormal high values. There were some abnormal high values of cholesterol and LDL cholesterol in both treatment groups, but no trends were evident over the treatment periods. These high values like those of the liver enzymes probably reflect the conditions of the relatively older group of patients who have been recruited for the study.

The cardiovascular and other vital signs (Table 22) likewise reflect those expected the type of patients who were recruited in this study, and the comparison of changes from baseline to end of study did not differ between OST and PLA. Aside from some missing values, there were in mean no obvious abnormalities in vital signs that occurred during the study (Table 23) and presumably none could be related to the treatments unless they appeared as adverse events. The intake of concomitant medications, particularly cardiovascular, anti-thrombotic and GI agents, reflects that expected in the study population, while other drugs are of little

Table 18 Incidences of adverse events on the basis of SOCs

SOC	OST [N = 84]		PLA [N = 82]		X^2 test
	N	%	N	%	p-value
Skin and appendages disorders	9	10.7	6	7.3	0.445
Musculo-skeletal system disorders	9	10.7	15	18.3	0.165
Central and peripheral nervous system disord.	5	6.0	7	8.5	0.520
Vision disorders	2	2.4	2	2.4	0.981
Hearing and vestibular disorders	2	2.4	1	1.2	0.574
Psychiatric disorders	5	6.0	5	6.1	0.969
Gastro-intestinal system disorders	11	13.1	17	20.7	0.189
Liver and biliary system disorders	1	1.2	1	1.2	0.986
Metabolic and nutritional disorders	4	4.8	2	2.4	0.423
Endocrine disorders	–		1	1.2	0.310
Cardiovascular disorders, general	4	4.8	7	8.5	0.328
Myo-, endo-, pericardial and valve disorders	1	1.2	2	2.4	0.560
Heart rate and rhythm disorders	–		1	1.2	0.310
Vascular (extracardiac) disorders	3	3.6	2	2	0.670
Respiratory system disorders	7	8.3	12	1.6	0.202
Red blood cell disorders	–		1	1.2	0.310
White cell and reticuloendothelial system disorders	1	1.2	–		0.322
Platelet, bleeding and clotting disorders	2	2.4	–		0.160
Urinary system disorders	4	4.8	3	3.7	0.724
Reproductive disorders, male	1	1.2	–		0.322
Reproductive disorders, female	–		2	2.4	0.150
Neoplasm	–		1	1.2	0.310
Body as a whole—general disorders	20	23.8	19	23.2	0.923
Resistance mechanism disorders	6	7.1	7	8.5	0.738
Operations	–		1	1.2	0.310
Injuries	4	4.8	8	9.8	0.214

consequence for safety. No drug–disease or drug–drug interactions were noted, and no deaths were reported.

The assessments of local tolerability assessed by the patients and investigators (Table 24) showed that the injections were overall acceptable and well tolerated with no reports of "bad" tolerability. At study end, 78 of 79 patients treated with OST and 77 of 80 patients treated with PLA rated the tolerability 'good' or 'very good'. There were four serious adverse events in the patients who received Osteochondrin and four who were in the placebo group (Table 25). It was considered that the relationship with the study group was excluded or rated unlikely in all these cases. The outcomes for most of these cases were either complete recovery after intervention or in the case of the arthritic conditions would be expected to be unresolved because of the progressive nature of the disease.

Table 19 Deviations of normal laboratory ranges

	OST		PLA	
Parameter	*N*	−···+	*N*	−···+
Leucocytes	66	1···2	70	1···4
Erythrocytes	69	6···2	66	6···3
Haematocrit	68	1···1	65	2···3
Haemoglobin	70	1···1	61	2···1
Platelets	68	1···2	74	– 2
Neutrophils	60	6···–	62	3···1
Basophils	60	–···3	60	2···1
Eosinophils	59	1···4	61	1···5
Lymphocytes	54	6···6	57	5···1
Monocytes	58	–···1	66	1···4
Others	69	–···–	67	–···2
Quick's time	66	–···1	67	1···1
PTT	58	– 2	53	–···–
Sodium	62	2···–	70	–···1
Potassium	65	–···1	72	2···2
Calcium	67	5···–	71	1···2
Phosphate	40	4···–	39	4···–
Glucose	58	1···4	57	3···4
Total cholesterol	28	– 8	26	– 5
LDL cholesterol	38	–···5	36	–···3
HDL cholesterol	36	4···1	32	2···2
Triglycerides	55	– 8	50	– 2
Creatinine	68	1···5	68	1···2
Uric acid	56	– 6	69	– 4
Urea	57	1···5	55	–···3
Blood-urea nitrogen	6	–···2	2	–···1
GOT (AST)	60	–···3	60	–···2
GPT (ALT)	64	– 7	68	– 2
γ-GT	54	–···5	62	–···4
Alk. phosphatase	67	–···2	68	1···5
Total bilirubin	67	–···3	68	– 5

Pre-post changes of the laboratory parameters are represented by means of shift tables. In case of normal pre-treatment values, changes to abnormal occurred in the following frequencies
N number of patients with normal pre-treatment values. – = abnormal low after treatment. + = abnormal high after treatment

It is worth noting that the study population had a considerable number of concomitant circulatory, endocrine, nutritional and metabolic diseases and immunity disorders (Table 26). The occurrence of other chronic conditions (e.g. malignancies, hyperthyroidism, GI and mental conditions) whether treated or untreated was relatively low and probably reflected the population at large. Since no drug–disease interactions were noted, it is unlikely that the presence of these conditions appreciably influenced the outcome of responses to the treatments.

Table 20 Laboratory findings from clinical chemistry and haematological screening

Parameter		OST			PLA			t test (*p*-value)
		N	*X*	SD	*N*	*X*	SD	
Leucocytes	[10^9/L]	74	0.32	2.36	75	–0.02	1.76	0.332
Erythrocytes	[10^{12}/L]	74	0.01	0.40	75	–0.02	0.34	0.617
Haematocrit	[%]	74	–0.05	3.14	75	0.22	2.33	0.546
Haemoglobin	[g/dL]	74	–0.08	1.04	75	0.07	0.82	0.351
Platelets	[10^9/L]	74	–8.62	58.27	76	1.91	69.20	0.316
Neutrophils	[%]	70	–0.10	8.51	70	0.59	8.17	0.622
Basophils	[%]	70	0.16	1.31	70	–0.07	0.78	0.214
Eosinophils	[%]	70	–0.25	1.70	70	–0.00	2.55	0.503
Lymphocytes	[%]	70	0.11	7.14	70	–0.30	6.85	0.731
Monocytes	[%]	70	–0.14	3.07	70	–0.07	2.07	0.882
Others	[%]	70	0.19	1.27	70	–0.17	1.72	0.161
Quick’s time	[%]	68	0.81	7.26	67	–0.81	6.95	0.190
PTT	[s]	61	–0.23	3.25	61	–0.54	5.23	0.698
Sodium	[mmol/L]	70	1.51	4.46	76	0.84	4.13	0.346
Potassium	[mmol/L]	71	–0.16	0.87	76	–0.20	0.67	0.762
Calcium	[mmol/L]	71	–0.03	0.15	73	0.01	0.30	0.314
Phosphate	[mg/L]	44	–0.11	0.98	45	–0.04	1.11	0.736
Glucose	[mg/dL]	72	–5.51	40.05	75	0.89	20.52	0.222
Total cholesterol	[mg/dL]	74	–5.91	36.03	75	–12.43	28.98	0.225
LDL cholesterol	[mg/dL]	55	–9.38	29.74	56	–7.50	20.56	0.699
HDL cholesterol	[mg/dL]	54	–0.62	10.39	54	3.40	12.19	0.068
Triglycerides	[mg/dL]	72	9.19	62.82	74	–7.47	53.21	0.086
Creatinine	[mg/dL]	74	0.00	0.14	76	0.00	0.10	0.986
Uric acid	[mg/dL]	73	–0.02	1.23	76	0.04	1.01	0.723
Urea	[mg/dL]	58	2.05	8.50	63	0.32	9.89	0.308
Blood-urea nitrogen	[mg/dL]	6	0.60	5.50	5	4.80	8.29	0.340
GOT (AST)	[U/L]	74	–0.10	3.22	72	0.85	5.29	0.193
GPT (ALT)	[U/L]	72	1.13	4.70	76	0.24	7.62	0.398
γ-GT	[U/L]	74	0.65	12.04	76	4.92	35.16	0.323
Alk. phosphatase	[U/L]	71	1.51	30.01	74	7.12	59.02	0.474
Total bilirubin	[mg/dL]	68	–0.04	0.19	69	0.03	0.20	0.036

Changes from baseline to endpoint in each individual laboratory parameter were compared between the trial group. *N* = number of patients

Table 21 Display of clinically significant abnormalities of laboratory findings (bold type: abnormal, in brackets: clinically significant)

Group	Parameter	V1	V3	V5	V7
OST	Total cholesterol [mg/dL] LDL cholesterol [mg/dL] HDL cholesterol [mg/dL]	**264** 146 **42**	– – –	– – –	(**290**) (**160**) (**36**)
OST	Total cholesterol [mg/dL] LDL cholesterol [mg/dL] HDL cholesterol [mg/dL]	(**245**) (**160**) (**41**)	– – –	– – –	**260** 140 **40**
OST	Glucose [mg/dL]	(**185**)	–	–	(**196**)
OST	Total cholesterol [mg/dL] LDL cholesterol [mg/dL]	(**343**) (**239**)	– –	– –	**229** 120
OST	Glucose [mg/dL] Total cholesterol [mg/dL] LDL cholesterol [mg/dL] HDL cholesterol [mg/dL] Triglycerides [mg/dL] Uric acid [mg/dL] GOT (AST) [U/L] γ-GT [U/L]	(**297**) (**258**) (**217**) (**32**) (**376**) (**11.7**) (**21**) (**290**)	– – – – – – – –	– – – – – – – –	65 **263** **159** 44 **233** **6.6** 15 **223**
OST	Triglycerides [mg/dL] γ-GT [U/L]	**268** **37**	– –	– –	(**461**) (**68**)
OST	Triglycerides [mg/dL]	(**404**)	–	–	(**324**)
OST	Neutrophils [%] Lymphocytes [%]	**43.9** 39.2	(**71.2**) (**22.3**)	– –	**42.5** **45.3**
OST	Total cholesterol [mg/dL] LDL cholesterol [mg/dL]	(**250**) (**182**)	198 138	– –	198 117
OST	γ-GT [U/L]	(**109**)	**81**	–	–
OST	Glucose [mg/dL]	(**130**)	–	–	**130**
OST	Glucose [mg/dL]	(**143**)	**118**	–	**101**
OST	Glucose [mg/dL]	(**292**)	–	–	**180**
PLA	Total cholesterol [mg/dL] LDL cholesterol [mg/dL] HDL cholesterol [mg/dL] Triglycerides [mg/dL]	(**250**) (**160**) (**36**) (**205**)	– – – –	– – – –	(**240**) (**155**) (**38**) (**196**)
PLA	Glucose [mg/dL] Total cholesterol [mg/dL] LDL cholesterol [mg/dL] HDL cholesterol [mg/dL] Triglycerides [mg/dL]	(**166**) (**339**) (**160**) (**32**) (**210**)	– – – – –	– – – – –	(**176**) (**350**) (**165**) (**30**) (**235**)
PLA	Triglycerides [mg/dL] Uric acid [mg/dL] γ-GT [U/L]	(**723**) (**11.4**) (**163**)	**322** **9.3** **72**	**505** **7.2** **171**	**688** **10.9** **241**
PLA	HDL cholesterol [mg/dL]	(**49**)	–	–	**51**
PLA	Eosinophils [%] Lymphocytes [%] Total cholesterol [mg/dL]	2.1 30.8 (**324**)	(**6.3**) (**17.2**) 179	– – –	3.4 32.5 **219**

(continued)

Table 21 (continued)

Group	Parameter	V1	V3	V5	V7
PLA	Total cholesterol [mg/dL] LDL cholesterol [mg/dL] Triglycerides [mg/dL]	(**249**) 137 104	(**315**) (**189**) (**244**)	– – –	**221** 149 53
PLA	Leucocytes [10^9/L]	8.7	(**13.8**)	–	5.1
PLA	Potassium [mmol/L]	4.7	4.4	(**3**)	3.7
PLA	Haemoglobin [g/dL]	(**7.5**)	–	–	–
PLA	Glucose [mg/dL]	(**173**)	–	–	80
PLA	γ-GT [U/L] Alk. phosphatase [U/L]	**33** **176**	– –	– –	(**313**) (**579**)
PLA	Glucose [mg/dL] Total cholesterol [mg/dL] LDL cholesterol [mg/dL] HDL cholesterol [mg/dL] Triglycerides [mg/dL]	(**120**) (**323**) (**197**) (**39**) (**389**)	– – – – –	– – – – –	**137** **267** **176** **40** **233**
PLA	Glucose [mg/dL]	(**272**)	–	–	–

13 patients in the OST-group and 13 patients in the PLA-group were concerned with at least one clinically significant abnormality (V1 and control value at V3, V5 or V7 present)

Table 22 Vital signs (Heart rate, HR; Systolic blood pressure, BPS; Diastolic blood pressure, BPD) in the course of study. Comparison between OST and PLA of changes from baseline to end of study in vital signs

Parameter	OST			PLA			t test (*p*-value)
	N	*X*	SD	*N*	*X*	SD	
HR [bpm]	75	0.20	6.24	74	1.20	8.27	0.405
BPS [mmHg]	75	–2.92	13.12	74	–3.01	14.80	0.968
BPD [mmHg]	75	–0.15	7.37	74	–0.84	6.89	0.555

Table 23 Vital signs (Heart rate, Systolic and Diastolic blood pressure) in the course of treatment

Heart rate after 5′ in sitting position [bpm]: OST-group				
Stat. estimate	V1	V3	V5	V7
N	75	54	42	70
Mean	73.9	72.9	74.3	74.0
SD	8.7	8.0	8.4	7.1
Median	72.0	72.0	76.0	73.0
Heart rate after 5′ in sitting position [bpm]: PLA-group				
N	74	48	46	70
Mean	73.2	73.9	75.5	74.5
SD	6.8	8.8	8.3	9.0
Median	72.0	72.0	76.0	73.0

(continued)

Table 23 (continued)

Heart rate after 5′ in sitting position [bpm]: OST-group				
Stat. estimate	V1	V3	V5	V7
Systolic blood pressure after 5′ in sitting position [mmHg]: OST-group				
N	75	55	42	70
Mean	140.7	138.0	138.1	138.2
SD	13.3	16.1	12.1	12.7
Median	140.0	140.0	140.0	140.0
Systolic blood pressure after 5′ in sitting position [mmHg]: PLA-group				
N	74	49	46	71
Mean	142.2	140.3	138.7	139.4
SD	16.5	14.7	17.0	15.1
Median	143.5	140.0	140.0	140.0
Diastolic blood pressure after 5′ in sitting position [mmHg]: OST-group				
N	75	55	42	70
Mean	82.8	81.9	82.1	82.7
SD	6.1	8.1	6.6	7.1
Median	80.0	80.0	80.0	80.0
Diastolic blood pressure after 5′ in sitting position [mmHg]: PLA-group				
N	74	49	46	71
Mean	83.9	83.7	82.8	83.1
SD	7.7	6.2	7.3	6.8
Median	81.0	80.0	80.0	80.0

Table 24 Global assessment of tolerability

Patients assessment (p = 0.674)				
Judgement	OST		PLA	
	Frequency	(%)	Frequency	(%)
Very good	50	63.3	52	65.0
Good	28	35.4	25	31.3
Moderate	1	1.3	2	2.5
Bad	–	–	1	1.3
Investigators assessment (p = 0.571)				
Very good	52	65.8	56	70.0
Good	26	32.9	21	26.3
Moderate	1	1.3	2	2.5
Bad	–	–	1	1.3

High placebo responses as shown in this study were already known in osteoarthritis studies especially when the medication was applied by injection (Zhang et al. 2008; Doherty and Dieppe 2009; Zeidler 2011; Abhishek and Doherty 2013). Since this is an unsolved problem in clinical studies, the proved safety of Osteochondrin S might be of high significance.

Table 25 Serious adverse events

Treatment group	Item	Findings
OST	WHO preferred term	Arthrosis
	AE	Acute exacerbation of coxarthrosis
	Age of patient	73 years
	Gender	Female
	Drug relationship	No
	Outcome[a]	AE persisting/still under treatment
	Follow-up	Completely recovered at follow-up dated 471 days after onset of the event
OST	WHO preferred term	Haematemesis
	AE	Gastric haemorrhage
	Age of patient	62 years
	Gender	Male
	Drug relationship	Unlikely
	Outcome[a]	Recovered completely
OST	WHO preferred term	Diabetes mellitus
	AE	Exacerbation of Diabetes mellitus
	Age of patient	71 years
	Gender	Female
	Drug relationship	No
	Outcome[a]	Recovered completely
OST	WHO preferred term	Arthralgia
	AE	Acute pain in the left knee
	Age of patient	46 years
	Gender	Male
	Drug relationship	Unlikely
	Outcome[a]	Recovered completely
PLA	WHO preferred term	Fistula of the bladder
	AE	Vesical fistula
	Age of patient	74 years
	Gender	Female
	Drug relationship	No
	Outcome[a]	Recovered completely
PLA	WHO preferred term	Chest pain
	AE	Unclear chest pain
	Age of patient	74 years
	Gender	Female
	Drug relationship	No
	Outcome[a]	Recovered completely
PLA	WHO preferred term	Arthrosis
	AE	Joint effusion of the knee
	Age of patient	63 years
	Gender	Female
	Drug relationship	No
	Outcome[a]	AE persisting/still under treatment
	Follow-up	Knee joint endoprosthesis at both knees within 2 years after onset of the event

(continued)

Table 25 (continued)

Treatment group	Item	Findings
PLA	WHO preferred terms AE Age of patient Gender Drug relationship Outcome[a]	Uterine carcinoma, Uterine disorder nos Ca. in situ, descensus uteri vaginae 71 years Female No Recovered completely

[a]Outcome at individual discontinuation of study

Table 26 Rate of concomitant diseases at study onset

ICD-9 Code	Diagnosis	Frequency (%)
ICD VII	Diseases of the circulatory system	54.8
ICD III	Endocrine, nutritional, and metabolic diseases and immunity disorders	35.5
ICD IX	Diseases of the digestive system	10.8
ICD XVI	Symptoms, signs, and Ill-defined conditions	10.2
ICD VI	Diseases of the nervous system and sense organs	9.6
ICD VIII	Diseases of the respiratory system	9.6
ICD X	Diseases of the genitourinary system	8.4
ICD V	Mental disorders	3.6
ICD XIII	Diseases of the musculo-skeletal system and connective tissue	3.0
ICD XII	Diseases of the skin and subcutaneous tissue	2.4
ICD XVII	Injury and poisoning	1.2
ICD I	Infectious and parasitic diseases	0.6
ICD IV	Diseases of the blood and blood-forming organs	0.6

4 Future Developments and Conclusions

Micro-RNA research is expected to induce the development of new medicinal products showing specific effects in the cellular metabolism with high benefit in patients with degenerative diseases and cancer. These products will be based on well-characterised synthetic RNA entities, detailed descriptions of pharmacological properties and proven efficacy in clinical applications. Natural RNA extracts from animal tissues or yeast provide mixtures, like most natural extracts from plants, which were not yet characterised in detail but with clinically proven relevance and with a background of decades of experience in a safe application in patients with chronic and degenerative diseases. Since natural RNA extracts contain a variety of micro-RNAs, further progress in micro-RNA research might lead to a better understanding of the pharmacological properties of natural RNA extracts confirming decades of clinical experience with these products.

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Perna canaliculus (Green-Lipped Mussel): Bioactive Components and Therapeutic Evaluation for Chronic Health Conditions

Samantha Coulson, Talia Palacios and Luis Vitetta

Abstract *Perna canaliculus* (Green-Lippped Mussel) is found only in New Zealand waters and is cultivated and manufactured for both the food and nutraceutical industry world-wide. *P. canaliculus* has traditionally been used as a therapeutic to treat various arthralgias in both humans and animals; however, clinical research reports provide conflicting results. Numerous in vitro studies have reported anti-inflammatory activity of the mussel under various conditions and also demonstrated a synergistic effect with pharmaceutical medications such as non-steroidal anti-inflammatory drugs (NSAIDs) with *P. canaliculus* protecting the gastrointestinal mucosal lining against such medications. It is proposed that the anti-inflammatory activity demonstrated by *P. canaliculus* is predominantly due to the lipid fraction, however, among the major classes of compounds found in mussel meat, proteins and peptides are the largest with isolates demonstrating various anti-microbial, anti-inflammatory, anti-oxidant, bioadhesive and anti-hypertensive activities. A review of the bioactive components, their function and therapeutic application is outlined in this chapter. Furthermore, we hypothesise and provide supportive evidence that the gastrointestinal microbiota play an important role in disease processes such as Rheumatoid arthritis and Osteoarthritis and also in the efficacy of *P. canaliculus* in chronic inflammatory conditions. The metabolic capacity of intestinal microbiota can modify bioactive food components altering the hosts' exposure to these components, potentially enhancing or diminishing their health effects. Understanding the interaction of the bioactive compounds in *P. canaliculus* with commensal and pathogenic bacteria may facilitate the development of novel interventions to control intestinal and extraintestinal inflammation.

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Keywords *Perna canaliculus* · Green-Lipped mussel · Anti-inflammatory · Gastrointestinal tract · Microbiota

Abbreviations

AA	Arachidonic acid
ARA	American rheumatism association
5-HETE	5-Hydroxyeicosatetraenoic acid
CRP	C-reactive protein
DHA	Docosahexaenoic acid
ESR	Erythrocyte sedimentation rate
EPA	Eicosapentaenoic acid
E/LFT	Electrolytes/liver function test
FFAs	Free fatty acids
FBC	Full blood count
GIT	Gastrointestinal tract
GC-MS	Gas chromatography-mass spectrometry
GSRS	Gastrointestinal symptom rating scale
HAQ	Health assessment questionnaire
Hb	Haemoglobin
IBD	Inflammatory bowel disease
IgG	Immunoglobulin G
IL-1	Interleukin-1
KOH	Potassium hydroxide
LCPUFA	Long chain polyunsaturated fatty acids
LOX	Lipoxygenase
NSAIDs	Non-steroidal anti-inflammatory drugs
NMR	Nuclear magnetic resonance
NF-kB	Nuclear factor-kappa B
OA	Osteoarthritis
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
PMN	Polymorphonuclear leukocytes
PG-PS	Peptidoglycan-polysaccharides
RA	Rheumatoid arthritis
RBC	Red blood count
SCFA	Short chain fatty acids
SFE	Supercritical fluid extraction
SF-12V2™	SF-12 heath questionnaires
TNF-α	Tumour necrosis factor—α
TXB2	Thromboxane-2
WOMAC	Western Ontario McMaster Universities arthritis index
VAS	Visual analogue scale

1 Introduction

Perna canaliculus belongs to the class *Bivalvia*, the phylum *Mollusca* and family *Mytilidae*. The genus *Perna* contains species of both green and brown mussels located predominantly in the Southern Hemisphere but also found in North Africa and the northern coasts of South America, with Paleontological data dating the genus back to the Eocene period (60 million years ago) (Wood et al. 2007). Three well-defined species are recognised in the *Perna* genus that includes *P. viridis* (Asian green mussel) found through Indo-Pacific regions, *P. perna* (brown or rock mussel) found through Atlantic regions and *P. canaliculus* which is endemic to New Zealand waters only and has been commercially and sustainably farmed since the early 1970s (Wood et al. 2007). *P. canaliculus* is distinguished from other mussel species by the bright green stripe around the posterior ventral margin and inside the lip of its shell (see Fig. 1) (Wolyniak et al. 2005). Numerous bioactive compounds have been identified in both the *Mytilus* and *Perna* genera of mussels, but it is *P. canaliculus* that has been most comprehensively studied for medicinal purposes. It has supported the development of commercial therapeutic products to treat arthralgia in humans and animals. It has also been assessed as an adjunct therapy for rheumatoid arthritis (RA), asthma and gastrointestinal tract (GIT) complaints (Gibson et al. 1980; Gibson and Gibson 1998; Coulson et al. 2012; Mickleborough et al. 2013). *P. canaliculus* is manufactured in New Zealand as unadulterated freeze-dried whole (i.e. without shell) extract of the mussel meat; as whole with the lipid fractions removed and as a concentrated lipid extract only.

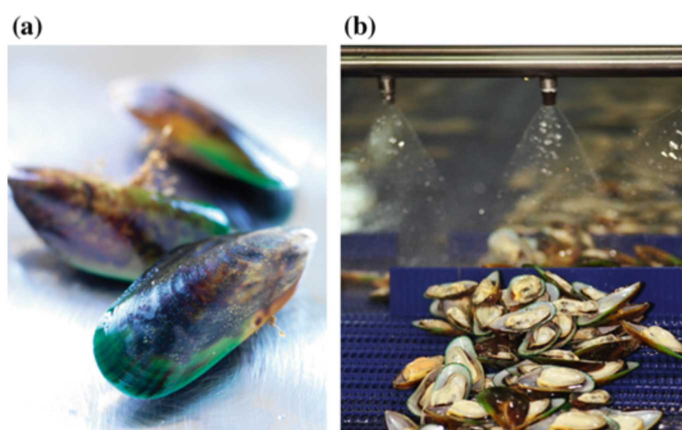

Fig. 1 *Perna canaliculus* **a** mussel shell **b** mussel in shell, note green lip on inside and outer posterior ventral margin (used with permission from Aroma New Zealand)

1.1 *Growing and Harvesting* Perna canaliculus

Interest in the application of *P. canaliculus* for arthritic conditions began in the 1960s, when research was undertaken to discover new natural compounds from marine organisms, which included *P. canaliculus* to treat cancer. *P. canaliculus* extract did not provide significant results for cancer outcome measures, but it was found that the study participants who also suffered from arthritis reported less pain and stiffness and improved mobility when taking the extract (Kendall 2000). It was also observed that coastal Maoris of New Zealand, whose staple diet consisted of *P. canaliculus* had a lower incidence of arthritis than Maoris residing in land (Halpern and Georges 2000; Brien et al. 2008a). Research has therefore focused on the anti-inflammatory capabilities of *P. canaliculus* extract and its fractions. Mussels are farmed in New Zealand using long-line technology around sheltered, in-shore areas such as the Marlborough Sounds. The spat, or seed, is collected from farmers suspending the spat catching lines or by collection of seaweed that spat (<5 mm in size) have naturally adhered to. The collected spats are then resettled onto nursery lines and grown for 3–6 months (10–30 mm) after which the juveniles are resettled onto thicker ropes and grown to maturity (90–120 mm) for another 12–18 months depending on the growing conditions. Once harvested, mussels are quickly transported to the processing plant where they are chilled (<10°C) in holding tanks. Mussels are then placed in a continuous centrifuge that separates the meat from the shell, which is then placed in refrigerated tanks and a natural anti-oxidant is added to improve stability. Mussel meat is then freeze-dried (lyophilised) at −20°C for 20–22 h. The freeze-dried product is then milled into a fine powder (FAO 2014; Aroma 2014). The lipid extract from the stabilised freeze-dried mussel powder is obtained by a supercritical fluid extraction process (SFE) using liquefied carbon dioxide (CO_2).

1.2 *Nutritional Content of* Perna canaliculus

Whole *P. canaliculus* extract consists of a complex mixture of compounds being predominantly 55–60 % protein, 5–15 % carbohydrates, 5–15 % glycosaminoglycans (including chondroitin sulphate and heparin), 3–5 % lipids, 5 % minerals and 0.5–4 % water (Ulbricht et al. 2009). Vitamins A, D3, E and B12 are also present. The concentrated lipid extract contains a complex profile of fatty acids classes including sterol esters of cholesterol and desmosterol/brassicasterol, triglycerides, free fatty acids (FFAs), sterols and phospholipids (Ulbricht et al. 2009; Whitehouse et al. 1997; Murphy et al. 2003). Analytical assessment of aqueous and lipid metabolomes by nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometry (GC-MS) can clearly demonstrate constitutive differences in mussel species, for example, between *P. canaliculus* and *Mytilus galloprovincialis* (Australian Blue mussel). There are distinguishing patterns of

amino acids, several metabolites, glucose and lipids between the two species, although some of these differences could in part be due to location rather the species (Rochfort et al. 2013). Heavy metals that accumulate in water such as arsenic, mercury, cadmium and lead are also present in the whole mussel meat due to their filter-feeding behaviour; however, heavy metal limits are rigorously monitored (see Table 1). Furthermore, the growing waters from which the mussels are harvested are monitored weekly for biotoxins. If levels exceed the legislational limit in New Zealand, no harvesting of the mussels can take place.

Table 1 Whole *P. canaliculus* extract: typical nutritional evaluation (*Source* Aroma NZ Ltd and Biolane®)

Components	Results and (reference range)
General	
Crude protein (g/100 g)	56–61 (40–70)
Carbohydrate (g/100 g)	9.6–12 (NLT 5.0)
Lipids (g/100 g)	10–10.8 (6–15)
Omega 3 fatty acids (EPA/DHA per 100 g)	2.8–4.5 (NLT 2.0)
Saturated fat %	3.3
Glycosaminoglycans %	3.0 (NLT 1.0)
Ash (g/100 g)	18–21 (4–25)
Moisture (g/100 g)	0.6–4 (0–5)
Vitamins	
Vitamin A (IU/100 g)	131.5–329
Vitamin D3 (IU/100 g)	272–1640
Vitamin E (IU/100 g)	2.8–10.6
Vitamin B12 (μg/100 g)	116
Minerals	
Copper (mg/kg)	4.5–5.6
Zinc (mg/kg)	57–62
Manganese (mg/kg)	15–24
Boron (mg/kg)	28
Chromium (mg/kg)	1.4
Iron (mg/kg)	380–670
Calcium (g/100 g)	1.3–1.5
Phosphorus (g/100 g)	0.84–1.25
Sodium (g/100 g)	3.6–4.8
Potassium (g/100 g)	1.2
Magnesium (g/kg)	4.9–6.8
Nickel (ppm)	1.3
Selenium (mg/kg)	2.5
Iodine (mg/kg)	15.4
Sulphur (g/100 g)	3.9

(continued)

Table 1 (continued)

Components	Results and (reference range)
Amino acids (mg/g)	
Aspartic acid	42.8–44.0
Glutamic acid	51.8–58.8
Serine	18.9–19.5
Histidine	7.8–8.5
Glycine	40.9–43.8
Threonine	18.9–21.2
Arginine	27.0–35.9
Alanine	18.3–24.5
Valine	14.6–16.3
Methionine	8.5–9.5
Phenylalanine	14.8–16.2
Isoleucine	16.0–17.7
Lysine	29.2–51.3
Leucine	17.9–23.6
Proline	14.5–19.7
L cysteine	6.1–6.6
Tyrosine	13.9–15.4
Tryptophan	4.9–5.2
Heavy metals (mg/kg)	
Lead	0.89 (NMT 2.0)
Cadmium	0.56 (NMT 5.0)
Mercury	0.08 (NMT 1.0)
Total arsenic	11 (NMT 15.0)

NLT Not lower than; *NMT* Not more than; *EPA* Eicosapentaenoic acid; *DHA* docosahexaenoic acid

There are no standardisation procedures in place for *P. canaliculus* raw material suppliers. This may result in potential variability between the nutrient profiles and stability of the raw materials. Temperature and season both affect the nutrient profile of the mussels during harvest. As the key constituent responsible for the observed therapeutic activity is not definitively known, lack of standardisation practises may influence therapeutic efficacy between marketed products (Whitehouse et al. 1997, 1999). Early human clinical studies for both osteoarthritis (OA), RA and animal studies have resulted in variable assessments of the efficacy of *P. canaliculus* whole extract powders, reporting both positive (Gibson et al. 1980; Audeval and Bouchacourt 1986) and negative outcomes (Highton and McArthur 1975; Huskisson et al. 1981; Caughey et al. 1983; Larkin et al. 1985). It was only in 1986 when New Zealand manufacturers began stabilising the whole mussel extracts with 3 % tartaric acid (a metal chelator and anti-oxidant)

immediately after removing the flesh from the shell, preventing auto-oxidation, when the activity of *P. canaliculus* whole extract powders began to demonstrate more potent activity (Whitehouse et al. 1997).

2 Clinical Therapeutic Activity

Current opinion concerning the therapeutic efficacy of *P. canaliculus* whole extract and/or the lipid fraction is that, the existing evidence is rather inconclusive for the treatment of OA symptoms, with overall evidence for RA suggesting inefficacy (Brien et al. 2008b; Cobb and Ernst 2006; Ulbricht et al. 2009). Individual studies to assess *P. canaliculus* (whole and lipid extract) for treating joint symptoms of OA have all reported a clinically relevant reduction in joint pain and stiffness. Clinical studies assessing *P. canaliculus* for OA, RA and asthma are presented in Table 2. Systematic reports of little or no conclusive evidence from the available studies are generally due to poor methodological rigour, variations in product stability and dosing, lack of raw material standardisation and use of inappropriate placebos, such as dried fish powder. The majority of studies assessing *P. canaliculus* for RA were conducted in the mid-1970s and early 1980s; the results of which may have been influenced by the lack of product stability during this period. Assessment of efficacy is also difficult due to the variable prescribed dosing patterns (dose and duration) used for both OA and RA symptoms. Importantly, the use of rescue pain medication, in the form of either acetaminophen (paracetamol) or non-steroidal anti-inflammatory (NSAID) medications were inconsistent and poorly reported in these earlier studies and may have further influenced the interpretation of the results. It is now recognised; however, that *P. canaliculus* may have credible pharmacoactivity as demonstrated in animals and in vitro studies, which requires further rigorous scientific investigations to assess efficacy and optimal dosage in humans (Rainsford and Whitehouse 1980; Brien et al. 2008b).

The lipid fraction is obtained by supercritical fluid extraction (CO_2-SFE) from the stabilised, freeze-dried mussel powder that is then combined with olive oil and vitamin E as an anti-oxidant. By using CO_2 as an extracting medium, high temperatures and solvents are not used for extraction, thus maintaining therapeutic activity of the extract. Processing with high temperature activates degrading enzymes within the mussel, namely phospholipases and lipoxygenases that degrades the lipid components (Grienke et al. 2014; Wakimoto et al. 2011). Further, fractionation and analysis of the active components in the whole lipid fraction is difficult due to their instability and concomitant decomposition during the bioassay process (Wakimoto et al. 2011). It is reported that the stabilised SFE lipid fraction significantly improves asthmatic symptoms (hyperpnea-induced bronchoconstriction and mild to moderate atopic asthma) when compared to placebo in humans (see Table 2) (Mickleborough et al. 2013; Emelyanov et al. 2002). The lipid extract has also been assessed for its anti-inflammatory activity with the assessment of serum inflammatory markers such as TNF-α and IL-1β, but with non-significant results

Table 2 Human clinical studies assessing the therapeutic activity of *P. canaliculus* whole extract and SFE lipid-rich fraction

Author GLM/dose per day	Design	Cohort Age (mean ± SD)	Comparator/placebo	Duration	Use of analgesic medication	Outcome measures	Clinical trial results
Osteoarthritis							
Coulson et al. (2012) Whole extract powder (3,000 mg/day)	Non-blinded, non-randomised pilot	21 Knee OA 8 M/13 F 61.1 ± 12.2 years	None	8 weeks	NSAIDs/paracetamol (variable doses)	WOMAC Lequesne algofunctional index GSRS SF-12v2™ Analytical blood safety assessment (FBC, E/LFT, CRP, ESR)	The standardised extract significantly improved knee joint pain ($p < 0.001$), stiffness ($p = 0.002$) and mobility ($p < 0.001$) Furthermore, GIT symptoms were also significantly improved ($p = 0.005$) Adverse events included reflux (10 %), abdominal pain (5 %), diarrhoea (5 %) and gout (10%)
Coulson et al. (2013) Whole extract powder (3,000 mg/day)	Non-blinded, randomised, comparator-controlled, parallel-group	40 knee OA 12 M/16 F 58.6 ± 8.9 years	Glucosamine sulphate 3,000 mg/day	12 weeks	NSAIDs/paracetamol (variable doses)	WOMAC Lequesne algofunctional index GSRS SF-12V2TM Analytical blood safety assessment (FBC, E/LFT, CRP, ESR) Faecal bacterial profiles	Both the he standardised *P. canaliculus* extract ($p < 0.001$) and Glucosamine ($p = 0.001$) treated groups significantly improved knee joint pain, stiffness and mobility. The GLM group also demonstrated significant improvement in GIT symptoms ($p = 0.02$). Improvement in the Glucosamine group was borderline ($p = 0.044$) *Clostridia* and *Staphylococcus* species were observed to decrease from baseline to week 12 in both treatment groups Adverse events included gastrointestinal symptoms, infections (URTI, gastroenteritis, cutaneous), headache, migraine, falls and angina

(continued)

Table 2 (continued)

Author GLM/dose per day	Design	Cohort Age (mean ± SD)	Comparator/placebo	Duration	Use of analgesic medication	Outcome measures	Clinical trial results
Gibson et al. (1980) Whole extract powder (1050 mg/day)	Double blinded, randomised controlled	38 OA 1 M/37 F Mean age 69.6 (active) and 68.6 (placebo) years 28 RA 1 M/27 F Mean age 54.1 (active) and 60.6 (placebo) years	Inactive fish capsules [dose not specified]	3–6 months	NSAIDs (Type/dose not specified)	OA and RA outcomes: • Morning stiffness • VAS • Functional index • Time taken to walk 50 feet • Range of joint movement • Patient and physicians global assessment of improvement Additional RA outcomes: • Articular index of joint tenderness • Grip strength Analytical blood safety assessment (Hb, WBC, ESR, rheumatoid serology, serum biochemistry, urine analysis)	The whole extract improved pain and stiffness in 76 % of RA patients and 45 % OA patients. From 66 patients in the trial, 44 suffered from night pain. This was relieved in 17 patients on active treatment and 2 in the placebo group. There was no significant improvement in range of movement or grip strength Adverse events included exacerbation of joint symptoms (9 %), increased joint stiffness (3 %), epigastric discomfort (2 %), increased flatulence (2 %) and nausea (6 %)

(continued)

Table 2 (continued)

Author GLM/dose per day	Design	Cohort Age (mean ± SD)	Comparator/placebo	Duration	Use of analgesic medication	Outcome measures	Clinical trial results
Audeval and Bouchacourt (1986) Whole extract powder (6 capsules/day) (Dosage not specified)	Double blinded, randomised placebo controlled	53 knee OA 16 M/37 F 66 ± 11 years (placebo) 65 ± 10 years (active)	Placebo (not specified)	6 months	NSAIDs (type/dose not specified)	Functional capacity ARA (stage I–IV) VAS Intensity of pain (I–IV) Amplitude of joint mobility Distance of heel-cheek Utilisation of a walking stick Maximum distance walked Patient and Physicians global assessment of improvement	The whole extract significantly improved functional capacity ($p < 0.001$), pain intensity ($p < 0.01$), patients assessment ($p < 0.05$), physicians assessment ($p < 0.01$) compared to placebo. However, morning stiffness improved significantly in the placebo group compared to the treatment group ($p < 0.01$) No adverse events were reported
Zawadzki et al. (2013) Lipid extract 4 capsules (1,200 mg/day)	Blinded, comparative controlled in stage I, non-blinded in stage II	50 knee and/or hip OA 6 M/44 F 65.6 ± 9.5 years (active) 66.7 ± 8.4 years (fish oil)	Fish oil 1,200 mg/day	3–6 months	Paracetamol (dose not specified)	VAS (100 mm) HAQ Analytical blood assessment (WBC, RBC, FBC, LFT, ESR) Adverse reactions	In stage I, the patients from Group A, treated with the lipid extract, showed a statistically significant reduction of pain, improved levels of mobility and activity and 100 % tolerance with no side effects noted. In comparison, patients from Group B, who were treated with fish oil, did not show a notable reduction in pain, there was no significant improvement of mobility or activity and only 71 % tolerance. In stage II, the patients knew that they had taken the lipid extract and those

(continued)

Table 2 (continued)

Author GLM/dose per day	Design	Cohort Age (mean ± SD)	Comparator/placebo	Duration	Use of analgesic medication	Outcome measures	Clinical trial results
							patients also showed a reduction of pain Adverse events included diarrhoea, stomach ache, increased blood pressure, nausea, constipation, headaches and pain in the kidney and liver areas in the fish oil group only
Gibson and Gibson (1998) Lipid extract (210 mg/day)	Double-blinded randomised for first 3 months; Non-blinded, randomised for last 3 months	30 OA 8 M/22 F Mean age 57.3 (lipid) and 52.8 (powder) years 30 RA 2 M/28 F Mean age 46.8 (lipid) and 47.1 (powder) years	GLM extract powder 1150 mg/day	3–6 months	NSAIDs (type/dose not specified)	Articular index Morning stiffness Grip strength VAS Presence/absence of night pain Patient and physicians global assessments Analytical blood assessment (FBC, ESR, rheumatoid factor)	In RA patients, significant improvements were obtained in articular index ($p < 0.01$), morning stiffness ($p < 0.05$) and functional index ($p < 0.01$) in both the lipid and GLM extract powder groups. Grip strength and the VAS did not improve significantly. Night pain improved In OA patients, significant reductions were obtained in articular index ($p < 0.01$), morning stiffness ($p < 0.01$) with improved function index ($p < 0.01$). The VAS significantly improved in the GLM extract powder group only. Grip strength did not change. Night pain improved No obvious difference was demonstrated between the stabilised mussel powder and the lipid extract

(continued)

Table 2 (continued)

Author GLM/dose per day	Design	Cohort Age (mean ± SD)	Comparator/placebo	Duration	Use of analgesic medication	Outcome measures	Clinical trial results
Lau (2004) Lipid extract 4 caps/day for 2 months then 2 caps/day for 4 months Dosage not specified	Blinded, randomised placebo controlled	80 knee OA	Olive Oil capsules	6 months	Paracetamol 2 g/day plus additional amounts for rescue medication		Significant improvement in pain and global assessment
Cho et al. (2003) Lipid extract (4 caps/day) Dosage not specified	Non-blinded, non-randomised, multi-centre	60 knee and hip OA 2 M/52 F Average age 6.4 years	None	8 weeks	NSAIDs discontinued prior to starting study Use of pain relief medication not specified	VAS Lequesne algofunctional index Patient and physicians global assessment	After 4 weeks, 53 % of patients experienced pain relief and improved joint function; this number increased to 80 % after 8 weeks. The grade of pain and functional impairment were reduced significantly ($p < 0.05$) and patient and physician global assessments improved, 87 and 90 % respectively Adverse events included initial transient worsening of arthritic pain (4 %)
Rheumatoid arthritis							
Huskisson et al. (1981) Whole extract powder (900 mg/day)	Blinded, randomised, placebo, cross-over, controlled	30 RA Gender/age not specified	Dried fish capsules (dose not specified)	8 weeks	Analgesics and NSAIDs (dose and type not specified)	VAS Duration morning stiffness Articular index Proximal interphalangeal joint circumference Analgesic requirements	No significant difference obtained between the whole extract powder and placebo for all outcome measures Adverse events included headache, abdominal pain, diarrheoa and constipation

(continued)

Table 2 (continued)

Author GLM/dose per day	Design	Cohort Age (mean ± SD)	Comparator/placebo	Duration	Use of analgesic medication	Outcome measures	Clinical trial results
Caughey et al. (1983) Whole extract powder (1050 mg/day)	Blinded, randomised, placebo controlled	47 RA 11 M/36 F 29–68 years (median 49.4 years)	Dried fish capsules (dose not specified)	12 weeks	Group I: Naproxen 750 mg/day plus *P. canaliculus* extract from week 1–6 Group II: naproxen 750 mg/day plus placebo from week 1–6 From weeks 7–12, naproxen was replaced by a placebo in each group	VAS Grip strength Ritchie articular index Returned analgesic count Patient weekly record of subjective symptoms (severity day/night pain, severity morning stiffness, analgesic requirements and global assessment) Analytical blood assessments (included ESR)	No significant difference obtained between treatment and placebo for all outcomes measures Following withdrawal of naproxen after week 6, there was a high dropout rate with no significant difference between the active and placebo groups Adverse events included skin rash, diarrhoea and upper gastrointestinal symptoms
Larkin et al. (1985) Whole extract powder 6 caps/day (920 mg/day for 3 months then (1180 mg/day) for a further 3 months	Double-blinded, randomised, placebo controlled	35 RA 54–77 years (median 60 years) (active) 48–70 years (median 60 years) (placebo)	Placebo (not specified)	6 months	NSAIDs and prednisolone (n=1)	Ritchie index Grip strength Morning stiffness VAS (100 mm) Analytical blood assessment (ESR, Hb, platelets, globulins, Immunoglobulin's, rheumatoid factor, serum biochemistry)	No significant difference observed between treatment and placebo group for any of the measured parameters. There were a significant number of patients who reported arthritis symptoms worsening while taking the *P. canaliculus* extract ($p < 0.05$)

(continued)

Table 2 (continued)

Author GLM/dose per day	Design	Cohort Age (mean ± SD)	Comparator/placebo	Duration	Use of analgesic medication	Outcome measures	Clinical trial results
Highton and McArthur (1975) Whole extract powder (Dosage not specified)	Double-blinded, randomised, cross-over, placebo controlled	6 RA 1 M/5 F 34–59 years (median 46.5 years)	Placebo (not specified)	12 weeks	Maintenance gold and low dose steroids continued NSAIDS withdrawn 14 days prior to participating Paracetamol permitted for pain relief	Ritchie articular index Number swollen joints Joint range of movement Joint circumference Joint swelling grip strength Time taken to walk 10 m Analytical blood analysis Patient diary record of morning stiffness duration, pain and number paracetamol tablets required	No significant difference observed between treatment and placebo group for all measured parameters. Paracetamol consumption increased during trial Adverse events included increased severity of symptoms
Gruenwald et al. (2004) Lipid extract combined with Ω-3 fish oil (140 and 1832 mg/day respectively)	Non-blinded, controlled	50 RA 25 M/25 F 29–73 years	None	12 weeks	Existing pharmacotherapy continued but not specified	Morning stiffness Joint pain Joint swelling Joint pain intensity	After 12 weeks supplementation there was a significant reduction in the duration of morning stiffness ($p \leq 0.001$), the number of painful joints ($p = 0.001$) and the number of swollen joints ($p = 0.001$). A highly significant reduction in the number of painful small joints was achieved ($p = 0.002$). Successive reduction in pain intensity was observed over the course of the study

(continued)

Table 2 (continued)

Author GLM/dose per day	Design	Cohort Age (mean ± SD)	Comparator/placebo	Duration	Use of analgesic medication	Outcome measures	Clinical trial results
							Further, 34 (68 %) of patients required pharmacotherapy prior to and during the course of the trial. One of most important outcomes in this trial is that pharmacotherapy could be reduced for 21 of these patients by the end of the treatment period and eliminated for 13 Adverse events included mild nausea
Anti-inflammatory activity							
Murphy et al. (2006) 2 ml/day of the lipid extract containing 241 mg Ω-3 LCPUFA supplying 97 mg EPA and 72 mg DHA. Preparation combined with olive oil and dl-α-tocopherol	Double-blinded, randomised, parallel intervention study	30 healthy people 14 M/16 F	2 ml/day of fish oil containing 181 mg Ω-3 LCPUFA supplying 87 mg EPA and 50 mg DHA. Preparation combined with olive oil and dl-α-tocopherol	6 weeks	None	Serum inflammatory markers—TXB2, PGE2, IL-1β and TNF-α Levels of fatty acids in neutrophils determined at day 0, 42 and 56	Following supplementation, there were no significant changes in inflammatory markers in either of the marine oil-fed groups or between the groups of apparently healthy volunteers. There was a very wide spread of values for most analytes which may have obscured changes with treatment or between treatments

(continued)

Table 2 (continued)

Author GLM/dose per day	Design	Cohort Age (mean ± SD)	Comparator/placebo	Duration	Use of analgesic medication	Outcome measures	Clinical trial results
Anti-asthmatic							
Mickleborough et al. (2013) 8 caps/day (1,200 mg/day) of the lipid extract containing ~72 mg EPA and 48 mg DHA	Double-blind, placebo controlled randomised crossover study	20 asthmatic people with hyperpnea-induced bronchoconstriction (HIB) 12 M/8 F	Olive oil (1,200 mg/day)	8 weeks	Inhaled short acting β2-agonists	Eucapnic voluntary hyperventilation (EVH) challenge (surrogate for an exercise challenge test) Fraction of exhaled nitric oxide (F_ENO) Pulmonary function tests Eucapnic voluntary hyperventilation (EVH) Symptoms of rescue β-agonist use and peak flow measurements Exhaled breath condensate (EBC)	The lipid extract significantly reduced pre- and post-EVH, asthma symptom scores and EBC rescue medication when compared to the placebo group
Emelyanov et al. (2002) 4 caps/day (600 mg/day) of the lipid extract	Double-blind, parallel group, randomised placebo-controlled study	46 people with mild to moderate atopic asthma	Olive oil (600 mg/day)	8 weeks	Inhaled short-acting β2-agonists	Peak expiratory flow rate (PEF) Hydrogen peroxide (H_2O_2) in expired breath condensate (marker of airway inflammation)	There was a significant decrease in daytime wheeze, the concentration of exhaled H_2O_2 and an increase in morning PEF in the lipid extract group compared to the placebo group

WOMAC Western Ontario McMaster Universities Arthritis Index; *GSRS* Gastrointestinal symptom rating scale; *SF-12V2™* SF-12 heath questionnaires; *FBC* Full blood count; *E/LFT* Electrolytes and liver function test; *RBC* Red blood count; *CRP* C-reactive protein; *ESR* Erythrocyte sedimentation rate; *VAS* Visual analogue scale; *Hb* Haemoglobin; *ARA* American Rheumatism Association; *HAQ* Health assessment questionnaire; *LCPUFA* Long chain polyunsaturated fatty acids; *EPA* Eicosapentaenoic acid; *DHA* Docosahexaenoic acid; *TXB2* Thromboxane-2; *PGE2* Prostaglandin E2; *IL-1* Interleukin-1; *TNF-α* tumour necrosis factor—α; *VAS* Visual analogue scale

(Murphy et al. 2006). The cohort recruited, however, were healthy individuals that did not demonstrate any signs of inflammation, with serum cytokine markers within normal reference ranges before intervention. Such a design is unlikely to answer the question of whether the lipid extract reduces markers of inflammation.

2.1 Dosing

The optimal therapeutic dose of either the whole or lipid extract has not been clearly ascertained and can only be estimated from previous clinical research. It is clear that carefully designed Phase I dose-ranging studies are required to ascertain what the effective prescribed dose should be for both extracts and in various disease contexts. Clinical studies have used a dose range between 1,050 and 3,000 mg/day for the whole extract and 210–1,200 mg/day for the lipid extract in OA patients with dose duration between 8 and 24 weeks. A dose between 900 and 1,180 mg/day of the whole extract and 140 mg/day of the lipid extract has been assessed in RA patients with a dose duration between 8 and 24 weeks and a dose between 300 and 1,200 mg/day of the lipid extract has been assessed for asthma patients with a dose duration of 8 weeks (see Table 2). The recommended dose from the manufacturer for the *P. canaliculus* whole extract is typically 1,500 mg/day and 200 mg/day for the lipid extract. It is unclear, however, if these are the effective therapeutic doses for each extract based on the presented research dose variations.

2.2 Adverse Effects and Toxicity

Adverse events reported in clinical studies assessing both the whole and lipid extract have included mild gastrointestinal events such as reflux, flatulence, epigastric discomfort, fluid retention, nausea and altered bowel habits, headaches and a transient increase in knee symptoms. Apart from these minor events, the intake of *P. canaliculus* is not associated with any serious adverse events and is generally well tolerated. The use of *P. canaliculus* should, however, be avoided by people with allergies to shellfish. Heavy metal poisoning is unlikely to occur (Ulbricht et al. 2009); however, biotoxins may be found in shellfish due to their filter-feeding behaviour and ingestion of large amounts of algae. The majority of the mussel's diet consists of nutrient-rich eukaryotic microalgae, typically diatoms and dinoflagellates, but it is mainly when they ingest harmful algal blooms from the surrounding water that toxins become a serious threat to both the health of the consumer and the mussel industry (Grienke et al. 2014). In New Zealand, the growing waters from which the mussels are harvested are tested weekly for biotoxins and if the levels exceed the National limit harvesting is prohibited. The very strict guidelines now in place ensure that there is very low risk of the mussel products containing any biotoxins. To date, there are no reports of either GLM

extract or the lipid extract interacting adversely with pharmaceutical or nutraceutical medications, but rather they may enhance their therapeutic effect (Rainsford and Whitehouse 1980; Whitehouse and Butters 2003).

2.3 *Gastroprotective Activity*

The SFE lipid-rich fraction has exhibited synergistic anti-inflammatory therapy when combined orally with NSAIDs and analgesic pharmaceutical medications such as prednisone, pentoxifylline or meloxicam in rat models. When administered as tandem therapies to reduce paw swelling in adjuvant-induced arthritis and zymosan-induced paw inflammation in rats, the result was more effective than using therapy alone (Whitehouse 2004; Whitehouse and Butters 2003). The whole stabilised extract powder also demonstrates equally synergistic anti-inflammatory activity when combined with prednisone or meloxicam. Both extracts have NSAID and steroid-sparing effects when administered concomitantly, reducing the effective dose required for the drug and also protecting the gastrointestinal tract (GIT) from the adverse effect of such medications. The stabilised whole extract powder reduced the occurrence of gut lesions in rats, more than the lipid fraction, when combined with NSAIDs in an animal model (Whitehouse and Butters 2003). Lipid and whole extracts reinforce the anti-inflammatory therapeutic activity of acetylsalicylic acid and indomethacin while concomitantly exhibiting gastroprotective activity (Rainsford and Whitehouse 1980). Supportive data indicates that supplementation with whole *P. canaliculus* extract may support GIT function and even show gastroprotective activity when administered with anti-inflammatory and analgesic medications in patients with OA (Coulson et al. 2012, 2013). Further, preliminary evidence has demonstrated that the SFE lipid-rich fraction significantly reduced colonic damage in an inflammatory bowel disease (IBD) animal model (Tenikoff et al.2005) and partially improved selected indicators of intestinal inflammation and intestinal morphology in an animal model of chemotherapy-induced mucositis.

3 Bioactive Metabolites of *Perna canaliculus*

Studies to identify bioactive metabolites within *P. canaliculus* products has led to the evaluation of extracts, hydrolysates and purified components from the fractionated lipids, carbohydrates and proteins present in the mussel meat. A review of these fractions is discussed in depth by Grienke 2014 (Grienke et al. 2014). The fraction(s) responsible for the therapeutic efficacy demonstrated in the OA disease model, both human and animal, is not yet fully defined. Previous claims suggest that the lipid fraction represents the dominant anti-inflammatory component of *P. canaliculus* (Ulbricht et al. 2009; Halliday 2008). Early clinical trials reported mixed results with whole extract powder. Stabilisation of the mussel meat with 3 %

tartaric acid in the 1980s resulted in a more active product (Whitehouse et al. 1997). Furthermore, while Gibson and Gibson (1998) comparing the lipid extract to the whole stabilised powder extract in treating joint symptoms of both RA and OA, both mussel preparations demonstrated significant therapeutic activity with no substantial difference found between either treatment (Gibson and Gibson 1998). The major classes of compounds found in mussel meat (peptides, carbohydrates and lipids) have demonstrated various anti-microbial, anti-inflammatory, anti-oxidant, bioadhesive and anti-hypertensive activities (Grienke et al. 2014).

The content of bioactive metabolites in mussel meat is influenced by the season, life cycle, diet and habitat in which the mussels are grown and can therefore vary between harvests (Fearman et al. 2009; Narvaez et al. 2008). Furthermore, there are evident metabolic differences between mussel species and also within the same species when collected from different locations. For example, metabolomic assessment of the Australian Blue mussels (*Mytilus galloprovincialis*) and *P. canaliculus* found taurine, glycine, lactate, succinate, homarine, ATP, ADP, valine and leucine were elevated in *P. canaliculus* while betaine, isoleucine, acetoacetate and glucose were elevated in *M. galloprovincialis*. Also, analysis of lipid methyl ester derivatives indicted a clear separation between the species, with significantly higher levels of palmitic acid methyl ester (C16:0), cis-5,8,11,14,17 eicosapentaenoic acid methyl ester (C20:5n3) and palmitoleic acid methyl esters (C16:1) obtained from *P. canaliculus,* which overall contained a higher lipid level. These differences are likely due in part to the different environments that each species are grown in lower water temperatures correlating with higher degrees of unsaturated lipids (Rochfort et al. 2013). Experimental studies of bioactive carbohydrate compounds from *P. canaliculus*, is limited, with one report of a glycogen isolate demonstrating anti-inflammatory activity (after *i.v.* administration) against carrageenan-induced arthritis in the footpad of rats (Miller et al. 1993). The authors, however, confirmed the anti-inflammatory activity was lost when the glycogen isolate was treated with either potassium hydroxide (KOH) or proteinase-K, proposing that the anti-inflammatory activity was actually due to the protein moieties associated with the glycogen macromolecule.

3.1 Bioactive Proteins, Peptides and Amino Acids

Approximately 70 % of whole mussel meat is protein. The anti-inflammatory and immunomodulating activity of the fractionated extracts of whole extract powder in animal and in vitro models have suggested that the predominant active agent is associated with a protein moiety or is itself a protein macromolecule; however, supportive research for a bioactive high molecular weight protein is currently limited (Couch et al. 1982; Miller et al. 1993; Mani and Lawson 2006; Grienke et al. 2014). Current research has reported anti-bacterial, antifungal, anti-inflammatory, anti-hypertensive, anti-oxidant, anti-thrombin and anticoagulant bioactive proteins, peptides and amino acids from various mussel species.

The only bioactive protein identified from *P. canaliculus* is pernin from the cell-free haemolymph. It is an aggregating, non-pigmented, glycosylated protein extract composed of 497 amino acids with a particularly high content of histidine and aspartic acid residues. Pernin can act as a serine protease inhibitor but only demonstrates weak anti-thrombin activity. The pernin content from homogenise whole mussel meat averages 0.2 mg per mussel (Scotti et al. 2001).

Anti-microbial peptides (AMPs) are also present in the mussel haemolymph and are a vital part of the mussel's innate immunodefense system, protecting it from bacterial, fungal and viral attack. AMPs have been a focus in marine mussel research, particularly in the Blue mussel (*Mytilus edulis)* and the Mediterranean (or Blue) mussel (*Mytilis galloprovincialis)* species. Several cysteine-rich peptides from *M. edulis* were reported to be potent bactericides (i.e. against both Gram-positive organisms, e.g. *Enterococcus faecalis, Staphylococcus aureus* and Gram-negative bacteria, e.g. *Escherichia coli* bacteria) and anti-fungal (i.e. *Neurospora crassa* and *Fusarium culmorum*) (Charlet et al. 1996). The AMPs were identified as isoforms from the peptide families of defensins, mytimycin and mytilins with big-defensins (and mytimacins) also being described (Charlet et al. 1996; Grienke et al. 2014). Crustacean haemolymph, particularly from the crab, contains a multitude of AMPs that participate not only as endogenous antibiotics but may also have a role in inflammation, wound repair and regulation of the adaptive immune system. This has generated some interest in using marine peptides for pharmaceutical developments (Fredrick and Ravichandran 2012). Furthermore, fermented *M. edulis* is reported to contain peptides that inhibit angiotensin I converting enzyme (ACE) with the anti-hypertensive activity confirmed in vivo rat models (Je et al. 2005). An anti-coagulant peptide has also been identified in *M. edulis* (Jung and Kim 2009). Anti-inflammatory activity of proteinaceous fractions in carrageenan-induced footpad swelling in rats was expressed only when following *i. p.* or *i.v*. injections (see Table 3). One study (Miller and Ormrod 1980) compared *i. p.* to orally administered whole extract powder which may or may not have been a stabilised extract. *P. canaliculus* extract powder may also inhibit the production of prostaglandins in rats (Miller and Wu 1984). The therapeutic activity of mussel protein and peptide fractions has not yet been investigated in humans.

3.2 Bioactive Lipid Fractions

The concentrated lipid extract of *P. canaliculus* contains a complex profile of five main lipid classes that include sterol esters (cholesterol and desmosterol/brassicasterol), triglycerides, free fatty acids (FFAs), sterols and phospholipids (Ulbricht et al. 2009; Whitehouse et al. 1997; Murphy et al. 2003). The fatty acid and sterol composition of the mussel lipid is influenced by water temperatures in which the mussel is grown and also the mussels' diet which includes marine phytoplankton, dinoflagellates and zooplankton (Rochfort et al. 2013; Murphy et al. 2003). Approximately 90 fatty acids are present in the

Table 3 Pharmacological activity of protein fractions from *P. canaliculus* in animal and in vitro models

Author	Design	Fractions	Comparator	Method of delivery	Outcome measures	Results	Duration
Miller and Ormrod (1980)	Male and female dark Agouti rats injected with 0.1 ml of a 2 % carrageenan solution into footpad to induce oedema	Freeze dried powder ground (ORA-C) and suspended in saline at parenteral doses ranging from 100 to 1500 mg/kg	Control group with saline injected into footpad Comparator anti-inflammatory used: aspirin (200 mg/kg), phenylbutazone (200 mg/kg) and indomethacin (5 mg/kg) dosed orally	Administered either IP using 18G needle or by gastric lavage	Footpad thickness measured with an engineer's micrometer gauge. Detectable swelling was demonstrated 2 h after challenge and peaked at 4 h Measurement of footpad thickness made before the injection and 2, 4 and 6 h later	Higher doses of ORA-C (1500–500 mg/kg) administered IP produced significant reductions in foot pad swelling compared to untreated animals. Lower doses of ORA-C (200 mg/kg) showed significant anti-inflammatory activity but only if pre-treatment at the same dose had been carried out for several days prior to the carrageenan challenge i.e. a cumulative effect No significant anti-inflammatory activity was seen when ORA-C was administered orally	Foot pad swelling measured before carrageenan challenge and 2, 4 and 6 h after

(continued)

Table 3 (continued)

Author	Design	Fractions	Comparator	Method of delivery	Outcome measures	Results	Duration
Couch et al. (1982)	Female dark Agouti rats injected with 0.1 ml of a 2 % carrageenan solution into footpad to induce foot oedema	1. ORA-C—unfractionated mussel prep dosed at 300 mg/kg 2. ORA-F1—granular, water insoluble material removed from ORA-C and dosed at 150–500 mg/kg (a) ORA-F1—denatured by autoclaving (b) ORA-F1—proteolysed (NH3HCO3/trypsin) (c) ORA-F1—proteolysed (NH3HCO3/no trypsin)-control (d) ORA-F2 - ORA-F1 dialysed, freeze-dried at 185–500 mg/kg	Control group with saline injected into footpad	ORA-C (300 mg/kg), ORA-F1 (150–500 mg/kg), ORA-F2 (500 mg/kg), saline (control) administered IP injection Foot pad measurements 4 h after challenge	Hind footpad thickness measured 2 and 4 h after challenge	ORA-C, ORA-F1, ORA-F2: significantly reduced foot pad swelling Autoclaving and trypsin treated ORA-F1 destroyed most of the anti-inflammatory activity	IP injection 48, 24 and 2 h before carrageenan challenge, foot pad swelling measured 2 and 4 h after challenge

(continued)

Table 3 (continued)

Author	Design	Fractions	Comparator	Method of delivery	Outcome measures	Results	Duration
Miller and Wu (1984)	Male and female dark Agouti rats grouped for breeding	Whole extract powder dosed orally in chow at a ratio of 12.5 g extract to 1 kg of chow	Normal chow diet group	Oral	Conception, Parturition Foetal development	Parturition and foetal development was delayed in animals fed the whole extract diet. There was no significant difference in offspring weights. These results indicate whole extract powder demonstrates an NSAID-like effect inhibiting prostaglandin biosynthesis (prostaglandin inhibitors interfere with ovulation and prolong gestation period in the rat)	30 days
Miller et al. (1993)	Female dark Agouti rats with carrageenan-induced footpad oedema	Phenol—extracted mussel glycogen (0–25 mg) Protein degraded glycogen extract (10 mg) Lipid degraded glyco gen extract (10 mg)	Untreated control group	Intravenous injection	Foot pad oedema	Glycogen extract significantly reduced foot pad swelling but activity was lost when protein or lipid fractions were removed	Not indicated

(continued)

Table 3 (continued)

Author	Design	Fractions	Comparator	Method of delivery	Outcome measures	Results	Duration
Mani and Lawson (2006)	In vitro Cytokine and IgG producing cell lines (V2E9, THP-1, L-929, U-937, A375. S2, Jurkat E6-1, EL-4, CTLL-2, LS174T, and 7TD1)	1. Freeze-dried whole powder—HCL treated (0, 5, 15, 20 and 25 μg) 2. Freeze-dried whole powder—tween 20 treated (0, 5, 15, 20 and 25 μg) 3. Proteinase-K treated tween 20 extracts 4. Glycogen extract of whole powder	Untreated control	In vitro	Secretion of IgG, TNF-α, IL-1, IL-2, IL-6 and colorimetric ovine cyclooxygenase (COX) assay	Both HCL and tween 20 significantly reduced IgG expression. Tween 20 extract significantly reduced cytokine expression and COX-1 and COX-2 activity. Activity was lost when treated with proteinase. Glycogen rich extract decreased COX-1 and COX-2 activity but less so than tween 20 extract. Activity was lost when it was treated with proteinase	

IP Intraperitoneal; *ORA-C* Oedema reducing agent-crude; *ORA-F* Oedema reducing agent-fraction; *HCL* Hydrochloric acid; *IgG* Immunoglobulin G; *COX* Cyclooxygenase

concentrated lipid extract with the omega-3 (Ω-3) FFAs making up approximately 40 % of the fatty acid content in the lipid extract with docosahaexanoic acid (DHA) and eicosapentaenoic acid (EPA) accounting for 84 % of the Ω-3 PUFA (Murphy et al. 2003; Wolyniak et al. 2005; Lee et al. 2009). The FFA fractions and to a lesser extent the triglyceride fractions, are reported to be the only lipids inhibiting cyclooxygenase (COX) isoforms (McPhee et al. 2007; Wakimoto et al. 2011; Whitehouse et al. 1997; Macrides 1997). Furan fatty acids (F-acids) have been identified as minor components of the fatty acids in the lipid extract and have shown anti-inflammatory activity in rat models of adjuvant-induced arthritis, more than that of EPA (Wakimoto et al. 2011). Further, another single phospholipid compound was isolated from freeze-dried whole powder and identified as lyso-lecithin which demonstrated anti-histamine activity in an ex vivo experiment (Kosuge et al. 1986). The investigation of single lipid components is extremely difficult due to the instability of the extract during the purification process; analytical studies have tendered to focus in characterising lipid extracts rather than identifying single lipid compounds (Grienke et al. 2014).

3.2.1 Anti-inflammatory Activity

The anti-inflammatory activity of the CO_2-SFE lipid-rich fraction isolated from stabilised freeze-dried mussel meat has been demonstrated using in vitro analysis, particularly decreasing leukotriene (LTB4 and 5-HETE) and COX metabolite (COX-1 and COX-2) synthesis using variable doses of the SFE lipid-rich extract or further fractionations from this preparation dosed orally (Whitehouse et al. 1997; Dugas 2000; McPhee et al. 2007; Treschow et al. 2007; Macrides 1997). Adjuvant-induced arthritis rat studies have further analysed the anti-inflammatory and pain reducing effects of whole CO_2-SFE lipid-rich fractions and some fractions administered orally, demonstrating the SFE lipid-rich fractions reduce paw swelling and pain when compared to control groups being equal or superior to oral NSAIDs such as naproxen (see Table 4) (Whitehouse et al. 1997; Lee et al. 2009; Whitehouse 2004; Wakimoto et al. 2011; Butters and Whitehouse 2003). Further, it is reported that a fractionated FFA class exhibited greater anti-inflammatory activity at a lower dosage (30 mg/kg) and for a shorter duration (5 days) when compared to the significant anti-inflammatory activity demonstrated for the crude lipid component, a SFE lipid-rich fraction (100 mg/kg) administered for 15 days to adjuvant-induced arthritis rats via subcutaneous injection (Singh et al. 2008).

A further hypothesis for the anti-inflammatory activity demonstrated in in vitro and animal models, beyond that of reducing inflammatory cytokines (LTB4, 5-HETE, COX-1), is that the lipid-rich fraction may beneficially influence change in protein expression related to arthritis (Lee et al. 2008). Lee et al. (2008) conducted a proteomic study examining the effect that the lipid-rich fraction had on protein expression in splenocytes from adjuvant-induced arthritis rats. They found that in rats administered the lipid-rich fraction, six particular proteins (related to metabolism) were decreased while malate dehydrogenase (MDH), which is

Table 4 Therapeutic activity of SFE lipid-rich fractions from *P. canaliculus* in animal and in vitro analysis

Author	Design	Fractions	Comparator	Method of assessment	Outcome measures
Experimental arthritis					
Whitehouse et al. (1997)	Wistar and dark Agouti rats with either (a) adjuvant-induced polyarthritis or (b) collagen(II)-induced auto-allergic arthritis	An SFE lipid-rich extract from stabilised mussel powder 15 mg/kg Oral administered to rats with (a) aqueous dispersions prepared with 0.2 % Tween-20 as a suspending agent or (b) un-emulsified lipids diluted into olive oil	Naproxen Ibuprofen Dried mussel powder (300 mg/kg) Plant oils	Rear paw inflammation was measured with a micrometer. Forepaw inflammation was assessed arbitrarily on a scale of 0-4+ An independent observer assigned an overall arthritis score to all animals based on paw/tail inflammation and overall condition/mobility	Dried mussel powder and the SFE lipid extract both substantially reduced rear and front paw swelling when compared to no treatment and naproxen
Rainsford and Whitehouse (1980)	NSAID-induced gastric damage Wistar rats				
Butters and Whitehouse (2003)	Adjuvant-induced polyarthritis in rats	Various GLM extract preparations including both stabilised and un-stabilised whole mussel powder (300 mg/kg) and SFE lipid-rich extract 20 mg/kg) dosed orally	NSAIDs	Rear and front paw swelling after 4 days of supplementation	The SFE lipid extract substantially reduced paw swelling with the whole extracts demonstrating mixed results with the stabilised whole powder showing greatest paw swelling inhibition
Whitehouse and Butters (2003)	Collagen-induced polyarthritis (relevant to RA) in Wistar rats Persistent inflammation engendered with insoluble calcium salts	SFE lipid-rich extracts from stabilised mussel powder (20 mg/kg) and stabilised whole mussel powder (300 mg/kg) dosed orally	NSAIDs Prednisone	Rear and front paw swelling	Both the SFE lipid extract and stabilised whole mussel extract substantially reduced paw swelling when used alone or when combined with steroid or NSAID medications. Less gastric injury were observed in

(continued)

Table 4 (continued)

Author	Design	Fractions	Comparator	Method of assessment	Outcome measures
	in Wistar rats relevant to OA in rats				the rats treated with the whole mussel extract
Whitehouse (2004)	Rats with pre-established adjuvant arthritis Rats inflamed with zymosan (chronic irritant and, activator of complement and inducer of fibrosis)	SFE lipid rich extract from stabilised mussel powder (20 mg/kg) dosed orally	Pentoxifylline (PTX) Prednisone	Rear and front paw swelling	When prednisone, PTX and SFE lipid extract were each given alone, they were not able to reduce established arthritis or prevent fibrosis and the co-administration of prednisone and PTX has limited effect. But co-administration of the SFE lipid extract amplified the anti-inflammatory potency of either prednisone or PTX The SFE lipid extract would seem a suitable PTX synergistic agent for anti-TNF therapy
Lee et al. (2009)	Adjuvant-induced arthritis using *Mycobacterium butyricum* in male Sprague-Dawley rats	SFE lipid-rich extract dosed orally at 25 mg/kg	Olive oil (300 µl) and Naproxen (20 mg/kg) were fed as vehicle and positive control	Measurement of pain scores: The number of pain-related responses, represented by vocalizations, was recorded during 10 flexions of the tarsotibial joints of the adjuvant-injected paw. Results were expressed as the mean number of vocalizations	The SFE lipid-rich extract was able to control pain at the initial phase of its administration; with similar efficacy to that observed with Naproxen. The pain scores slowly increased again in the group of rats treated with the SFE extract after day 9–14. The Naproxen-treated rats remained pain-free while treated. Both Naproxen and the SFE extract decreased the levels of the

(continued)

Table 4 (continued)

Author	Design	Fractions	Comparator	Method of assessment	Outcome measures
					pro-inflammatory cytokines TNF-α and IFN-g, and increased that of IL-10. Administering olive oil had no effect on cytokine secretion. Rats treated with the SFE extract were apparently cured after 1 year
Wakimoto et al. (2011)	Adjuvant-induced arthritis using *Mycobacterium butyricum* in female Wistar rats	Isolated furan fatty acids from the SFE lipid-rich extract 1–10 mg/kg administered orally	Control Naproxen (10 mg/kg) SFE lipid-rich extract (200 mg/kg)	Paw swelling	Furan fatty acids (minor component of the fatty acids in the lipid extract) exhibited more potent anti-inflammatory activity than EPA but the whole SFE lipid-rich extract demonstrated the most potent AI activity compared to the furan fatty acids
Singh et al. (2008)	Adjuvant-induced arthritis using *Mycobacterium* tuberculosis in male long Evans rats	SFE lipid-rich fraction from stabilised freeze-dried mussel powder s.c. injection dosed at 50 and 100 mg/kg (crude lipid) via s.c. injection Further fractionation of a novel FFA class (C18:4, C19:4 and C21:5) s.c. injection at a dose of 30 mg/kg	Olive oil control Piroxicam (2 mg/kg)	Rear paw swelling Daily body weight changes Subjective disease activity in both the fore and rear paws	Compared to the olive oil control groups, the crude lipid group (50 and 100 mg/kg) did not alter paw swelling or body condition after 5 days, but at 15 days administration, the 100 mg/kg group did respond significantly both in reduced paw swelling and improved body condition

(continued)

Table 4 (continued)

Author	Design	Fractions	Comparator	Method of assessment	Outcome measures
					In contrast, the FFA class group (30 mg/kg) significantly reduced paw swelling and deterioration of body condition at 5 days administration and was equipotent to piroxicam (2 mg/kg/day) Overall, the FFA class exhibited greater AI activity at a lower dose (30 mg/kg) and for a shorter dosage period (5 days) when compared to the significant AI activity obtained for the crude lipid component (100 mg/kg/15 days)
In vitro studies					
McPhee et al. (2007)	In vitro	• SFE lipid-rich extract • Purified PUFA (saponified by KOH hydrolysis) from the lipid extract • Protease lipid extract from whole mussel meat • Protease-lipase lipid extract Various doses used	Fish oil *M. edulis* lipid extract Purified PUFA (saponified by KOH hydrolysis) Indomethacin	COX inhibition assay and COX metabolite analysis	The SFE lipid-rich extract exhibited strong concentration dependent inhibition of both COX-1 and COX-2 and was more inhibitory than indomethacin at comparable concentrations Hydrolysed lipid extract demonstrated 10 times more inhibitory activity against COX than non-hydrolysed lipid extract

(continued)

Table 4 (continued)

Author	Design	Fractions	Comparator	Method of assessment	Outcome measures
					Hydrolysed fish oil exhibited similar activity to that of the hydrolysed mussel lipid extract The SFE free fatty acid fraction, and to a lesser extent the SFE triglyceride fraction, were the only lipid classes to exhibit strong inhibition of the COX isoforms The *P. canaliculus* and *M. edulis* total lipid extracts moderately inhibited COX-1 and COX-2. Inhibition was increased after the mussel extract were saponified by KOH hydrolysis, indicating that the FFA fraction is likely to be in part responsible for the anti-COX activity
Treschow et al. (2007)	In vitro	FFA class isolated and purified from the SFE extract and then rigorously fractionated to isolate bioactive constituents		LOX inhibition assay involving human neutrophils challenged with AA and assayed for LTB4 and 5-HETE	The FFA fraction that demonstrated high bioactivity in inhibiting LTB4 and 5-HETE synthesis was identified as DHA. Other novel compounds with AI activity were identified as C18:4, C19:4, C20:4 and C21:5

(continued)

Table 4 (continued)

Author	Design	Fractions	Comparator	Method of assessment	Outcome measures
Macrides et al. (1997)	In vitro	Ω3 Tetraenoic fatty acids: C18:4, C19:4 and C20:4 PUFAs	None	Inhibition of Leukotriene ((LTB4) produced from stimulated human neutrophils was used as an in vitro screening method to test the efficacy of purified PUFAs for AI activity	The two most active fractions obtained from the separations inhibited LTB4 formation by 64 and 47 % respectively (at 1:100 dilution)
Whitehouse et al. (1997)	In vitro	Fatty acid methyl esters prepared from SFE lipid extract subfractions at various concentrations	None	Leukotriene biosynthesis by human PMNs. Inhibition of arachidonate induced leukotriene-B4 and 5-HETE synthesis in A-23187-activated human PMN	Four of the fractions were effective in inhibiting 5-LOX and 5-HETE with the active fractions containing polyunsaturated acids with 4, 5 or 6 double bonds The unfractionated SFE material also inhibited prostaglandin E2 production from endogenous arachidonate by stimulated human blood monocytes The SFE lipid extract is a potent but slow acting anti-inflammatory agent
Dugas (2000)	In vitro	An SFE lipid-rich extract from stabilised mussel powder Dose NS	None	Leukotriene biosysnthesis by human monocytes (normal and allergic donors). Production of LTB4 by normal and allergic human monocytes when challenged with IL-4	The SFE lipid extract inhibited 5-LOX pathway in both normal and allergic human monocytes when challenged with IL-4

(continued)

Table 4 (continued)

Author	Design	Fractions	Comparator	Method of assessment	Outcome measures
Gastrointestinal protection					
Tenikoff et al. (2005)	Dextran (2 %) sulfate sodium—induced IBD in male C57BL/6 mice	SFE lipid rich-fraction from stabilised freeze-dried mussel powder dosed orally (5 mg lipids/day)	Olive oil Fish oil (55 mf EPA/DHA/day)	Myeloperoxidase (MPO) activity = a measure of neutrophil infiltration Body weight Rectal bleeding Stool consistency Overall condition (disease activity index—DAI) Colon damage—histopathology	SFE lipid-rich fraction significantly reduced body weight loss, DAI scores, crypt area losses and cecum and colon weights compared to the fish oil group. MPO activity was not significantly affected by any treatment
Torres et al. (2008)	Intestinal mucositis model in female dark Agouti rats injected with 5-fluorouracil (5-FU) (150 mg/kg i.p.)	High dose lipid extract—800 µl/day Low dose lipid extract—400 µl/day	Fish oil Olive oil Saline	Small intestinal weight Food intake MPO activity Histological damage Sucrose breath test (small intestinal sucrase activity and absorptive function) Biochemical sucrase activity (biomarker of total small intestinal sucrase activity and absorptive function)	SFE lipid-rich fraction treatment in rats with 5-FU-induced mucositis only minimally decreased indicators of intestinal integrity producing lower histological damage and high dose Lyprinol group had longer crypts and increased proliferation in the mid small intestine

AI Anti-inflammatory activity; *PMN* Polymorphonuclear leukocytes; *LOX* Lipoxygenase; *5-HETE* 5-Hydroxyeicosatetraenoic acid; *KOH* Potassium hydroxide; *AA* Arachidonic acid; *s.c.* Subcutaneous

specifically related to glucose, metabolism was increased, possibly equating to a decline in glucose levels available for the activation of major histocompatibility complex class I (MHC-I). MHC-1 gene expression contributes to autoimmune diseases. Elevated levels of MDH can possibly decrease free glucose in the cytoplasm by converting pyruvate into malate (Lee et al. 2008).

Rat studies reporting anti-inflammatory activity with the SFE lipid-rich fraction have typically orally administered 20 mg/kg of body weight per day (highest 100 mg/kg/day) to rats with adjuvant-induced arthritis. The current dosage recommendation for humans, however, is 100–200 mg per day of the SFE lipid-rich fraction (administered with olive oil as a carrier). When comparing the dosages, using a 70 kg person for example, the doses used in rat studies are typically 7–14 times higher than that which is recommended for relief from arthritis-induced inflammation and pain in humans. How the low dose for human consumption was recommended, when rat studies demonstrated anti-inflammatory and pain relief at high doses, is not clear. It may be due to variations of metabolic capacity between rats and humans.

3.2.2 Anti-asthmatic Activity

The inhibition of leukotriene and prostaglandin E series production by the lipid-rich fractions has led to its assessment in both animal and human asthma models. Significant reductions in the development of allergic inflammation and airway hyperresponsiveness (rat model) (Wood et al. 2010) and asthma symptoms were attained in humans (Mickleborough et al. 2013; Emelyanov et al. 2002). Mickleborough et al. (2013) conducted a placebo controlled, double-blind randomised crossover study in patients with mild to moderate asthma (n = 20) who were given either eight capsules per day of a stabilised SFE lipid-rich extract (providing 72 mg EPA and 48 mg DHA) or placebo (olive oil) for 3 weeks duration, then followed by a 2 week 'washout' period before treatments were crossed over. The study showed that the lipid-rich fraction significantly reduced airway inflammation and the bronchoconstrictor response to dry air hyperpnea. The lipid-extract group also benefited by reduced asthma symptom scores and their lesser use of rescue medication compared to the placebo group (Mickleborough et al. 2013). Emelyanov et al. (2002) also demonstrated in a double-blind, parallel group, randomised, placebo-controlled study (n = 46) that the stabilised SFE lipid-rich fraction when supplemented at four capsules/day for 8 weeks duration, significantly decreased day time wheeze, concentration of exhaled H_2O_2 and an increase in morning peak expiratory flow in patients with atopic asthma compared to the placebo group (olive oil) (see Table 2) (Emelyanov et al. 2002).

3.3 *Gastrointestinal Protection*

The therapeutic efficacy of the CO_2-SFE lipid-rich fraction has also been assessed in gastrointestinal disorders, with significant efficacy in a dextran sodium sulphate-induced inflammatory bowel disease (IBD) model in rats (Tenikoff et al. 2005). The lipid fraction significantly limited body weight loss, reduced disease activity indices and overall morphology of the inflamed intestinal tissue reducing crypt area loss preventing cecum and colon weight loss, all providing potential evidence for successful management of IBD. The lipid fraction was also assessed as a potential treatment in chemotherapy-induced intestinal mucositis in a rat model, using 5-fluorouracil as the toxin; however, the lipid fraction demonstrated only limited efficacy in reducing the symptoms (Torres et al. 2008) (see Table 4).

4 The Role of Intestinal Microbiota in the Therapeutic Activity of *Perna canaliculus* for Inflammatory Conditions

Humans and commensal bacteria coexist in a usually symbiotic relationship with a host to microbe cell ratio of 10:90 %, respectively. It is estimated that the GIT microorganisms collectively make up to 100 trillion cells, tenfold the number of human cells (Lederberg 2000). The collective microbial community is termed the microbiota or the microbiome and it populates specific human environments (e.g. the skin, mouth, nasal cavity, GIT and the urogenital tract). The GIT, skin, urogenital and respiratory systems are extensively colonised by symbiotic microorganisms (Singh et al. 2013). In the human GIT, there is a gradual increase (proximally to distally) in the density and diversity of the microbiota, with the large bowel microbiota representing the most dense, diverse and complex microbial ecosystem known (Tremaroli and Backhed 2012). The genomic content of the GIT microbiome is reported to encode 3.3 million unique bacterial genes, out-numbering the human genome by a factor of approximately 150 (Qin et al. 2010). The human genome, together with its associated microbiome, shares a mutually symbiotic relationship. The microbiota that colonise the GIT regulate normal development and function of the mucosal barriers; assist with maturation of immunological tissues, such as gut-associated lymphoid tissues, promoting immunological tolerance to antigens (foods, environment, pathogens); induce chemical communication to target tissues such as the liver, brain, muscle, adipose tissue, heart and GIT; prevent propagation of pathogenic microorganisms as well as control nutrient uptake and metabolism (Shen et al. 2013).

The GIT microbiota contributes to the metabolism of ingested compounds during the digestive process, including both foods and pharmaceutical drugs, to produce numerous metabolic products. Such metabolites function as signalling molecules between the bacteria and host cells. Metabolites that regulate

host–microbiota dialogue include short-chain fatty acids (SCFA), bile acids (e.g. choline) and lipids (i.e. LPS and peptidoglycan). The genetic richness of the GIT microbiota allows the expression of specific metabolic activities that are not encoded by human DNA (Gill et al. 2006; Egert et al. 2006; Laparra and Sanz 2010), including the hydrolysis and fermentation of dietary polysaccharides (Tremaroli and Backhed 2012). Therefore, the commensal GIT microbiota plays a critical role in human GIT metabolism. The metabolism of *P. canaliculus* by commensal microbial species has not been well explored. However, in vitro analyses have indicated that certain commensal bacteria ferment and metabolise the popular anti-arthritic medication D- glucosamine (Foley et al. 2008; Koser et al. 1961; Wolfe and Nakada 1956; Lutwak-Mann 1941; Faulkner and Quastel 1956). The metabolic capacity of intestinal microbiota can modify bioactive food components altering the hosts' exposure to these components and potentially enhancing or diminishing their health effects. Furthermore, a number of microbiota-based interventions have shown to contribute to human health through maintaining normal microbial composition, improving metabolism and immunity of the gut and by enhancing mucosal integrity and barrier function (Turnbaugh et al. 2006; Gigante et al. 2011). Functional food components such as inulin, are known to influence the growth and metabolic activity of the GIT microbiota and thus its composition and subsequent metabolic capacity (Laparra and Sanz 2010; Campbell et al. 1997; Gibson et al. 2005). The intestinal microbiota is a target of nutritional interventions such as *P. canaliculus*, influencing bacterial viability, growth and metabolic activity (Coulson et al. 2013). Bacterial microbiota may consequently influence biological activity of nutritional supplements. It is proposed, therefore, that the therapeutic activity of *P. canaliculus* is potentially, or in part, due to its interaction with gut bacteria and consequential influence on the host immune system.

The implication of the GIT microbiota in rheumatic diseases has been recognised in in vivo studies. The discovery of a wide variety of bacterial species and bacteria-derived peptidoglycan-polysaccharides (PG-PS) present in synovial fluid from not only reactive arthritis, but also chronic forms of arthritis including RA and OA, indicates that arthritic joints are not sterile as previously thought (Kempsell et al. 2000b). The presence of bacterial antigens within the synovial fluid may play a role in the pathogenesis of several forms of arthritis, other than septic arthritis, such as triggering or exacerbating joint inflammation (Gerard et al. 2001; Kempsell et al. 2000b; van der Heijden et al. 2000; Siala et al. 2009a; Olmez et al. 2001; Carter et al. 2009). Analysis of synovial fluid from OA patients using various polymerase chain reaction (PCR) methods including reverse-transcriptase PCR, broad-range PCR and 16S rRNA PCR, has detected DNA from various bacterial strains. Bacterial DNA that has been detected in OA patients includes *Pseudomonas sp., Shigella sp., Escherichia coli, Chlamydia trachomatis* and *Chlamydia pneumonia* (Olmez et al. 2001; Carter et al. 2009; Gerard et al. 2001). From OA synovial fluid, immunoglobulin G (IgG) antibodies have been detected against *Porphyromonas gingivalis, Prevotella intermedia and Bacteroides forsythus* using enzyme-linked immunosorbent assays (ELISA), while IgA antibodies against *B. forsythus* have also been detected (Moen 2003). Additionally, there is evidence

suggesting that host MHC genes may affect the microbiological milieu of the gut (Vaahtovuo et al. 2003; De Palma et al. 2010). High levels of antibodies directed against antigens of certain gut bacteria in RA patients propose a pathogenic relationship between these bacteria and RA (Scher and Abramson 2011). The exposure to bacterial cell walls may increase the susceptibility to develop arthritis as shown in animal studies (van den Broek et al. 1988; Jonsson et al. 2003). Evidence that bacterial DNA can be detected in OA joints, albeit not as frequently as in RA joints, highlights the importance of patient genetic variability and tolerance. Furthermore, in the case of *Chlamydia trachomatis*, there are several different serotypes that may predict various pathogenic outcomes (Carter et al. 2009). The source of bacteria detected in synovial fluids is not known, but it is suggested they may be derived from environmental sources or from the enteric microbiota (Siala et al. 2009). Periodontal pathogens may also be implicated in arthritic joint inflammation with antibodies to Gram-negative, anaerobic periodontal pathogens such as *Porphyromonas gingivalis, Prevotella intermedia, Prevotella melaninogenica* and *Tannerella forsythia* detected in the serum and synovial fluid of RA patients (Ogrendik 2012). These investigations support the hypothesis that in genetically susceptible subjects, exposure to degraded products of the gut bacteria locally in synovial fluids may cause inflammation. Consequently, it is proposed that bacteria may cause or influence joint disease in a number of ways such as creating persistent infection, inducing autoimmune pathophysiology, producing bacterial antigens or the induction of immune dysfunction (Kempsell et al. 2000a). However, the exact role and the full clinical implications of finding bacterial DNA in arthritic joints are still unknown.

RA patients have altered intestinal microbial profiles that may be relevant to the aetiopathogenesis of RA (Gul'neva and Noskov 2011; Toivanen 2003; Vaahtovuo et al. 2008). RA patients demonstrate decreased *Bifidobacterium*, Lactobacillus and *Bacteroides-Porphyromonas-Prevotella* species, elevated opportunistic *Enterobacteria* and *Staphylococci* species and variable reports of high or low *Clostridium* profiles. Evaluation of gut microbial profile compositions are limited in OA patients, however elevated *Clostridium* and *Staphylococcus* profiles have been found (Coulson et al. 2013). Pain relief medications used by rheumatic patients (i.e. acetaminophen and NSAIDs) may also contribute to altered bacterial profiles in conjunction with their known gastrotoxic effects (Upreti et al. 2010; Cuzzolin et al. 1994; Al-Janabi 2010). In genetically predisposed individuals, environmental factors such as diet, infections and smoking can cause dysbiosis in the GIT microbiota recognised as a microbial imbalance in which one or more bacterial phyla, genus or species overgrow and negatively impact on other beneficial bacteria (Taneja 2014). This dysbiosis may be related to the production of some metabolites as well as the activation of Nuclear Factor-kappa B (NF-kB) pathway that mediates the release of anti-inflammatory cytokines, compromising the integrity of the colonic epithelial cells, increasing gut permeability and consequently affecting health (Chen and Kasper 2014). Therefore, modification of inflammatory conditions such as OA, RA, asthma and IBD may be achieved in part through the refinement of GIT bacterial profiles to reflect a more homeostatic status (Coulson et al. 2012, 2013).

5 Discussion/Conclusion

The therapeutic efficacy of *P. canaliculus* for the treatment of OA, RA and asthma has been a contentious issue with a lack of conclusive evidence-based research. Further scientific investigations are required to evaluate product stability, optimal dosage, novel bioactive compounds and GIT microbiota profiles when assessing the efficacy of *P. canaliculus*. The predominant compound of *P. canaliculus* is protein, which has shown anti-inflammatory and immunomodulating activity in in vitro studies; however, its efficacy has not been investigated in humans yet. Understanding the interaction of the bioactive compounds in *P. canaliculus* with commensal and pathogenic bacterial may facilitate the development of novel interventions to control intestinal and extraintestinal inflammation.

While only two bacterial divisions (*Bacteroidetes* and *Firmicutes*) have been reported to dominate the gut microbiome, thousands of bacterial genera and species inhabit the human gastrointestinal tract. Hence the administration of compounds such as *P. canaliculus* and glucosamine to ameliorate the symptoms of OA and perhaps also RA may involve the actions of the gut microbial cohort to down regulate gut mucosal inflammatory sequalae. Recent clinical data (Coulson et al. 2012, 2013) plausibly suggests that these nutraceuticals may act as prebiotics in the gut, attenuating musculoskeletal inflammatory pain via interactions with the gastrointestinal microbiome.

Advanced sequencing tools/methodologies and experimental approaches have brought novel insights into the mechanisms that promote and maintain gut inflammatory processes that also include auto-inflammatory processes such as in RA. Indeed, it is now possible to locate the site and identity of thousands of bacteria (as well as their functions). This understanding has provided a previously unmatched level of bacterial communities and species detail. For example, animal models studying RA have shown the capacity of specific commensal bacteria to activate pro-inflammatory signalling, which in turn initiate and progress deleterious effects in the joints. The clinical implications of these findings, in parallel with reports that demonstrate humans harbour distinct enterotypes, strongly suggest that musculoskeletal diseases such as RA and the perpetuation of OA may originate in the gut. Certainly this can be plausibly pre-empted for RA from well-characterised studies utilising DNA-parallel sequencing in animal models elucidating possible dysbiosic states (Scher 2010).

If a distinct microbiota profile or pathogen promoted enterotype can be identified, it would then be possible to speculate whether a particular microbiome triggers or drives autoimmunity in genetically predisposed individuals or progresses pro-inflammatory sequale from the gut to the systemic circulation and the musculoskeletal joints. The identification of gut pathogenic commensal profiles could provide insights into the environmental triggers of musculoskeletal diseases and lead to a new understanding of disease pathogenesis, perhaps leading to novel approaches for adjunctive thereby.

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Celery Seed and Related Extracts with Antiarthritic, Antiulcer, and Antimicrobial Activities

Michael C. Powanda, Michael W. Whitehouse and K.D. Rainsford

Abstract Celery preparations have been used extensively for several millennia as natural therapies for acute and chronic painful or inflammatory conditions. This chapter reviews some of the biological and chemical properties of various celery preparations that have been used as natural remedies. Many of these have varying activities and product qualities. A fully standardized celery preparation has been prepared known as an alcoholic extract of the seeds of a plant source derived from northern India. This is termed, Celery Seed Extract (CSE) and has been found to be at least as effective as aspirin, ibuprofen, and naproxen in suppressing arthritis in a model of polyarthritis. CSE can also reduce existing inflammation in rats. CSE has also been shown to provide analgesia in two model systems. CSE, in addition to acting as an analgesic and inflammatory agent, has been shown to protect against and/or reduce gastric irritation caused by NSAIDs, as well as act synergistically with them to reduce inflammation. The CSE was fractionated by organic solvent extractions, then subjected to column chromatography followed by HPLC and was characterized by mass spectrometry. This yielded a purified component that had specific inhibitory effects on *Helicobacter pylori* but was not active against *Campylobacter jejuni* or *Escherichia coli*. Additionally, toxicology studies did not reveal any clear signs of toxicity at doses relevant to human use. Also, unlike many dietary supplements, the available data suggest that CSE does not significantly affect the p450 enzyme systems and thus is less likely to alter the metabolism of drugs the individual may be taking. CSE may be a prototype of a natural product that can be used therapeutically to treat arthritis and other inflammatory diseases.

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1 Introduction

The prevention or inhibition of inflammation and pain is of significant concern, particularly for those afflicted with arthritis and other musculoskeletal ailments, including sports-related injuries. Pain usually accompanies inflammation and vice versa.

Acute and chronic pain and inflammation are often treated with anti-inflammatory/analgesic compounds such as aspirin, ibuprofen, and naproxen (Rainsford 1996, 2004, 2012). There are, however, appreciable risks from these drugs, in particular, gastrointestinal ulceration and bleeding (Rainsford 1996; Kean et al. 2008). While another commonly-used pain-killing drug, acetaminophen, does not have this side effect, its benefits and range of uses are limited because it does not have anti-inflammatory activity (Towheed et al. 2006; Kean et al. 2008. Paracetamol is less effective in control of pain because it does not control the inflammatory reactions that often accompany pain, especially that from chronic conditions such as arthritis (Rainsford 1996). Furthermore, there is considerable concern in recent years about the safety of paracetamol (acetaminophen), especially at over-the-counter doses, because of the risks of severe and, rarely, irreversible liver damage that can lead to death (Rapp et al. 1997; Rainsford and Powanda 1998; Bateman et al. 2014; Whitehouse and Butters 2014).

2 Historical Uses of Celery Preparations

Celery (Apium graveolens, Linn; Family: UMBELLIFERAE) roots, leaves, stems, and seeds have long been used as herbal remedies (Kapoor 1990; Lawless 1992; Wright 2005; De Vos 2010). In recent years, there has been interest in components of celery being useful as "food-based" medicines or neutraceuticals (Sowbhagya 2014). From a medicinal aspect through history, there have been many claims for therapeutic benefits from celery preparations. Thus, in Ayurvedic medicine there are descriptive references to Rasa-katu, Guna-Teekshna, Veerya-ushna, and Vipaka-katu. In Sanskrit and Hindu, Celery has the vernacular name, Ajmoda, in Nepalese, Ajumu, in Arabian Bazr-ul-karafs and in Persian, Tukhm-e-karafs, all reflecting long-standing uses in medicine, (Kapoor 1990). Its natural habitat (presumably of what is described as "wild celery") used in Ayurvedic medicine is at the base of the northwestern Himalayas and outlying hills in the Punjab and western India. There are extensive references to what is described as Chinese Umbelliferae in Chinese medicine (Wright 2005). The infamous English Herbalist, Nicholas Culpeper of some four centuries ago refers to Smallage or Wild Celery growing on marshes and damp land, and being used for rheumatism and arthritis (Potterton 1983). In this reference it is stated that "… the leaves… eaten in the spring, sweeten and purify the blood, and help the scurvy." It is also stated that the "medicinal virtues [of smallage] roots provoke the urine and are effective where there is

stoppage, or for removing stone and gravel. They also open obstructions of the liver and spleen, help dropsy and jaundice and remove female obstructions." Also, "The seeds are hot and carminative, and are therefore good for the wind." Some of these properties might have relevance for some of the biochemical and cellular actions of celery components referred later in this chapter (e.g., hepatocellular protection). The pharmacological actions include those referred to in Ayurvedic medicine including actions as a tonic, carminative, diuretic, and stimulant (Kapoor 1990). It has been successfully employed in rheumatoid arthritis, probably acts as an intestinal antiseptic, while the essential oil is used as an antispasmodic and nerve stimulant (Kapoor 1990). Reference in Culpepper's book to its use in scurvy may have some relevance to its relatively high content of Vitamin C (Kapoor 1990). The well-known volatile oil, sometimes referred to as an essential oil, has had a long history since Roman and medieval times in Europe as a condiment (Livarda and Van der veen 2008). Other herbal references exist for celery seed being used in bladder and kidney complaints (Podlech 1987; Lawless 1992), digestive upsets, menstrual problems, skin ailments, and as a remedy for hepatobiliary disorders and liver regeneration (Lawless 1992). Also, of particular interest is its actions in eliminating uric acid and as a remedy for gout, as well as for rheumatoid arthritis and neuralgia (Lawless 1992).

There are many parts of celery that have been used therapeutically and so these may have variable actions. There are references to toxic actions or adverse reactions to celery preparations in some herbal guides. In the German commission E Monograph, Therapeutic Guide to Herbal Medicines (Blumenthal 1998), risks are stated that celery can cause allergic reactions, even ending in anaphylactic shock (so-called celery-carrot-mugwort syndrome) and celery containing phototoxic furancoumarin. Unfortunately, the exact part of celery associated with the allergic reactions is not stated. This guide also states that celery preparations are used as a diuretic, for rheumatic complaints, gout, stone diseases, "blood purification," loss of appetite, and malnutrition as well as unrest. It also cautions that documentation is weak for claimed applications, although this is surprising in view of the extensive literature on herbal remedies over centuries making some of these claims.

3 Chemical Composition and Some Properties of Celery Preparations

A wide variety of pharmaceutical or nutraceutical grades and extracts of celery preparations have been reported. The chemical composition of these can, as would be expected, vary considerably, and this depends on the source of the celery, methods of preparation, and extraction techniques employed. The celery seed preparations, which are of interest in the present chapter comprise about 2 % of volatile or essential oils (Marongiu et al. 2013; Sowbhagya 2014). These oils

contain about 60 % limonene and 20 % selinene. These give rise to the typical aromas as well as those from 3-*n*-butyl-4,5-dihydrophthalide (sednenolide), 3-*n*-butyl phthalide, and sedanonine anhydride, which are present in quantities ranging from 1 to 3 % (Sowbhagya 2014). Typically, variations in the components of celery seed vary according to the organic solvent mixing process, time of distillation, and whether the celery is fresh or frozen (Falzari and Menary 2005). Studies with a fresh Tasmanian herbal preparation of celery have shown the presence in the distilled oil of 67.95 % limonene, 7.79 % sedanolide, 4.16 % α- and β-selinene, 3.68 % pinene, 3.41 % *n*-pentyl-cyclohexadiene, 2.82 % sedanenolide, 1.45 % myrcene, and 0.62 % *n*-butylphthalide.

High antioxidant activity in celery seed oil has been attributed to limonene (74.6 % composition) (Wei and Shibamoto 2007). Antioxidant activity has also been reported in other essential oil components of celery seed (Shiratsuchii et al. 2012).

In addition to the volatile oils, celery seeds extracted with aqueous methanol have shown the presence of a variety of celerioside glucosides and 3-phthalide glycosides (celephthalide A-C) along with some aromatic and lignan glucosides (Kitajima et al. 2003; Lin et al. 2007).

4 A Novel Celery Seed Alcohol Extract

Celery Seed Extract, an alcoholic extract of green Indian celery seed (A-CSE) as supplied by Beagle International Pty Ltd (Nerang, QLD, Australia) has a combination of analgesic, anti-inflammatory, and gastro-protective qualities in one herbal preparation, without evidence of side effects often associated with NSAIDs, aspirin or acetaminophen. CSE appears to have synergistic anti-inflammatory activity with the NSAIDs. Supercritical extracts of CSE (S-CSE) maintain activity. Various fractions and purified components of CSE also exhibit antimicrobial activities.

It should be noted that not all celery-based products may be equal in composition, range of biological activities, safety, or potency. This may be due to the source (country of origin), physical state (fresh or dried), component of the plant chosen, mode of extraction process, and stability of the product. Much of the following data pertain to an alcohol extract of fresh green seeds sourced from Amritsar, Punjab, India, which have been tested for pesticide residues, heavy metal contaminants, and microbial contamination, and which, in some cases, have been subjected to further fractionation/purification.

4.1 Efficacy of CSE in Controlling Chronic Inflammation

An experimental polyarthritis was induced in rats by injecting a mycobacterial arthritogenic adjuvant. The animals were then treated daily for at least 14 days,

beginning at the time of initiating polyarthritis (Day 0) and continuing until the arthritis was optimally expressed in untreated controls (Days 14–16). Paw thickness was measured using a micrometer screw gauge.

The data in Table 1 indicate that repeated oral treatment with CSE (Beagle International Pty Ltd) to rats whether in liquid, capsule, or tablet form, is equally as effective as repeated doses of naproxen and more effective than either aspirin or ibuprofen in suppressing polyarthritis (Whitehouse et al. 1999). Since 2500 mg of celery seed yields approximately 190 mg of active extract, on a potency basis the extract is three times more efficacious than aspirin and roughly comparable to ibuprofen. The data are not shown in this table, but the celery seed extract exhibited no gastrotoxicity in rats at 50–70 times the normal human dose, whereas aspirin, ibuprofen, and naproxen are known to cause gastric lesions at human therapeutic doses (Whitehouse et al. 1999, 2001).

The celery seed extract can not only suppress the development of arthritis, but can also ameliorate its severity, in a dose-dependent fashion, when given after the onset of disease. The data in Table 2 reflect the effect of various doses of CSE and given for 4 days after arthritis first observed, usually on day 10. The polyarthritis was induced as above. Twenty-two celery-based products were tested in either the therapeutic or inflammation suppression models. Four products had no effect in treating or suppressing inflammation. Only eight products elicited more than a 50 % response in treating or suppressing inflammation. Six of those eight products contained various doses and forms of the extract of fresh green celery seeds sourced from Amritsar, Punjab, India (Whitehouse et al. 1999).

Table 1 Efficacy of celery seed in various formulations compared to conventional anti-inflammatory agents in suppressing an experimental polyarthritis in rats

Product	TGA no.	Format	Dose (mg/kg)	Inhibition of swelling (%)		Wgt change
				Rear paw	Front paw	
CS-extract	L 39931	Liquid	2700	67	59	++
CS-Extract	L 46524	Capsules	2500	79	54	+
Celltech CT	L 46525	Capsules	2500	84	48	0
CT-Forte	L 55831	Tablets	2500	94	64	+
Aspirin	R 19793	Tablet	300	34	22	0
Ibuprofen	R 13818	Tablet	50	44	30	0
Naproxen	R 10175	Tablet	25	77	67	++

Table 2 Efficacy of celery seed in various doses in treating experimental polyarthritis in rats

Treatment	Increase in mm		AS	Inhibition (%)		
mg/kg	Rear paw	Front paw		Rear paw	Front paw	AS
Control	0.64	1.5	2	–	–	–
CTF 2500	−0.13	0.7	0.8	100	53	60
CTF 1500	0.01	0.6	1.0	98	60	50
CTF 500	0.32	0.8	1.7	50	47	15
CSO 5000	0.77	1.4	2	−20	07	0

Animals dosed for 4 days only (Days 10–14); measurements made on Day 14
Dose of celery products refer to original weight of seed; 2,500 mg of seed yields approximately 190 mg of active extract
AS Arthritis score, assessed by an independent observer
CTF CT-Forte (Beagle)
CSO Nature's way (Roche) celery seed oil

4.2 Efficacy of CSE in Treating Acute Pain

The pain model used was that based on the standard model of Randell and Selitto (1957) in which rat paws were pre-swollen with a carageenan injection and then pressure sensitivity assessed with or without a dose of celery extract.

Nurofen™ (OTC ibuprofen) at 200 mg/kg completely abolished the pain as determined by lack of vocalization when a standard pressure was applied to rat paws swollen by pre-injected carrageenan (0.6 mg/paw). This analgesia lasted at least for 3.5 h. The celery extract gave the same response at 500 mg/kg but there was no attempt to define a dose response. A concentrate produced by supercritical fractionation, S-CSE, was equipotent at 70 mg/kg, and therefore on a weight basis was superior to Nurofen™. These data indicate that the original alcohol extract (A-CSE) can relieve acute pain, but that a supercritical extract (S-CSE) is more active, i.e., has a sevenfold higher specific activity.

4.3 Efficacy of CSE in Treating Chronic Pain and Inflammation

Rats were injected in the left hindpaw with M. butyricum after taking baseline measurements of mechanical paw withdrawal threshold (PWT), paw thickness, ankle size, and body weights. PWT, paw thickness, ankle size, and body weights were measured on day 5 post inflammation. The rats were then dosed orally from day 5 through 12 with methylcellulose vehicle, naproxen 30 mg/kg, ibuprofen 100 mg/kg, or CSE 1,500 mg (seed equivalent)/kg. Measurements of PWT, paw thickness, ankle size, and body weights were conducted 4.5 h each day after dosing.

Both naproxen and ibuprofen increase PWT within hours after administration. However with time CSE also increases PWT and by day 10 CSE appears to have the same analgesic efficacy as either naproxen or ibuprofen (Fig. 1).

The changes produced by CSE on mean paw thickness showed that this had a delayed effect compared to the comparator NSAIDs; by day 12 it appears to have an effect comparable to ibuprofen on this measure of inflammation (Fig. 2). A similar trend is evident when ankle size is used as a measure of inflammation (Fig. 3).

Measurement of body weight can be used both as measure of inflammation and as a sign of adverse side effects. Body weight decreases from day 0 to 5 in all groups, as a consequence of inflammation. Body weight continues to decrease in the naproxen treated group, despite the reduction in inflammation, consistent with the drug's known propensity for inducing gastric irritation. Body weights trend toward baseline values in both the ibuprofen and CSE treated groups consonant with the known lower gastric toxicity of these agents (Fig. 4).

Other authors have investigated the mechanism of anti-nociceptive activity in mice of a hydro-alcoholic extract of the fruit of *A. graveolans* (Nasri et al. 2012). Using naloxone, dextramethorphan, and L-NAME as selective antagonists, the authors found that the mode of analgesic action of the extract is NMDA receptors with some involvement of nitric oxide.

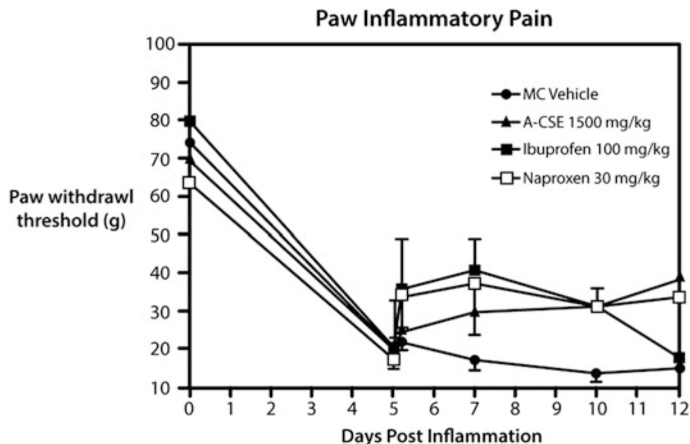

Fig. 1 Anti-nociceptive effects of oral administration of an alcoholic extract of celery seeds (A-CSE) (1,500 mg/kg) compared with ibuprofen (100 mg/kg), naproxen (30 mg/kg) or aqueous methocellulose (as a control) in rats previously injected in the left hindpaw with *M. butyricum*, and flexion-induced algesia employed for eliciting pain (Randall and Sellito 1957). The analgesic response produced by A-CSE was comparable with that from ibuprofen and naproxen but slightly slower in onset than these two NSAIDs

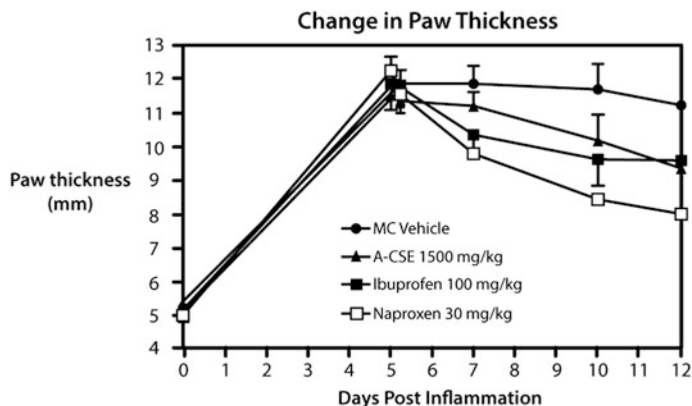

Fig. 2 Anti-inflammatory effects measured by changes in paw thickness, following oral administration of A-CSE (1,500 mg/kg), compared with ibuprofen (100 mg/kg), naproxen (30 mg/kg), or aqueous methocellulose (as a control) in rats previously injected in the left hindpaw with *M. butyricum.* A-CSE produced analgesic effects in a comparable time-course with that from the NSAIDs but with lower lesser effects than with ibuprofen and naproxen

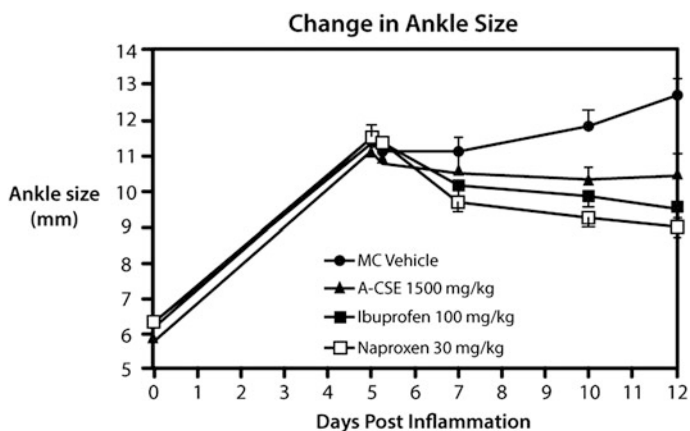

Fig. 3 Anti-inflammatory effects measured by changes in ankle thickness, following oral administration of A-CSE (1,500 mg/kg), compared with ibuprofen (100 mg/kg), naproxen (30 mg/kg), or aqueous methocellulose (as a control) in rats previously injected in the left hindpaw with *M. butyricum.* A-CSE produced analgesic effects in a comparable time-course with that from the NSAIDs but with lower lesser effects than with ibuprofen and naproxen. The results are comparable with the data from paw swelling shown in Fig. 2

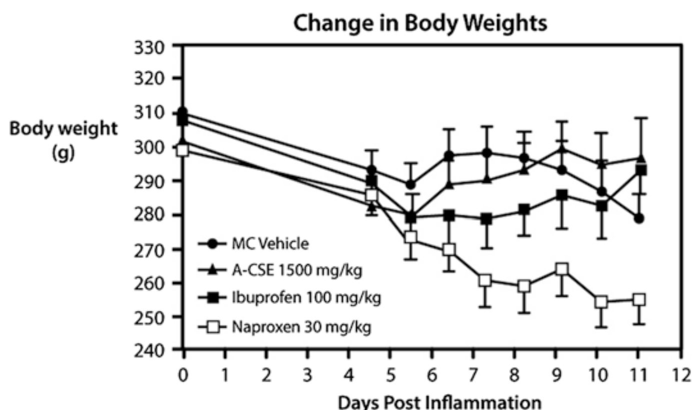

Fig. 4 Changes in body weights of the arthritic rats given daily oral doses of A-CSE (1,500 mg/kg) compared with ibuprofen (100 mg/kg), naproxen (30 mg/kg) or aqueous methocellulose (as a control). Naproxen animals showed the greatest loss in body weight while those given ibuprofen had less loss in body weight. The loss in body weights might be related to intestinal injury from the drugs with accompanying loss of blood. The animals dosed with A-CSE, like that of controls, did not show this loss of body weight seen with the NSAIDs

4.4 Efficacy of CSE in Treating Acute Inflammation in Combination with NSAIDs

In the commonly employed test for evaluating rapid-acting NSAIDs based on suppressing the carrageenan-induced paw oedema, it was found that the effective dose for 50 % reduction of the paw oedema (ED_{50}) was 200 mg/kg for aspirin, 45 mg/kg for ibuprofen, and 6 mg/kg for ketoprofen (data not shown). The corresponding ED50 values for biologically active celery seed extract preparations used alone (without an NSAID) were greater than 500 mg/kg for CSE (data not shown).

Lower doses of ibuprofen (15 mg/kg) and ketoprofen (1 mg/kg) gave only 19 and 12 % inhibition of paw swelling, respectively, not statistically significant for the small number of animals (n = 6). However, when either the ibuprofen or ketoprofen was co-administered with modest amounts of CSE (50 mg/kg) in these same experiments, the inhibition of paw swelling was significantly amplified to 61 and 46 % respectively. Ibuprofen, given for four successive doses orally at 30 mg/kg, still only suppressed arthritic paw swelling by 38 %. Supercritical fractionation of A-CSE to yield S-CSE in this study increased specific activity tenfold. Combining this dose of ibuprofen with 50 mg/kg of A-CSE or 5 mg/kg S-CSE yielded inhibition of the arthritic inflammation that was consistently greater than 90 %.

4.5 Efficacy of CSE in Preventing Gastrointestinal Irritation

To evaluate gastric injury caused by aspirin/NSAIDs, administered alone or in combinations with celery seed extract, these drugs were given (orally or parenterally) to rats that had been pre-sensitized by disease stress (induced 5 days previously either by injecting an arthritigenic adjuvant or 0.1 ml oleyl alcohol into the tail base to incite severe local inflammation). Rats were permitted free access to water but routinely fasted for 16 h prior to dosing them for the gastrotoxicity assay, to facilitate inspection of the gastric lining post mortem. The animals were sacrificed 2.5 h after administering the aspirin/NSAID.

Ibuprofen given to pre-inflamed female Wistar rats as either the free acid (obtained from Sigma Chemical Co. USA) or as a proprietary formulation for over-the-counter use (Nurofen™) regularly causes gastric bleeding with the mean number of hemorrhagic lesions (±SD) being 42 ± 06 (n = 90 rats) for a standard dose of 50 mg/kg administered as a suspension in a volume of 5 ml/kg.

Co-administration of the celery extract in doses ranging at 50, 150, and 250 mg/kg administered in a volume of 10 ml/kg, dose dependently reduced (25, 39, 85 %) the mean number of gastric lesions elicited by 50 mg/kg ibuprofen (Whitehouse et al. 2001). Furthermore, the severity as well as the total number of lesions was significantly reduced from a mean grade of 3.4+ to 1.9+ (on a scale of 0 to 4+) by the co-administration of the celery extract. More strikingly, the number of animals that had no macroscopically detectable gastric bleeding was increased from 7 % (ibuprofen with no celery, n = 90 rats) to 29 % with celery extracts (n = 67 rats). The efficacy of the celery supplement was attested by these three criteria, namely reduction in number of lesions, reduction in severity of lesions, and total absence of lesions in a significant proportion of the celery-dosed rats.

In parallel studies, aspirin (100 mg/kg), naproxen (10 mg/kg), and ketoprofen (5 mg/kg) given orally and piroxicam (5 mg/kg) given intraperitoneally to pre-inflamed rats, also caused significant gastric lesions and bleeding (90 % incidence), the severity of which was reduced to 70 % with these same doses of CSE. In earlier studies, CSE (500 mg/kg) also was shown to reduce the extent of gastric lesions induced by alcohol in rats. In other studies, it was found that the optimal effect of the gastroprotective celery extract was obtained when it was given concurrently with the NSAID, preferably in a specifically formulated admixture.

The gastroprotective effects of celery extracts appears not to be limited to an alcohol extract of fresh green seeds sourced from Amritsar, Punjab, India. Pretreatment with an ethanol extract of the aerial parts of celery, presumably grown in Saudi Arabia, produced dose-dependent reductions in gastric lesions in rats irrespective of whether ulcers were induced by indomethacin, or gastroirritant agents (80 % ethanol, 0.2 M NaOH and 25 % NaCl) or cold restraint stress (Al-Howiriny et al. 2010). This celery extract prevented the depletion in gastric wall mucus and gastric mucosal nonprotein sulfhydryl groups, as well as reducing malondialdehyde production. The authors posit that this extract's gastroprotective effect may in part be due to antioxidant activity.

Gastroprotection in fact appears to be a general property of celery, aerial parts, as well as seeds, water extracted or methanol extracted. Antiulcerogenic activity was evaluated in rats by the HCl/EtOH method; the methanolic extract as well as the aqueous extracts used at 300 mg/kg dose exhibited a highly significant inhibition of gastric lesions (91 and 95 %, respectively), which was similar to that induced by omeprazole (94 %) (Baananou et al. 2013). Considering that gastroprotection is a seemingly common property of celery extracts, the concept of using such extracts in combination with aspirin and nonsteroidal anti-inflammatory drugs to lessen gastrointestinal side effects appears reasonable. If indeed the anti-inflammatory effects of celery extracts are additive or synergistic, one may achieve the same efficacy at lower doses of NSAIDs.

4.6 Efficacy of CSE as an Antimicrobial Agent

The alcohol extract of celery seeds sourced from Amritsar, Punjab, India, is a dark green oily liquid which is microbiologically stable with yeast, mold, and *Enterobacter* counts of less than 10 per gram. Initially, it was thought that the viscosity prevented microbe growth, but it appears there may be inherent antimicrobial activity in celery extracts, which can be affected by method of processing, as well as source.

Celery plant material, both fresh and dried, harvested in Algeria was subjected to hot water extraction and the water vapor laden with essential oil collected. The fresh and dried oils were compared to antimicrobial activity. Oil extracted from fresh plant material had detectable antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli, Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Candida alibicans*. The dried plant material extracted oil displayed diminished or no activity against these microbes (Benbelaid et al. 2013).

The extracts of the essential oil of *A. graveolens* that exhibited antiulcerogenic activity were also strongly inhibitory against *Escherichia coli* and moderately inhibitory against *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Baananou et al. 2013). In contrast, the Indian derived extracts appear to have little or no effect against *Campylobacter jejuni* or *E. coli*, but did significantly inhibit the growth of *Helicobacter pylori* (Zhou et al. 2009). Furthermore, a purified component of the Indian derived celery seed extracts had potent bactericidal effects against *H. pylori*; the minimum inhibitory concentration and minimum bactericidal concentration were 3.15 mg/ml and 6.25–12.5 mg/ml, respectively, activity comparable in these assays to tetracycline. The MS (Figs. 5 and 6) and NMR data for the compound were consistent with a dimeric phthalide structure incorporating either sedanenolide (compound 1, Fig. 7), *n*-butylphthalide (compound 2, Fig. 7) or sedenolide (compound 3, Fig. 7). The results appeared to rule out mechanisms that operated solely by loss of membrane integrity or inhibition of protein or nucleic acid synthesis (Zhou et al. 2009).

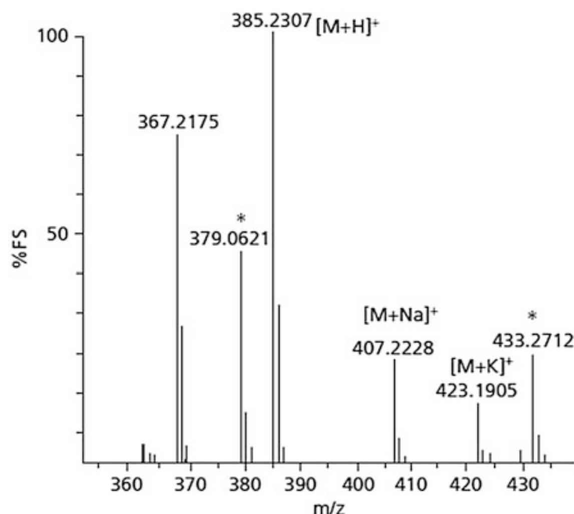

Fig. 5 Matrix Laser Desorption [MALDI] mass spectrum of a purified component (known as Compound with Anti-Helicobacter Activity, or CAH) from A-CSE. Peaks originating from the matrix are shown in *asterisks*. The main component has masses of 423.19, 407.22, 385.23, and 367.22. From Zhou et al. (2009)

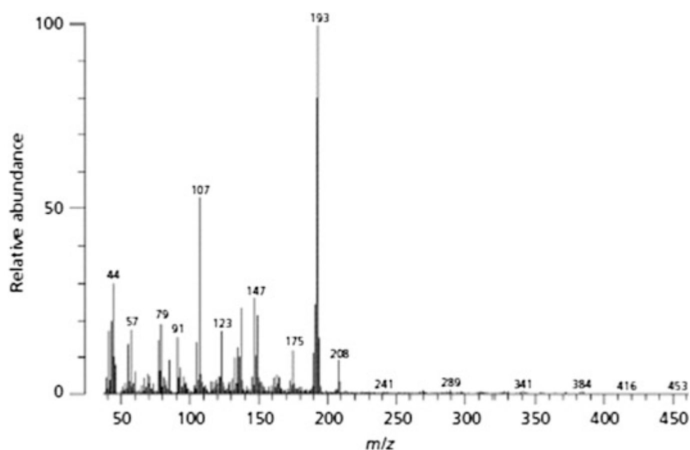

Fig. 6 Electron ionization mass spectrum of CAH showing a principal component with m/z of 193. Rationalizing this mass with the values found in Fig. 5 suggests that the principal component of CAH is a dimer based on two components shown in Fig. 7

Volatile fractions of *A. graveolens* collected in natural populations in Portugal and Italy were evaluated for their potential as antifungal agents (Marongiu et al. 2013). The volatile oils were obtained by hydrodistillation. The oils were analyzed by gas chromatography-flame ionization detector and gas chromatography-mass

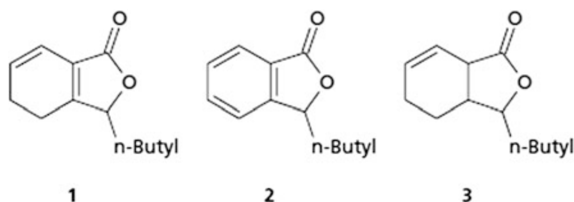

Fig. 7 Structures of phthalide compounds suspected of being components of the dimeric CAN compound

spectrometry. The results showed the presence of sedanenolide, neocnidilide, and neophytadiene as main components. The minimal inhibitory concentration (MIC) was used to evaluate the antifungal activity of the oils against *Candida albicans*, *Candida tropicalis*, *Candida krusei*, *Candida guilliermondii*, *Candida parapsilosis*, *Cryptococcus neoformans*, *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *T. mentagrophytes var. interdigitale*, *Trichophyton verrucosum*, *Microsporum canis*, *Microsporum gypseum*, *Epidermophyton floccosum*, *Aspergillus niger*, *Aspergillus fumigatus*, and *Aspergillus flavus*. The oil from Italy rich in neophytadiene is the more active, with MIC values of 0.04–0.64 μL/mL.

The following are examples of some additional activities ascribed to extracts of celery other than the extract of green Indian celery seed supplied by Beagle International Pty Ltd (Nerang, QLD, Australia). The list is not all inclusive, but rather indicative of the potential applications of celery seed extracts or components thereof.

4.7 Safety of CSE (Beagle International Pty Ltd)

A toxicity study was undertaken in 18 adult male and 18 adult female Sprague Dawley rats (Powanda and Rainsford 2010). These were randomly assigned to three treatment groups of six rats/sex/group and were administered doses of A-CSE of 0, 150, or 5,000 mg/kg per day. The A-CSE used was an alcoholic extract of green Indian celery seed supplied by Beagle International Pty Ltd (Nerang, QLD, Australia). Methylcellulose (0.5 %) was used as the control and to dilute the A-CSE. Food was provided ad libitum during the study except the night prior to necropsy. Water was provided ad libitum during the study. The animals were observed twice daily (a.m. and p.m.) for morbidity and moribundity. Body weights, food consumption, and detailed clinical observations were recorded prior to initiation of treatment and at least weekly thereafter. During each detailed clinical observation interval, performed in conjunction with body weight measurements, each animal was assessed and findings recorded. Ophthalmology examinations were performed prior to study initiation and during week 4. After 28 days of treatment, all study animals were fasted overnight and urine was collected for 16–18 h. Just prior to

necropsy, animals were anesthetized with carbon dioxide and blood samples were collected via the vena cava for hematology, clinical chemistry, and coagulation evaluation. At necropsy, terminal body weights and macroscopic observations were recorded and organ weights and tissues were collected. Histopathology was performed on all tissues from the vehicle control and the high dose (5,000 mg/kg per day) animals. In addition, the kidney tissues of the low dose animals were also processed and evaluated by light microscopy. Alpha-2-l-globulin immunohistochemistry was performed on kidney tissues from all animals. Total CYP450, total form-specific activity, and total microsomal protein were determined on liver tissue from all animals.

Treatment-related macroscopic changes were not observed at necropsy and microscopic findings were limited to minimal increases in gastric eosinophils in several male and female rats in the 5,000 mg/kg per day treatment groups. Minimal focal degeneration of renal tubules was observed sporadically in both sexes assigned to all treatment groups, including control, and was consistent with early spontaneous nephropathy of laboratory rats and thus was not considered to represent a pathologic change associated with the CSE.

There were no statistically significant changes in hematology or measures of coagulation and only a few significant differences in clinical chemistry. Male rats in the 5,000 mg/kg per day group had a statistically significant increase in serum globulin concentration and an increase in total serum protein consistent with the observed increase in serum globulin. The increase in serum phosphorus in male rats appeared not to correlate with any other chemical measure or histological observation. Female rats exhibited significantly decreased levels of serum triglycerides at both the 150 and 5,000 mg/kg per day doses. Male rats also exhibited a decrease in mean serum triglycerides values of similar magnitude in both treated groups, but due to the large standard deviations these were not statistically significant. No effect on cholesterol was seen in either sex. Total cytochrome P450 did not appear to be different between groups nor affected by treatment.

Under the conditions for this study, the no adverse effect level for systemic toxicity was considered to be 5,000 mg/kg per day. Using a generally accepted formula for converting the animal dose in mg/kg into the human equivalent dose in mg/kg by correcting for interspecies differences in body surface area (CDER 2005), the 5,000 mg/kg dose in rats equals 810 mg/kg in adult humans or 56.76 g/day for a 70 kg person. Though these data represent only one preparation of celery seed extract, the results may apply to other alcohol extracts of celery seed. Another ethanol extract of celery that demonstrated antigastric ulcer activity in rats at 250 and 500 mg/kg displayed no deleterious or toxic symptoms or mortality over a period of 14 days. The LD_{50} in mice was found to be 7.55 g/kg (Al-Howiriny et al. 2010).

In the chronic model of pain and inflammation in the rat previously described, 1,500 mg/kg per day was shown to be as effective as either naproxen (30 mg/kg) or ibuprofen (100 mg/kg), but with a lag time of about 4–5 days before a similar degree of efficacy was observed (Powanda and Rainsford 2010). Using the same body surface area correction factor used above, this would translate into a human

dose of 243 mg/kg or 17.03 g/day for a 70 kg person. However, in a small clinical trial of 15 patients, a dose of 1,360 mg per day provided a 45–50 % decrease in pain (Paul Sweeney, unpublished observations). Thus, as regards safety versus efficacy in animals there is at least a threefold margin and in the case of humans, if the clinical trial is representative, then this would denote an approximate 40-fold margin.

The CSE supplied by Beagle International Pty Ltd (Nerang, QLD, Australia) also was tested in 64 ligand binding and enzyme interaction assays, as part of the General Side Effect Profile array, as well as in the glucocorticoid assay. CSE at concentrations up to 40 μM gave no evidence of activity in any of these assays.

In addition, this CSE was tested in a set of 88 primary and secondary in vitro and in vivo assays to screen for potential activity in major therapeutic systems. Much of these data are consistent with CSE showing anti-inflammatory or analgesic activity, but provide no clear mechanism(s) of action.

Other authors have also shown that extracts of celery leaves and a major component, apiin, have anti-inflammatory activity in mice (Mencherini et al. (2007).

5 Miscellaneous Properties of Various Celery Preparations

5.1 Blood Pressure Reduction

The hexanic, methanolic, and aqueous-ethanolic extracts of celery seed were administered intraperitoneally to normotensive and deoxycorticosterone acetate-induced hypertensive rats and their effects on blood pressure (BP) and heart rate (HR) were evaluated in comparison with spironolactone as a diuretic and positive control (Moghadam et al. 2013). The results indicated that all extracts decreased BP and increased the HR in hypertensive rats, but had no effect on normotensive rats. The data showed that administration of 300 mg/kg of hexanic, methanolic, and aqueous-ethanolic (20/80, v/v) extracts of the celery seed caused 38, 24, and 23 mmHg reduction in BP and 60, 25, and 27 beats per minute increase in the HR, respectively. HPLC analysis data revealed that the content of *n*-butylphthalide (NBP) in the hexanoic extract was 3.7 and 4 times greater than methanolic and aqueous-ethanolic extracts. The authors concluded that celery seed extracts have antihypertensive properties, which appears to be attributable to the actions of its active hydrophobic constitutes such as NBP. The human equivalent dose for a 60 kg person would be approximately 3,000 mg.

A pilot study in 30 patients was conducted to evaluate the efficacy of a standardized extract of celery seed, 150 mg/d, supplying 85 % 3-*n*-butylphthalide (3*n*B) in mild to moderate hypertensive patients (Madhavi et al. 2013). There was statistically significant decrease in both systolic blood pressure (SBP) and diastolic blood pressure (DBP) at week 3 and week 6 compared to baseline. The change at

week 6 for the SBP was 8.2 mmHg (SD = 3.6, $P < 0.005$) and for the DBP was 8.5 mmHG (SD = 2.9, $P < 0.005$). While the data indicate a seemingly modest reduction in both SBP and DBP, the changes were comparable to those seen with some calcium channel blockers. It would be interesting to determine if there is a dose response, and if use as an adjunct treatment in patients with moderate to severe hypertension might be beneficial.

5.2 Cholesterol-Lowering Properties

Tsi and colleagues found that an aqueous extract caused a significant reduction in serum total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), and triglyceride (TG) concentrations in Wistar rats fed a high fat diet for eight weeks to induce hyperlipidemia (Tsi et al. 1995). They noted that the extract did not contain 3-*n*-butylphthalide (BuPh), a compound in celery that has previously been reported to have lipid-lowering action. Moreover, when the aqueous celery extract was administered intraperitoneally to genetically hypercholesterolemic and normocholesterolemic rats over a 13-day period, the serum cholesterol concentration of the hyperchlosterolemic rats was found to be significantly lower ($P < 0.05$) than the control rats. The aqueous celery extract was effective in preventing the rise of cholesterol level in the hyperchlosterolemic rats, but had no effect in control rats (Tsi and Tan 1996). Subsequent studies indicated that the cholesterol-lowering effect appeared to be due to enhanced excretion of cholesterol and metabolites via bile acids rather than altered synthesis (Tsi and Tan 2000).

Both in the above studies and in the safety study reported below neither aqueous nor alcoholic celery extracts caused a change in cholesterol in normal animals.

5.3 Insect Repellency

A number of studies have indicated that celery extracts have the ability to repel insects. This property is of general biological interest.

A hexane fraction of celery seeds not only repelled mosquitoes, but also provided protection against mosquito bites for 3.5 h when applied, in the laboratory, at a concentration of 250 mg/ml (Tuetun et al. 2004). Under field conditions, the hexane fraction showed strong repellent activity against a wide range of mosquito species belonging to various genera (*Ae. gardnerii*, *Ae. lineatopennis*, *Armigeres subalbatus*, *Culex tritaeniorhynchus*, *Cx. vishnui group*, *Cx. quinquefasciatus* and *Mansonia uniformis*). It appeared not to cause dermal irritation or any other adverse effect, either during 6 months of use or in the following 3 months of follow-up. Further studies demonstrated that the ethanolic preparation of hexane-extracted *A. graveolens* displayed a significant degree of repellency in a dose-dependent manner with vanillin added (Tuetun et al. 2005). Ethanolic *A. graveolens* formulations (10–25 %

with and without vanillin) provided 2–5 h protection against female *Aedes aegypti*. Repellency that derived from the most effective repellent, 25 % of hexane-extracted *A. graveolens* with the addition of 5 % vanillin, was comparable to the value obtained from 25 % of DEET with 5 % vanillin added. Chemical identification by gas chromatography coupled with mass spectrometry discovered that the major constituents of Apium graveolens hexane extract (AHE) were 3-*n*-butyl-tetrahydrophthalide (92.48 %), followed by 5.10 % beta-selinene and 0.68 % gamma-selinene (Tuetun et al. 2008). The best AHE-developed product provided remarkable repellency with a median protection time of 4.5 h (4.5–5 h), which was greater than that of ethanolic DEET solution (25 % DEET, 3.5 h) and comparable to that of the best commercial repellent, Insect Block 28 (28.5 % DEET, 4.5 h).

5.4 Amelioration of Neurological Diseases

The effects of L-3-*n*-butylphthalide (L-NBP), an extract from seeds of *A.graveolens* Linn. (Chinese celery), on learning and memory was studied in a triple-transgenic Alzheimer's disease (AD) mouse model (3xTg-AD) (Peng et al. 2010). This model develops both plaques and tangles with aging, as well as cognitive deficits. Ten-month-old 3xTg-AD mice were given 15 mg/kg L-NBP by oral gavage for 18 weeks. L-NBP treatment significantly improved learning deficits, as well as long-term spatial memory, compared with vehicle control treatment. L-NBP treatment significantly reduced total cerebral amyloid-beta (A-beta) plaque deposition and lowered A-beta levels in brain homogenates but had no effect on fibrillar A-beta plaques, suggesting preferential removal of diffuse A-beta deposits. L-NBP markedly enhanced soluble amyloid precursor protein secretion (alpha APPs), alpha-secretase, and PKC alpha expression but had no effect on steady-state full-length APP. Thus, L-NBP may direct APP processing toward a non-amyloidogenic pathway and preclude A-beta formation in the 3xTg-AD mice. The effect of l-NBP on regulating APP processing was further confirmed in neuroblastoma SK-N-SH cells overexpressing wild-type human APP(695) (SK-N-SH APPwt). L-NBP treatment in 3xTg-AD mice also reduced glial activation and oxidative stress compared with control treatment. The authors concluded that L-NBP shows promising preclinical potential as a multi-target drug for the prevention and/or treatment of Alzheimer's disease.

Subsequent studies examined the effect of L-NBP on learning and memory in AβPP and presenilin 1 (PS1) double-transgenic AD mouse model (AβPP/PS1) and the mechanisms of L-NBP in reducing Aβ accumulation and tau phosphorylation (Peng et al. 2012; Xiang et al. 2014). Twelve-month-old AβPP/PS1 mice were given 15 mg/kg L-NBP by oral gavage for 3 months. L-NBP treatment significantly improved the spatial learning and memory deficits compared to the vehicle-treated AβPP/PS1 mice, whereas L-NBP treatment had no effect on cerebral Aβ plaque deposition and Aβ levels in brain homogenates. However, L-NBP induced reduction of tau hyperphosphorylation at Ser199, Thr205, Ser396, and Ser404 sites in

AβPP/PS1 mice. Additionally, the expressions of cyclin-dependent kinase and glycogen synthase kinase 3β, the most important kinase involved in tau phosphorylation, were markedly decreased by L-NBP treatment. The effects of L-NBP on decreasing tau phosphorylation and kinase activations also were further confirmed in neuroblastoma SK-N-SH cells, overexpressing wild-type human AβPP695 (SK-N-SH AβPPwt) (Peng et al. 2011b).

Peng et al. (2011a) have also shown that a common chemical component of celery extracts, L-3-*n*-butyl-phthalide (BTH) reduces amyloid precursor protein processing via mitogen activated protein kinase (MAPK) and protein kinase C pathways as well as attenuating hydrogen peroxide-induced apoptosis in human neuroblastoma cells (Peng et al. 2011b; Lei et al. 2014). Huai et al. (2013) showed that BTH reduces the activation of the AKT kinase pathways which is involved in development of vascular dementia. The authors state that BTH has been approved by the State Food and Drug Administration of China for the treatment of ischemic stroke These cellular properties indicate that BTH and other components of celery seed extracts may have multiple modes of action in neuroprotection and treatment of cerebrovascular conditions such as stroke.

6 Conclusions

Celery and celery extracts have a long history of use in many cultures to treat joint pain, gout, hysteria, nervousness, headache, weight loss due to malnutrition, loss of appetite, and exhaustion. Celery is also used to promote relaxation and sleep; to kill bacteria in the urinary tract; as a digestive aid and for regulating bowel movements; to start menstruation; to control intestinal gas (flatulence); to increase sexual desire; to reduce the flow of breast milk; for stimulating glands; treating menstrual discomfort; and for "blood purification." See the following websites for examples (http://www.webmd.com/vitamins-supplements/ingredientmono-882-CELERY.aspx?activeIngredientId=882&activeIngredientName=CELERY).

In general, the supporting clinical data for these claims are anecdotal and reference a range of products, some of which include materials other than celery. The preclinical data are somewhat more rigorous, but again encompass a range of products from a variety of sources. That said, some of the preclinical and clinical data suggest that extracts of celery seed, or specific components of such extracts, should be pursued as alternatives and/or adjuncts to conventional drugs and biologics for a number of diseases. As the rules for dietary supplements, at least with regard to chemistry, manufacturing and control, as well as safety, migrate toward the rigor of pharmaceuticals, there is increasing likelihood of the production of reproducible, defined quality product(s) that could achieve a greater role in world healthcare, as well as market share.

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Traditional Medicinal Oils Sourced from Birds: Anti-inflammatories and Potential Immunoregulants

Athol Turner, Gerald Hancock, John Wells and Michael Whitehouse

Abstract This chapter describes medicinal oils of animal origin, used in Africa and Australasia both for nutritional and medicinally for treating pain and inflammation. Analytical studies of composition, bio-efficacy and their remarkable safety are described. For obtaining reproducible benefits, it is very important to introduce Quality Controls whenever possible. These should cover all stages of production, storage and certify the 'truth in their advertising': to help eliminate adulterated products and false claims for purity and potency.

Keywords Emu · Ostrich · Mutton bird · (goanna) · Anti-inflammatory/ immunoregulant · Restorative medicine

List of Abbreviations

AI	Anti-inflammatory
AO	Anti-oxidant
EO	Emu oil
FAME	Fatty acid methylester
GO	Goanna oil
HPLC	High pressure liquid chromatography

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IS	Immunosuppressant
MBO	Mutton bird oil
OO	Ostrich oil
NZ	New Zealand
PUFA	Polyunsaturated fatty acids
SSF	Solid substrate fermentation
16:0	Palmitic acid
18:1	Oleic acid
18:2	Linoleic acid
18:3	α-linolenic acid
20:4	Arachidonic acid

1 Introduction

> It takes great grace for independent thinkers to acknowledge the truth that can flow in channels other than their own
>
> Donald Guthrie (MacDonald 1985)

Modern western medicine is historically founded on herbal products: many contemporary drugs still being sourced from the plant kingdom, e.g. digitalis, taxol, colchicine, morphine, etc. Supplementing these phytopharmaceuticals has been a smaller parcel of drugs/hormones derived from animals, the zoopharmaca; outstanding examples being ACTH, insulin, pancreatic hydrolases and thymus hormones. Another source of zoopharmaca is cold-water marine animals as a source of omega-3 fatty acids (for treating cardiac, arthritic and dermal disorders) and active principles from certain shellfish (see chapter on NZ green-lipped mussel).

Another range of traditional zoopharmaca is those sourced from birds, the avi-pharmaceuticals.[1] They are almost never mentioned in contemporary pharmacological texts although their historical use by many cultural dates back to times well before medical records were compiled and circulated in accessible languages. Here we discuss three, sourced from birds native to Africa and Australasia.

The chemistry of these 'avi-pharmaceuticals' is still largely unexplored. Their medicinal benefits have been attested for several centuries in the traditional wisdoms of various cultures and many indigenous languages. They offer scientific (and regulatory) challenges—being poorly defined pharmaca, yet providing significant clinical benefits with *minimal* toxicity. They need to understood more as multi-component, multi-potent medicinal aids; rather than as unique single drug entities.

[1]Avis(Latin) = bird; pharmaco(n) (sing.), pharmaca(plural, Greek) = drug.

An intriguing recent development has been the realisation that some (or even much) of their value arises from the action of micro-organisms (Turner 2012[2]) inducing solid substrate fermentation (SSF) in the fats (Raimbault 1998), from which the oils are derived.

They are awkward products to classify and regulate, not falling neatly into the categories used by drug-regulatory agencies to classify bio-activity and acceptability. Consequently, they are frequently either ignored altogether or derided as belonging to the ages of ignorance (i.e. pre-patent and before mega-marketing). Also they are largely unpatentable, being 'natural products' of ancient origin. Yet if a headcount was to be conducted of their regular users, their use would match that of many heavily promoted proprietary anti-inflammatories—but at low cost in terms of both price *and* adverse responses.

A major problem with many natural products used as pharmaceuticals is to ensure reproducible Quality and Availability, despite variable seasonal factors, fluctuating supply lines and minimal financial investments. Another setback has been some unscrupulous marketing involving either false claims or significant product adulteration. This has been a problem with many other alternate remedies when introduced into markets larger than their traditional user base. The difficulty is to ensure consistent quality without such stringent controls as to regulate them out of existence.

A compelling factor in adjudicating value is the remarkable safety record (and therapeutic index) of many of these avi-pharmaca used by so many generations of indigenous people as both medicines and a part of their normal nutrition. Their widespread use in cosmetics indicates their dermal compatibility and relative safety for topical use.

The low incidence of rheumatoid arthritis has been noted in both Australian Aborigines (Roberts-Thomson and Roberts-Thomson 1999) and the Eskimos/Inuits (Horrobin 1987). These people use emu oils and seal or fish oils, respectively, suggesting that nutritional lipids may increase resistance to arthritigenic agents (perhaps additional to other factors, e.g. human genetics).

Table 1 provides a summary (for time-pressed readers).

NOTE: The term 'oil' refers here to 'fixed', i.e. triglyceride nutritional oils, not to be confused with volatile, mainly hydrocarbon 'essential' oils (i.e. essences) from plants used as perfumes, flavourants, insect-repellents, solvents, etc.

2 Emu Oils

2.1 Historical Background

The evolution of emus and other ratites has been brilliantly summarised by Carroll and Martine (2011). The flightless emu, *Dromaius Novae Hollandiae*, has

[2]In this context consider the fermentation products associated with wine-making, brewing and baking. Or the essential microbial factors used to manufacture cheese, generate flavours and food additives e.g. from soya bean etc., as well as producing mainline antibiotics.

Table 1 Synopsis: traditional medicinal oils from some birds and Australian lizards (goannas)

Animal	
Emu (*Dromaius Novaehollandiae*)	• Flightless bird, origin = Australia but also farmed, Canada, China, NZ and the USA • Height up to 2 m • Has three toes and body temperature = 38.1 °C • Some excavated eggshells date back 6000 years • Long-lived, up to 80 years • Its oil (EO) = pale triglyceride oil from body fat (intestinal, rump) • Source of medicinals = antoxidants and oxylipids (triglycerides and others) • Used as anti-inflammatory (transdermal, oral) • Good[1] oils also gastroprotectant
Ostrich (*Struthiio camelus*)	• Flightless bird, origin = Africa and Middle East (Palestine)[2] now farmed in Europe, N. America and Australia and NZ • The largest bird now living (≥2.5 m tall) • Eggshells used as cups/vases in Mesopotamia 5000 years ago • Has only two toes and body temperature = 39.9 °C. Can run up to 80 km/h • Other unique features = eyelashes, large eyes, a urinary bladder (but no gall bladder) and particularly long white tail feathers (males) • Its oil (OO) = a pale triglyceride oil from body fat used as emollient and healing salve • Good[1] oils also gastroprotectant
Muttonbird (*Puffinus tenuirostris*)	• Migratory seabird, nesting in Southern Australia and New Zealand • Its oil (MBO) = preventricular (stomach) lipids from young chicks • Used orally as anti-inflammatory, externally for water-proofing and healing skin abrasions • High content of wax (60–70 %) and carotenoid pigment(s)
Goanna (*Varanidae* sp.) aka monitors also found in emu's natural habitats	• Australian lizard (other monitors found in Africa and Asia) • Its oil (GO) = brown oil from perirenal and caudal (tail) fat depots • Variable content of stabilising antoxidants • Powerful anti-inflammatory and anti-arthritic agent • Formerly widely used as liniment with/without eucalyptus oils

Notes

[1]"Good" implies not all EO or OO carry these properties, depending on (a) bird nutrition and some genetic factor(s) and (b) possibly some post-harvest (fungal) transformations of the fat before rendering → oil

[2]Many references in the Hebrew/Old Testament, Holy Bible dating from before 300 BCE and also some classical Roman and Greek authors (Turner 1544)

co-existed with Aboriginal peoples occupying the Australian continent for at least 16,000 years (attested by carbon-dated cave painting in N.Australia). They are named in nearly all surviving Aboriginal languages. Their images frequently appear in ancient bark paintings and contemporary traditional art, reflecting their veneration by the first Australians.

The bird's footprints were noted by the visiting Dutch sailors looking for fresh water in Western Australia in 1696. The earliest written records of European settlers only date back to the 1790s, but thereafter there are many descriptions by early explorers and subsequent settlers relying on Aboriginal medicines (Bennett 1860; Ghosh et al. 1995; Snowden and Whitehouse 1997). These included emu oils as a 'must have' liniment to treat surface wounds, ease strained muscles and alleviate stomach cramps. Emu eggs were also an important dietary supplement.

Intensive emu farming began in Western Australian in the late 1970s using birds sourced from Aboriginal settlements in Central Australia (Davies 2002). In the 1980s, emus began to be seriously farmed in Northern America and Europe, the main gene pool being derived from birds originally kept in zoos and fed diets of convenience. Many of the American oil products possess lesser anti-inflammatory activities (Whitehouse et al. 1998) and had triglyceride profiles more closely resembling those of commercial poultry fats (low antioxidants, low linoleate), than the Australian emu oils, derived from either feral emus or birds raised within captivity with access to natural feedstuffs.

As in so many vogue industries, the boom in emu farming soon led to a 'bust' with investors retreating as financial returns failed to meet expectations.

In Australia and New Zealand, the industry is sustained by a few tenacious farmers conserving pedigree flocks bred to resist microbial disease and increase meat and oil production (O'Malley and Snowden 1999).

2.2 *Processing of Emu Fats*

There are two main fat depots in healthy birds. One is primarily functional, protecting the internal organs (particularly when the emu is running at speed). The second one in the rump is a metabolic fuel reserve, considerably expanded when feed is plentiful. The rump fat is greatly depleted in the winter months, when the females lay several eggs and the males cease foraging while they protect and hatch the eggs (Kalaya 1990; Minnaar and Minnaar 1992; Davies 2002; Thompson 2002).

For farmed birds, oil yield and composition is largely determined by their genetics, nutrition and husbandry. Attention to these factors, e.g. diminishing stress produces healthy birds achieving maturity at 12–14 months weighing 45–60 kg and yielding 10–15 kg fat from strains of emus either captured in the wild or bred in Southern Australia. They can be typed by DNA analyses and also recognised by certain physical features, e.g. physique and neck plumage (Lacey and Lacey 1996). [These authors noted they had never seen an emu with arthritis, even among farm-reared birds.]

Both fat depots may be in contact with the skin and the transdermal roots of the bird's feathers. This particular anatomical feature might allow certain external fungi to become symbionts within the subdermal fat.

Frozen emu fat is guillotined and minced, then placed in a stainless steel vessel, heated to 75 °C while mixing until all the fat tissue is melted. For optimal activity, the oil is filtered through a 1-micron filter with no further processing.

2.3 Oil Composition

The fatty acid composition of the oil triglycerides is similar to that of olive oil and palm oil (Appendix C), notably with a high oleate (18:1), low linoleate (18:2) content. These three oils, if minimally processed contain significant antioxidants which help preserve the low levels of linoleate ex vivo for further transformation in vivo to arachidonate (20:4), the precursor of eicosanoids that both promote inflammation in the short term *and* then terminate it to stimulate healing in the longer term (Buckley et al. 2013). But unlike palm oil and most olive oils, quality emu oils contain other lipophilic entities (Dromaiols™, A, B & C). These are often absent from low quality oils, i.e. those with minimal pharmaco-activity in inflamed/arthritic rats. In fractionating oils from various sources, it was found that a rare component might behave as an antagonist, effectively nullifying the beneficial activities of the Dromaiols (Turner and Whitehouse, unpublished observations).

The fatty acid profile of emu oil is usually obtained by FAME analysis using gas chromatography to separate fatty acid methyl esters; the results normally being presented as area percent. This type of analysis does not give sufficient evidence for predicting therapeutic/biological activity of triglyceride type oils. It is also not much of use either for distinguishing fake oils, e.g. chicken oil sold as emu products. Much of the activity of emu oils is contained in the other compounds which exist in oils and can be detected by HPLC but not by triglyceride analysis (Fig. 1).

Oils used primarily in cosmetics and for massage are usually subjected to considerable refining (R), bleaching (B) and deodorising (D) and may contain additives, e.g. synthetic antioxidants to replace lost vitamin E. These RBD products are often poor sources of medicinals for treating inflammatory disorders and for aiding wound healing.

Oil 'quality' is often measured by quantifying free fatty acids (FFA) and 'peroxide content'; acceptable levels being <1 % FFA and peroxide <10 PV units (determined by methods adopted by the American Oil Chemists Society). These indices may indicate undue exposure to heat, light or moisture, (bacteria) and oxygen. As yet, there is little evidence that these measured impurities significantly reduce oil's bio-efficacy: though they may affect shelf-life and customer acceptability.

Dromaiols™ (*the signature components of anti-inflammatory emu oils*).

During 10 years of intensive investigation, emu oil samples were found to show enormous variation in therapeutic activity. Field experiments indicated this was not solely due to variations in the birds' nutrition. It was considered that if symbiotic

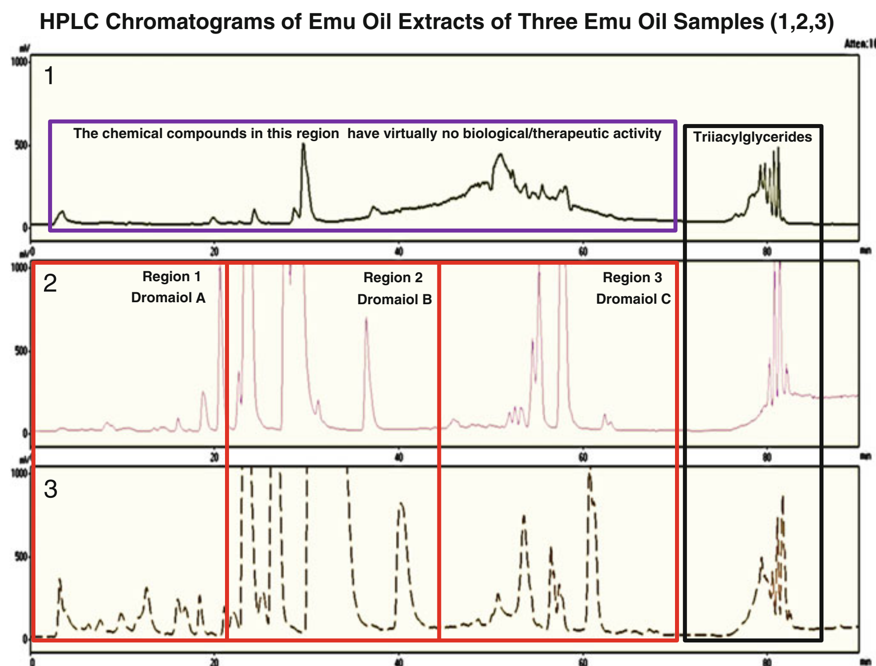

Fig. 1 HPLC Chromatograms of Three Emu Oil Samples: *Chromatogram 1* Emu oil sample from Victoria without anti-inflammatory (AI) activity. *Chromatogram 2* Emu oil sample from Western Australia. Active AI (harvested 10 years previously). *Chromatogram 3* Emu oil sample from New South Wales with AI and analgesic activities. These three chromatograms show that the chemical composition of these oil samples were vastly different

fungi/bacteria were present in the unrendered fat, they might alter the structure of some lipids prior to rendering the fat to yield oil.

To test this hypothesis, three different slabs of emu fats, samples 1, 2 and 3 from a cold store were sliced (using a band saw) and inspected prior to rendering: two samples showed significant differences in the number and colour of fungi (no bacteria were present). Aliquots of the three samples of emu fat were then rendered and the derived oil was analysed using reverse phase high performance liquid chromatography (Fig. 1) followed by in vivo testing for anti-inflammatory activity. The data showed that the changes in the chemical composition of the oil, associated with increased anti-inflammatory activity, were due mainly to fungal metabolism. For the HPLC analysis, equal volumes of emu oil and methanol were warmed, mixed for 30 s and then placed overnight in a refrigerator at 4 °C. The supernatant was decanted and stored for injection into the HPLC system.

Dromaiol fractions are identified by their bio-efficacy as anti-inflammatory agents. They are not single entities (i.e. a specific compound) but (a) clusters of molecules within a given chromatic fraction, which may contain up to 50 or more entities separable by negative ion electrospray mass spectrometry and (b) provisionally characterised by:

(i) low nitrogen content, most likely excluding alkaloids and acyl ethanolamides;
(ii) being relatively rich in oxylipids; and
(iii) low content of phenolic antioxidants.

Their further chemical characterisation is still a 'work in progress', rather dependant on continuing bioassays. The Dromaiols may not be of wholly avian origin, i.e. endobiotics synthesised in toto by emus—but rather, xenobiotics produced elsewhere—either (a) ingested with their feed or (b) generated by transformations ex vivo e.g. by fungal 'fermentation'. Paradoxically, their production may be enhanced by both (i) best practise husbandry, particularly appropriate nutrition and parasite control but also (ii) less meticulous management to exclude microbial 'ripening'. [This also seems to be true for the goanna and neatsfoot oil, used as comparators. q.v.] Active pharmaca present in 'good' oils or generated from emu fat preparations ex vivo, i.e. the Dromaiols, may be likened to special qualities in microbial processed milk, e.g. yoghourts, ripened and flavoured cheeses.

The lipid composition of chicken fat is quite similar to the emu's; but the 'oil' from the rendered factory-farmed chicken fat is not anti-inflammatory in rats. Significantly, it contains minimal amounts of Dromaiols.

The most potent emu oils obtained from relatively unstressed birds did not contain measurable quantities of cortisol (hydrocortisone) or corticosterone.

2.4 Medicinal Applications

Emu oil contains antioxidants in larger quantity than found in most ostrich or rhea oils (Bennett et al. 2008). These both help preserve the oil and are also made more bio-available by the oil acting as their carrier.

The greatest use for emu oil has been to relieve symptoms of (i) rheumatoid arthritis, mild asthma and inflammatory bowel diseases when ingested and (ii) treat skin wounds and inflammation of superficial muscles when applied topically. There is a large body of testimonials to support these applications. Studies in laboratory rats and mice with various forms of experimental inflammation have validated this testimonial evidence. Other therapeutic uses are described in the patent literature (Ghosh et al. 1995; Fein et al. 1995; Ferrante 2005).

The anti-inflammatory properties have been evaluated in inflammatory bowel diseases, NSAID-enteropathy, chemotherapy-induced mucositis, infective enteritis (fungal, bacterial and viral gastroenteritis) (Howarth et al. 2008; Lindsay et al. 2010; Turner 2012–2014). Emu oil generally has little antimicrobial activity but may be useful in Candida infections (Riley and Carson 1999). However, emus feeding on antibacterial plants (and lower tree foliage) in their native environments (Williams 2011) may accumulate bioactive terpenoids in their fat depots. [These are largely conserved when the rendering processes involve minimal heating and minimal treatment with filter aids.]

For dermal application, emu oils are often mixed with 5–15 % v/v terpenoids, e.g. cineole from eucalyptus leaves. These both reduce the oil's viscosity, allowing more

efficient spreading over the skin and *also* enhance skin penetration across/through the epidermis (Smith and Maibach 1995). Some of these percutaneous enhancers are themselves antibacterial (Cock 2008; Wilkinson and Cavanagh 2005).

Certain drugs may penetrate the skin through the shaft of the human hair follicles (Grice et al. 2010). This may also be a route of entry for some components of pharmaco-active emu oils when applied dermally.

3 Ostrich Oils

3.1 History

Ointments, unguents and cosmetic products have been prepared from ostrich fat for at least 3,000 years (Gavenji et al. 2013).

Ostriches, *Struthio camelus*, are the fastest running birds still extant and do not lend themselves readily to small scale farming. Nevertheless, broadrange flocks were established for commercial harvesting. The invention of an egg incubator by Arthur Douglas in 1869 led to development of commercial ostrich farming in many countries.

Originally, it was the long tail feathers of the male birds which were more in demand (for embellishing headgear, etc.) and birds were selected and bred for this feature. Although ostriches range throughout much of Africa, intensive farming is largely restricted to southern Africa, Israel and the USA. A rather stunted ostrich (originally from Africa) was farmed in southern Australian for feathers. After 1914, the collapse of the feather market led to many birds being released into the wild to breed as feral animals for a further 50 years, throughout the less arid parts of Central Australia. Here they competed with emus, feral camels and goats for food and water until being captured to re-establish intensive ostrich husbandry—mainly for meat and hides.

With the transition of ostrich farming from mainly providing plumes to providing durable leather and low fat red meat, the yield of carcass fat has declined. Farmed ostriches slaughtered aged 12–15 months weighing 100 ± 10 kg will deliver 25–35 kg boneless meat and approximately 3 kg fat (Sales and Franken 1996). By contrast, the fat yield from emus is usually 10–20 % of its live body weight (Minnaar 1998). The lower fat yield from ostriches may be partly due to conditions of 'finishing', adding high fibre to the diet to improve meat and leather qualities.

Ostriches have a long hind gut harbouring bacteria that digest plant fibre to provide a source of energy, so their feed costs are usually less than that for farmed emus. Having no gall bladder to store bile detergents, ostriches cannot utilise dietary fats as efficiently as emus or rheas (Minnaar 1998).

The main market for ostrich fat sourced from Israel, South Africa, USA has been the cosmetic industry and the limited unregulated medicinal applications, e.g. burn creams, arthritis rubs, sports liniments. Traditionally, it was used in Africa as a rub-on remedy for 'rheumatism' and acute pain (de Mosenthal and Hartung 1877).

3.2 Oil Quality

The fatty acid composition of some ostrich fats (Appendix C) sourced from West Africa, South Africa and Europe is distinct from emu fats, e.g. having less oleate (18:1) and more palmitate (16:0) and arachidonate (20:4) (Sales 1999; Horbanczuk et al. 2003). It is not clear if this only reflect differences in the feed and other environmental factors, rather than genetic constitution.

Analysis of low triglyceride fractions derived from African ostrich oils indicate the presence of two types of pharmaco-active principles (Struthiols™ A and C) and other signature components (Struthiols B and D) (Turner and Whitehouse unpublished).

There is an interesting contrast between South African–Zimbabwe ostrich oils and those recently farmed in Australia; the African birds were the sources of oils (Saols™) with significant anti-inflammatory activities (evaluated in rats developing experimental polyarthritis)—but the Australian birds, mainly bred from captured feral ostriches, yielded oils with minimal/negligible activity (Appendix B). These Australian ostriches retained characteristics of birds originally bred for ornamental feathers until the market collapsed in 1914. For many years, the strict quarantine regulations in Australia effectively minimised the transfer of South African breeding stock to raise the calibre of Australian-farmed ostriches (still largely persisting in an earlier time capsule).

4 Mutton Bird Oil

4.1 Natural History

The mutton bird (*Puffinus Tenuirostris*) aka yolla, short-tailed shearwater or moon bird, is a seabird belonging to the petrel family. It inhabits the shoreline and islands throughout SE Australia, notably the Bass Straits islands and also the southern coasts of New Zealand.

Eggs are laid in sand burrows in late November and the chicks hatch approximately 6 weeks later. The parent birds feed them until early April with krill, zooplankton, algae and minute fish sourced from the Southern Ocean and Antarctica waters. This feed is regurgitated by the adults into the open beaks of the juveniles and digested first into a reddish oil within the stomach and then laid down as subdermal fat around the body. [Eventually the young birds will fly north to the Arctic Circle.]

The local Indigenous peoples (Aboriginals, Maoris) take the juveniles now about 160 grams as part of their winter diet, but retain the stomach (yolla) oil for use in liniments etc. This oil was also rubbed over the skin for thermal insulation by divers and long distance swimmers, before wetsuits became available. It is still used as a waterproofing agent on wood and leather.

4.2 Oil Composition

The stomach (proventricular) oil is used both internally and externally. It differs in composition from the body fat (Cheah and Hansen 1970; Warham et al. 1976; Woodward et al. 1995) having a high content of waxes (≥60 %) with a low content of triglycerides (30 %). The fatty alcohol components of the wax are approximately 60 % saturated, 40 % monounsaturated. Polyunsaturated C_{16} fatty acids have also been found in the stomach oil (Clarke 1989; Woodward et al. 1995).

The stomach oil in petrels may arise from the slow digestion of an unusual food which the birds have learned to use with difficulty but confers significant advantages in providing energy and water reserve, food and water for chicks and an offensive/defensive weapon. Utilisation of stomach oil as energy and water sources complement other energy-savings adaptations that fit petrels for their lifestyle, such as their low body temperature and the ability of many species to travel by dynamic soaring (Warham 1977).

The carcass fat containing approximately 60 % monounsaturated acid, 10 % omega-3 PUFA and 3 % omega-6 PUFA is readily melted (<40 °C) to use as a nutritional supplement and also as a lamp oil and for making soaps.

Other distinctive feature of yolla oil are (a) its red carotenoid pigments, mainly astaxanthin (≥0.2 % w/v) derived from krill, that is a powerful antioxidant, immunomodulator and natural preservative (Jyonouchi et al. 1993; Capelli and Cysewski 2012); and (b) its minimal processing; in contrast to most commercial triglyceride oils. These usually require heating and refining by bleaching, deodorising, degumming with steam or solvent extraction and exposure to alkali etc. (Erasmus 1993[3]; Gillespie 2012). Some of these operations generate free fatty acids and unhealthy artefacts—notably *trans*-unsaturated, toxic epoxides and hydroperoxides.

4.3 Pharmacology

Horse trainers in Australia and New Zealand have used the oil both as a feed supplement and a rub-on liniment to bring racehorses into condition for strenuous racing. Oil efficacy is judged very objectively by extra race winning, i.e. prize monies.

Testimonials from patients indicate its value for treating weight loss (from tuberculosis), asthma, bronchitis, rheumatism, juvenile arthritis, hypercholesterolemia, eczema, sunburn and as a skin moisturiser. The yolla oil mixed with

[3]Udo Erasmus (1993) noted ‘the most easily destroyed oils are also *nutritionally* the most valuable’ and that ‘refined white fats and oils are nutritionally equivalent to refined white sugar and white flour’. But this may not be true for *medicinal* oils from animals and birds, which predominantly contain saturated or mono-unsaturated lipids (before fungal fermentation).

methylated spirits (denatured ethanol) is used by bird harvesters as a liniment to treat skin abrasions, dermal inflammation, etc.

The efficacy of Australian and New Zealand yolla oils has been compared with commercial polyunsaturated oils from seals, squid and many cold water ocean fish species. It is ranked as the most potent of over 30 marine oils tested as an anti-inflammatory (AI) and immunosuppressant (IS) agent when applied dermally or given orally to rats developing experimental arthritis (see Appendix B). This is probably the sum of both the efficacy of the AI lipids together with some antioxidant (AO) activities from the carotenoid pigments. Parallel assays show that antioxidant β-carotene, luteins (from marigolds) and lycopene (from tomato) dispersed in olive oil, can also exhibit some anti-inflammatory activity in rats developing polyarthritis (Schlipalius and Whitehouse unpublished).

5 Comparison of Bird Oils with Other Animal 'Oils'

These comments are based on both (a) subjective testimonials from past authors and many patients and (b) objective assays with laboratory rats developing inflammation and chronic arthritis (the methodology used preclinically to evaluate modern NSAIDs since the late 1950s).

5.1 Goanna Oil

This is another traditional Aboriginal remedy, also much valued by the early European and Chinese settlers in Australia. Of the 40 identified species of goannas, also known as monitors or varanids, at least 25 are found on the Australian continent (Swanson 1976). Being cold-blooded animals, they are fairly easy to capture during the cooler seasons and are not poisonous. They have adapted to many environments: some living in trees, others in bushland; some being virtually amphibious and others living in arid regions. Like emus, goannas are fairly omnivorous consuming small animals, birds, snails, insects, fish, crustacea and eggs. [Larger goannas invading golf courses are known to swallow the golf balls.] The sand monitors (N.Queensland) for many years sustained a commercial goanna oil industry based in Brisbane (Gregory 1985).

Goannas are now a protected species outside Aboriginal Reserves. Their numbers have notably declined in recent years attributed to less feed, more pesticides, more predators (e.g. feral pigs) and climatic change. Goanna eggs are laid and incubated in termite mounds, a source of warmth for their long incubation; but in many inhabited parts of Australia these natural hatcheries are disappearing.

The oil is sourced from perirenal fat and extensive caudal fat depots, which may be considerable in good seasons. [Fat from an animal one metre long may deliver half a litre of oil.] This oil is usually brown, pink or yellow—depending on

dominant food sources, being traditionally collected as the whole animal was roasted. Mixed with eucalyptus oils (5–50 % v/v), it was extensively used as a liniment for treating burns, abrasions and relieving sore muscles; effectively combining antiseptic with anti-inflammatory activities to promote early healing. The eucalyptus oil effectively 'thinned' the goanna oil, facilitating both its dermal application as a 'rub-on' and subsequent percutaneous absorption. The whole animal fat was also consumed for its nutritional *and* medicinal benefits.

Laboratory studies indicated that several samples of goanna oil were more potent than many emu oils (Appendix B). Some unrefined dark oil samples have retained activity for at least 20 years, when stored in the dark at ambient temperatures (Hancock and Whitehouse unpublished).

5.2 Neatsfoot Oil

This was also used medicinally by stockmen and other residents of the Australian bush. It is a liquid fat derived from the hooves of cattle and horses. Its composition distinguishes it from the more saturated depot fats/tallows from these animals. It was often used by country people for treating inflammation in horses and themselves, particularly valued as a water-proofing agent and a skin conditioner. Some samples have shown remarkable anti-inflammatory activity when applied dermally to rats; others have not. These variable properties of neatsfoot oil probably reflect their provenance, particularly the fodder (antioxidant status), conditions of rendering the hoof fat and subsequent storage. Hilditch (1956) has emphasised how both feed and the gut flora may determine linoleate (18:3) content of the hoof oil. In some instances, the 18:3 content may exceed the 18:2 content (Gunstone et al. 1986).

Unlike most liquid fats which deteriorate with age (rancidification), older neatsfoot oils stored for 5 years or more at ambient temperatures (5–35 °C), though sometimes malodorous, still retain their potency. Contamination with traces of iron, either haematogenous or from using sheet iron plates for melting hoof fat, may promote aerobic oxidation ex vivo forming hydroxy-lipids, reactive aldehydes, etc. that confer further anti-inflammatory benefit. [This may also be the situation in primitive processing of emu fat.]

5.3 Other Animal Sources

Depot fats from a number of Australian animals, farmed or feral, living in emu and goanna country were generally too 'hard', i.e. saturated to produce much oil at reasonably low temperatures. These included free-ranging sheep, cattle, wild boars, goats and camels together with domesticated pigs, ducks, geese and chickens. A few samples of fat from feral camels browsing in Central Australia did yield

anti-inflammatory oils but in such low yields (<10 %) to be not a reliable and productive source. This situation might be changed by modifying both the camel's nutrition and/or their gastrointestinal symbiont microflora.

One conclusion from these miscellaneous observations is that the activity of anti-inflammatory animal oils may largely depend upon (i) the quality of the animal feed, especially antioxidant content; (ii) genetic factors and (iii) likely transformation(s) of the fat ex vivo, e.g. fungal hydrolysis and/or oxygenation; effectively increasing the yield of non-steroid anti-inflammatory agents.

6 Conclusions

This survey of some traditional treatments for inflammation also raises some compelling questions:

(i) Can we afford to lose/overlook them?
(ii) Can we improve them (stability, potency)?
(iii) How can more rigorous clinical studies be carried out, allowing honest comparisons with vigorously promoted products from the pharmaceutical industry?

(A) **Concerning oils**: Many oils—of mineral, vegetables or animal origin—are potential immunological stimulants (adjuvants); that is they can raise the profile of 'non-self' components to the immune system, eliciting 'anti-self' responses that underlie many auto-immune diseases. These non-self products (crypto antigens) may be exogenous, e.g. microbial immunogens or of endogenous origin, e.g. cartilage breakdown products released from inflamed/damaged joints. They trigger experimental autoimmune responses/disease when co-presented with mineral oil or many vegetable oils, acting as adjuvants.

By contrast, these bird (and goanna) oils are remarkably feeble adjuvants and useful medicines—but only under conditions that conserve their active constituents, for example:

(i) They are minimally processed, retaining natural preservatives and acquiring minimal contaminants (no solvent residues);
(ii) When farmed, the birds and lizards should have access to a range of feed components, much the same as they have in their native environment;
(iii) Their genetic constitution;
(iv) Possible exposure to beneficial fungi, able to transform less active lipid precursors into more potent pharmaco-active species (Turner 2012).

In summary, these animal/bird oils are virtually harmless—in contrast to so many undigested plant and indigestible mineral oils that may become adjuvant-active and therefore pro-pathogenic (Whitehouse et al. 1974; Whitehouse 2012; Vera-Lastra et al. 2013).

(B) **Some consequences**: These desirable features need to be controlled constructively to ensure reproducible qualities of avian medicinal oils and conserve lack of pathogenicity (Appendix B). This would include preventing rancidification, i.e. aerobic destruction of essential unsaturation, and also avoiding gross chemical manipulations, e.g. hydrogenation.[4]

(C) **Oils as Carriers**: What they carry may determine their value. Fractionation of pharmaco-active emu, ostrich and goanna oils has shown that much of their anti-inflammatory potential is contained in a largely delipidated fraction, constituting less than 10 % v/v of the whole oil (Turner 2012). Diluting these lipophilic active concentrates into inert olive oil or even admixing them with some toxic mineral oils, creates anti-arthritic pharmaca for transdermal or oral administration. These 'concentrates' also carried much of the gastroprotectant activity of the whole oils (See Appendix B). Such 'molecular transplants' may resolve some problems of supply, demand and costs of bulk transportation; allowing (i) active *concentrates* to be more readily and rapidly distributed from sources of supply to distant centres for reformulation but (ii) may create a problem with those drug-regulatory agencies which might now interpret these concentrates to be no longer a foodstuff and therefore considered a drug! [Preparation of concentrates is commonly practised in the cordial, fruit juice, tea and coffee industries—so why not conserved natural medicinals?]

(D) **Further reflections**: looking back *and* looking forward

This chapter has discussed some aspect of 'farming for pharmaca' to derive medicinally potent emu, ostrich and even neatsfoot oils; showing that animal resources may still be important in contemporary medicine beyond providing hormones and other regulatory factors, e.g. insulin, heparin, etc. These avian resources are renewable and sustainable when sensibly farmed. Harvesting oils from free-ranging goannas and mutton birds continues the tradition of ancient hunter-gathering populations to obtain both foods and medicines, i.e. before the development of intensive agriculture and commodification of medicines.

By commercial standards focussed on volume production, these therapeutic oils may be deemed insignificant. But their long history as *nutritional* medicines—and continuing use today—indicates they may still be just as helpful as the most recently trumpeted advance in synthetic/biological pharmaceuticals. Moreover, they can still provide novel vistas for the development of further, less toxic, anti-inflammatory and restorative agents. As such, they deserve respect—until disproven in rigorous trials. Prejudice, i.e. *pre*-judgment should have no place in contemporary integrated medicine.

[4]As Erasmus also noted, "Healers and manufacturers head in opposite directions regarding oils", adding the sensible *nutritional* advice, "Eat things that spoil, but eat them before they do".

(E) **Some unfinished business**: This survey has only indicated *why* some particular animal-sourced products, long used as traditional medicines, are still with us today; providing significant benefits when due attention is paid to some critical factors that determine their pharmaco-activity.

But this review has not described *how* these zoopharmaca act at the molecular and cellular levels. The challenge may seem simple but the answers will probably be complex. This is because these natural anti-inflammatories are:

(i) Poly pharmaceuticals, i.e. mixtures of components—some being intrinsically pharmaco-active—with others that may be supportive/synergistic (but nonetheless essential for optimising bioavailability and medicinal activity); and
(ii) Only effective because of particular genetic and environmental factors primarily associated with the animal *donors* (and their symbionts); and
(iii) Able to meet the needs of the *recipients*, particularly patients with nutritional deficits, dysregulated immunity and other physiological imbalances underlying persistent pain, sustained inflammation and retarded healing.

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Appendix A

Avian-sourced anti-inflammatories: some positive features and negative factors

Positive	Negative
Renewable tresource	Sustainable(?)
Extended experience (safety, efficacy, etc.)	This ‘wisdom’ is often derided/ignored, having little profit value
Favourable attributes for rural production: – Minimal energy input – Minimal polluting output – Marketable by-products, e.g. meat, hides, feathers – low labour component	
Extra-urban farming/sourcing	
Suitable for countries with limited resources (energy, raw materials)	
Low financial investment for production, i.e. fences rather than factories	Insufficient investment for rigorous clinical trials—if made obligatory
Scope for quality improvement in:	Erratic quality and lack of Quality controls[a]

(continued)

(continued)

Positive	Negative
– Breeding (birds and symbiont microbes) – Efficient feed stuffs – Parasite control – Product stability – Pharmaco-availability, e.g. improving transdermal delivery	

[a]Particularly to eliminate bad/fake products being marketed as substitutes for genuine medicinal oils

Appendix B

A comparison of (i) anti-arthritic and gastroprotective (GP) activities with (ii) the safety of some bird and goanna oils in rats developing experimental arthritis

Doses = ≤2 ml/kg oil

Efficacy scores = asterisks (*), toxicity scores = + on a scale 0–4; as indications of a potential therapeutic index, i.e. a benefit: risk ratio to be derived here as anti-arthritic/pathogenic scores, i.e. (*/+)

Source of Oil	Anti-arthritic efficacy			GP activity[3]	Pathogenicity[4]	
	Dermal[1]	Oral-ST[2]	Oral-LT[2]	Oral (%)	AIA	CIA
Emu fat, Australia						
WA (Kalaya)	3*	3.1*	2.6*	58	0.6+	0.3+
Qld (Cherburg)	3.3*	1.2*	3*	68	+	0.5+
NSW (Turner)	3.7*	3.8*	2.8*		0.2+	
Vic (Baramul)	3.5*	3.8*	3.4*	100	0.5+	1.2+
Others (n = 23)	<2.0*	0.9*	<0.5*	<23	1.7–3.3+	
Ostrich fat from birds in						
Africa (n = 3)[5]	3.6*	1.2*	1.7*	82	0.4+	1.7+
Vic (n = 1)	2.1*	1.8*		63	2.3+	
NSW, Qld, SA, WA (n = 7)	<1.1*		07*	07	3+	
Muttonbird (proventricular oil)						
Tas (Yolla, n = 3)	33*	0.7*	0	84	0.2+	

(continued)

(continued)

Source of Oil	Anti-arthritic efficacy			GP activity[3]	Pathogenicity[4]	
	Dermal[1]	Oral-ST[2]	Oral-LT[2]	Oral (%)	AIA	CIA
						0.5 +
SA (Bickford)	1.4*				2.3+	
NZ (Stewart Is)	3*		0.3*		0.3+	
Goanna fat						
Qld (n = 3)	4*			54	0.2+	0.3 +
NSW (n = 1)	2.7*				0.4+	0.7 +

Data = mean values from replicate studies (n = 2–7), each with 3–5 rats per group. Experimental details given in published reports (Whitehouse et al. 1998, 2001; Turner 2012) also outlined below.

Key to Table:

1. Rubbed into shaved dorsal skin after mycobacterial-induced (adjuvant) arthritis was established for 10 days. Effects quantified by *reduction* in arthritic scores, compared to store-bought light olive oil (**** = very effective, i.e. no arthritis and 0 = no effect).
2. Given orally once daily for short term (ST) dosing = 4 days only beginning after first sign of arthritis expression (usually day 10) as in (1) above, or long-term (LT) dosing = for 15 days from time of arthritis inception.
3. Inhibition of gastric bleeding from oral ibuprofen (80 mg/kg) in arthritic rats (Whitehouse et al. 2001), oils administered orally 5 min previously: data here = mean of three studies.
4. Adverse immunostimulant (potential adjuvant) activity for inducing arthritis with mycobacterial (AIA) or collagen type-II (CIA) arthritigens (see text). These scores are toxicities on a scale of 0–4+; 0 = no induction of arthritis (4 + = maximum severity when mineral oil was the adjuvant oil).
5. Two from South Africa, one from Zimbabwe.

Sources of traditional medicinal oils

- Emu. *Dromaius novaehollandiae* = flightless bird native to Australia.
- Ostrich. *Struthio camelus* = flightless bird, originally from Africa and the Middle East.

- Muttonbird. *Puffinus tenuirostris* = seabird from Southern Australia and New Zealand
- Goanna (Monitor lizards) of *Varanidae* family found in North and Central Australia. (Other monitors inhabit Asia and Africa)

Key to sources:
NSW, Qld, SA, Tas, Vic, WA = Australian States; NZ = New Zealand

Appendix C

Fatty acid composition of some triglyceride oils

Data = % w/w of total fatty acids from triglycerides (and waxes)

Fatty acid	Human skin		Emu oils from					Olive oils			Palm oil[7]
	S.Africa (SD)[1]	USA[2]	USA[2]	S. Aus[3]	Vic[4]	WAust[5]	Aus (SE)[6]	CDX[7]	Malta[8]	ESP[3]	
12:0	0.4 (0.2)						0.4 (0.7)				
14:0	3.4 (1.2)	2.1	0.4		0.3	0.4	0.4 (0.1)				0.5 = 5.9
16:0	22.7 (2.6)	20.2	22.0	24	19.8	26.0	20.4 (2.3)	7.5–20	15.9	10.4	32–59
16:1 ω7	8.4 (2.2)	3.8	3.5	4.3	2.8	4.7	3.7 (1.0)	0.3–3.5	1.4	0.7	
18:0	4.3 (1.6)	11.2	9.6	8.5	8.5	9.8	9.3 (1.2)	0.5–3.5	2.6	3.1	1.5–8.0
18:1 ω9	45.4 (4.1)	30.8	47.4	49.1	45.6	48.0	48.4 (4.6)	56–83	65.9	73.9	27–52
ω7							2.1 (0.3)				
18:2 ω6	9.7 (3.6)	15.1	15.2	9.5	19.4	8.6	11.4 (3.7)	3.5–20	11.7	8.4	5–14
α18:3	0.7 (0.4)	0.3	0.9	1.1	1.4	0.4	2.4 (5.5)	<1.5	0.8	0.7	<1.5
18:4					–	–					
20:0	0.9 (0.5)				0.1	0.2	0.2 (0.1)		0.5		
20:1 ω9						0.3	0.3 (0.2)		0.3		
20:4 ω6	≤0.5										
20:5 ω3											
22:6											

Fatty acid composition of some triglyceride oils

Data = % w/w of total fatty acids from triglycerides (and waxes)

Fatty acid	Ostrich oils from			Muttonbird oils from						Neatsfoot oils		
	W.Africa[9]	S.Africa[10]	Poland[11]	NZ[12]	*P.ten.*[13]	Yolla-1[13]	Yolla-2[13]	Tas(P)[14]	Tas(A)[14]	Horse[15]	Cattle[16]	X[17]
12:0												
14:0	0.9	0.8	0.7 (0.1)	4.0	5.4	3.3	3.9	2.5	6.1	0.8	1.0	1.5
16:0	24.8	28.4	20.3 (0.9)	7.7	15.8	8.7	7.2	3.7	14.6	17.9	18.2	12.6
16:1 ω7	5.6	8.4	0.4 (0.1)	9.9	15.3	13.9	9.5	9.3	7.9	18.8	11.9	9.7
18:0	5.9	6.3	0.6 (0.1)	1.3	1.8	1.1	1.3	3.7	3.6	2.5	3.6	2.4
18:1 ω9	39.7	16.9	36.4 (1.2)	25.2	24.8	35.4	26.6	25.0	29.9	34.3	60.5	64.4
ω7				4.8		5.4	5.3					
18:2 ω6	17.0	13.3	16.2 (1.4)	1.9	1.8	1.8	2.0	3.1	4.0	5.1	2.9	2.1
α18:3	3.8	4.9	16.0 (1.2)	0.5	0.9	–	0.5	4.0	1.7	16.9	0.7	2.0
18:4				2.3	2.6	1.9	2.5					
20:0	0.3				0.2			0.1			0.1	
20:1 ω9				2.9	5.5	4.4	2.7	0.1	5.5			
20:4 ω6			6.7 (1.2)	0.7	1.2		0.7	1.8	2.1			
20:5 ω3				14.0	7.5	5.3	13.5	13.2	6.1			
22:6 ω3				10.2	9.1	3.0	10.9	5.7	14.4			

Footnotes to Appendix C (Parts 1 and 2)

1	Subcutaneous adipose tissue: mean value (±SD) for 137 South Africans, 3 racial groups (Krut and Bronte-Stewart 1964)
2	American Emu Association (Minnaar 1998; Craig-Schmidt 1998)
3	South Australia (Abimosleh et al. 2013)
4	Baramul Castlemaine, Vic
5	Kalaya, Wiluna W.A.
6	Pan-Australian Survey: mean values (±SD) for 48 oils (Turner 2014, unpublished)
7	Codex Committee on Fats and Oils (Gunstone et al. 1986)
8	Wardija = indigenous Maltese monoculture (reputed to be 2000 years old)
9	Kept in captivity UK (Gunstone and Russell 1954)
10	Abdominal fat (Sales 1999; Sales and Franken 1996)
11	Culled females, n = 6 (Horbanczuk et al. 2003)
12	Stewart Island, New Zealand
13	Bass Strait Islands, Tasmania (Warham et al. 1996) and recent harvesting 2011, 2013 (Y-1, Y-2)
14	P = proventricular (stomach) oil; A = adipose tissue (Clarke 1989; also see Woodward et al. 1995)
15	Washpool Stud, Qld. Oil aged 6 months at room temperature
16	Irish cattle (Hilditch 1956)
17	Source unspecified; also cite data from Argentina (Barr et al. 1970)

Appendix D

Some pharmacogenic fungi that transform emu fat (SSF)
Epicoccum purpurescens
Mucor BB12
Mucor BB16
Penicillium chrysogenum
Rhizopus stolonifer
Absidia sp
Alternaria alternata
Chaetomium globosum
Chaetomium sp
Cryptococcus albidus
Mucor BB13
Mucor BB14
Mucor BB15

(continued)

(continued)

Some pharmacogenic fungi that transform emu fat (SSF)
Mucor BB18
Mucor Black
Mucor spp
Nigrospora sphaerica
Penicillium janczewski
Penicillium sclerotiorum
Rhodotorula mucilaginosa
Trichosporon pullulans

MacGee and Turner (unpublished)

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The Genus Aloe: Phytochemistry and Therapeutic Uses Including Treatments for Gastrointestinal Conditions and Chronic Inflammation

I.E. Cock

Abstract Plants of the genus Aloe have perhaps the longest recorded history of medicinal usage and are amongst the most widely used plants for traditional medicinal purposes worldwide. *Aloe vera*, *Aloe ferox*, *Aloe arborescens* and *Aloe perryi* are the best known and most widely used, but many other species are also used for their therapeutic properties. The Aloes have been used since ancient times, particularly for the treatment of microbial infections, gastrointestinal disorders and inflammatory conditions. In addition to their myriad uses in traditional therapeutics, the Aloes have also been used as components of cosmetic formulations, and in the food and beverage industries. Despite their wide acceptance, studies from different laboratories often report wide variations in the therapeutic bioactivities from within the same Aloe species, even when the same extraction procedures are used. Furthermore, leaves from individual Aloe plants within the same species may have widely varying levels of the bioactive phytochemicals. Phytochemical analyses have shown that many Aloe species contain various carbohydrate polymers (notably glucomannans) and a range of other low molecular weight phenolic compounds including alkaloids, anthraquinones, anthrones, benzene and furan derivatives, chromones, coumarins, flavonoids, phytosterols, pyrans and pyrones. There has been a wealth of information published about the phytochemistry and therapeutic potential of the Aloes (especially *Aloe vera*). Much of this has been contradictory. Intra- and interspecies differences in the redox state of the individual Aloe components and in the ratios of these components may occur between individual plants. These factors may all affect the physiological properties of Aloe extracts. Due to the structure and chemical nature of many of the Aloe phytochemicals, it is likely that many of the reported medicinal properties are due to antioxidant or prooxidant effects. The antioxidant/prooxidant activities of many

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Aloe phytochemicals depend not only on their individual levels, but also on the ratios between the various components and their individual redox states. Therefore, discrepancies between bioactivity studies are likely when using different crude mixtures. This report aims to summarise the phytochemistry of the Aloes and (a) examine how their constituents may be responsible for their medicinal properties and (b) some possible reasons for the wide variations reported for their medicinal properties and (c) their therapeutic mechanisms. Some future areas of research into the medicinal activities of this important genus are also highlighted.

Keywords *Aloe vera* · Inflammation · Anticancer · Antioxidant · Anthraquinone · Anthrone

1 Introduction

Aloe (Asphodeloideae) is a genus of flowering succulents consisting of over 500 known species including trees, shrubs and perennials. The genus is predominately native to Africa, with species also found on the Arabian Peninsular and Jordan, as well as on several islands including Madagascar off the African coast (Fig. 1). Whilst species overlap occurs across regions, the greatest biodiversity of Aloe species is in Southern Africa (Angola, Botswana, Lesotho, Malawai, Mozambique, Namibia South Africa, Swaziland, Zambia and Zimbabwe), with approximately 290 species reported (Reynolds 2004). East Africa (Burundi, Djibouti, Eritrea, Ethiopia, Kenya, Rawanda, Somalia, South Sudan, Tanzania, Uganda) also has a remarkable Aloe biodiversity, with approximately 200 species present, whilst Madagascar and the Indian Ocean Islands contain approximately 90 species. The Arabian Peninsula is the habitat of approximately 50 species and nearly 30 species are present in central and western African countries.

There is much confusion regarding Aloe systematics and taxonomy. The genus was previously assigned to the families Liliaceae and Aloeaceae (Viljoen 1999). More recently, the genus was classified to the family Asphodelaceae, until this was later merged into the family Xanthorrhoeaceae as a subgrouping. Confusion also exists regarding the naming of individual species. The best-known Aloe species (*Aloe vera* (L.) Brum. f.) was originally classified by Carl Linnaeus as *Aloe perfoliata* var. *vera* in 1753 and is still occasionally referred to under this name. In 1768 it was independently classified as *Aloe vera* (L.) Brum. f. and as *Aloe barbadensis* Miller in two books published within days of each other. Both names persist and are commonly found in the botanical and scientific literature. Furthermore, Aloe species are also often referred to by common names, further confusing the taxonomy. *Aloe vera,* for example is often reported as Barbados Aloe, Burn Aloe, Chinese Aloe, First Aid Plant, Indian Aloe and True Aloe. Furthermore, it is not uncommon for lay persons to mistakenly refer to multiple species under the broad name *Aloe vera*, further confusing the taxonomy. Whilst it is important to always note the classification system

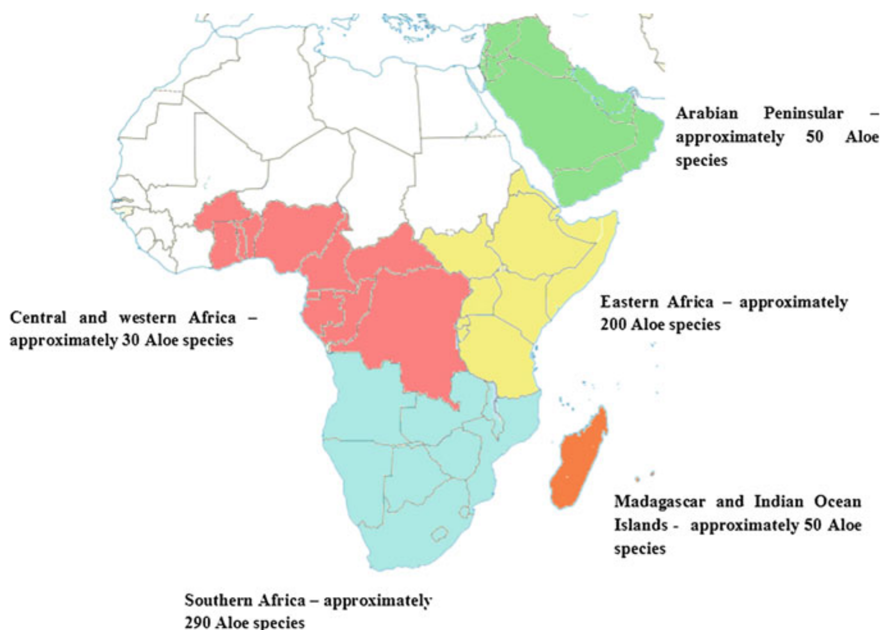

Fig. 1 Aloe species biodiversity distribution by region, based on the number of species per country reported by Reynolds (2004)

used in technical literature, this is especially true for the genus Aloe. In this review, I will refer to the most common species using Brum. systematics (i.e. *Aloe vera* (L.) Brum. f., or *Aloe vera* for simplicity). The classification system will be noted for all other species described when first mentioned.

All Aloe species are evergreen with most species having distinct rosettes of thick, fleshy, often sword-shaped leaves terminating at the trunk or branches (in branched species). The leaves of individual species vary greatly in size, colour and the presence and distribution of prickles on the leaf margins and faces. The flowers (usually shades of yellow, orange or red) are generally tubular to narrow bell shaped and are borne densely clustered at the apex of long stemmed spikes. Following fertilisation, oval fruits (up to 5 cm long) form. These usually change from green to brown as the fruit ripens.

The Aloe leaf is the portion of the plant most commonly used medicinally. Structurally, the leaf can be divided into three major parts (Fig. 2):

- The outer green rind primarily consists of structural components, but also contains anthraquinones, preanthraquinones, and their glycosides (Reynolds 2004)
- The outer pulp region below the rind consists of vascular bundles and is where the bitter latex or sap is derived. The latex consists predominantly of phenolic compounds including anthraquinones and preanthraquinones, anthrones, chromones, coumarins, flavonoids and pyrones (Gutterman and Chauser-Volfson 2000).

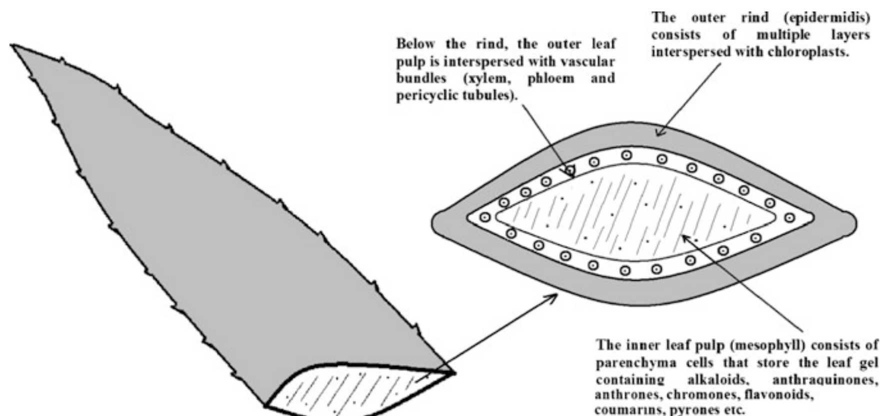

Fig. 2 A schematic representation of Aloe leaf morphology showing a leaf cross section

- The inner leaf pulp consists of the aloe gel containing parenchyma cells. The pulp of all Aloes has a high water content (approximately 99 % for *Aloe vera*; Hamman 2008; Eshun and He 2004) and contains a high acemannan polysaccharide content, as well as a variety of phenolic phytochemicals including alkaloids, anthraquinones, anthrones, chromones, coumarins, flavonoids and pyrones (Reynolds 2004; Dagne et al. 2000). The pulp also contains vitamins, minerals, enzymes and proteins (Boudreau and Beland 2006).

Aloes have been used therapeutically for thousands of years, with written evidence of their usage recorded in the herbal Dioscordes Codes Aniciae Juliana in 512 AD. This text also provides the first published illustration of any Aloe species, although much earlier South African rock paintings have been reported to depict *Aloe ferox* Miller and *Aloe broomii* Schönland in ceremonial and therapeutic usage (Reynolds 2004). It is likely that Aloes have been part of the traditional healing systems of a wide variety of cultures since before written history. Indeed, a recent study has indicated that several Aloe species were incorporated into the pharmacopoeas of the Khoikhoi (Hottentot) and San (Bushman) of South Africa (Van Wyk 2008a). As these populations are amongst the oldest surviving ethnic groups in Africa (dating back approximately 60,000 years; Tobias 1957), it is likely that plants of this genus are amongst the earliest recognised medicinal plants. Interest in the therapeutic properties of this genus has increased dramatically in recent years, with Aloe now a familiar component in a wide variety of healthcare products and cosmetics worldwide.

Despite the widespread usage and recent commercial appeal of Aloe products, only a few species are currently traded internationally. The most important of these in terms of volume traded is undoubtedly *Aloe vera* which has been domesticated and is now extensively grown commercially in many countries. This species has also attracted the most extensive scientific study and will therefore be highlighted in this review. However, other Aloe species (including *Aloe ferox* Miller and *Aloe*

arborescens Miller) are also commercially important components of many products. *A. ferox* (commonly known as cape aloe) is the main species used to produce the purgative drug 'cape aloes', whilst *A. arborescens* (often called Japan aloe after its widespread introduction and usage in Japan) leaf gel is a common component of many tonics and cosmetic products. *Aloe marlothii* Berger (traded as Natal aloe) and various unknown east African species (collectively traded as Kenya aloe) are other commercial products. Other species of Aloe, whilst not widely traded outside of traditional healers markets, are also known to have medicinal properties and will therefore be considered in this review.

The commercially most important species, *Aloe vera* (L.) Brum. f. is a stemless or short stemmed species of Aloe growing 60–100 cm tall. *Aloe vera* has been widely cultivated internationally for thousands of years resulting in its origin and natural range becoming unclear. Recent DNA sequence comparisons have suggested that it is closely related to *A. forbessi* Balf. f., *A. inermis* Forssk., *A. perryi* Baker, *A. scobinifolia* Reynolds & Bally and *A. sinkatana* Reynolds (which are all native to Somalia, Sudan and Yemen), indicating that it may have originated from the Arabian Peninsular or northern Africa (Grace et al. 2008; Darokar et al. 1999; Truetlein et al. 2003). Today, its use is widespread, not only amongst practitioners of complimentary and alternative medicine, but also by individuals in western countries who would otherwise only utilise allopathic medicines. As well as its pharmaceutical uses, *A. vera* is also used in the food and beverage industry and in the cosmetic and toiletry industry. Indeed, with its wide commercialisation, nowadays it would be difficult to find an individual who had never used *A. vera* or a product containing *A. vera* leaf components. Thus, *A. vera* arguably bridges the gap between herbal medicine and allopathic medicine more effectively than any other plant species.

2 Ethnopharmacology

Members of genus Aloe have been used for a broad range of medicinal purposes by traditional healers from a wide variety of ethnic and cultural groupings worldwide (Table 1). The most widely used and best documented is the species *A. vera*. The history of *A. vera* usage as both a curative/therapeutic agent as well as a component of cosmetics dates from ancient times (Reynolds 2004; Bancroft and Myskja 2003). Many cultures including African, Arabic, Asian, European and American have used *A. vera* for a wide variety of therapeutic purposes for hundreds to thousands of years. In most of these areas *A. vera* is an introduced species, although it has now been incorporated into the local pharmacopoeas.

The dried leaf exudate was traditionally the main medicinal agent used for most treatments, although the fresh gel also had many uses (Reynolds 2004). Since the time of the Roman empire (and likely before), *A. vera* latex had been prescribed on the Arabian Peninsula in small doses as a tonic and to improve digestion. In larger doses, it is useful as a laxative and purgative and as an emmenagogue. *A. vera*

Table 1 A summary of the origin, ethnomedical uses and major classes of phytochemical constituents for selected Aloe species

Species	Common name	Origin/distribution	Indications for medicinal use	Known constituents	References
Aloe affinis	Unknown	Occurs on rocky slopes in Mpumalanga Province of South Africa	To relieve pain	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008), Grace et al. (2008)
Aloe africana	Uitenhage aloe	Grows at sea level around the Port Elizabeth area, Eastern Cape Province, South Africa	Juice used by the Khoi-San as a laxative and purgative	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008), van Wyk (2008a, b)
Aloe arborescens	Candelabra aloe, Krantz aloe, inkalane (Zulu)	Widely distributed although endemic to southern Africa (South Africa, Malawai, Mozambique, Zimbabwe)	Pulp is used to treat wounds and to reduce microbial growth. Leaf extracts used to treat X-ray burns. The leaf gel is used as a general tonic and in cosmetics. Also has purgative and laxative properties	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008), Grace et al. (2008)
Aloe barberae	Tree aloe, boomaalwyn (Afrikaans), inKalane enkulu (Zulu)	Subtropical coastal regions and valleys of south eastern Africa including South Africa, Swaziland and Mozambique	The leaf sap is used for the treatment of burns, sores and wounds. Also has purgative and laxative properties. It	acetylated mannans, polymannans, anthroquinones and anthraquinone	Ndhlala et al. (2009), van Wyk and Smith (2008), Grace et al. (2008)

(continued)

Table 1 (continued)

Species	Common name	Origin/distribution	Indications for medicinal use	Known constituents	References
			also has antiinfective and antiinflammatory properties	glycosides, anthrones, lectins	
Aloe broomii	Bergaalwyn or Slangaalwyn (Afrikaans)	Occurs on rocky slopes of large areas of the interior of South Africa	Boiled leaf infusions was used to treat ear ailments in sheep	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008), Grace et al. (2008), Reynolds (2004)
Aloe buettneri	Unknown	Western African savannahs, especially in Nigeria, Senegal and Togo. Also reported as far south as Malawi and Zambia	Leaves used to treat burns, wounds and insect bites. The dried leaves are used in Burkina Faso to treat malaria. The roots are used similarly in Côte d'Ivoire and Togo. Leaf also used to treat rheumatism and in Nigeria as an antihelmintic	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	Burkill (1995)
Aloe broomii	Snake aloe	Indigenous to South Africa and Lesotho	Used as a disinfectant, and as an ear remedy as well as veterinary uses to purge ticks from livestock	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	Grace et al. (2008), van Wyk et al. (2008)
Aloe chabaudii	Unknown	Widely distributed across South Africa, Swaziland,	Used as an abortifactant	acetylated mannans,	

(continued)

Table 1 (continued)

Species	Common name	Origin/distribution	Indications for medicinal use	Known constituents	References
		Zimbabwe, Zambia, Malawi and Tanzania		polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008), Grace et al. (2008)
Aloe christianii	Unknown	Southern Africa	Used as an abortifactant	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008), Grace et al. (2008), Steenkamp (2003)
Aloe ciliaris	Intelezi, Ikalene (Xhosa)	Eastern Cape Province, South Africa	Chopped leaves were placed onto inlamed sores to reduce swelling. Has antibacterial and antiinflammatory effects	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008), Grierson and Afolayan (1999)
Aloe commixta	Table Mountain aloe	Very limited distribution being endemic to the Cape Town/Cape Peninsula region of South Africa	Used by the early Khoi inhabitants of the Cape Peninsula to treat burns, wounds and insect bites	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008)

(continued)

Table 1 (continued)

Species	Common name	Origin/distribution	Indications for medicinal use	Known constituents	References
Aloe cooperi	Unknown	Grassland regions of north-eastern South Africa	To ease labour and as an abortifactant	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008), Grace et al. (2008), Hutchings et al. (1996)
Aloe cryptopoda	Geelaalwyn (Afrikaans), ngafane (Sekhukhueeland)	Oopen regions of north-eastern South Africa	As a tonic and to treat general maladies	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008), Grace et al. (2008), Hutchings (1996), Watt and Breyer-Brandwijk (1962)
Aloe dichotoma	Quiver tree (English), kokerboom (Afrikaans)	Indigenous to South Africa and Namibia	To treat tuberculosis in Namibia	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008)
Aloe ecklonis	Unknown	Widely distributed in eastern regions of South Africa	As a pugrative and laxative in Lesotho. Also to ease labour.	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	Grace et al. (2008), van Wyk and Smith (2008), Hutchings et al. (1996), Watt and Breyer-Brandwijk (1962)

(continued)

Table 1 (continued)

Species	Common name	Origin/distribution	Indications for medicinal use	Known constituents	References
Aloe excelsa	Zimbabwe aloe	Indigenous to a relatively small area encompassing the Limpopo province mountains in South Africa, north to Zimbabwe and Malawi and east to Mozambique	To treat depressed fontanel in infants	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008), Grace et al. (2008)
Aloe ferox	Bitter aloe	Indigenous to South Africa and Lesotho	Purgative and laxative. Pulp used to treat wounds and to reduce microbial growth. Leaf extracts used to treat X-ray burns. Also to relieve rheumatic arthritis and treat conjunctivitis. The leaf gel is used as a general tonic and in cosmetics	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008), Grace et al. (2008), Hutchings et al. (1996)
Aloe globuligemma	Knoppiesaalwyn (Afrikaans)	Occurs at low altitudes in Zimbabwe and north-eastern regions of South Africa	The Ndebele people drink a leaf preparation as a general tonic and for microbial infections	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008), Grace et al. (2008)
Aloe greatheadii	Kgopane (Tswana), Kizima-bupiwa (Congo)	Distributed widely across sub-Saharan Africa with significant populations in Botswana, Congo, Malawi,	Leaf sap is used for the treatment of burns, sores and wounds. Also has purgative and laxative properties.	acetylated mannans, polymannans, anthroquinones	van Wyk and Smith (2008)

(continued)

Table 1 (continued)

Species	Common name	Origin/distribution	Indications for medicinal use	Known constituents	References
		Mozanbique, South Africa, Zambia and Zimbabwe	Reputed to be effective for treating snake bite	and anthraquinone glycosides, anthrones, lectins	
Aloe hereroensis	Sandaalwyn or Vlakte-aalwyn (Afrikaans)	Indigenous to South Africa, Namibia and Angola	Diluted sap in used in Namibia to treat infections including STIs. To treat conjunctivitis and chest complaints	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	Grace et al. (2008), Watt and Breyer-Brandwijk (1962)
Aloe humilis	Unknown	Occurs mainly in arid parts of the Little Karoo region of South Africa	Administered internally to treat tapeworm, roundworm and other parasites	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	Grace et al. (2008), van Wyk and Smith (2008), Watt and Breyer-Brandwijk (1962)
Aloe kraussii	Unknown	Occurs on grassy slopes in KwaZulu Natal Province of South Africa	An abortifactant and to ease labour as well as to relieve muscular pain	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008), Grace et al. (2008), Watt and Breyer-Brandwijk (1962), Reynolds, (2004)

(continued)

Table 1 (continued)

Species	Common name	Origin/distribution	Indications for medicinal use	Known constituents	References
Aloe littoralis	Mopane aloe (English), bergaalwyn (Afrikaans)	Widely distributed in southern Africa including Angola, Botswana, Mozambique, Namibia, South Africa, Zambia and Zimbabwe	Leaf decoctions are used to treat infections (including STIs)	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	Grace et al. (2008)
Aloe maculata	Bontaalwyn (Afrikaans), lekhala (Sotho)	Indigenous to South Africa, Swaziland and Lesotho	To treat infections including STIs and internal parasites. Leaf pulp is applied to the skin to treat ringworm. Also used by the Zulu as a laxative, purgative and traditional remedy for colds and fever. Some annecdotal evidence in treating diabetes	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008), Grace et al. (2008)
Aloe marlothii	Mountain aloe (English), bergaalwyn, umHlaba (Zulu), Kgopha (Sotho)	Mountainous regions and flat elevated rocky places in southern parts of Africa including South Africa, Botswana, Malawi, Mozambique, Swaziland, Zimbabwe	Administered internally to treat tapeworm, roundworm and other parasites. Infusion of the chopped leaves used for stomach complaints	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008), Grace et al. (2008), Hutchings et al. (1996), Watt and Breyer-Brandwijk (1962)
Aloe peglerae	Red hot poker (English), bergaalwyn,	Limited distribution, mainly within Gauteng Province, South Africa	To heal wounds, burns and to treat infections	acetylated mannans, polymannans, anthroquinones	van Wyk and Smith (2008), Grace et al. (2008), Hutchings et al. (1996), Watt and

(continued)

Table 1 (continued)

Species	Common name	Origin/distribution	Indications for medicinal use	Known constituents	References
	vuurpylaalwyn (Afrikaans)			and anthraquinone glycosides, anthrones, lectins	Breyer-Brandwijk (1962), Reynolds (2004)
Aloe perticola	Unknown	Narrow distribution around Nelspruit in South Africa	To heal wounds and burns. Also to treat stomach ailments	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008)
Aloe perryi	Perry's aloe	Endemic to rocky areas of Yemen	To heal wounds, burns and treat infections	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	Mpala et al. (2010), Adwadh Ali et al. (2001)
Aloe plicatilis	Fan aloe	Endemic to the steep rocky slopes of the Western Cape region of South Africa	Juice used by the Khoi-San as a laxative and purgative	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008), Grace et al. (2008)
Aloe pubescens	Unknown	Occurs in north-eastern Africa including Ethiopia	Leaf and flower used in Ethiopia to treat wounds,	acetylated mannans, polymannans,	Wondimu et al. (2007)

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Table 1 (continued)

Species	Common name	Origin/distribution	Indications for medicinal use	Known constituents	References
			stomach disorders, muscle cramps and anthrax	anthroquinones and anthraquinone glycosides, anthrones, lectins	
Aloe rupestris	Kraalaalwyn (Afrikaans), inKalane (Zulu)	Common in KwaZulu Natal Province of South Africa as well as in Swaziland and Mozambique	To enhance fertility in women and to treat impotence in men. Also was used as a general strengthening tonic for Zulus	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008), Grace et al. (2008)
Aloe spicata	Lebombo	Indigenous to South Africa, Swaziland and Mozambique	To treat digestive problems, stomach disorders and as an abortifactant	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008), Grace et al. (2008), Hutchings et al. (1996)
Aloe succotrina	Fynbos aloe	Naturally occurs in the Fynbos habitats of Table Mountain and the Cape Peninsula	To heal wounds, burns and treat infections	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	Köhler (1887)

(continued)

Table 1 (continued)

Species	Common name	Origin/distribution	Indications for medicinal use	Known constituents	References
Aloe striatula	Ingcelwane (Xhosa)	Occurs in the arid interior regions of South Africa	The leaves applied as poultices to wounds and burns.	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008), Grierson and Afolayan (1999)
Aloe teniior	iKhalene (Xhosa), inTelezi (Fingo)	Occurs in Swaziland and eastern regions of South Africa	Administered internally to treat tapeworm, roundworm and other parasites. Leaf tinctures used to treat haemorrhoids	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008), Grace et al. (2008), Hutchings et al. (1996), Watt and Breyer-Brandwijk (1962)
Aloe thraskii	Dune aloe, strand aloe (English), Strandaalwyn (Afrikaans)	Occurs on sand dunes and coastal areas of the east coast of South Africa	To enhance fertility	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008), Grace et al. (2008), Hutchings et al. (1996)
Aloe vera	*Aloe barbadensis*, Barbados aloe, burn aloe, Chinese aloe, first	The natural range is not clear as this species has been widely cultivated worldwide, although it is likely to	Broad range of medicinal uses including purgative and laxative. The pulp is used to treat wounds and to reduce microbial growth. Leaf	acetylated mannans, polymannans, anthroquinones and anthraquinone	Sirdaarta and Cock (2010), Sirdaarta and Cock (2008), Tai-Nin Chow et al. (2005),

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Table 1 (continued)

Species	Common name	Origin/distribution	Indications for medicinal use	Known constituents	References
	aid plant, Indian aloe, true aloe	originally be from northern Africa/Middle East	extracts used to treat X-ray burns. Also to relieve rheumatic arthritis and to treat conjunctivitis. Has antiinflammatory and immunomodulatory activity. The leaf gel is used as a general tonic and in cosmetics	glycosides, anthrones, lectins	Langmead et al. (2004), Ferro et al. (2003)
Aloe veriegata	Kanniedood (Afrikaans)	Widely distributed in arid regions of South Africa and Namibia	To relieve toothache. Leaf tinctures to treat haemorrhoids	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008), Grace et al. (2008), Watt and Breyer-Brandwijk (1962), Reynolds (2004)
Aloe viridiflora	Unknown	Endemic to dry savannah regions of Namibia	Can produce hallucinations when ingested	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	Dagne et al. (2000)
Aloe vryheidensis	Bruinaalwyn (Afrikaans)	Mountainous regions of northern South Africa	To heal wounds, burns and infections	acetylated mannans, polymannans, anthroquinones	van Wyk and Smith (2008), Grace et al. (2008), Hutchings et al. (1996), Watt and

(continued)

Table 1 (continued)

Species	Common name	Origin/distribution	Indications for medicinal use	Known constituents	References
				and anthraquinone glycosides, anthrones, lectins	Breyer-Brandwijk (1962), Reynolds (2004)
Aloe zebrina	Unknown	Widely distributed in the northern regions of South Africa, Botswana, Namibia, Angola, Zambia, Zimbabwe and Mozambique	The pulp was used in Botswana to treat infections including STIs. Leaf mesophyll used to treat eye infections	acetylated mannans, polymannans, anthroquinones and anthraquinone glycosides, anthrones, lectins	van Wyk and Smith (2008), Grace et al. (2008)

Ethnopharmacological uses, constituents and common names are derived from searches of Google Scholar and PubMed databases
STI sexually transmitted diseases

preparations have also been used for the treatment of multiple skin disorders, mouth infections, wounds and burns due to their antiseptic properties and cell proliferative effects.

A. vera is still an important component in the traditional medicine systems of many contempory cultures. For example, Indian Ayurvedic medicine prescribes the use of *A. vera* leaves and exudates as an antihelmintic and cathartic agent as well as for the treatment of stomach and digestive complaints (Govindarajan et al. 2005; Kapoor 1990). *A. vera* is also used in traditional Chinese medicine (TCM) for similar purposes (Tan and Vanitha 2004). Traditional Mexican ethnomedicinal systems use wild harvested leaves to treat burns, bruises and skin irritations (Heinrich et al. 1998). *A. vera* is used widely in Central America and throughout the Carribean region for similar purposes (Morton 1981). More recently, *A. vera* has been incorporated into modern western complementary and alternative medicinal systems, particularly homoeopathy and herbalism. It is frequently used as a topical household remedy to treat burns and scalds to promote tissue healing and for pain relief. It is also commonly used to treat sunburn, and when ingested, as a general tonic. A wide variety of other medicinal properties have also been described for *A. vera*, such as the treatment of rheumatic and inflammatory disorders including rheumatoid arthritis and gout (Yagi et al. 2002; Malterud et al. 1993; Azfal et al. 1991), as well as uses as an immune system stimulant (Hamman 2008) and to treat diabetes (Rajasekaran et al. 2005) and cancer (Hamman 2008). It also is reputed to assist gastrointestinal function, lower blood pressure and have general antimicrobial properties (Hamman 2008; Reynolds 2004).

Other Aloe species originating from the Arabian Peninsula also have a well-documented history of medicinal usage. Of these, the Yemeni species *Aloe perryi* Baker (commonly known as Perry's aloe) has perhaps the widest usage. It is used for the topical treatment of skin diseases, burns and wounds (Al-Fatimi et al. 2005). Several papers also document its use to treat eye infections, stomach problems and constipation as well as in the treatment of malaria (Mothana et al. 2012; Mothana et al. 2009). It is also reputed to have antiseptic properties, although wide differences in efficacy have been reported. One publication reported *A. perryi* to have good antibacterial activity, although this study did not report minimum inhibitory concentrations (MICs) or minimum bactericidal concentrations (MBCs) (Awadh Ali et al. 2001). In contrast, other studies have reported only mild antibacterial activity against a narrow panel of bacteria (Mothana et al. 2009). *Aloe dhufarensis* Lavranos has also been reported to be useful in promoting wound healing and as a purgative (Marwah et al. 2007). The species *Aloe fleurentinorum* Lavranos, *Aloe inermis* Forsk., *Aloe niebuhriana* Lavranos, *Aloe officinalis* Forsk., *Aloe rivieri* Lavranos, *Aloe rubroviolacea* Schweinf., *Aloe sabeae* Schweinf., *Aloe tomentosa* Deflers and *Aloe vaccillans* Forsk. have also been listed as having significance in traditional Yemeni medicinal systems, although their specific uses were not discussed (Fleurentin et al. 1983).

Given the high degree of biodiversity in the Southern African region, it is not surprising that numerous traditional healing systems from that region use Aloe species in the treatment of many disorders. Different species occur in different

regions and their usage, whilst often widespread, is often associated with specific cultural/ethnic groupings. Thus, a species used by one cultural group for treating a specific disease or disorder may have had different therapeutic uses (or no uses) by the same or other cultures in different regions. Despite such variation, two South African Aloe species (*Aloe arborescens* Miller and *Aloe ferox* Miller) stand out as having the widest usage and most commercial potential after *A. vera*.

A. arborescens has been used traditionally in much the same ways and for similar disorders and conditions as *A. vera* is used. In South Africa, it is used for the topical treatment of burns and wounds, insect bites and other skin irritations (Mabona and van Vuuren 2013; Watt and Breyer-Brandwijk 1962). It is also a treatment for gastrointestinal complaints in the traditional healing systems of several ethnic groups, of which Zulu usage has been particularly well documented (Hutchings et al. 1996; Watt and Breyer-Brandwijk 1962). *A. arborescens* has also been widely introduced internationally and is now incorporated into many medicinal systems. In Japan in particular, *A. arborescens* is used as a folk remedy to treat burns, skin disorders, intestinal complaints and as a general antiseptic (Kameyama and Shinho 1980).

Aloe ferox also has a long history in Southern African medicinal systems, with ancient rock paintings depicting its use (Reynolds 2004). A recent ethnobotanical study documents its usage by Khoi-San and by other cultures of the greater Karoo region of South Africa (van Wyk et al. 2008) as a laxative and purgative agent. Early Cape Dutch settlers learned from these earlier inhabitants and subsequently incorporated *A. ferox* into their own folk medicinal system for similar purposes (van Wyk 2008b). These studies also noted that *A. ferox* has a widespread distribution in Southern Africa and a corresponding widespread usage by many other ethnic groups, including the Sotho and Nguni cultures (van Wyk et al. 2008). *A. ferox* is traditionally used for the topical treatment of burns and wounds, psoriasis, eczema, skin cancers and other skin irritations (Mabona and van Vuuren 2013; Watt and Breyer-Brandwijk 1962). It is also used in some traditional South African medicinal systems as an abortifactant (Steenkamp 2003) and in the treatment of sexually transmitted infections including syphilis, gonorrhoea and *Candida albicans* (De Wet et al. 2012; van Vuuren and Naidoo 2010; Hutchings 1996; Watt and Breyer-Brandwijk 1962). It has also been utilised for the treatment of arthritis, digestive problems and diabetes by ethnic groupings in the Eastern and Western Cape provinces of South Africa (Reynolds 2004). *A. ferox* has also been disseminated internationally and is now commonly used throughout Africa and in Europe for its laxative, antimicrobial, anti-inflammatory and anticancer properties (Chen et al. 2012).

Many Southern African Aloe species have narrower geographic distributions and correspondingly narrower ethnobotanical usages. *Aloe africana* Miller, *Aloe broomii* Schönland, *Aloe marlothii* Berger, *Aloe plicatilis* Miller and *Aloe variegata* L., are amongst the other southern African species with documented ethnopharmacological uses, particularly as laxatives (van Wyk 2008a, b). *A. marlothii* also is used for the treatment of respiratory disorders (York et al. 2011), whilst *Aloe chabaudii* Schönland, *Aloe christianii* Reynolds and *Aloe rupestris* Baker have

been used as abortifactants (Steenkamp 2003). Several other species including *Aloe striatula* Haw. and *Aloe ciliaris* Harvey are used in rural communities to treat septic and inflamed wounds (Grierson and Afolayan 1999). Whilst less well documented, *Aloe barberae* Dyer is used to treat sores, wounds and skin conditions (Grace et al. 2008) and to have antimicrobial and anti-inflammatory properties (Ndhlala et al. 2009). Anecdotal evidence also exists that *Aloe striata* Haw. was used to treat rheumatism and leg pain, and that *Aloe variegata* L. was used in the treatment of callouses and bunions, although very little published information is available (van Wyk 2008a, b).

Eastern Africa is also a region of high Aloe biodiversity and thus the rich tradition of Aloe therapeutic usage in this area is not surprising. *Aloe secundifolia* Engl. has particularly broad ethnobotanical uses. This species is used by the Kikuyus of central Kenya for the treatment of skin diseases including pimples, rashes, warts and ringworm, as well as in the treatment of wounds and burns (Njoroge and Bussmann 2007). It has further uses in South Kenya to alleviate pain and for the treatment of rheumatism and inflammation (Wambugu et al. 2011). It has also been used to treat ectoparasites such as ticks (Nanyingi et al. 2008) and to treat malaria (Muthee et al. 2011).

Several other Eastern African Aloe species are also used as antimalarial therapeutics. These include *Aloe nyeriensis* Christian & Verd. (Kigondu et al. 2009), *Aloe deserti* Berger, *Aloe macrosiphon* Bak. (Nguta et al. 2010a; Nguta et al. 2010b) and *Aloe kedongensis* Reynolds (Jeruto et al. 2008). *A. kedongensis* is also used to treat wounds and skin conditions, as well as in the treatment of typhoid, colds and flu, and for ear problems (Jeruto et al. 2008) by the Nandi people of Kenya. East African Aloes have also been used in the treatment of other infective diseases and conditions. Several Aloe species including *Aloe secundiflora* Engl. (Wagate et al. 2010) and *Aloe harlana* Reynolds (Asamenew et al. 2011) have been reported to be good antibacterials. *Aloe lateritia* Engl. has been reported to have good antifungal activity (Hamza et al. 2006). There is also a report of Aloe species in Uganda being used by traditional healers for the treatment of HIV/AIDS (Lamorde et al. 2010): the species used was not identified so it is not clear if it was a local species or the introduced species *A. vera*.

3 Pharmacology—Bioavailability and Toxicity

Aloe products are used medicinally in a number of ways to treat different ailments. Topical treatments may be used to treat wounds and skin conditions, whilst the leaf gel and/or exudates may be ingested for the treatment of internal ailments. However, the ingestion of Aloe products is associated with diarrhoea and an increased intestinal rate of passage (Ishii et al. 1994). Indeed, one of the major therapeutic uses of Aloes at higher doses is as a laxative/purgative and as an emmenagogue. The high content of anthraquinones and anthrones in Aloe leaf exudates stimulate intestinal motility, increasing the rate of passage through the

digestive system. This impacts on the bioavailability of the Aloe compounds ingested (as well as any other co-administered medications), with the increased rate of passage resulting in lower intestinal absorption rates. Given the widespread usage of Aloe products, it is perhaps surprising that limited information is available on the bioavailability and intestinal transport of Aloe phytochemicals.

A recent study demonstrated that intestinal absorption of aloin, aloe emodin and aloesin is relatively low (ranging from 5.5–6.6 %, 6.6–11.3 % and 7.6–13.6 %, respectively) in an intestine absorptive model system using a Caco-2 human colon carcinoma cell line (Park et al. 2009). Many of the anthraquinones have poor affinity for phospholipid membranes, resulting in inefficient cellular uptake by passive diffusion (Alves et al. 2004). It is noteworthy these studies used a Caco-2 model system and thus do not take into account the decreased exposure time that would occur with the increased motility in whole animals. In vivo absorption rates would be expected to be further impacted by the decreased time for absorption with more rapid intestinal passage time, potentially resulting in substantially lower percentage absorptions.

The bioavailability of ingested Aloe compounds is also affected by their degradation in the digestive system. Aloenin A and aloe emodin are stable in salivary, gastric and small intestine model systems with close to 100 % recovery of the intact compounds after digestion (Shim and Kwon 2010). In contrast, aloin is rapidly degraded by the digestive enzymes, with less than 50 % remaining following passage through the digestive system, despite a low absorption rate. Thus, whilst ingested aloin, aloe emodin and aloesin all have low bioavailability due to their low absorption rates, the bioavailability of aloin is further decreased due to its rapid degradation.

Interestingly, despite the poor intestinal absorption of the Aloe compounds, *Aloe vera* gel and whole leaf extracts substantially enhance the transmembrane transport of other poorly absorbed drugs (Chen et al. 2009). This suggests that the Aloes may have potential as absorption enhancers for orally administered drugs with poor bioavailability. This may also have profound adverse effects if the increased bioavailability of the co-administered drugs results in toxic plasma levels. Furthermore, oral administration of *Aloe vera* leaf gel rapidly enhances the bioavailability of co-administered vitamin B_{12} and ascorbic acid as much as twofold in healthy middle aged and elderly people (Yun et al. 2010), potentially enhancing the antioxidant activities of these cofactors.

Despite the laxative/purgative bioactivities resulting from intestinal irritation, ingestion of Aloe is relatively safe. Indeed, it is perhaps these same laxative/purgative bioactivities which account for the safety of the Aloes (Ishii et al. 1994). Gastrointestinal motility increases coincidently with increased concentrations of the anthraquinones. Thus, increased anthraquinone levels initiate a physiological mechanism which increases intestinal passage rates and thus decreases the intestinal anthraquinone level. Perhaps for this reason, there are limited reports of acute Aloe toxicity. Several single case studies do exist of individuals suffering acute toxicity from self-medicating with *Aloe vera*. However, the reported toxicity

is generally due to drug interactions between the self-prescribed *Aloe vera* and another medication (Lee et al. 2004).

Several studies have also reported on the in vitro toxicity of *Aloe vera* leaf extracts (Cock and Ruebhart 2004, 2009). These studies report toxicity to be low and have determined that it is due to the induction of oxidative stress (Cock and Sirdaarta 2011; Sirdaarta and Cock 2008, 2010). Similarly, low toxicities have also been reported for *A. elgonica*, *A. pruinose* and *A. daiyana* (Mpala et al. 2010). This study also reported a panel of other Aloe species to be completely nontoxic. It is noteworthy that these studies reported on the toxicity of concentrated extracts with significantly higher concentrations of Aloe polyphenolic compounds than in untreated Aloe leaves or exudates.

4 Phytochemistry and Antioxidant Content

Aloe leaves have significant antioxidant activity, one study showing that extracts from *A. vera* leaves had higher antioxidant activity than either pure α-tocopherol or butylated hydroxytoluene (BHT) (Hu et al. 2003). The same study also showed that the antioxidant activity of the leaves depended on the age of the plant, with 3-year-old plants having the greatest antioxidant content. A common feature of the genus Aloe is the relatively high level of phenolic compounds including anthraquinones, anthrones, chromones, coumarins, saponins and polysaccharides in the leaf gel. Phenolic compounds are generally strong antioxidants (Rice-Evans and Miller 1997). They protect cell constituents against oxidative damage by scavenging free radicals, averting their deleterious effects on nucleic acids, proteins and cellular lipids (Rice-Evans and Miller 1997).

The anthraquinone aloe emodin (Fig. 3a) is a widespread leaf constituent of many Aloe species (Dagne et al. 2000). This compound has well-established antioxidant activity via its ability to scavenge hydroxyl radicals (Tian and Hua 2005). The free radical scavenging activity of aloesin (Fig. 3b) and its derivatives has also been extensively documented (Yagi et al. 2002). The oxygen scavenging activity of 2′-O-p-coumaroylaloesin (Fig. 3c) and 2′-O-feruloylaloesin (Fig. 3d) were subsequently reported (Beppu et al. 2003). A high consumption of polyphenolic antioxidants may prevent the development of chronic and degenerative diseases such as cancer (Hertog et al. 1996), cardiovascular diseases (Vita 2005), neural degeneration (Youdim et al. 2002), diabetes and obesity (Tsuda et al. 2003) and is likely to be responsible (at least in part) for many of the myriad of therapeutic effects reported for the Aloes.

The leaf gels from several Aloe species are good sources of inorganic minerals including magnesium, zinc, calcium, potassium, sodium, iron, phosphorous, manganese, copper and molybdenum (Dagne et al. 2000). Aloe leaf gel also contains a number of vitamins including ascorbic acid (vitamin C; Fig. 3e) as well as vitamins

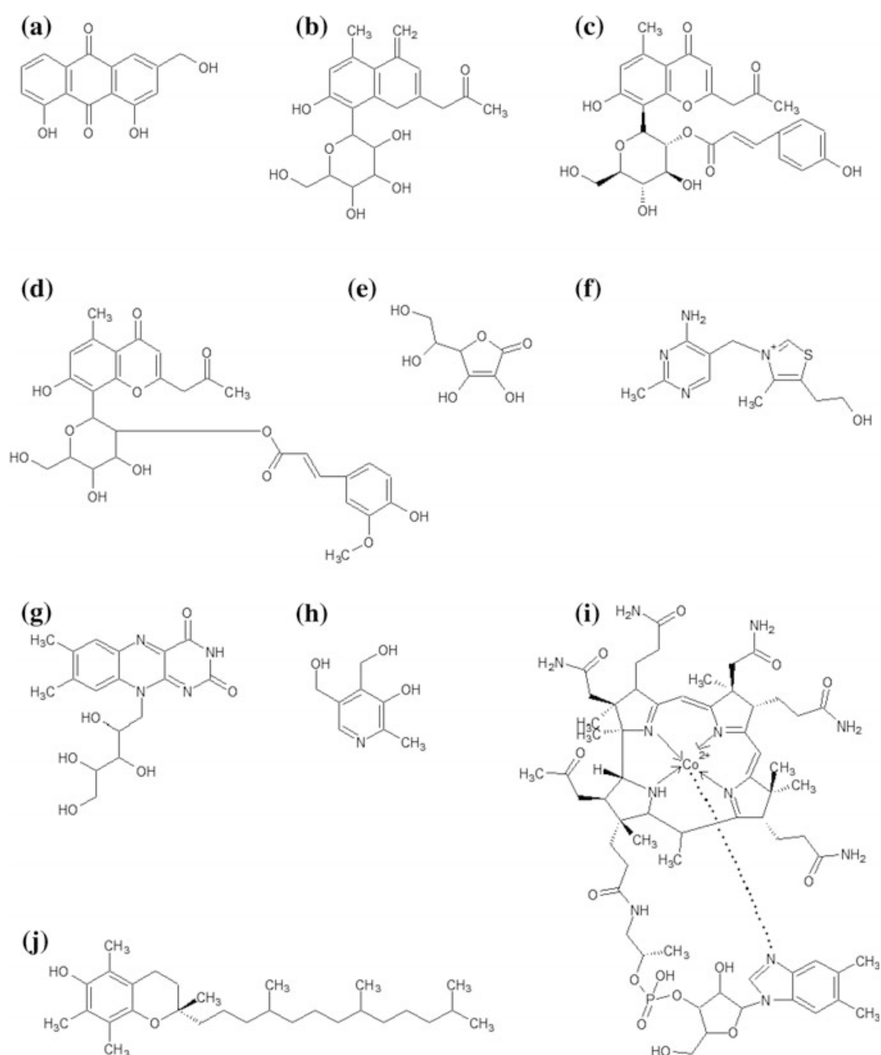


Fig. 3 Chemical structures of selected molecules with antioxidant potential identified in Aloe spp. leaf gels: **a** Aloe emodin, **b** Aloesin, **c** 2′-O-p-Coumaroylaloesin, **d** 2′-O-Feruloylaloesin, **e** Ascorbic acid (vitamin C), **f** Vitamin B1 (thiamine), **g** Vitamin B2 (riboflavin), **h** Vitamin B6 (pyridoxal phosphate), **i** Vitamin B12, **j** α-Tocopherol (vitamin E)

B1 (Fig. 3f), B2 (Fig. 3g), B6 (Fig. 3h), B12 (Fig. 3i) and α-tocopherol (vitamin E; Fig. 3j) (Dagne et al. 2000), all of which may contribute to the antioxidant activity of Aloe leaf gels.

4.1 Anthraquinones

Many phytochemicals have been isolated from Aloe leaves and their bioactivities extensively examined. The anthraquinones, anthrones and chromones have been particularly well studied and shown to be effective in modulating various disease states (Dabai et al. 2007; Malterud et al. 1993). Anthraquinones such as aloe emodin (Fig. 4a) and aloin (Fig. 4b) probably manifest their therapeutic effects via an antioxidant mechanism (Tian and Hua 2005). For example, aloe emodin has high-inhibitory free radical scavenging activity and inhibits lipid peroxidation (Yen et al. 2000).

Interestingly, aloe emodin and aloin are capable of behaving as either antioxidants or as a prooxidants, their action being dependent upon their concentration (Tian and Hua 2005). Aloe emodin exerts antioxidant behaviour at lower concentrations, yet acts as a prooxidant at high concentrations. In contrast, aloin has an antioxidant effect at higher concentrations, yet a prooxidant effect at low concentrations. Thus, the variable medicinal effects reported for crude *A. vera* extracts may be largely due to differing level of aloe emodin and/or aloin present in the extract.

A number of other anthraquinones are present in the leaves of Aloe spp. They include aloesaponarin (Fig. 4c), chrysophanol (Fig. 4d) and its progenitor prechrysophanol (Fig. 4t), desoxyerythrolaccin (Fig. 4f), 1,5-dihydroxy-3-hydroxy methylanthraquinone (Fig. 4g), helminthosporin (Fig. 4 h), 7-hydroxyaloe emodin (Fig. 4i), isoxanthorin, (Fig. 4j) laccaic acid D methyl ester (Fig. 4k), nataloe emodin (Fig. 4l) and its ester nataloe emodin-8-methyl ester (Fig. 4m), aloechrysone (Fig. 4o) and aloesaponol (Fig. 4p). Furthermore, Aloe anthraquinones are often present as O-glycosides such as aloe emodin-11-O-rhamnoside (Fig. 4b), nataloe emodin-2-O-glucoside (Fig. 4n), aloesaponol-6-O-glucoside (Fig. 4q), aloesaponol-8-O-glucoside (Fig. 4r) and aloesaponol-O-methyl-4-O-glucoside (Fig. 4s). Anthraquinone dimers such as asphodelin (Fig. 4u) and bianthracene (Fig. 4v), and the glycosylated dimer derivative elgonicardine (Fig. 4w) may be present in the leaf exudates of *Aloe saponaria* Haw. and *Aloe elgonica* Bullock (Dagne et al. 2000).

4.2 Anthrones

Anthrones are a class of compounds mainly responsible for the laxative and purgative effects associated with Aloe leaf exudates. The history of the detection and structural elucidation of the anthrones has been previously reported in detail (Dagne et al. 2000; Viljoen 1999) and will only be summarised here. The first Aloe anthrones to be

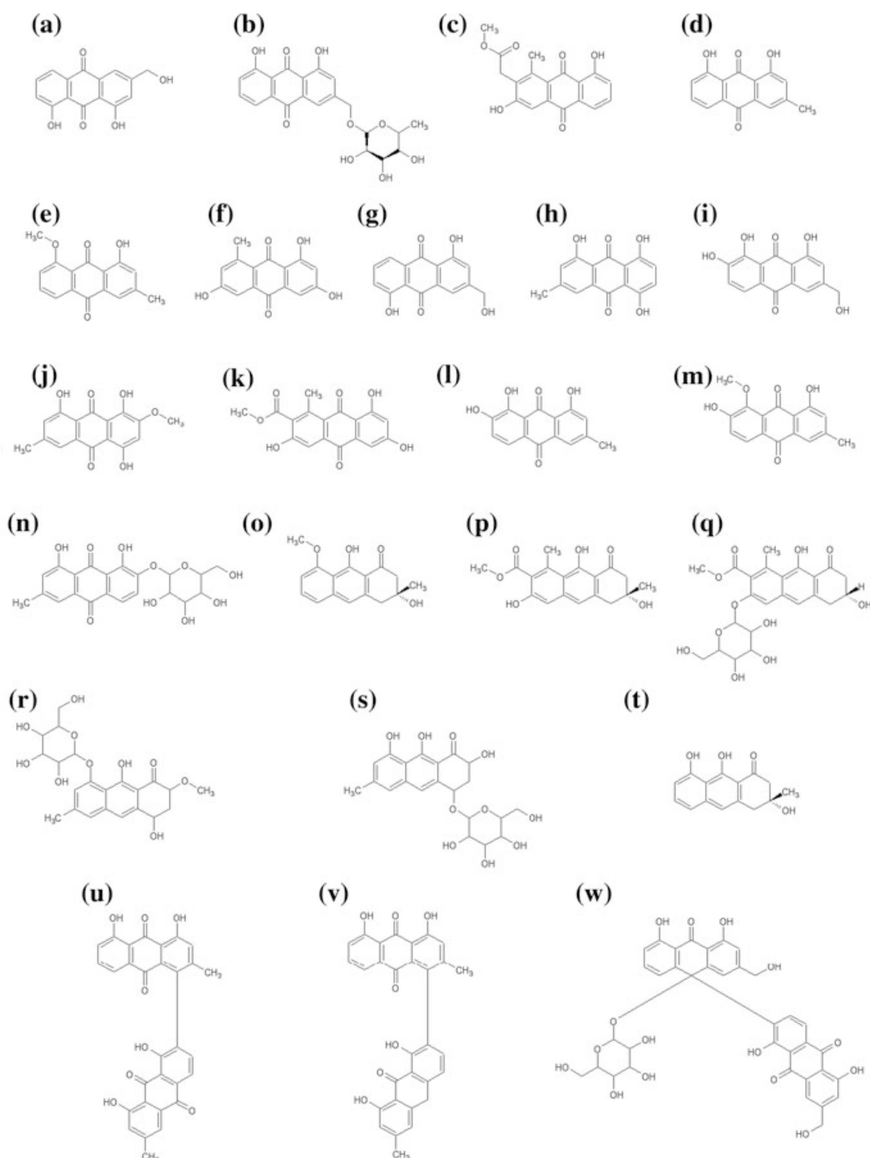
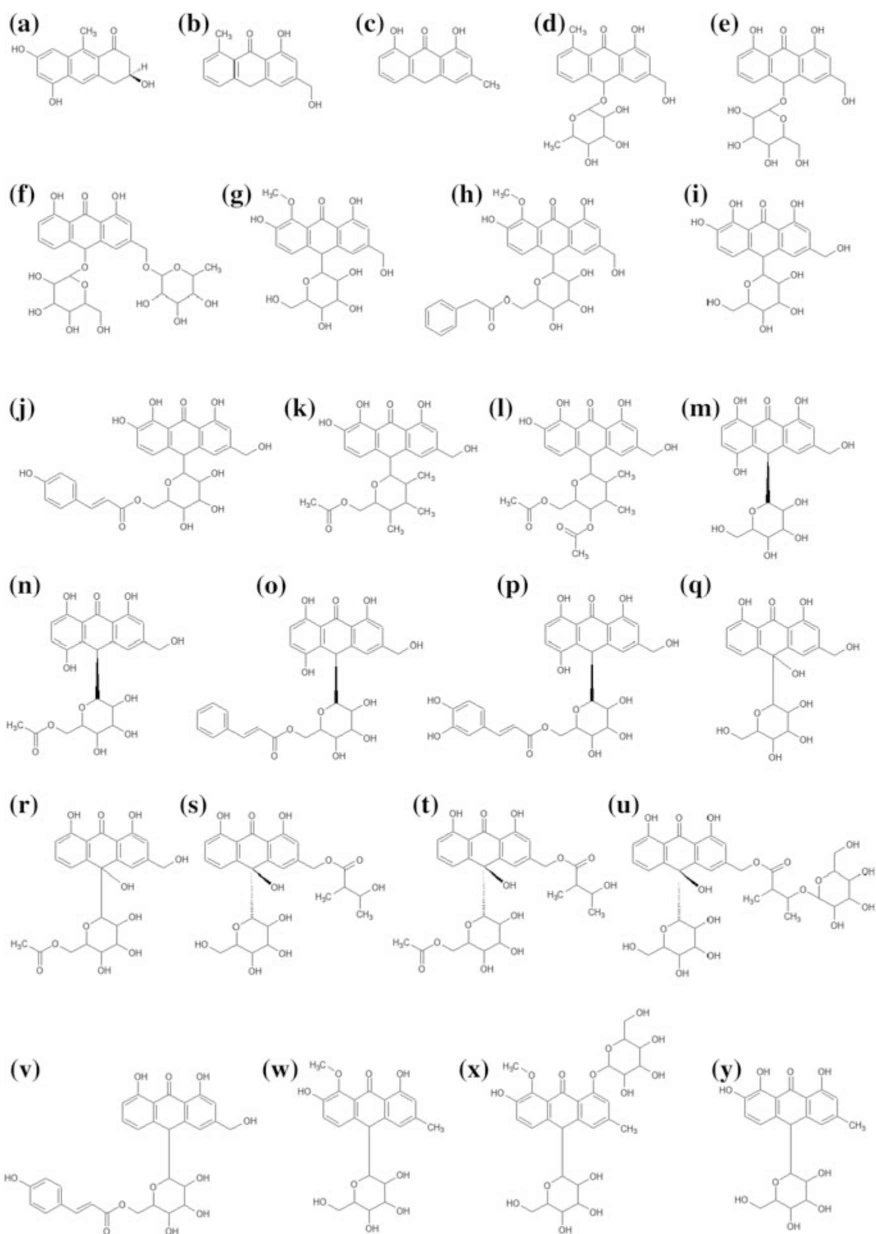


Fig. 4 Chemical structures for the Aloe anthraquinones. **a** Aloe emodin, **b** Aloe-emodin-11-O-rhamnoside, **c** Aloesaponarin, **d** Chrysophanol, **e** Chrysophanol-8-methyl ether, **f** Desoxyerythrolaccin, **g** 1,5-Dihydroxy-3-hydroxymethylanthraquinone, **h** Helminthosporin, **i** 7-Hydroxyaloe emodin, **j** Isoxanthorin, **k** Laccaic acid D methyl ester, **l** Nataloe emodin, **m** Nataloe emodin-8-methyl ester, **n** Nataloe emodin-2-O-glucoside, **o** Aloechrysone, **p** Aloesaponol, **q** Aloesaponol-6-O-glucoside, **r** Aloesaponol-8-O-glucoside, **s** Aloesaponol-O-methyl-4-O-glucoside, **t** Prechrysophanol, **u** Asphodelin, **v** Bianthracene, **w** Elgonicardine

isolated were the C-glycosyl anthrone isomers aloin A and aloin B (collectively named aloin and also often referred to as barbaloin in Fig. 5e). They were initially isolated from *A. vera* as early as the mid-nineteenth century, although their structures were not completely elucidated until 150 years later (Dagne et al. 2000; Viljoen 1999). The presence has since been detected in several other Aloe species. Indeed, the presence of aloin has been reported in nearly 100 species of Aloe, usually at relatively high levels of 10–20 % in concentration (Dagne et al. 2000). *A. ferox* leaf exudates in particular have very high aloin contents (up to 30 %; van Wyk et al. 1995). Aloin may be transformed into the hydroxylated derivatives 5-hydroxyaloin A (Fig. 5m), 7-hydroxyaloin (Fig. 5 i) and 10-hydroxyaloin B (Fig. 5q) as well as their acetate derivatives 5-hydroxyaloin A 6′-O-acetate (Fig. 5n), 7-hydroxyaloin-6′-O- mono-acetate (Fig. 5k) and 10-hydroxyaloin-6-O-acetate (Fig. 5r), respectively, under alkaline conditions (Dagne et al. 2000).

Homonataloin (Fig. 5w) and nataloin (Fig. 5y) were subsequently isolated from *Aloe marlothii* Berger (Dagne et al. 2000). Aloinoside (Fig. 5f), which differs from aloin by the presence of an additional rhamnose moiety, was also reported. Other anthrones isolated from the leaves of Aloe spp. include aloe barbendol (Fig. 5a), aloe-emodinanthrone (Fig. 5b), chrysophanolanthrone (Fig. 5c), aloe emodin-10-C-rhamnoside (Fig. 5d), 8-O-methyl-7-hydroxyaloin (Fig. 5g), 6′-O-cinnamoyl-8-O-methyl-7-hydroxyaloin (Fig. 5h), 6′-O-p-coumaroyl-7-hydroxyaloin (Fig. 5j), 7-hydroxyaloin-4′,6′-O- diacetate (Fig. 5l), 6′-O-cinnamoyl-5-hydroxyaloin A (Fig. 5o), microstigmin A (Fig. 5p), deacetyllittoraloin (Fig. 5s), littoraloin (Fig. 5t), littoraloside (Fig. 5u), microdontin (Fig. 5v) and homonataloside (Fig. 5x). A number of these anthrones contain cinnamic and coumaroyl moieties. Studies indicate that cinnamic acid and coumaroyl derivatives have concentration-dependent antioxidant/prooxidant activities similar to those of the anthraquinones (Maurya and Devasagayam 2010). Cinnamic acid and coumaroyl derivatives may behave as antioxidants at lower concentrations, but convert to prooxidants at concentrations above 5 μM.

In contrast, Yen et al. demonstrated that the chemical structure of the other anthrones predisposes them to function as electron acceptors (electrophiles), hence as strong antioxidants, independent of their concentration within an extract (Yen et al. 2000). It therefore remains possible that *A. vera* extracts with high anthrone concentrations may maintain antioxidant potential, even under conditions which would otherwise predispose the extract to function as a prooxidant. For example, *A. vera* extracts containing high aloe emodin and low aloin concentrations (both of which favour prooxidant bioactivity) may still function as an antioxidant if high enough levels of anthrone are present to maintain the redox state of the anthraquinones. Conversely, low levels of anthrone may predispose an extract to display prooxidant activities. It is therefore likely that the redox character of an extract is not only dependent on the levels of the different phytochemicals present, but also on the ratios of several important components within the mixture.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◀ **Fig. 5** Chemical structures for the Aloe anthrones. **a** Aloe barbendol, **b** Aloe-emodinanthrone, **c** Chrysophanolanthrone, **d** Aloe emodin-10-C-rhamnoside, **e** Aloin (barbaloin), **f** Aloinoside, **g** 8-O-Methyl-7-hydroxyaloin, **h** 6′-O-Cinnamoyl-8-O-methyl-7-hydroxyaloin, **i** 7-hydroxyaloin, **j** 6′-O-p-Coumaroyl-7-hydroxyaloin, **k** 7-hydroxyaloin-6'-O-monoacetate, **l** 7-Hydroxyaloin-4′,6′-O-diacetate, **m** 5-hydroxyaloin A, **n** 5-hydroxyaloin A-6'-O-acetate, **o** 6′-O-Cinnamoyl-5-hydroxyaloin A, **p** Microstigmin A,**q** 10-hydroxyaloin, **r** 10-Hydroxyaloin B 6′-O-acetate, **s** Deacetyllittoraloin, **t** Littoraloin, **u** Littoraloside, **v** Microdontin, **w** Homonataloin, **x** Homonataloside, **y** Nataloin

4.3 Chromones

Chromones are the most abundant class of phenolic compounds in Aloe leaves (Dange et al. 2000). Aloesin (formerly called aloeresin B; Fig. 6j) and aloeresin A (Fig. 6k) are some of the most common constituents of Aloe leaves, being present in a significant number of the Aloe species screened (Reynolds 2004). A number of isomeric and substituted isomeric forms including aloeresin C (Fig. 6l), aloeresin D (Fig. 6m), aloeresin E (Fig. 6n), aloeresin F (Fig. 6o), iso-aloeresin A (Fig. 6p) and iso-aloeresin D (Fig. 6q) have been described (Dagne et al. 2000). Since early studies resulting in the detection and structural elucidation of aloesin in the early 1970s, a wide variety of chromones have been reported for the Aloes (Figs. 6 and 7). Methylated derivatives including 7-O-methylaloesin (Fig. 6a), 7-O-methylaloesinol (Fig. 6b), 7-O-methylaloeresin A (Fig. 6c), 8-[C-B-D-[2-O-(E)-cinnamoyl]glucopyranosyl]-2-[(R)-2-hydroxypropyl]-7-methoxy-5-methylchromone (Fig. 6d), 8 -C-glycosyl-7-O-methylaloediol (Fig. 6g), -C-Glycosyl-7-O-methyl-S-aloesol (Fig. 6i), 2-acetonyl-7-hydroxy-8-(2-furanonyl)-7-hydroxy-5-methylchromone (Fig. 6u), 7-Hydroxy-2,5-dimethylchromone (Fig. 7a), 2′-p-O-methlcoumaroylaloesin (Fig. 7p) are also common chromone components of Aloe leaves (Dagne et al. 2000).

As with the anthraquinones and anthrones, many of the chromones including 8-C-glycosyl-(2′-O-cinnamoyl)-7-O-methyl-aloediol (Fig. 6f), 8 2-acetonyl-8-(2′,6′-di-O,O-coumaroyl)-glucopyranosyl-7-hydroxy-5-methylchromone (Fig. 7j), 2-acetonyl-8-(2′,cinnamoyl)-glucopyranosyl-7-hydroxy-5-methylchromone (Fig. 7l), 6′-O-coumaroylaloesin (Fig. 7m) and 2′-p-O-methlcoumaroylaloesin (Fig. 7p) contain cinnamic and coumaroyl moieties (Dagne et al. 2000). As for the anthrones, it is possible that the chromone cinnamic acid and coumaroyl derivatives may behave as antioxidants at lower concentrations, but convert to prooxidants at higher concentrations (Maurya and Devasagayam 2010).

The antioxidant activity of aloesin and various other chromones have also been extensively studied (Gomes et al. 2009; Araya-Maturana et al. 2008). In contrast, a literature search was unable to find any studies examining the potential prooxidant activity of these compounds. Several chromones may have higher reducing power than ascorbic acid (Gomes et al. 2009). The relatively high reducing power of ascorbic acid is believed to determine its ability to function as a prooxidant (Joel 1995). It is therefore possible that aloesin and other Aloe chromones may have a similar antioxidant/prooxidant profile to ascorbic acid (i.e. antioxidant activity at lower concentrations and prooxidant activity at higher concentrations).

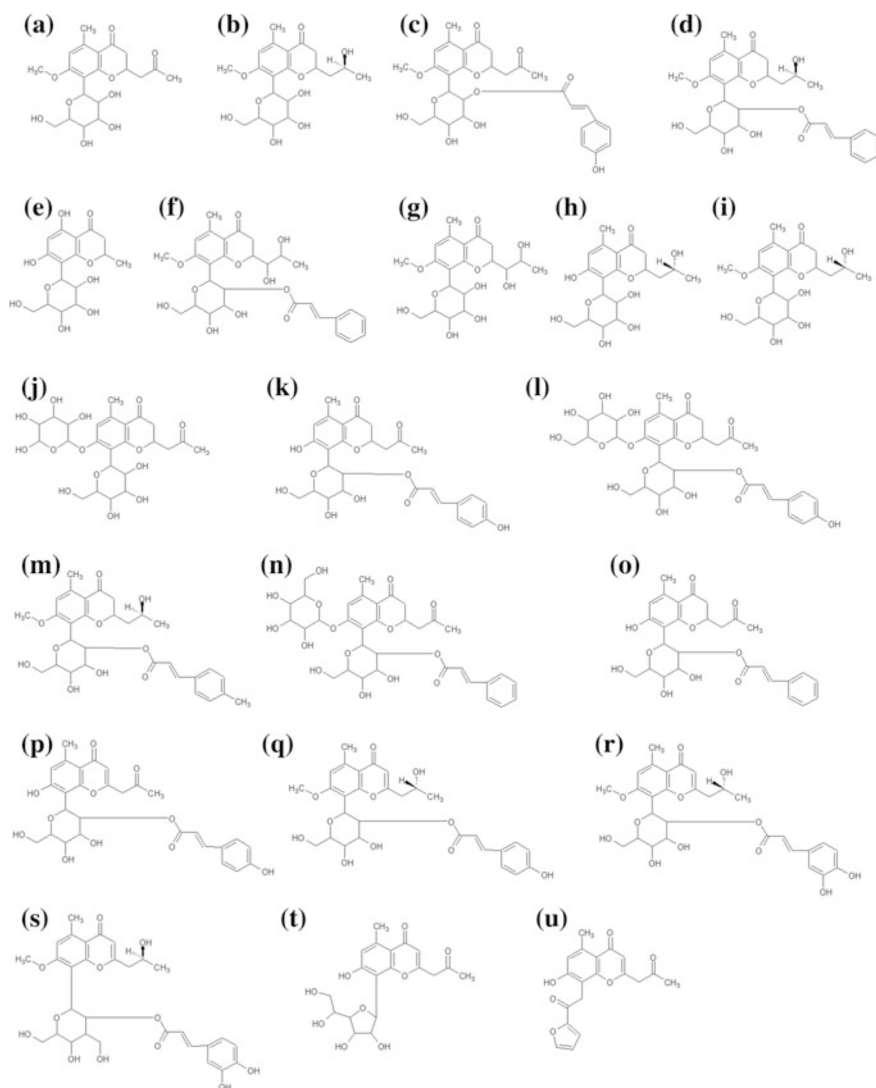

Fig. 6 Chemical structures for the Aloe chromones. **a** 7-O-Methylaloesin, **b** 7-O-Methylaloesinol, **c** 7-O-Methylaloeresin A, **d** 8-[C-B-D-[2-O-(E)-cinnamoyl]glucopyranosyl]-2-[(R)-2-hydroxypropyl]-7-methoxy-5-methylchromone, **e** 8-C-Glucosyl-noreugenin, **f** 8-C-glycosyl-(2′-O-cinnamoyl)-7-O-methyl-aloediol, **g** 8-C-glycosyl-7-O-methylaloediol, **h** 8-C-Glycosyl-S-aloesol, **i** 8-C-Glycosyl-7-O-methyl-S-aloesol, **j** Aloesin (aloeresin B), **k** Aloeresin A (2′-O-p-coumaroylaloesin), **l** Aloeresin C, **m** Aloeresin D, **n** Aloeresin E. **o** Aloeresin F, **p** Iso-aloeresin A, **q** Isoaloeresin D, **r** Isorabaichromone, **s** Rabaichromone, **t** Neoaloesin A, **u** 2-acetonyl-7-hydroxy-8-(2-furanonyl)-7-hydroxy-5-methylchromone

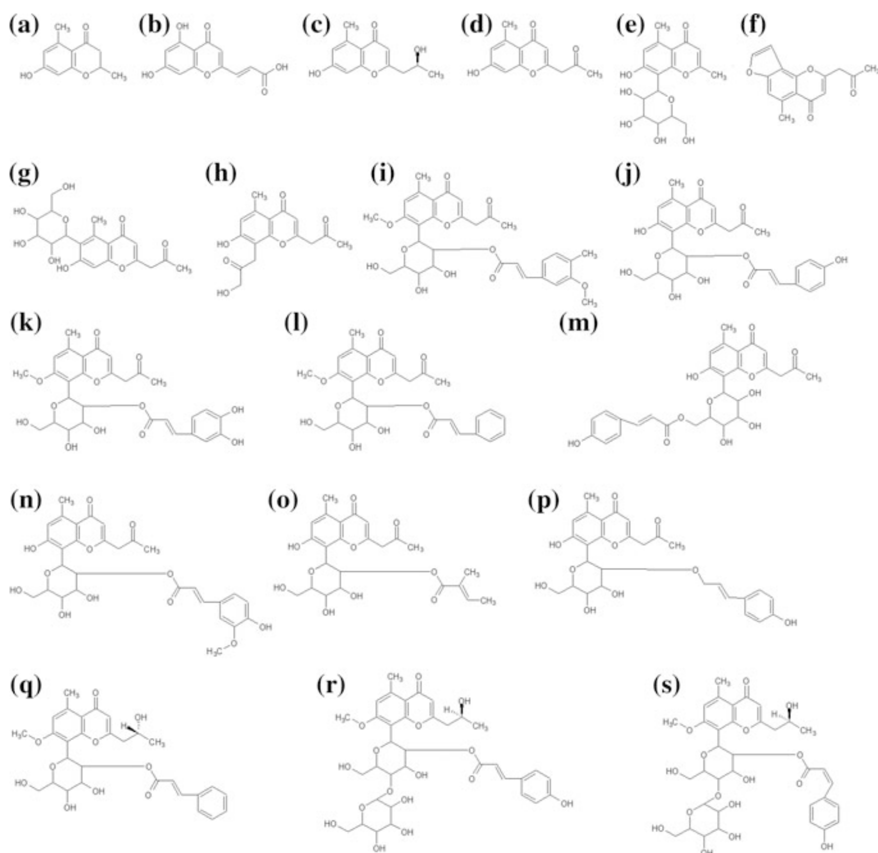

Fig. 7 Chemical structures for the Aloe chromones **a** 7-Hydroxy-2,5-dimethylchromone, **b** 2-(Carboxyethenyl)-5,7-dihydroxychromone, **c** Aloesol, **d** Aloesone, **e** Deacetylaloesin, **f** Furoloesone, **g** Isoaloesin, **h** 2-Acetonyl-7-hydroxy-8-(3-hydroxyacetonyl)-5-methylchromone, **i** 2-Acetonyl-8-(2-furoylmethyl)-7-hydroxy-5-methylchromone, **j** 2-acetonyl-8-(2′,6′-di-O,O-coumaroyl)-gluco pyranosyl-7-hydroxy-5-methylchromone, **k** 2-acetonyl-8-(2′,caffeoyl)-glucopyranosyl-7-hydroxy-5-methylchromone, **l** 2-acetonyl-8-(2′,cinnamoyl)-glucopyranosyl-7-hydroxy-5-methylchromone, **m** 6′-O-Coumaroylaloesin, **n** 2′-O-Feruloylaloesin, **o** 2′-O-Tigloylaloesin, **p** 2′-p-O-methl coumaroylaloesin, **q** 7″-Deoxyaloeresin D, **r** 4′-O-glucosyl-isoaloeresin DI, **s** 4′-O-glucosyl-isoaloeresin DII

This possibility is based on the reported higher reducing power of the chromones compared to their free radical scavenging activity (Gomes et al. 2009) and has not been adequately tested (Fig. 8).

4.4 Coumarins, Pyrans and Pyrones

The coumarins feralolide and dihydroisocoumarin glycoside have been found in *A. ferox* (Speranza et al. 1993) and *A. hildebrandtii* (Veitch et al. 1994) respectively.

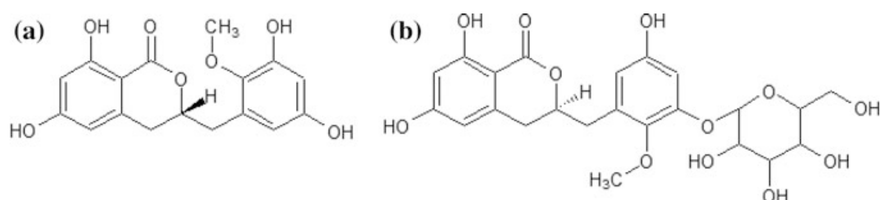

Fig. 8 Chemical structures for the Aloe coumarins. **a**, Feralolide, **b** Dihydroisocoumarin glycoside

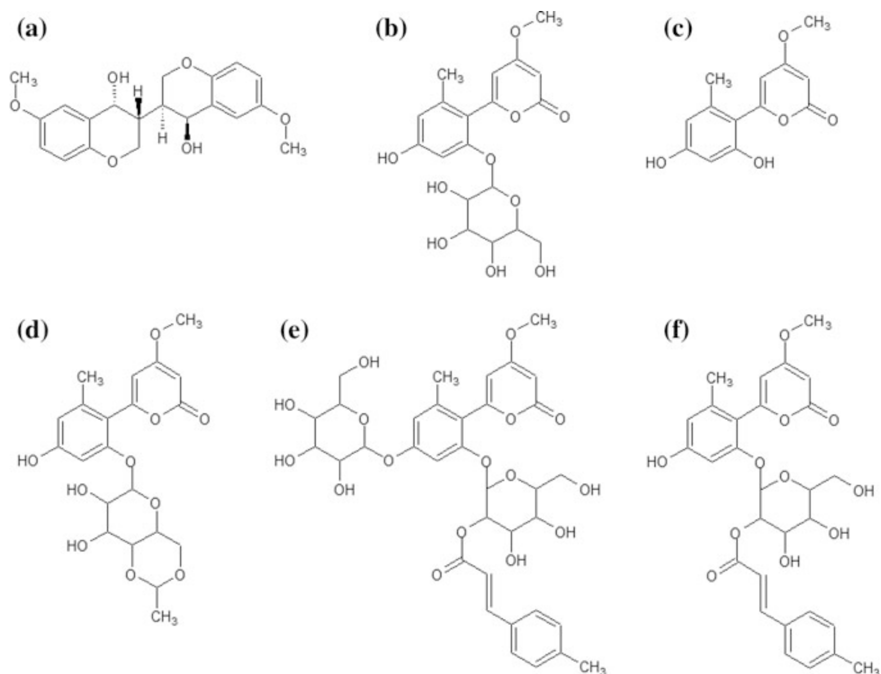

Fig. 9 Chemical structures for the Aloe pyrans and pyrones. **a** Bisbenzopyran, **b** Aloenin, **c** Aloenin aglycone, **d** Aloenin acetal, **e** Aloenin B, **f** Aloe-2″-p-O-coumaroyl ester

Related coumarin compounds in other plants have been reported to be bitter tasting appetite suppressants (Aslam et al. 2010). They may also have a number of therapeutic properties including macrophage stimulation and the subsequent degradation of extracellular albumin, allowing more rapid reabsorption of fluids in individuals with oedema (Casley-Smith et al. 1993). Coumarins have also been shown to be vitamin K antagonists and therefore function as anticoagulants (Whitlon et al. 1978).

Bisbenzopyran (Fig. 9a) and the pyrones aloenin (Fig. 9b), aloenin aglycone Fig. 9c), aloenin acetal (Fig. 9d), aloenin B (Fig. 5e) and aloe-2″-p-O-coumaroyl

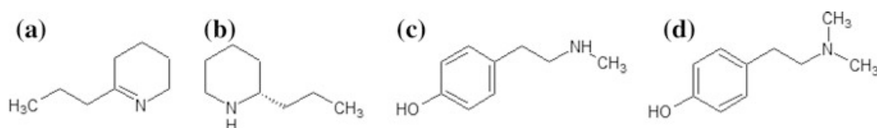

Fig. 10 Chemical structures for the Aloe alkaloids. **a** γ-Coniceine, **b** Coiine, **c** N-Methyltryamine, **d** O,N-Dimethyltryamine

ester (Fig. 5f) are also found in the leaf exudates of several Aloe species (Dagne et al. 2000). The benzopyrones have similar macrophage stimulatory bioactivities as the coumarins (Casley-Smith et al. 1993), and so may also be potential medications for the treatment of oedema.

4.5 Alkaloids

Alkaloids have been detected in several Aloe species with N-methyltryamine (Fig. 10c) and O,N-dimethyltryamine (Fig. 10d) being the most common Aloe alkaloids (Blitzke et al. 2000). In contrast, γ-coniceine (Fig. 10a) has only been detected in a few species (Dagne et al. 2000), whilst coniine (Fig. 10b) has only been reported to occur in the single species *Aloe viguieri* Perrier (Dring et al. 1984). These alkaloids are toxic. There are reports of the use of Aloe leaf exudates as arrow poisons (Reynolds 2005; Blitzke et al. 2000; Neuwinger 1998). They block nicotinic receptors on the postsynaptic nerve membrane, resulting in muscle paralysis similar to that of curare-induced paralysis (Reynolds 2005).

4.6 Benzene, Naphthalene and Furan Derivatives

A number of benzene, naphthalene and furan based compounds have been widely reported as Aloe constituents. The benzene derivative protocatechuic acid (Fig. 11a) has been detected in several Aloes (Dagne et al. 2000). Several studies have reported protocatechuic acid to have antioxidant activity via free radical scavenging (Lodovici et al. 2001; Masella et al. 1999). In contrast, other studies have reported that protocatechuic acid acts as a prooxidant (Babich et al. 2002). The same study reported the induction of apoptosis in human leukaemia and HSG1 cell cultures. Another study showed that the antioxidant/prooxidant properties of protocatechuic acid are dose dependent, with low concentrations resulting in antioxidant activity and concentrations higher concentrations behaving as prooxidants (Nakamura et al. 2000). Other benzene derivatives isolated from Aloes include methyl-p-coumarate (Fig. 11d) and pluridone (Fig. 11e). Pluridone, which was isolated from *Aloe pluridens* Haw., is the only sulphur containing compound isolated from the Aloes.

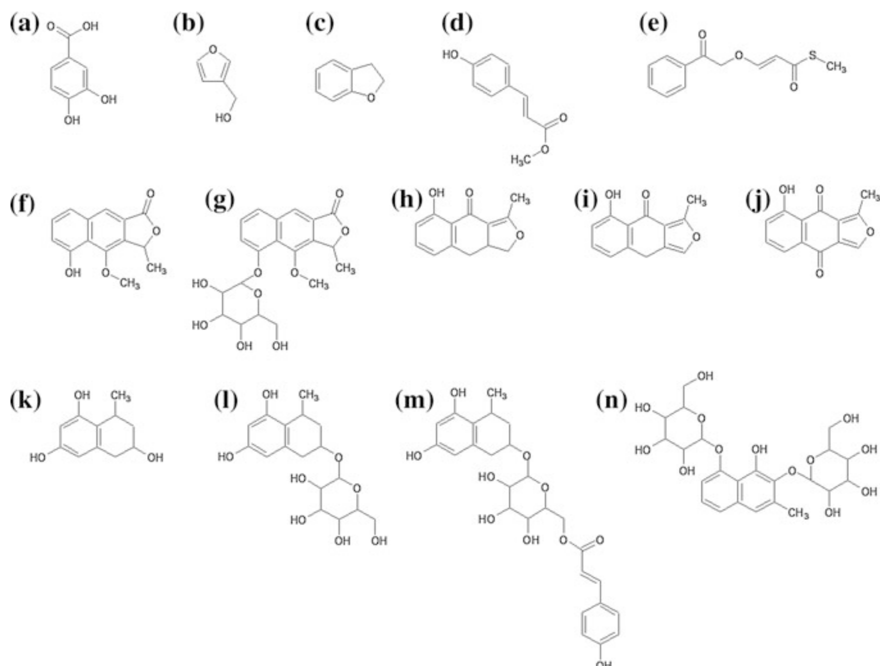

Fig. 11 Chemical structures for the Aloe benzene, naphthalene and furan compounds **a** Protocatechuic acid, **b** 3-Furanmethanol **c** Dihydrocoumarone (2,3-dihrdrobenzofuran), **d** Methyl-p-coumarate, **e** Pluridone, **f** Isoeleutherol, **g** Isoeleuthrol-5-O-glucoside, **h** 5-OH-3-Methylnaphto[2,3-c]furan-4(1H)-one, **i** 3-Methylnaphto[2,3-c]furan-4(9H)-one, **j** 3-Methylnaphto[2,3-c]furan-4,9-dione, **k** Feroxidin, **l** Feroxidin A, **m** Feroxidin B, **n** Plicataloside

The aglycone isoeleutherol (Fig. 11f) and isoeleutherol-5-O-glucoside (Fig. 11g) have been reported in the roots of several Aloe species (Dagne et al. 2000). Other naphthalene compounds isolated from Aloes include feroxidin (Fig. 11k), feroxidin A (Fig. 11l), feroxidin B (Fig. 11m) and plicataloside (Fig. 11n). Several of these compounds, including 5-OH-3-methylnaphto[2,3-c]furan-4(1H)-one (Fig. 11h), 3-methylnaphto[2,3-c]furan-4(9H)-one (Fig. 11i) and 3-methylnaphto[2,3-c] furan-4,9-dione (Fig. 11j) also contain a furan moiety. In general, the naphthaloenes possess insecticidal properties (Daisy et al. 2002).

4.7 Flavonoids

Most phytochemical studies of the Aloe genus have concentrated on their content of anthraquinones, anthrones and chromones but several flavonoids have been noted also in Aloes. However, of the species examined, flavonoids have only been

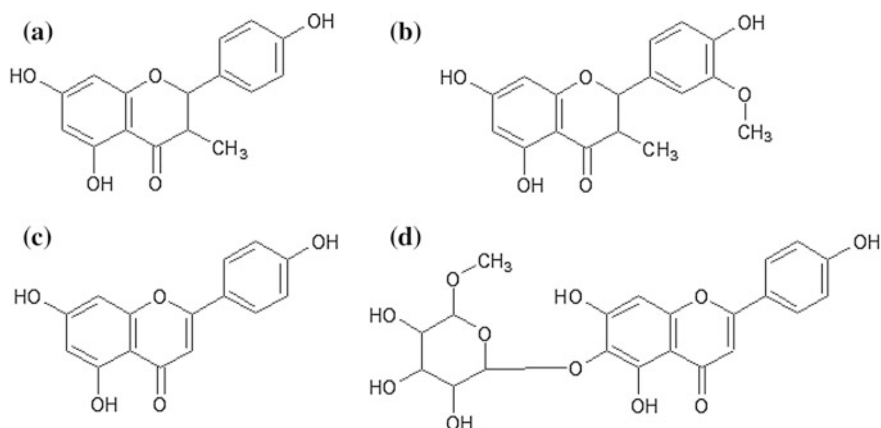

Fig. 12 Chemical structures for the Aloe flavonoids. **a** Naringenin, **b** Dihydroisorhamnetin **c** Apigenin, **d** Isovitexin

reported for a relatively small number. The major flavonoids are naringenin (Fig. 12a), dihydroisorhamnetin (Fig. 12b), apigenin (Fig. 12c) and isovitexin (Fig. 12d) (Dagne et al. 2000). Flavonoids have many therapeutically important bioactivities generally associated with their antioxidant activity. Similar bioflavonoids prevent oxidation of LDL cholesterol via their free radical scavenging activity (Furman and Aviram 2001), inhibit endothelial activation (Carluccio et al. 2003) and also inhibit platelet aggregation (Ruff 2003). They also inhibit cyclooxygenases and can thus prevent thrombosis (Ruff 2003). Evidence exists that high dietary levels of flavonoids are inversely proportional to the risk of coronary artery disease (CAD) (Martikainen et al. 2007; Middleton et al. 2000; Peluso 2006). As with several classes of phytochemicals already considered, the therapeutic potential of the flavonoids may be concentration dependent, with individual flavonoids behaving as either antioxidants or prooxidants at different concentrations (Rahman et al. 1990).

4.8 Sterols

Aloe leaves also contains a number of medicinally important phytosterols including cholesterol (Fig. 13a), campesterol (Fig. 13b), β-sitosterol, (Fig. 13c) and lupeol (Fig. 13d) as well as their glucosides (Dagne et al. 2000). These phytosterols have been shown to promote arterial endothelial cell proliferation (Moon et al. 1999). They also promote the expression of proteins involved in angiogenesis and thus have potential applications in the management of chronic wounds. Recently, β-sitosterol has also been proposed for the treatment of breast cancer (Awad et al.

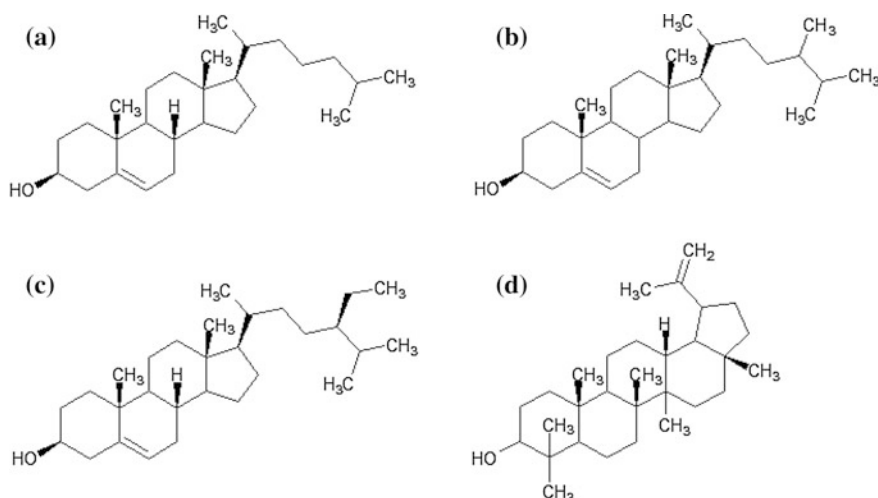

Fig. 13 Chemical structures for the Aloe sterols. **a** cholesterol; **b** campesterol, **c** β-sitosterol, **d** lupeol

2008) and diabetes (Gupta et al. 2011), although its efficacy is still under investigation.

It appears that these therapeutic bioactivities may be due, at least in part, to the redox state of the molecule. A recent study has indicated that β-sitosterol treatment results in reduction of glutathione as well as maintaining the antioxidant enzymes superoxide dismutase and glutathione peroxidise in a reduced state (Vivancos and Moreno 2005). This bioactivity in turn is related to the redox state of the sterol. Interactions between the various components within the crude extracts may convert otherwise antioxidant molecules into prooxidants or vice versa.

4.9 Other Phenolic Aloe Vera Constituents

A number of other secondary metabolites, including tannins (Arunkumar and Muthuselvam 2009), have also been reported in Aloe leaves. Inorganic physiological nutrients including calcium, magnesium, zinc, iron, and copper have also been identified in Aloe leaf extracts (Cock 2011; Dagne et al. 2000). Previous studies have shown that transition metal ions such as copper or iron can enhance the conversion of many phenolics from the antioxidant to the prooxidant state (Wu et al. 2008; Lastra and Villegas 2007). The prooxidant/antioxidant effect of plant extracts is due to a balance between the free radical scavenging activities and reducing power of their phytochemical components. This can be explained using the antioxidant ascorbic acid as an example. Although ascorbate has well characterised antioxidant bioactivities, it also acts as a prooxidant at high concentrations

(Joel 1995). This is due to the greater reducing power of ascorbic acid compared to its free radical scavenging activity. In the presence of transition metal ions, ascorbic acid reduces the metal ions, being itself converted to a prooxidant (ascorbyl radical). Therefore, high dietary intake of ascorbic acid in individuals with high iron levels (e.g. premature infants) may cause unexpected health effects by inducing of oxidative damage within susceptible biomolecules (Halliwell 1996; Herbert et al. 1996; Samuni et al. 1983). Thus Aloes growing in soil containing elevated levels of metallic ions would be expected to have higher concentrations of metal ions, and thus tend towards prooxidant rather than antioxidant bioactivities. Other molecules (such as vitamins, amino acids and proteins) may also have an effect on the redox state of the phytochemical components.

4.10 Non-phenolic Components

Aloe leaf gels also contain high levels of polysaccharides including acemannan (Fig. 14), a long chain polymer of β (1 → 4) linked galactomannan saccharides (Ni et al. 2004; Dagne et al. 2000). Many of the therapeutic properties of Aloe leaf gels have been attributed to acemannan and similar polysaccharides (Ni and Tizard 2004; Ni et al. 2004). Recent publications have suggested that these components act

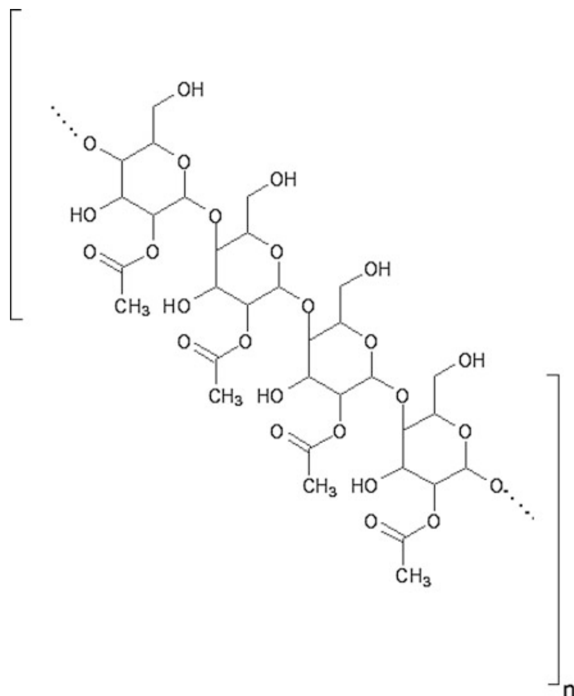

Fig. 14 The structure of acemannan (a major polysaccharide component of *A. vera* leaves) consists of a polymer of β (1 → 4) linked galactomannan sugars

synergistically with many of the other compounds already described, and no single compound is solely responsible for these effects (Cock 2011; Dagne et al. 2000). Acemannan accelerates wound healing (Choi et al. 2001; Chithra et al. 1998a, b, c) and activates macrophages (Zhang and Tizard 1996; Ramamoorthy et al. 1996). It also shows synergistic antiviral activity in combination with azidothymidine and acyclovir (Kahlon et al. 1991). Acemannan has antioxidant properties which may be responsible for acemannans therapeutic activities (Wu et al. 2006). Furthermore, the antioxidant potential of Aloe polysaccharides depend upon their concentration and the degree of acetylation of the monomeric units (Chun-hui et al. 2007). High polysaccharide concentrations (>8 mg mL^{-1}) were found to be necessary for *Aloe vera* polysaccharides to display antioxidant activity. The same study also showed that increased acetylation enhances the antioxidant activity of *A. vera* polysaccharides. However, the polysaccharide components within Aloe leaves are not constant and there are large variations between different individual plants. The composition and concentration of the polysaccharides also fluctuates with changes in the season, growing environment and conditions (Femenia et al. 1999).

Some of the medicinal properties associated with plant extracts require the concerted action of several bioactivities. The following discussion examines some therapeutic properties of Aloe extracts that require the synergistic action of several bioactivities, each of which may be reliant on multiple phytochemicals. This is not a comprehensive examination of the therapeutic properties of Aloe extracts, but only discusses the bioactivities that have been the most extensively studied.

5 Medicinal Uses

The vast majority of these documented uses relate to a relatively minor portion of the Aloe genus. It has been estimated that only approximately a quarter of the recognised Aloe species account for nearly all of the documented uses of the Aloes (Grace et al. 2009). Of these, *A. vera* in particular, and to a lesser extent, *A. ferox*, *A. arborescens* and *A. perryi* dominate these documented uses. The following sections summarise some of the main therapeutic activities.

5.1 Gastrointestinal Activities

One of the most frequently cited therapeutic uses of Aloe spp. is for digestive ailments. Laxative/purgative effects have been particularly well documented for many species. Aloe leaf exudates are used for this purpose due to their high content of anthraquinones and anthrones (van Gorkom et al. 1999). It is generally believed that these compounds act exclusively in the colon in several ways, particularly by:

- stimulating colonic motility (Leng-Peschlow 1986)
- increasing electrolyte and fluid transport, resulting in increased fluid transport into the intestine (Wienbeck et al. 1988) and
- decreasing the readsorption of these fluids by accelerating the intestinal transit rate (Wienbeck et al. 1988; Leng-Peschlow 1986).

The secretion of intestinal fluid is driven by the induction of increased Cl^- transport. Chloride ions enter the basolateral side of the epithelial intestine cells via a Na^+, K^+, $2Cl^-$ cotransport mechanism and are secreted into the intestinal lumen via a transmembrane Cl- channel (Yang et al. 2011). As both the Na^+ and Cl^- ions are transported into the intestinal lumen, there is nett secretion of isotonic fluid that increases the volume of intestinal fluid which in turn increases intestinal motility and thus the laxative/purgative effects attributed to Aloe exudates.

A. vera gel has also been used as a curative agent for gastric ulcers and to protect the stomach lining (Eamlamnam et al. 2006; Yusu et al. 2004). These antiulcer properties of *A. vera* gel are probably due to several individual therapeutic effects including antiinflammatory activity, promoting cell proliferation and wound healing properties, stimulating mucus secretion and via its regulation of gastric secretions (Suvitayavat et al. 2004). This particular study also showed that *A. vera* extracts inhibit gastric acid secretions in rats only at low concentrations. At higher concentrations this effect is lost.

5.2 Antimicrobial Activity

The use of Aloes to treat infections is perhaps the most frequently cited use for the leaves of this genus. Most if not all cultures in regions of Aloe biodiversity have utilised Aloes for the treatment of infections and microbial diseases. The interruption of the external epidermal barrier by a wound, burn or other such event allows microbes to enter and infect the wound. The invasion of microorganisms may cause or intensify inflammation, hindering wound healing and/or cause systemic disease. *A. vera* leaf extracts display good antibacterial (Pandey and Mishra 2010; Cock 2008; Barrantes and Guinea 2003) and antifungal bioactivities (Cock 2008; Ali et al. 1999) against a variety of microbes. Similarly, other Aloe species have also been reported to be potent antimicrobials (van Vuuren and Naidoo 2010; Ndhlala et al. 2009).

Early antibacterial studies of *A. vera* extracts have proved confusing. Some of them indicated that the bioactive agent(s) are anthraquinones (Levin et al. 1988; Anton and Haag-berrurier 1980) whilst other studies found *A. vera* anthraquinones to be inactive (Lorenzetti et al. 1964). Numerous subsequent studies have demonstrated the antibacterial activity of isolated anthraquinones from Aloes (Alves et al. 2004; Ubbink-Kok et al. 1986) and various other plant species (Park et al. 2006; Manojlovic et al. 2002; Hatano et al. 1999). Whilst the mechanism of their antibacterial activity is still subject to investigation, it has been suggested that aloe

emodin and aloesin act by disrupting bacterial membranes (Alves et al. 2004). This study also showed that the form of aloe emodin and aloesin being tested affects their antibacterial activity. Thus anthraquinone loaded liposomes had strong antibacterial activity, whilst the purified free anthraquinones did not. The effect of concentration was not extensively examined. Minimum inhibitory concentration (MIC) values were determined by testing across a range of concentrations, but only relatively low concentrations were tested. It is possible that higher concentrations may have a very different effect, analogous to the concentration effects already described for anthraquinone antioxidant/prooxidant activity.

Other Aloe leaf components have also been implicated in their antibacterial activity. A recent study examined anthraquinone-free leaf gel extracts and isolated components (Lawrence et al. 2009), finding that cinnamic acid, coumaric acid, ascorbic acid and pyrocatechol (all purified from *A. vera* gel) all display good antibacterial activity, especially against Gram positive bacteria. It was proposed that these phenolic antibacterial agents functioned by disrupting bacterial cell membranes, as well as by denaturing bacterial proteins. Cinnamic acid is known to block bacterial glucose uptake and ATP production (Kouassi and Shelef 1998), therefore inhibiting bacterial growth. Coumaric acid inhibits several bacterial enzymes (Weir et al. 2004). A number of other phenolic components were also found to have low to moderate antibacterial activity.

As well as having direct inhibitory effects on bacteria, Aloe leaf components may also selectively modulate cells of the immune system (Boudreau and Beland 2006). Furthermore, acemannan also inhibits bacteria from adhering to epithelial cells and establishing an infection (Azghani et al. 1995). It is therefore likely that the antibacterial activity of Aloe leaf extracts in vivo is due to the synergistic effects of several bioactive components, each acting by one or more mechanisms.

Antifungal activity has received less attention although some studies have demonstrated the ability of *A. vera* extracts to inhibit fungal growth (Joseph and Raj 2010; Cock 2008; Ali et al. 1999). Anthraquinones, especially aloe emodin and aloesin, were implicated in this anti-fungal activity (Ali et al. 1999), although the identity of antifungal components and their mechanisms of action have not been extensively examined. Similarly, the antiviral activity of *A. vera* leaf extracts has been demonstrated (Cock and Kalt 2010; Saoo et al. 1996) although detailed purification, identification and mechanistic studies are required.

5.3 Immunomodulation

Manipulation of the immune system is important for the treatment of a variety of diseases. *A. vera* leaf extracts have been reported to have both good immunostimulant (Ramamoorthy and Tizard 1998) and immunosuppressant activities (Boudreau and Beland 2006). Several studies of the immunomodulatory potential of *A. ve*ra extracts have focused on their immunostimulant activities, particularly of the polysaccharide components. Whilst numerous *A. vera* polysaccharide

components are known to be immunostimulants (Im et al. 2005; Pugh et al. 2001; Qiu et al. 2000), acemannan has been particularly well studied. Its immunostimulant effects are thought to be due to activation of macrophage cells and antigen processing. The activated macrophages secrete cytokines including IL-1, IL-6, interferon, GM-CSF and TNF-α in vitro (Ramamoorthy et al. 1993). The release of these cytokines is itself associated with further pathology through the induction of inflammation. Acemannan also further enhances macrophage sensitivity to IFN-γ, inducing apoptosis (Ramamoorthy and Tizard 1998). Neither acemannan nor IFN-γ alone was capable of inducing apoptosis. Both are required and this synergistic effect appears to function by the inhibiting the expression of Bcl-2 proteins (Ramamoorthy and Tizard 1998).

A few studies have also highlighted the immunomodulatory properties of the smaller phenolic components of Aloe leaves. Aloe emodin and other anthraquinone derivatives have an immunosuppressant effect by blocking lymphocyte proliferation (Huang et al. 1992; Wang et al. 1987). Aloe emodin also reduced IL-1, IL-2 and IL-2 receptor expression (Huang et al. 1992). It was suggested that aloe emodin suppresses both macrophages and lymphocytes. Further studies have identified 37 other anthraquinones with the ability to block the induction of cytolytic T lymphocytes and the to prevent antibody production (Wang et al. 1987). The effect of concentration and the ratio between anthraquinones were not examined in these studies.

Aloe leaf extracts may also exert immune modulatory effects functioning as antioxidants, inhibiting/stimulating the production of free radicals (Hu et al. 2003). Treating streptozotocin-induced diabetic (Rajasekaran et al. 2006) or gamma-irradiated rats (Saada et al. 2003) orally with *A. vera* leaf extracts reduces lipid peroxidation and the formation of hydroperoxides whilst increasing the levels of antioxidant enzymes (e.g. reduced glutathione, glutathione peroxidase, glutathione-S-transferase, catalase, superoxide dismutase) in the liver, lungs and kidney. Similarly, *A. vera* gel has been shown to inhibit ROS production in colorectal mucosa cells (Langmead et al. 2004). This particular study also found that the Aloe gel extract lost their activity at either higher or lower concentrations, indicating that the immune modulatory activity may be concentration dependent similar to that of the isolated *A. vera* components (Tian and Hua 2005). It is therefore quite probable that the variable immune modulatory effects reported for *A. vera* extracts in different studies may be due to the concentrations, ratios and redox states of several active components in the tested extracts; extract conditions favouring antioxidant bioactivity being associated with immune stimulation. Conversely, conditions favouring prooxidant activity might be expected to result in immune suppression, although this has not been extensively tested.

5.4 Antiinflammatory Activity

Inflammation is a normal response by the body to injury. It is not a single event, but involves a complex sequence of metabolic events triggered by a variety of insults including burns, wounds, bites and stings, etc. It is characterised by a wide variety of symptoms including swelling, redness, heat and pain (Macpherson 1992). These effects are the result of vasodilation and increased membrane permeability, allowing leukocyte migration and fluid accumulation.

The inflammatory processes involves the cellular release of several classes of molecules. Vasoactive substances (e.g. bradykinin, prostaglandins and vasoactive amines) dilate blood vessels, opening junctions between cells to allow leukocytes to pass through capillaries. Any compound capable of blocking these vasoactive substances would potentially affect the symptoms of inflammation. β-sitosterol is the most abundant phytosterol in Aloe extracts. β-sitosterol stimulates smooth muscle cells to release of prostacyclin (PGI_2) (Awad et al. 2004). However, β-sitosterol treatment blocks the release of PGI_2 and prostaglandin E_2 (PGE_2) from macrophages (Awad et al. 2004). Thus, β-sitosterol treatment would be expected to affect vasodilation and therefore have a therapeutic effect on inflammation. The Aloe leaf chromone aloesin and its derivatives inhibit cyclooxygenase-2 and thromboxane A_2 synthesis through their antioxidant activities (Yagi et al. 2002; Hutter et al. 1996). Thus *A. vera* chromones have antiinflammatory effects. In contrast, anthraquinones have been shown to stimulate PGE_2 release (Beubler and Kollar 1985) and might therefore be expected to be proinflammatory.

The peptidase bradykinase has been isolated from *A. vera* leaves and shown to break down the vasoactive peptide bradykinin (Wynn 2005; Ito et al. 1993). As bradykinin treatment results in vasodilation, hydrolysing this polypeptide decreases vasodilation and therefore inhibit leukocyte passage and fluid leakage from the capillaries into the surrounding tissue. *A. vera* leaf bradykinase would therefore be expected to contribute a therapeutic effects on the symptoms of inflammation.

Chemotactic factors including several proteins and peptides are required to increase cell motility, especially the motility of leukocytes during inflammation. Blocking these chemotactic factors or blocking their effects prevents inflammatory swelling. Several compounds in *A. vera* extracts are able to block chemotaxis. Anthraquinones suppress cytolytic T lymphocytes in favour of suppressor cells (Huang et al. 1992; Wang et al. 1987). Furthermore, anthraquinones decrease cytokine production and IL-2 mRNA expression in activated T lymphocytes (Kuo et al. 2001), thereby decreasing chemotaxis. More recent studies have demonstrated that the anthraquinone emodin decreases plasma levels of the cytokines IL-2 and TNF-α whilst increasing IL-10 (which itself downregulates IL-2 and TNF-α cytokine activity) (Lin et al. 2010). None of these studies examined the relationship of the redox state of the anthraquinones with these effects. Furthermore, these studies have not rigorously examined the effects of a range of doses of these phytochemicals.

In contrast, Aloe polysaccharides (including acemannan) have a stimulatory effect on chemotaxis. Acemannan exposure stimulates cytokine production and activates lymphocytes (Tan and Vanitha 2004). Specifically, pure acemannan isolated from *A. vera* leaves has been shown to stimulate macrophages to release IL-1, IL-6, interferon, GM-CSF and TNF-α in vitro (Ramamoorthy et al. 1993). Similarly, *A. vera* lectins stimulate cytokine production. Aloctin A, the best characterised of the Aloe lectins, has been shown to stimulate the production of IL-2 (Lee 2006) and to enhance the production and activation of macrophages (Lee 2006). Therefore, *A. vera* extracts contain both chemotactic stimulatory and inhibitory compounds. The chemotactic effect of *A. vera* extracts would therefore be dependent on the levels and ratios of the factors affecting chemotaxis, as well as their redox state. Aloe leaf extracts contain multiple active phytochemicals. It is likely that several of these may be required to control different phases of the inflammatory process. Failure to consider this is probably responsible for past ambiguities about the efficacy of Aloe extracts as antiinflammatory agents.

5.5 *Wound Healing*

Promoting wound healing is a relatively well-studied therapeutic property of *A. vera*. It is the result of several bioactivities that include antiseptic activity, diminishing inflammation, stimulating matrix remodelling, cellular growth and proliferation. The growth of endothelial, epithelial and fibroblast cells are critical in wound healing. As a first step, a fibrin clot is formed as a temporary repair. This is vital to minimise microbial infection which may retard the healing process. The wound is subsequently invaded by a variety of cell types, some of which stimulate an inflammatory response, and others which are directly involved in the repair mechanism (Davis et al. 1987). Wound repair itself occurs in three phases: the migration of epithelial cells and fibroblasts into the wound site, proliferation of these cells and subsequent cellular maturation. The wound healing effect of Aloe extracts involves the synergistic action of multiple components on several pathways.

Aloe anthraquinones show contradictory effects on cell growth and proliferation. Aloe emodin stimulated a 2.5 fold increase in rat hepatocyte DNA synthesis with a corresponding increase in cell growth (Wolfle et al. 1990). Furthermore, aloe emodin has also been shown to protect hepatocytes from apoptosis (Lin et al. 2010). In contrast, other studies have shown that aloe emodin induces apoptosis in promyeloleukemic HL-60 cells (Chen et al. 2002) and human lung squamous cell carcinoma (Lee 2001; Lee et al. 2001) and also inhibits human neuroectodermal tumour growth (Pecere et al. 2000). Some studies have postulated that the proapoptotic effect of aloe emodin is due to an induction of caspase 3 activity, together with a decrease in the levels of the antiapoptotic protein Mcl-1 (Chen et al. 2002). Other studies have implicated caspase 8-mediated cleavage in the apoptotic activity of emodin (Yan et al. 2008). Studies into the proapoptotic mechanism of aloe

emodin are ongoing. Similarly, anthrones have also been shown to induce cell death. In a recent study, an anthrone from the Ethiopian medicinal plant *Kniphofia foliosa* was shown to induce rapid death in mouse and human cancer cells via necrosis (Habtemariam 2010).

Other Aloe phenolics and some non-phenolic compounds have also been implicated in the wound healing effects of Aloe extracts. β -Sitosterol and β-sitosterol glucosides promote endothelial cell proliferation and angiogenesis (Moon et al. 1999), although their activity appears to be dependent on their redox state (Vivancos and Moreno 2005). The reduced sterol has antioxidant activity and stimulates wound healing processes, whilst oxidised sterols are prooxidants and induce cell death. Therefore, β -sitosterol and β-sitosterol glucosides have potential applications in wound management in their reduced state. The Aloe chromone aloesin stimulates cellular proliferation (Yagi et al. 2002; Choi et al. 2001; Lee et al. 1997) possibly due to its high antioxidant activity (Gomes et al. 2009; Araya-Maturana et al. 2008). In contrast, cinnamic acid has been shown to downregulate expression of cell proliferation and anti-apoptotic gene products, although the effects of both high and low concentrations were not examined (Aggarwal et al. 2005; Liu et al. 1995).

The redox environment affects cellular signal transduction, DNA and RNA synthesis, protein synthesis, enzyme activation, regulation of the cell cycle, ligand binding, DNA binding and nuclear translocation and therefore ultimately cell proliferation/death (Makino et al. 1999; Simons and Pratt 1995). Transcription factors are active in their reduced form and their translocation to the nucleus is also redox dependent (Okamoto et al. 1999). A reducing environment favours cellular proliferation whilst an oxidising environment results in an increase in reactive oxygen species, initiating cell death (Kim et al. 1996; Hamilos et al. 1989). Therefore, extract conditions favouring antioxidant activity (e.g. low aloe emodin, high aloin, low cinnamic acid, low ascorbic acid, low transition metal and high anthrone concentrations) would be expected to favour cellular proliferation, whilst conditions favouring prooxidant activity (e.g. high aloe emodin, low aloin, high cinnamic acid, high ascorbic acid, high transition metal and low anthrone concentrations) might promote cell death.

The non-phenolic components, particularly acemannan, also have a role in wound healing. A stimulation of gingival fibroblast proliferation has been demonstrated when treating oral wounds with high doses of acemannan (Jettanacheawchankit et al. 2009). This stimulatory effect was found to be due an induction in expression of the growth factors KGF-1, VEGF and an increase in collagen expression. This study only examined the effects of relatively high concentrations of acemannan, in the range that would relate to antioxidant activity. As lower concentrations may relate to prooxidant activities, it is possible that the induction of fibroblast proliferation may not be seen at these concentrations. Indeed, as lower concentrations of acemannan correspond to prooxidant effects, it is possible that at lower concentrations, cell death may be induced. The concentration dependent redox effect of acemannan may also contribute to the discrepancies seen between proliferative studies of *A. vera* extracts.

As well as requiring cellular growth and proliferation, wound healing also requires matrix remodelling. *A. vera* gel extracts have been shown to stimulate and accelerate the production of the mammalian structural proteoglycans hyaluronic acid and dermatan sulphate (Chithra et al. 1998a). Activities of the enzymes β-glucuronidase and N-acetyl glucosaminidase are increased during wound healing, resulting in increased carbohydrate turnover at the site of the wound. Wounded diabetic rats treated with *A. vera* gel show increased collagen formation (Chithra et al. 1998b) and cross linking (Chithra et al. 1998c). It is evident that a synergistic action is required for several *A. vera* extract components to beneficially promote wound healing. The reported discrepancies between different studies may be due to differences in concentrations, ratios and redox states of these components. As high antioxidant contents are a feature of many Aloes, it is not surprising that several species show wound healing properties.

5.6 Anticancer Activity

The growth and development of healthy cells depends on fine regulation of growth promoting and growth inhibiting pathways. Proto oncogenes and tumour suppressor genes encode the proteins that regulate cell division/cell cycle, as well as for the repair of damaged DNA and cell programmed death by apoptosis. Mutations within these genes have been implicated in the onset of cancer (Hanahan and Weinberg 2000) causing cells to no longer require external signals to proliferate. These cells also fail to recognise signals that restrict cell division, resulting in uncontrolled cell growth. In tumour genesis, multiple genes may be altered and transmitted to daughter cells, which subsequently escape normal growth restraints and form a tumour, that may be benign or malignant.

The induction of oxidative stress has been linked with several types of cancer (Brown and Bicknell 2001; Tome et al. 2001). Chromosome instability is also a common feature of many of the cancers that have been linked with oxidative stress, suggesting that increased oxidative stress may contribute to development of genetic instability. Oxidative stress leading to genetic instability would result in the emergence of new tumour phenotypes. In such populations, a decrease in apoptosis but an increase in tumour growth and subsequent tumour progression is observable.

Many of the currently used anticancer agents (e.g. doxorubicin, daunorubicin, mitomycin C, etoposide, cisplatin, arsenic trioxide, ionising radiation, photodynamic therapy) depend exclusively or in part on the production of ROS for cytotoxicity. Sensitivity of tumour cells to oxidative stress and/or apoptosis may affect treatment success (Davis et al. 2001; Ruefli et al. 2001). Studies indicate that WEHI7.2 mouse thymoma cells overexpressing catalase (CAT38) or thioredoxin (THX) are resistant to glucocorticoid induced apoptosis in vitro (Freemerman and Powis 2004; Baker et al. 1997). This suggests that glucocorticoid induced apoptosis occurs by a ROS dependant/independent mechanism. Average tumour weights increased in severe combined immune deficient (SCID) mouse tumour xenografts

from cells over expressing catalase or thioredoxin (Baker et al. 1997). Tumours from both transfectants contained fewer apoptotic cells but mitotic cell numbers were similar. This suggests that antioxidant overexpression may increase tumour size due to a decrease in apoptosis.

ROS-based tumour therapy should cause tumour regression if the tumour cells are not apoptotic/oxidant-resistant cells. Therefore, if Aloe components are present in concentrations and ratios consistent with prooxidant activity, the extract should promote apoptosis and therefore would have anticancer activity. If the levels of components are consistent with a reducing environment, antioxidant activity would result and the extract would not have anticancer activity. Conversely, should this same treatment protocol be repeated on a tumour with apoptotic resistant/oxidant resistant cells, the converse would apply, and tumour progression would be more likely.

Studies into the antioxidant/prooxidant effects of Aloe extracts have demonstrated that the ability of a plant extract to exert antioxidant activity depends on multiple factors. *A. vera* antioxidant components, for example may function as either an antioxidant or an oxidant with their action being dependent upon their concentration (Cock 2011). The *A. vera* anthraquinone aloe emodin exerts antioxidant behaviour at lower concentrations, yet acts as a prooxidant at high concentrations. In contrast, a different anthraquinone (aloin) has an antioxidant effect at higher concentrations, yet a prooxidant effect at low concentrations. Thus, Aloe leaf extracts and components may act as either antioxidants or as oxidants, dependent on differing levels of the various constituents, and on their ratios. Although many Aloe species have high antioxidant contents reported, it is possible that the individual components may act as either antioxidants or as oxidants and thus may also be effective in the treatment of cancer, as well as in its prevention at different concentrations.

Similar prooxidant effects have been reported for other antioxidant phytochemicals including flavonoids (Rahman et al. 1990) and tannins (Singh et al. 2001a) which are present in Aloes. Previous studies have also shown that the presence of transition metal ions such as copper or iron in the extract can enhance the conversion of the antioxidant to the prooxidant state (Lastra and Villegas 2007; Wu et al. 2008). The prooxidant/antioxidant effect of plant extracts is due to a balance between the free radical scavenging activities and reducing power of their phytochemical components.

5.7 Antidiabetic Activity

Diabetes mellitus refers to a group of metabolic disorders that result in increased blood glucose concentrations, either because the pancreas does not produce enough functional insulin (type 1 diabetes), or because cells do not respond to the insulin which is produced (type 2 diabetes). The causes of diabetes mellitus include the autoimmune destruction of pancreatic cells (Betterle et al. 1984), viral infections

(Yoon et al. 1979), genetic and environmental factors (Pyke 1977), insulin or insulin receptor gene mutations (Tager 1984), and altered pancreatic prostaglandin metabolism Robertson and Chen 1977). Diabetes has significant health effects, impacting on the quality of life and life expectancy of those suffering with it.

Glycosylation of blood proteins including haemoglobin, albumin and lipoproteins is characteristic of diabetes mellitus (Klein 1995). Under the hyperglycaemic conditions of diabetes mellitus, blood glucose interacts with specific amino acids on the surface of proteins, forming glycosylated protein products. These may undergo a series of further chemical modifications, resulting in the production of advanced glycation end products (AGE) (Singh et al. 2001a, b). The binding of AGEs to their receptors results in altered cell signalling which in turn results in free radical production (Penckofer et al. 2002). Indeed, diabetes mellitus has been shown experimentally to be associated with an increase in free radical formation and an associated decrease in antioxidant potential (Davi et al. 1999; Vessby et al. 2002). Studies have directly linked oxidative stress with the impaired maintenance of glucose homeostasis and the enhanced lipid peroxidation seen in diabetes mellitus (Davi et al. 1999). Furthermore, increased total antioxidant levels have been measured in the blood and saliva of diabetic patients, further supporting the proposed role of oxidative stress in diabetes mellitus (Astaneie et al. 2005).

Oxidative stress induction has also been suggested to be the common link between the diverse medical complications (including cardiovascular disease, renal and neural degeneration, impaired vision and erectile dysfunction) seen in diabetes mellitus (Rahimi et al. 2005; Shih et al. 2002). Therefore, treatment with antioxidants would be expected to counteract many of these complications. High antioxidant contents are a common feature for plants of the Aloe genus. All Aloe species have a number of compounds (both phenolics and non-phenolic compounds) that can act as antioxidants. Many phenolic compounds could potentially behave as either antioxidants or prooxidants, dependant on their concentration, redox state and ratio between compounds (Cock 2011).

A number of studies have indicated the beneficial effects of *A. vera* extracts in diabetic patients (Rajasekaran et al. 2004; Okyar et al. 2001). Administration of *A. vera* extracts to Streptozotocin-induced diabetic rats results in a decrease in blood glucose and a corresponding increase in liver glycogen (Rajasekaran et al. 2004). The maintenance of glucose homeostasis by *A. vera* extracts in diabetic rats was shown to involve a number of mechanisms including altered activities of several enzymes: glycogen phosphorylase activity was decreased and glycogen synthetase was increased, resulting in increased hepatic glycogen stores (Rajasekaran et al. 2004). Hexokinase activity and mRNA levels are decreased in diabetic rats (Saxena et al. 1992), yet treatment with *A. vera* extract returned these to more normal levels (Rajasekaran et al. 2004). Similarly, increased lactate dehydrogenase, glucose-6-phosphatase and fructose-1, 6-bisphosphatase activities are seen in diabetic rats (Saxena et al. 1984). *A. vera* extract treatment significantly restored these enzyme activities (Rajasekaran et al. 2004).

6 Conclusions

Aloes have been used traditionally as folk remedies for a wide variety of diseases and medical conditions for thousands of years. This survey of the traditional medicinal and pharmacognosy literature has highlighted the therapeutic importance of this useful genus of plants. The usage of *A. vera* is particularly widespread. Indeed, this species is now cultivated worldwide and has found its way into the herbal remedies of many different cultures. Other Aloe species (*A. ferox*, *A. arborescens*, *A. perryi* in particular) have also been widely used in such diverse traditional medicine systems as Arabian, Ayurveda, Siddha, Traditional Chinese Medicine (TCM), and those of Eastern, Southern and Central Africa and the tropical regions of the Americas.

Despite this long history of traditional usage, rigorous scientific research had been confined to the study of only a few species. In these species, multiple therapeutic bioactivities have been documented. Extracts from these species have good antioxidant, anticancer, antidiabetic, antiseptic, cardiotonic and antiinflammatory effects as well as assisting in wound healing. In some cases the active phytochemicals have been established, although for many medicinal properties the active principles have been only partially characterised. Often the partially purified compounds of a crude extract are itemised yet the active component(s) not identified. In other studies, the active compounds have not been characterised and instead only the classes of compounds in the crude mixture have been determined. Furthermore, contradictory reports from different laboratories are often published, leading many to doubt the efficacy of the Aloes. Purified compounds often do not display the activity seen for crude extracts so it is likely that the therapeutic properties of the Aloes is the result of the synergistic action of a complex mixture of phytochemicals.

Individual extract batches may vary widely with regards to individual phytochemical profiles, ratios between various components, and the redox state of these components. These variances may have profound effects on the reported bioactivities and are likely to account for the reported discrepancies between different studies testing crude mixtures. Despite these difficulties, the use of crude extracts is often necessary as the individual components often do not show the same bioactivities, or have different efficacy to the crude extracts. This is true for the Aloes. Aloe juice or Aloe crude extracts often display higher efficacy than the purified components. It is likely that the biological activity of the Aloes is a synergistic action of the different classes of compounds present in Aloe plants, rather than a single constituent or just a few compounds. Furthermore, these compounds need to be present in the correct levels/ratios/redox states for bioactivity to be consistently observed. More studies will still be required to fully understand the medicinal properties and the mechanisms of action of the genus Aloe. The pharmacology of Aloes is still very much a work in progress.

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Silver Pharmacology: Past, Present and Questions for the Future

Michael W. Whitehouse

Disclaimer: The author declares no conflict of financial interest with any commercial organisations.

Dedicated to the memory of Douglas Perrin (1922–1998), a physical chemist from New Zealand, with a passionate interest in using metals in medicine.

Abstract Silver pharmacology is at the cross-roads. It has a long history as a chemosterilent but is currently denigrated by some vested interests and other 'knowledge monopolies'. It deserves better—particularly in these critical times of ever mounting incidence of antibiotic resistance. This reappraisal outlines some approaches to a dispassionate debate as to why we should, or should not, be reconsidering silver as an addition to (not a substitute for) other antibiotics at the front line of medicine. This will require more understanding about (i) the chemistry of silver in a biological environment; (ii) the different physical and bio-reactive properties of ionised silver (Ag(I)) and nanoparticulate metallic silver (Ag^o); (iii) the antibiotic potential of both Ag(I) and Ag^o; and (iv) establishing objective Quality Controls for potential silver therapies. Six appendices (A–F) provide some technical data and focus further upon the need to clearly define (a) procedures for manufacturing nanoparticulate metallic silver (NMS); and (b) the purity and properties of NMS preparations—especially stability, antibiotic efficacy and safety of products offered for clinical evaluation. A further appendix (G) deals with some political considerations currently impeding impartial clinical research on silver therapeutics.

Abbreviations

[ ]	Either concentration usually in water or indicating a metallo-complex
Ag^0	Zerovalent metallic silver
Ag(I)	Monovalent silver, oxidation No. 1
Ag(III)	Unstable trivalent silver, powerful oxidant
AgAc	Silver acetate
$AgNO_3$	Silver nitrate
ATP	Adenosine 5′-triphosphate

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A.W.	Atomic weight
BBF	Bacterial biofilm
Cu^0	Metallic copper
Cu(II)	Cupric ions, a stable solute
DIY	Do-it-yourself (e.g. home preparation of ‘colloidal silver’)
LED	Light- emitting diode
MBC	Minimum bactericidal concentration to kill a particular organism
MIC	Minimum inhibitory concentration, as an antimicrobial
nm	Nanometre i.e. 10^{-9} m
NPS	Nanoparticulate zerovalent (metallic) silver clusters
$p[Ag^+]$	Negative (base 10) logarithm of the silver cation concentration
pH	The negative (base 10) logarithm of the hydronium ion concentration
RA	Rheumatoid arthritis
SERS	Surface-enhanced Raman spectroscopy
WWW	World Wide Web i.e. electronic sources of information

> ‘There are many lies told in every society today about what and what does not have value, or can or cannot transform our lives. The challenge is to identify them.’
>
> Mary Evans 2013 (Teacher and Theologian, Ethiopia)

1 Prologue

This chapter discusses some of the chemical and physical properties of silver that might help us to (a) better understand its interactions with biological organisms and ecosystems and (b) separate the hard facts and science from the commercialism, ‘politics’, ignorance and hype surrounding this ‘hot topic’ of silver as a medicinal. But why is it so hot? Largely because of expectations that so-called ‘colloidal silver’ (CS) will be the healing remedy for many of our ills; particularly as an antibacterial, antifungal and anti-protozoal agent, able to combat many antibiotic-resistant organisms (especially virulent bacteria and protozoa). It is also cheap and available without prescription.

A flood of reports, speculations, sales promotions and rather unfounded optimism about CS assails us almost daily via the World Wide Web (WWW). What is the reality behind this phenomenon? We really should know this to sift the facts from the fantasies. Also we need to examine/question present government regulations, virtually excluding it as a legitimate drug—lest we make a terrible mistake, erroneously pre-judging the future and value of any *well-characterised* medicinal silver preparations.

For many health professionals, it is rather a surprise that silver even has a pharmacology. It is rarely mentioned in standard medical texts beyond occasional allusions to its value as an adjunct for promoting wound healing. Yet it is widely known for its medicinal potential as an antiseptic. As a noble or ‘coinage’ metal it is

classed together with copper and gold in Group 11 (formerly 1B) of the periodic table of the chemical elements. Their long history of use in coinage for jewellery and for making mirrors also reflects (sic) their chemical stability, durability and resistance to corrosion.

Copper and gold have long been recognised for their bio-utility (Whitehouse and Walker 1978; Shaw 1999; Whitehouse 2008) and see Table 1. Copper is also an essential micronutrient, being a constituent of many enzymatic redox systems. As an *endobiotic*, it strongly binds to several defensive proteins to combat the toxicity of dioxygen and its metabolites. Copper also has an essential anabolic role being involved in the biogenesis of the extracellular matrix and connective tissues in animals. (Frausto da Silva and Williams 1991).

Silver and gold are *xenobiotic* and therefore suspected toxins. In fact, their toxicity may be considerably less than that of copper, a notable irritant (unless strongly complexed).

Table 1 Synopsis of noble (coinage) metal pharmacology

Metal	As	Use	References
Copper Atomic No = 29	Cu^0	a. Antifouling, marine vessels b. Bracelets/bangle for arthritis (a) and (b) = slow-release depots for reactive Cu(II)	Walker and Keats (1976)
	Cu (II)	Algicide, molluscicide, Schistosomaticidal Anti-inflammatory (transdermal) CuO (copper bullet) = oral supplement for livestock	Walker et al. (1980) Costigan and Ellis (1980)
Silver At. No. = 47	Ag^0	Nanoparticles: Antibiotic Matrix for drug delivery	
	Ag (I)	Antiseptic Accelerated wound healing	Higginbottom (1826) Spadaro et al. (1974), Becker and Selden (1985)
Gold At. No. = 79	Au^0	Microparticulate *Swarma bhasma* (Ayurvedic medicine) Colloidal gold: Oral anti-inflammatory (Aurasol[R]) Parenteral anti-arthritic (rats)	Brown et al. (2007) Abraham and Himmel (1997) Brown et al. (2008)
	Au (I)	Injected aurothiolates (AuSR) and oral Auranofin Anti-arthritic anti-tumour anti-parasitic Aurocyanide—a pharmaco-active metabolite of AuSR	Kean and Kean (2008) Tiekink (2008) Madiera et al. (2012) Berners-Price and Filipovska (2011) Graham et al. (2008)

Note This is only an outline, indicating some recent advances

The chemistry of silver appears deceptively simple at least in vitro and geologically (Stwertka 2012; Browne 2013). But it is another story to discuss silver's interactions with, and transformations by, living systems; the consequences of which may be expressed over time periods ranging from seconds to centuries. With organic pharmaceuticals, there is relative certainty that they will ultimately be inactivated, being degraded somewhere within the biosphere.

Silver, unique even among inorganic pharmaceuticals (think lithium, bismuth, platinum) remains intrinsically bio-reactive until finally captured by detoxification with sulphide anions. Seems scary? But we have learned to live with—and beneficially harness other scary entities ranging from oxygen (atomic number 8) to uranium (At. No. 92). With its atomic number being 47, silver lies almost halfway between these extremes. Just as animals and mankind evolved to coexist with and harness oxygen to utilise foodstuffs first and fossil fuels later, so we have had to learn how to control uranium as another source of energy (nuclear fuel). Having met these challenges, unlocking and controlling the power of silver pharmaceuticals ought then to be relatively simple—but at present it is not. Which is why this area where xeno chemistry meets physiological chemistry is just as fascinating today as it was to Paracelsus and his fellow alchemists 500 years ago.

Silver perhaps has/should have an important para-pharmaceutical role, e.g. when used as a conductor of electricity within biocontexts favouring healing and tissue repair i.e. regenerative medicine. Some aspects of this may be better understood in terms of physics, rather than chemistry.

The first English Nobel Laureate for Literature, in 1907, Rudyard Kipling (1865–1936) wrote these pithy comments:

> 'Gold is for the mistress,
> Silver for the maid,
> Copper for the craftsman cunning at his trade.'

It is the maid who serves the wider numbers and more frequently. In a pharmaceutical context, will this ever legitimately be said about silver?

This survey is only an introduction, not a detailed review. It focuses first on some essential background information and *then* tries to help clarify what might be the essential Quality Controls for medicinal silver preceding any significant clinical studies, either as a therapeutic agent or a preventive medication—both now and in the foreseeable future.

2 Some History (and More Commentary)

Silver is one of the few metals occurring naturally and was known to the Chaldeans in the Fourth Century BCE. It is mentioned several times in the Christian Bible (Second Century BCE onwards). Table 2 highlights some notable advances in our understanding of silver pharmacology. Some of them were made not by physicians but by the military and by surgeons. Whilst they may not have understood the

Table 2 A brief history of silver medication/disinfection

• Both Cyrus the Elder, founder of the Persian Empire (600–529 BCE), and Alexander III ('The Great'), a Greek general (356–323 BCE), were each reputed to have owed their military success to carrying and disinfecting the drinking water for their troops, using silver containers
• Specific uses in medicine are known to the eminent Arabic physician Gerber, the 'Father of Chemistry', and his pupils (Mahomaden School 702–765 CE)
• Argyria, a side-effect affecting the skin (see later) noted by Avicenna (980 CE) (Hill and Pillsbury 1939)
• Silver cutlery and tableware adopted by those who could afford it, as an antidote to microbial contamination e.g. prevent putrifaction
• Surgical insertion of thin silver plates to cover/close battle injuries especially to the scalp was routinely practised by the Knight of St John at their hospital in Valetta, Malta (16C). This procedure was reported to both diminish mortality and increase rates of wound healing compared with other treatments then available
• John Higginbottom MRCS (1826), Nottingham UK advocates application of 'lunar caustic' aka silver nitrate 'in the cure of certain wounds and ulcers'
• Small silver articles e.g. US$1 coins inserted into water, milk and other beverages for preservation during long voyages e.g. Mormon migration across the American Prairies from Illinois to Utah (1861–1888)
• The Merck Index First Edition (1889) lists at least 18 silver salts for pharmaceutical use
• Henry Crooks (1914) introduced electrical methods for preparing metallic silver colloidal particles (hydrosols), then shown to be germicidal against the typhoid bacillus (Simpson and Hewlett 1914)
• Treatment of 'trench fever' in World War I, caused by lice carrying *B.quintana*, using injected collogol a silver-protein colloid (Sweet and Wilmer 1919).
• Antibacterial activities of silver preparations against defined microbes documented in the British Medical Journal during 1920s—and a large number of reports published elsewhere since the 1950s
• Treatment of 'chronic arthritis' in Vienna with a colloidal silver preparation collagel (Loewenstein and Fee 1928)
• Early Twentieth Century: Mandatory introduction in many states of the USA of 'colloidal silver' = Ag(I)-impregnated small proteins to prevent blindness in the newborn, associated with *Chlamydia* and other micro-organisms in the birth canal
• Robert Becker an orthopaedic surgeon (Syracuse NY) discusses the 'silver wand' i.e. metallic silver rods inserted into wounds (Becker and Selden 1985) and electrically stimulated to produce ionic silver locally that greatly

(continued)

Table 2 (continued)

accelerate healing of bone fractures (Spadaro et al. 1974)—possibly by inducing de-differentiation of connective tissue cells[a]
• Re-epithelialisation of skin grafts increased by locally released silver ions
• Proving superior to standard antibiotic treatment (e.g. neomycin and polymyxin) (Demling and De Santi 2002)

[a]For these and other original contributions contravening 'politically correct' dogmas, Becker was dismissed from the Veterans Administration Hospital and repeatedly denied further funding from the US government (Becker and Selden 1985)

mechanisms underlying their discoveries, they nonetheless realised their immense practical benefits.

There are some thoughtful short discussions of the historical use of silver and some particular formulations (Hill and Pilsbury 1939; Gibbs 1999; Jefferson 2003; Lansdown 2010; Hancock 2011; Browne 2013). Several others are available on the WWW, including those originating from

silvergenesis.com (South Africa); with an excellent bibliography,
cwahealth.com,
Eric J. Rentze via Immunogenic Research Foundation (oligodynamic.com/history.html), and
Colloidal-silver.com.au (Australia) to cite just a few.

Much of the recent science/technology of silver has centred on preparing (very) *stable* nanoparticulate silver for various industrial applications. New methods of manufacture, stabilisation, characterisation and application appear at almost weekly intervals. By contrast, there is far less progress being reported in establishing its medical applications; not surprising in view of the conservatism of some parts of the medical profession and its manipulators at the present time.

By contrast, many medicinal properties of nanoparticulate silver are better understood in terms of its *instability* in a bio-environment e.g. being a slow-release source of bio-reactive oxidised silver, Ag(I); the particle serving as a drug/toxin precursor—often described by pharmacologists as either a 'latent' drug (Harper 1958) or a pro-drug (Albert 1979).

Small is not only beautiful but may also be more effective. Silver nanoparticles, 20 nm diameter, are approximately 50 times smaller than many bacteria (1–2 μm diameter) allowing many particles to become attached to a single planktonic bacterium. So these present times with technologies for reproducibly preparing nanosilver particles, have enabled a new range of studies probing the future of silver in medicine, particularly as an antimicrobial agent.

3 Physical Properties of Silver (Ag^0)

The colour of metallic silver may be 'silver', grey/black or yellow according to particle size. For many centuries, it has been used as a yellow pigment in stained glass. It is physically soft and highly malleable. For daily use, it must be hardened by forming alloys e.g. with up to 20 % copper in jewellery and bracelets. [Sterling silver contains 7 % added copper] It forms an amalgam, dissolving in mercury, formerly much used in dental fillings.

The optical properties of metallic silver set it apart from other metals. It has a high lustre, efficiently reflecting visible light at frequencies above 420 nm. It absorbs light at 417 nm (necessitating the use of powdered aluminium instead in some astronomical telescopes). Silver ions absorb electromagnetic radiation (EMR) most efficiently below 420 nm allowing their photo-reduction by hydrated electrons to generate atomic silver particles (Laroo 2013).

Molten silver absorbs molecular oxygen, which is liberated—sometimes explosively—when the metal resolidifies upon cooling. This compatibility/affinity with oxygen without concomitant oxidation underlies the catalytic—and possibly some of the bio-reactive—properties of finely divided metallic silver.

The physical properties of nano-sized silver particles are being intensively researched especially for applications in novel micro-devices e.g. for electrical (bio) sensing, electronic switching, etc. as well as potential therapeutic uses.

The *stability* of bulk silver metal (Ag^0) and its remarkable physical properties ensured its ever-increasing commercial importance for nearly 3 millenia. Metallic silver is no longer used in popular coinage, now being replaced by the cheaper and lighter alloys. Despite being superceded as an essential component of the photographic process, it is still in high demand for jewellery, dental amalgam, manufacturing mirrors and electronic devices. Fine films of metallic silver are being increasingly used prophylactically as an anti-infective aid for coating catheters, especially those inserted into blood vessels, lymphatics, ureters, etc. to minimise introduced infections.

A very practical use of nanoparticulate copper, silver and gold is facilitating physicochemical analyses using surface-enhanced Raman (infrared) spectroscopy (SERS); silver being particularly useful. This technique has the potential for indicating the specificity of a molecular fingerprint combined with the sensitivity for detecting single molecules (Cialla et al. 2012).

4 Silver Chemistry: An Outline

Useful discussions are to be found in texts by Mellor (1923), Thompson (1973), and the monumental compendium of inorganic chemistry (Gmelin 1971) written in German and some English. This section mainly focuses on transformations that may occur under physiological conditions i.e. at relatively low temperatures, in

saline media, amidst an abundance of ligands for ionic Ag(I) and of supramolecular structures able to interact with nano-sized metal particles, Ag^0.

Silver metal is sufficiently inert to cause no/minimal irritation when inserted into/under the skin e.g. nose rings and other body-piercing jewellery. This is in marked contrast to other metals e.g. copper, aluminium and even stainless steel.

Table 3 indicates some characteristics of four different states of silver, each with distinctive properties; some of which may overlap under certain conditions in vivo.

The redox potential (E^0) for Ag(I) aq → Ag^0 is +0.80 V, while that for Ag (III) → Ag(I) is 1.9 V; indicating that Ag(I) may be readily reduced to Ag^0 and that the argentic ion Ag(III) is a more powerful oxidant, akin to trivalent gold (for Au

Table 3 Biostatic/biocidal mechanisms of silver species

I. As silver metal, Ag^0
E.g. containers, cutlery, (undebased) coinage, germicidal coatings, surgical inserts, acting: As 'hostile' surfaces for microbial colonisation, By sorption of microbial toxins on container surfaces, By *local* oxidation when contaminated → diffusible bio-reactive Ag(I)
Note These surfaces are not always totally inert e.g. tarnishing with soluble or volatile sulphides
II. As nanoparticulate metallic silver (Ag^0), 2–50 nm diameter possessing
Very high sorptive activity involving: (a) van der Waal's bonding to bacteria/other bio-substrates (b) Coulombic interactions with anions e.g. HCO_3^-, acidic biofilm proteins and polysaccharides Catalytic activity (a) Well characterised for commercial oxidative syntheses e.g. methanol → formaldehyde at 500 °C (b) Less certain in physiological contexts e.g. at interfaces between biophases or on sorptive macromolecules: (but see Huang et al. 2014.) Lability, releasing bio-active (toxic) silver ions (Kittler et al. 2010; Liu et al. 2010)
III. As oxidised/ionic silver
(a) as univalent (Argentous) Ag(I),rarely ionic in vivo but rapidly interacting with a broad range of bioligands (Table 4) inactivating enzymes, denaturing proteins and polyphosphates and destabilising membranes (b) As trivalent (argentic) Ag(III), usually tightly complexed e.g. stabilised as the mixed valency complex $Ag(I)_2Ag(III)_2O_4$ (Tetrasil) used as a germicide in ointments; also claimed as a treatment (Imusil) for AIDS (Antelman 1997)

Notes
Some of these are well-established, others less so
Ag_4O_4 was formerly described as 'silver peroxide, Ag^0'. It is not a peroxide because it does *not* liberate hydrogen peroxide when acidified

(III) → Au^0,E^0 = 1.5 V). Ag(III) is only likely to exist transiently but might possibly be generated in a highly oxidant environment (Fischer and Jansen 1995). For reviews of Ag(III) chemistry, see McMillan (1962) and Tudela (2008).

The reactivity of *metallic* silver metal as nano-sized particles contrasts with that of the bulk metal. Indeed some physical chemists have deemed it another state of matter. This is because nano-sized materials are intermediate between the domain of quantum mechanics (describing the behaviour of lone molecules) and classical physics, governing the behaviour of bulk materials (Burcham 2010). These particles have a very large surface area relative to mass, which enormously increases their sorptivity, reactivity, etc. For example, silver does not normally react with hydrochloric acid, which dissolves nanoparticulate silver with the evolution of hydrogen (Li and Zhu 2006).

By contrast, oxidised/ionic silver (Ag(I)) is intrinsically unstable, being reduced to black metallic silver by light (the basis of photography) and also by a very large number of electron donors e.g. aldehydes qualitatively detected by forming a silver mirror. The black stain appearing on the skin after handling solutions of silver salts is another example of its bio-reduction.[1] This demonstrates both the reactivity of silver ions and the reducing i.e. electron-donating properties of many bio-substrates (also see Sect. 7).

A number of bio-ligands (L) can form complexes with Ag(I) in vivo, of varying stability and bioavailability (Table 4). The solubilities of some (model) silver complexes in water listed in Appendix A provide some guide to the relative stabilities of individual Ag-L bonds and comparative bioavailability of silver after forming these complexes. The actual *strength* of the Ag-L bond is indicated by their dissociation constants, K_m (Perrin 1979; Hogfeldt 1982). For the Ag-S bond, the K_m is 10^{-54}, which is considerably smaller in magnitude than the solubility of Ag_2S in water (1.4×10^{-4} M). Each of these figures indicates the very high affinity of silver and propensity to form Ag-S(R) bonds. Such measurements help us to understand more about the likely transit and distribution of Ag(I) not only within the body but also within the external bio-environment. For example, resolving the question of whether the low solubility of silver chloride might be selectively increased in the stomach by either local interaction with hydrochloric acid; perhaps forming the hydrated $[AgCl_2]^-$ anion (Forbes 1911; Forbes and Cole 1921) or binding with other gastric ligands from the diet or within the mucosal lining.

The bio-reactivities of both Ag^0 and Ag(I) are further discussed in Sect. 7 and 8.

[1]The author A.C. Doyle, a physician himself, has his character Sherlock Holmes identify Watson, his future associate, as a physician at his first meeting, by the black stain on his fingers after using silver nitrate as a sterilant.

Table 4 Some silver bioligands

Ligand	References
Thiols	Webb (1966), Jocelyn (1972)
Selenols	
Disulphides	Jocelyn (1972)
Oligophosphates e.g. • NAD(P) • Teichoic acids[a]	Kornberg and Pricer (1953)
Halides and thiocyanate (SCN^-) I^- > SCN^- > Br^-> Cl^-	
Phosphates Phospholipids and nucleic acids	
Carboxylates	
Nitrogenous ligands • Nucleobases: cytosine > thymine • Amines • Imidazole	Shukla and Sastry (2009), Czoik et al. (2008)
Unsaturated (ethenoid) fatty acids	Stahl (1965), Adlof and Emken (1985)

These are bioconstituents that bind Ag(I) listed in approximate order of diminishing affinity
[a]In cell walls of Gram-positive bacteria

5 Misapprehensions About Silver 'Biology'

Before attempting any rational discussion about silver pharmacology, we should be aware of four widely circulating 'untruths' concerning the properties of silver in various biosystems.

1. Silver is not a *trace* element, as the term is understood by agricultural and nutritional scientists. So far as we know today, silver is not an essential constituent of any biosystems; using the term 'essential' to imply loss or disruption of some biological function in its absence.
   Not so long ago boron, silicon, vanadium, molybdenum, arsenic (and a slowly increasing list of other elements) were not considered functional bioconstituents. Today we know otherwise. However, much nonsense is currently being circulated that we ourselves, our livestock, foodstuffs and the agri-environment are suffering silver depletion and therefore becoming 'sicker'. It is extremely doubtful that silver was ever widely distributed to be retained in biosystems. To continue to call it a 'trace element' implying it is a missing nutrient, totally obscures its utility for medicine as an xenobiotic (as most drugs are today).
   However, we should not ignore the biodynamics of metallic silver (Ag^0) as an oxidisable material in the natural environment, subject to metabolic transformations, just as any other xenobiotic.
2. Silver is often categorised as a *heavy* metal, an inaccurate generalisation considering its atomic weight (AW) is 108 i.e. much less than that of heavier toxic

metals such as mercury (AW 201), lead (207) and uranium (238); all justifiably classified as unselective poisons.
Silver is much less toxic than any of these heavy metals, being very rarely lethal. Nevertheless, silver does suffer 'guilt by association' when misclassified as 'heavy'—being implied to be much more toxic than it really is. [The presence and compatibility of other chemical elements in biosystems such as strontium, atomic weight (87), molybdenum (95), iodine (126), of similar atomic mass is usually accepted without comment.]

3. Describing a product as 'colloidal silver' is sufficient to 'say it all'. In fact, this is such an ambiguous, even misleading, appellation that it ought to be abandoned when and wherever possible. At least two different types of silver products have been/are being sold for pharmaceutical use under this unspecific designation. In general, they have somewhat different biological properties, particularly with regard to efficacies and in engendering toxic responses.

   (i) Historically the description 'colloidal' was used to specify water-soluble silver-impregnated macromolecules, particularly those classified as colloids (mid Nineteenth Century onwards). They were often prepared from casein (milk), serum proteins or some of their derived polypeptides, by interaction with silver salts. The products were either Ag(I)-polypeptides, or very fine Ag^0 particles embedded in a polypeptide coating—or a mixture of both valencies. In these products, silver was complexed chemically (or physically) and not precipitated by chloride, phosphate and other silver-insolubilising anions in physiological fluids (eyes, stomach, etc.).
   (ii) More recently (mid Twentieth Century), processes to manufacture extremely fine dispersible nanoparticles (NP)[2] made silver available as Ag^0 with much increased bio-reactivities compared to the bulk metal; able to act as a slow-release source of oxidised silver (Ag(I)) = a latent 'toxin'. The properties of these metallic nanoparticles are heavily influenced by their methods of production that determine their size, ionic character ('charge'), stability *and* content of impurities. Unless these preparative methods or the physical character (and chemical reactivities) of the NP products are clearly specified, it is both meaningless and foolish to imply/accept that they are somehow equivalent i.e. would have very similar bio-regulant properties in a particular context. This is one reason why it has been so difficult to interest drug regulators and medical practitioners in accepting the legitimacy of silver medications.

4. 'Silver will turn you blue'. This appalling half-truth is vigorously perpetuated by those who should know better. It turns up repeatedly (out of all proportion to its significance) when seeking information about colloidal silver on the WWW. There are two reasons for this; one honest and the other not so.

[2]Nano = 10^{-9}, 1-millionth of a mm.

(i) When 'colloidal silver' preparations became available in the late Nineteenth century, they usually were Ag(I)-impregnated proteins/polypeptides to be used topically e.g. as optical disinfectants to combat neonatal gonococcal infections. Although they were not prescribed, they were also often consumed for alimentary disinfection, nearly always being used at much higher silver dosage. A few people acquired a blue or grey skin pigment, Argyria. This was not surprising considering the photosensitivity of silver salts exposed to light. The effect was cosmetic, but fortunately infrequent. A careful analysis of over 600 clinical (and anatomical) reports (Hill and Pilsbury 1939) failed to establish any malfunction of an essential organ, but noted that Argyria was a consequence of

  (a) long-term, often non-prescription use;
  (b) the 'colloidal silver' products were nearly always poorly characterised patent medicines;
  (c) silver nitrate caused far higher incidence of Argyria (>50 %) than many colloidal silver preparations (6 %) or silver oxide (0.5 %) (Hill and Pillsbury 1939); and
  (d) the cumulative dose of silver consumed was of the order of grams—truly an overdose for sensible internal disinfection.

(ii) Various well-funded organisations, including the Food & Drug Administration USA (FDA), the Therapeutic Goods Administration Australia (TGA) and some less than honest private organisations e.g. QuackWatch, and Friends of Science in Medicine, have all repeatedly declared or implied that Argyria is a common side-effect of silver medication. Not only do they ignore the real incidence and general lack of harm from Argyria, but they also try to outlaw the current use of silver medications for healing; implying they are almost certainly serious health hazards.[3]

For some years, the FDA and TGA have maintained embargoes on claims for medicinal activity and therefore the sale (even clinical trials) of any form of colloidal silver as a medicine. Nevertheless both these government regulators concede that it may be used to sterilise water; surely a medicinal property!!

This simple fact was well recognised long before the TGA and FDA existed and came to adopt such unscientific postures as seemingly not wanting to know what is safe *and* what may be beneficial if it happens to concern silver; particularly if it is not patentable. Currently, the official attitude seems to be that the public must be shielded by outright bans from using, advertising (even researching), any form of silver for medicinal purposes.[4] This has undoubtedly helped the proprietary antibiotic industry and continues to do so—to the detriment of the very much larger

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[3]These regulatory agencies ignore the far greater health hazards, including mortality caused by heavily promoted drugs they had approved e.g. the NSAIDs, Vioxx^R and Prexige^R that ultimately had to be withdrawn. By contrast no one has died from silver medications.

[4]The legal penalties for breach of these regulations are truly an eye-opener.

number of potential beneficiaries, particularly the sick, the poor and the ailing if they happen to be infected with antibiotic-resistant, but possibly silver-responsive, micro-organisms (Also see Appendix G).

6 An overview of Silver Pharmacology (also See Sect. 7)

As discussed in textbooks, this is a minute topic: often totally ignored in many of the standard texts. When mentioned, it is nearly always in the context of treating burns and other superficial wounds; usually with the silver salt of sulphadiazine (an acidic sulpha drug dating from the 1940s). This is essentially an Ag(I) pharmaceutical, of defined composition and approved as a legitimate drug under various trade names (e.g. Silvadene, Flamazine, etc.). It is also biocidal to *Candida* and *Trepona palladum* (syphilis).

However, other silver preparations have many, already proven, uses in different biological contexts. These include antimicrobial, restorative and other aids for improving health or dealing specifically with chronic disease or dysfunctional wound healing. For many purposes, short-term therapy may be sufficient, e.g. as a germicide—with clear endpoints for determining efficacy and accepted methodologies to determine dosage and duration of activity. For other purposes, such as exploring the nexus between a prior infection and chronic debilitating diseases e.g. rheumatoid arthritis (RA), anklyosing spondylitis (AS) or multiple sclerosis (MS), see Ebringer's three books (2012, 2013, 2015), longer microbial eradication programs may be all-important. Sometimes these might be more effective and probably less harmful when used intermittently e.g. as a 'purge'. Chronic treatments necessitate using the safest possible yet effective doses. It is here that the concept of metallic silver as a slow-release toxin may be quite helpful.

The antibacterial activities of nanosilver preparations are often less pronounced against Gram-positive organisms e.g. Staphylococci, Streptococci. Gram-positive bacteria are distinguished by their content of anionic polyphosphates (teichoic acids) in their cell walls. The more silver-susceptible Gram-negative organisms (*E. coli; Proteus)* have a significant proportion of lipopolysaccharides and lipoprotein in their cell walls.

Bacteria with numerous flagellae e.g. *Proteus* also present an exocellular target for potential immobilisation by silver particles and Ag(I) released therefrom.

Shifting the focus back in time from *treating* established infections/inflammatory disorders i.e. therapeutic use; to using anti-infective silver preparations for *preventing* disease i.e. as prophylactics, then raises new concerns about long-term toxicities (See Sects. 8 and 9). Questions about (a) efficacy such as how much to use, or for how long (?); and (b) what are safe limits for extended use will have to be asked. Careful consideration must be given to preventing targeted microbes acquiring silver resistance by over- exposure. Suitable guidelines developed by consensus and some revision of present drug regulations will also be needed.

In the natural world, there are adaptive mechanisms whereby a potent metal cation may be detoxified by reduction to conglomerated metal. This bioreduction has been exploited to prepare nanosilver using *Enterobacteria* (Shahverdi et al. 2007), cyanobacteria (Legke et al. 2007), *Shewanella oneidensis* (Suresh et al. 2010) and many other micro-organisms. It is believed to also account for the geological formation of some gold deposits (Legke et al. 2006).

These examples of facilitated transformations within the biosphere indicate the relative safety of metallic silver and metallic gold as being ‘desirable’ end-products of reductive detoxication. They also suggest possibilities for reactivating metals by reversing this reductive detoxication process—or to put it more plainly—enhancing intoxication by controlled delivery by either (a) facilitated oxidations of inert metal → pharmaco-reactive cation(s) or (b) inhibiting the inactivating reductases e.g. with piperitone(an inhibitor of nitro-reductases) that potentiates nitro-furantoin antibiotics (Shahverdi et al. 2007) .

These considerations of safety versus efficacy show the need to clearly *distinguish* biological properties of Ag^0 as bulk metal or nanoparticles from those of its oxidation products, Ag(I) and even Ag(III); while also recognising the biodynamics of transition from one oxidation state to another. This suggests possibilities for controlled recycling i.e. inert → active entity and also the reverse transition, within particular pathological contexts (e.g. inflammatory loci). A clearer understanding of these contexts should open new vistas for silver as a medicinal, either as a prime drug or a therapeutic adjunct.

One example of this adjunctive role is interference with the protective biofilms created by chronically invasive bacteria such as *Pseudomonas* or *Enterococci* etc. These ‘films’ impede the antibacterial action of many antibiotics that effectively suppress populations of free-floating/planktonic micro-organisms. Disruptions of these biofilms, either by (a) adhesion of nano/micro-particulate silver (Ag^0) or (b) complexation of cationic silver (Ag(I)) with anionic protein/polysaccharide constituents of the bacterial biofilms (BBF), theoretically allows a double-pronged therapy—especially if adherent/trapped Ag^0 is then locally oxidised to intoxicant Ag(I) by electron withdrawal, either controlled or spontaneous (Table 5). Anti-BBF activities of silver preparations have been described (Chaw et al. 2005). These will surely be followed by many more (we hope) critical studies in coming years.

By contrast, the two million or more reports about medicinal silver on the Web are nearly all concerned with the antibacterial properties of metal silver usually in the form of nanoparticulate silver dispersions (NPS). In theory, this is an entirely different ‘drug’ from germicidal Ag(I); except that so many NPS preparations of medicinal Ag^0 may be contaminated with Ag(I); the degree of which is rarely specified but almost certainly determines their stability, efficacy *and* toxicity.

Currently, this is the core problem of nano-silver pharmacology; the reality being it is often that of a multi-component mixture. This is not unlike describing the medicinal properties of such traditional pharmaca as opium, cardiotonic glycosides, coffee, ephedra, etc.; all of which are composite drugs whose potency may be greater than that of any individual components (Weil 1996).

Table 5 Bacterial biofilms (BBF) and silver: some facts and speculations

Facts
(i) Silver-impregnated surfaces usually defy bacterial colonisation
(ii) BBF contain a range of micro-organisms, requiring a broad-spectrum microbicide
(iii) Most antibiotics mainly control replicating bacteria. But 'hibernating' bacteria lodged in BBF may still be Ag-susceptible
(iv) BBF present 'sticky' substrates for Ag^0 attachment, as NPS
(v) BBF contain many potential argentophilic 'docking' sites for Ag(I) including the 'extracellular polymeric substances'(EPS) composed of:
– Exocellular DNA
– Anionic glycans e.g. polysaccharide intercellular adhesin (PIA)
– Anionic glycolipids
– Proteins from host (fibrin, etc.) and colonising bacteria
– (Esterified) unsaturated fatty acids in phospholipids etc.
Speculations
(a) The presence of both (i) non-acylated D-glucosamine and (ii) anionic phosphate or hemisuccinate groups in the PIA of staphylococcal biofilms (Mack et al. 2009) may each provide exocellular 'docking sites' for silver to facilitate biofilm disruption.
(b) A quorum-sensing molecule = pyocyanine (aphenazine), a virulence factor in Ps. *aeruginosa*) (Lau et al. 2004), might be a target for silver deactivation
(c) Silver pharmaceuticals may augment the action of cationic agents used for wound debridement e.g. polihexanide
(d) Crafted Ag(I) complexes and appropriate delivery systems may confine/potentiate disinfection—*and* stimulate local healing (Becker and Selden 1985; Becker et al. 1998) of wounds, ulcers and other localised targets 'blanketed' with BBF

Under some circumstances, ionic Ag(I) 'impurities' in Ag^0 preparations may physically stabilise the metallic nanoparticles when adsorbed, so ensuring maximal ionic repulsion between the particles and minimising their spontaneous cohesion/precipitation. Another modification of the surface of nanoparticles may involve oxygen as an 'impurity' by forming Ag-O bonds i.e. an oxide skin (Roy et al. 2007), in essence diminishing the overall cationic character of the particles. So the impurities perhaps become a potential stabiliser, even an activator. But if we reverse the perspective, the NPS may also carry the more bio-reactive 'adsorbed' ionic Ag(I) or bound oxygen. [This is discussed further in Appendix F—speculation (iii).]

Summarising this dualism: Ag^0 as NPS *without* Ag(I) as cation or oxide = a potentially unstable package perhaps not widely bio-distributed; but when coated *with* Ag(I) = a stabilised, more dispersible, system combining a bio-reactive surface coating with a far less reactive supportive reservoir of Ag^0.

7 Mechanisms of Action of Silver Pharmaceuticals (Table 3)

These can be broadly categorised as properties of:

(a) readily available Ag(I);
(b) also of Ag(I) but only expressed after delayed delivery e.g. by local oxidation of Ag^0;
(c) unoxidised silver Ag^0, rapidly manifested by presenting surfaces inimical to bacterial growth e.g. silver-tipped catheters, silver amalgams used in dentistry, silver-impregnated bandages/underwear, etc.; and
(d) very small silver particles trapped in bacterial biofilms or ingested by phagocytic parasites as discussed (Sect. 6).

The significance of some reports about the pharmaco-activity of Ag(I), as studied in vitro, is difficult to assess. For example, treatment with silver salts (nitrate, sulphate) degranulates rat leukemic mast cells in vitro, a potentially noxious property (Suzuki et al. 2002). But is it likely to occur in vivo, particularly in the context of so many competitive ligands for Ag(I), ranging from plasma chloride and albumen to endo-cellular thiols? It might—but would it be significant i.e. with long-term consequences?

The main potential of current silver pharmaceuticals may lie with their composite character; for example being delivered as relatively inert micro Ag^0 particles but activated by local inflammation or other compartmentalised physico-chemical processes that might deliver 'payloads' of toxic Ag(I). An analogy here may be the antibiotic action of oral lactoperoxidase (LPO), which oxidises thiocyanate (SCN^-) actively secreted into saliva; *but* only when hydrogen peroxide becomes available from proliferating oral bacteria. In one sense, silver can replace this LPO-SCN^- combination, provided that the peroxide (or other oxidants) can be sourced from either a) the targeted bacteria in situ or b) delivered as an adjunct supplement. This scenario presents Ag(I) as a substitute for SCN^- but in fact it may reduce thiocyanate's availability and incipient toxicity [It is a precursor of cyanide.] by forming the highly insoluble AgSCN.

A fundamental biological property of free silver (I) ions is their interaction with, and usually inactivation of, bioregulant thiols both large and small e.g. enzymes, glutathione. The proton gradient established across energised biomembranes (mitochondria, chloroplasts) that sustains ATP synthesis (Mitchell 1966) is destroyed not only by lipophilic anions e.g. free fatty acids, many acidic NSAIDs but also by several thiolphilic reagents (Whitehouse and Leader 1966). Silver ions can interfere with mitochondrial ATP synthesis in vitro (Chappell and Greville 1954) and de-energise vital membranes of *Vibrio cholera* (Dibrov et al. 2002).

The question is: will the concentration of Ag(I) that poisons ATP production in vitro ever be attained in vivo i.e. in the presence of so many endocellular or exocellular silver bioligands e.g. adenosine nucleotides, chloride ions etc.? There may be two answers to this question—because ATP biogenesis in (a) prokaryocytes

is topographically more exposed to ambient poisons (and fewer detoxicants) than (b) eukaryocytes, where ATP is generated within endocellular mitochondria surrounded by all the intracellular constituents able to capture, bind, and detoxify Ag (I). This may be one of the keys to the relative selective toxicity of silver, being more damaging to vital energy production in invading bacteria than it is to that in the invaded host.

In summary: silver therapies may be (a) pluripotent—with several modes of action, (b) involve more than one silver species, and (c) act over different time spans ranging from rapid antibiosis to facilitating tissue repair (and ?regeneration).

8 Toxicity of Silver Pharmaceuticals

Three quotations help summarise this topic.

- "All things are poisons. It is only a matter of the dose".
  Paracelsus (1493–1541) Swiss physician and alchemist.[5]
- "Silver is an old problem and nanosilver is a new challenge".
  (Luoma 2008)
- "Silver is not carcinogenic……and should be placed in a No Risk category".
  Lansdown (2010)

So there are at least three questions we might ask:

1. Why is silver apparently such a feeble toxin; as compared to other metals of similar mass e.g. cadmium(=AW112) or thiophilic character e.g. lead and mercury?
2. Why are so many microbes so much more silver-sensitive than their host's epithelia/endothelia/blood cells? Glib phrases such as 'silver bullets' or 'selective toxicity' may describe, but do not explain, the host's relative insensitivity. Some comparative anatomy may help here: (a nuclear) prokaryocytes have relatively exposed DNA but eukaryotes do not. Moreover, their DNA is not usually associated with histone proteins, i.e. less protected.
3. Why is one relatively *benign* but much publicised side-effect of ingested silver, namely Argyria, officially considered so much more noxious—than self-imposed debilities and *mortality* from ingesting high doses of alcohol, salt, sugar or tobacco smoke? None of these are banned but using silver as an antibiotic, is! This is not a very enlightened attitude in the present circumstances of increasing antibiotic resistance.

It is difficult to find convincing records of silver being lethal, other than in (a) exceptional industrial circumstances (e.g. a worker falling into a vat of silver

[5]This is an apt quotation because Paracelsus himself experimented with and used silver in his medical practice and apparently was an ardent proponent of silver medications.

cyanide used for 'silver plating') or (b) after exposure to chronic inhalation of silver dust or fumes e.g. Bolivian miners working under bad conditions, extracting silver ores that may also contain lead, cadmium, etc. In other contexts, it is not considered to be an industrial hazard (Browning 1968; Venugopal and Luckey 1978 or a carcinogen.

Surveying the affinities of silver ions for so many bioligands (see Table 4 and Appendix A), it is remarkable that it appears so unreactive i.e. non-poisonous in many biosystems. One explanation is the ubiquitous distribution of chloride ions, rendering Ag(I) largely insoluble [the solubility of AgCl is normally about 2×10^{-3}g/L i.e. 2 ppm]. Another is the wide extracellular distribution of organic deactivators such as (a) endogenous bio-reductants e.g. ascorbate, methylglyoxal, glutathione, and (b) thiols both in the circulation, notably albumen and macroglobulins or in the liver and several other tissues e.g. glutathione and the metallothioneins (Vasak 2011). Metallothioneins in animals can be induced/increased with some metals (Zn or Cd) but whether this also occurs with silver is not clear at present. They are also present in some bacteria; notably M *tuberculosis*, some *Pseudomonas*, some aproteo bacteria and *Staphylococcos epidermidis* (Blindauer 2011, 2014).

These natural antidotes to silver intoxication are expended in much lesser quantities than for example the glutathione needed to help detoxify paracetamol/acetaminophen, used in doses greater than 1 g/day (Whitehouse and Butters 2014).

So we might anticipate cationic silver intoxication being a *serious* health hazard, as opposed to causing a cosmetic side-effect (Argyria), perhaps *only* when (a) these natural antidotes are severely depleted—e.g. after overdosing with paracetamol—or (b) as effects of very small silver particles (Ag^0); if and when these particles are taken up by non-phagocytic mammalian cells. The prevailing surface charge on the particles will largely determine their cellular attachment, subsequent retention and local membrane interactions or pinocytosis. In fact silver phagocytosis may be sometimes therapeutic if it helps 'quench' over-active inflammogenic leukocytes [a Trojan horse therapy] as the ingested Ag^0 slowly disrupts cellular function. Nevertheless we cannot afford to be complacent until such hazards as potential genotoxicity (AshaRani et al. 2009) have been properly evaluated for risk, reversibility etc. in vivo, not just in vitro—where detoxicant mechanisms may be absent from the test systems.

A number of exogenous thiols e.g. N-acetylcysteine, NMEA mesna (coenzyme M), D-penicillamine have been used to minimise intoxication by acrolein, cyclophosphamide, lead and other thiol-depleting xenobiotics. Supplementation with selenium is also recommended to overcome Ag(I) intoxication of essential seleno proteins e.g. glutathione peroxidase and thyroid peroxidases containing selenocysteine at their catalytic centres.

Thioredoxin reductases (TrxR) are another class of seleno-enzymes that regulate the redox homeostasis in animal cells, controlling the balance between oxidised and reduced forms of regulatory thiols e.g. glutathione (Fig. 1).

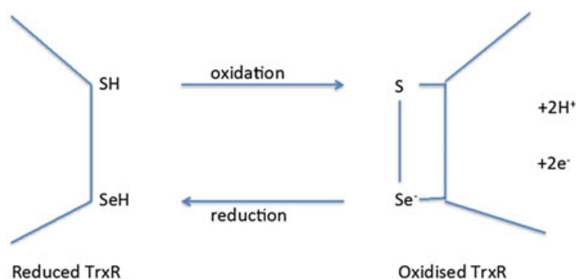

Fig. 1 The TrxR enzyme with adjacent thiol and selenol functions that participate in reversible 'redox shuttling' (*e* = electron)

These enzymes are a target for certain metallo-drugs notably anti-cancer agents containing gold (Omatu et al. 2006) or platinum (Arner et al. 2001). However, their silver sensitivity is not clear at present. This enzyme appears to be absent from many bacteria but may be present in other parasitic organisms.

So in the presently unlikely situation of physiological silver overload developing, there are some useful leads for detoxification and antidotal strategies.

9 Silver and the Environment

Sometimes this seems to be a topic with rather more theories and scare stories than 'hard' facts. A thoughtful review by Luoma (2008) provides a more reasonable perspective.

Much data have been generated concerning the toxicity of Ag(I) to fresh water fish and other organisms living in an aqueous environments with a low chloride content. One concern has been the irreversible poisoning of the fish's gills. This is not a problem unique to soluble silver, being replicated with other waste products from mining industries e.g. arsenic, tin, etc. Another problem is how valid are conclusions from *one* well-designed laboratory study of the effect of *one* type of NPS on *one* test organism, often separated from its natural environment—for understanding how *various* silver products might interact with *various* natural environments over *various* time spans.

There are no easy answers here. Nevertheless scientific studies conducted with silver-responsive organisms (e.g. zebra fish embryos) in the 'abnormal' environment of a laboratory can still provide insights for examining the nature and potency of silver detoxicants within the environment e.g. citrate and fulvic acids (Osborne et al. 2012).

By contrast, the potential eco-toxicity of nanosilver preparations in soil samples seems remarkably low, as the particles clump together spontaneously forming large aggregates—not so different from some native silver deposits (Maass 2014). Another natural detoxicant mechanism is oxysulphidation transforming nanosilver

particles into very insoluble silver sulphide after aerobic oxidation i.e. $Ag^0 \rightarrow Ag(I)$ (Liu et al. 2011).

The great debate is now, or certainly should be, about toxicities to 'good' bacteria present in the gastrointestinal tract or those constituting the essential microflora in sewage processing plants. If they are less susceptible to silver antibiotics than other microbiota, then why is this so? Does it imply they have already acquired silver resistance, particularly enzymic capacities to bio-reduce $Ag(I) \rightarrow Ag^0$? If so, is this transferable? This could present serious problems for human/animal therapeutics if silver were to join the very many organic antibiotics that are becoming evermore useless. Alternatively, if these good/beneficial bacterial are also silver-susceptible, how can they be protected from over-zealous use of NPS? The answers to these queries will be rather critical for determining the future acceptability of silver pharmaceuticals, beyond being used in crisis therapy e.g. to help treat super-infections.

10 Looking to the Future of Silver in Medicine

Again, there are a number of questions needing acceptable answers, sooner rather than later. For example:

(i) What are the basic 'truths' about silver pharmaceuticals (SP) that need to be *continually* re-evaluated e.g. its credible history, safety and efficacy?
(ii) What are the new opportunities for SP—perhaps seeming crazy/unrealistic today—but allowing further advances in health care tomorrow, particularly in the area of preventive medicine?
(iii) What are the real legal difficulties and other obstacles to researching SP and bringing them to clinical trials? Are these impediments still acceptable *today* or are they just *inherited* baggage from the past? (Maybe too much politics, too little science and commonsense.) The legal and financial disincentives currently hindering SP research and development need to be either justified—or *changed.* Retaining the *status quo* is indefensible, particularly at a time of ever-increasing microbial resistance to organic antibiotics.

Complementing these queries, Table 6 presents some other strategies for further research, some being more practical than others, at this time.

A useful guideline for changing attitudes about using silver pharmaca would be to adopt (or adapt) the Utilitarian Principle, originally proposed by the English philosopher, Jeremy Bentham (1748–1832), posing the question: 'What brings the greatest good/happiness for the greatest number?' This ought at all times to over-ride vested interests and negative mindsets, currently impeding honest enquiries about the future of silver as legitimate silver pharmaceuticals, even life-saving medicinal aids.

Table 6 Silver pharmaceuticals: exploring further applications

This brief list indicates that more research is needed, for example into:
(1) Better methods to dry, store and redisperse pharmaco-active nano-silver preparations *without* irreversible aggregation and/or significant losses of potency (2) Using Ag^0 or Ag(I) as supplements to other antibiotic and anabolic/restorative therapies i.e. in an Adjunctive role[1] (3) Producing silver nano- and micro-formulations of Ag^0 (or even Ag(I)?) designed to be slow-release/targeted delivery systems for Ag(I), either alone or together with other bio-regulants[2] (4) Ag^0 providing 'platforms' of controlled size, charge etc. to physically or chemically *carry*/deliver pre-bound pharmaca[3] e.g. as inserts into wounds or for controlling unhealthy intestinal microbes and parasites e.g. *Proteus*, *protozoa*, nematodes etc. (5) Continuing development for other pharmaceutical applications beyond human usage e.g. improved water purification, hydroponic horticulture, controlling protozoa and other parasites in farm animals, etc. (6) The presently unanticipated/unavailable new delivery systems that may be highly valued tomorrow e.g. beneficially harnessing some of silver's remarkable physical properties (light absorption, high thermal and electrical conductivities) as micro-thin inserts or using other bio-compatible technologies

[1]Perhaps patterned after silver sulphdiazine for burns or using NPS with membrane-permeating antibiotics e.g. polymyxin B (Ruden et al. 2009) or β-lactam antibiotics (Li et al. 2005)
[2]Furthering the well-tested tradition of 'drug latention', (Harper 1958) exemplified by cyclophosphamide, leflunamide, even aspirin
[3]Perhaps following the example of using thioligands to alter the physical characteristics, bio-distribution and reactivity of colloidal gold particles (Brust et al. 1994)

11 Postscript; on a More Personal Note

As an ignorant biochemist, 50 years ago, I wrote a chapter for Progress in Drug Research (Vol. 8, 1964) surveying drugs for inflammatory disease. It discussed gold but alas, ignored silver (including a seminal publication by Chappell and Greville 1954). This omission reflected first my ignorance that the noble metals were more than coinage and the basis for much fine craftsmanship—and secondly, my then lack of 'hands-on' experience with animal assays.

The literature as best as I could survey it, with the help of Chemical Abstracts (published by the American Chemical Society) was also pretty secretive about the history and potential of silver in medicine. The Bodleian Scientific Library in Oxford, was a treasure-trove but failed to diminish my ignorance about so-called natural medicines and their continuing importance for 'modern medicine', then just discovering auto-immune disease(s). A few journeys to London to investigate the

splendid library of the Royal Society of Medicine rewarded me with much information about the complexities of the inflammatory response and its regulation by patented pharmaceuticals. But metal pharmacology (other than haphazard reports about the efficacy of antimonials, bismuth preparations and 'gold salts') was definitely not on the radar at that time.

When at last (some 20 years later). I pondered the obvious question: why had other areas of medicine e.g. endocrinology, genetic disorders, etc. surged forward so dramatically but many chronic diseases including the whole auto-immune family still lacked a clear aetiology *and* an even clearer program for treatment/eradication, perhaps based on neutralising/eliminating defined causative agents. Didn't they exist? Meanwhile during the 1970–1980s at least, the treatment of chronic inflammation was frankly a mess—nearly always palliative, rarely curative and abounding in horrible side-effects e.g. gastric bleeding from poisonous NSAIDs and osteoporosis from the corticosteroids—not to mention the wretched consequences of aggressive immunosuppression, all too often employed as a treatment of last resort.

So it was encouraging to discover that eminent pioneers of modern medicine such as William Osler (1849–1919) and contemporary mavericks like Alan Ebringer (1938) were willing to consider infection as a prime cause of rheumatoid arthritis and ankylosing disease. They were of course derided for having no real evidence since ex vivo microbial analyses (applying Koch's postulates) had continually failed to unambiguously identify disease-causing agents. Historically the focus was upon cultivating viable pathogens from joints, certainly not upon extra-articular microbes establishing immune responses initially *outside* the joint, which then adversely affected joint physiology and function. Koch's original postulates certainly transformed medical microbiology but they also very much limited the search for etiological agents to those present and viable within the diseased tissue; ignoring any which had been and gone but left a memory of their presence. These were serious constraints if the search for causative agents focussed wholly on the wrong anatomical compartment.

Finally it all seemed to come together with the realisation that silver therapeutics needed to be re-assessed with an open mind, without prejudice in the context of a chronic inflammatory disease. There were historic precedents sufficient at least to ask the simple question: why not?

Animal responses were amazing, if silver was tested in the appropriate (slow-release) formulation and at the right stage of disease development. Nanosilver given orally prevented arthritis development at extraordinarily low doses but soluble silver salts and silver (I) oxide, given orally, were not anti-arthritic (Whitehouse et al. 2013).

But what was the relevance for RA etc. of these responses to NPS in rats? A preliminary study indicated that (arthritigenic) *Proteus* might be eliminated from the lower bowel and urinary bladder by a short course of ingesting an NPS preparation previously shown to be a powerful antibiotic against two species of *Proteus* in vitro (Disaanayake et al. 2014). An interesting finding maybe; but how would patients with RA respond? Might the silver prophylaxis be too late (the pessimistic

view)? Alternatively, if the RA pathogenesis needs to be restimulated intermittently, then this late silver therapy might be beneficial (the optimistic view).

Extrapolating from animal studies, however well-designed and carefully conducted, is always difficult. The sooner one can measure responses, both beneficial and/or toxic, in *real* patients with *real* diseases over *realistic* time frames, then the sooner will silver therapeutics be either vindicated *or* should be abandoned. The impediments to undertaking such an important clinical study should be clearly recognised and surmounted as soon as possible.

To quote Osler:

> 'The value of experience is not in seeing much but in seeing wisely.'
> (Peskett 2014)

In this survey of silver pharmacology we have seen much, mainly laboratory findings—but without more approvals for verifiable clinical studies, how do we become much wiser?

Acknowledgments This chapter owes much to (i) the enthusiasm of Gerald Hancock, Hans Laroo, John Petty, Ross Stevens, and Alan White who each taught me much about the practicalities of silver research, both within and outside the laboratory; and (ii) Desley Butters whose skills and patience ensured a typescript fit for publication.

Appendix A: Solubilities of Some Silver Salts/Complexes in Water

Section A, with physiological anions; Section B with some xenobiotic anions *S* = solubility (gm/L), temperature (*T*) = 25 °C except as noted.

	Anion	*S* (g/L)	*T*
A.	Nitrate	2.57×10^{3}	
	Acetate	11.1	
	Nitrite	4.2	
	Sulphate	2.2	
	Carbonate	3.3×10^{-2}	
	Phosphate	6.4×10^{-3}	
	Chloride	1.9×10^{-3}	
	Chloride	2.1×10^{-1}	100
	Stearate	6.5×10^{-4}	20
	Oxide (Ag_20)	2×10^{-4}	
	Sulphite	1.4×10^{-4}	
	Bromide	1.35×10^{-4}	
	Iodide	2.6×10^{-6}	
	Cyanide	2.3×10^{-6}	

(continued)

(continued)

	Anion	*S* (g/L)	*T*
	Oxalate	3.6×10^{-11}	
	Thiocyanate	1.0×10^{-12}	
	Sulphide	1.5×10^{-14}	
B.	Perchlorate	5.57×10^{3}	
	Fluoride	$>10^{3}$	
	Thiosulphate	7×10^{2}	20
	Bromate	1.6	
	Salicylate	0.95	
	Iodate	0.44	
	Chromate	3.5×10^{-2}	
	Azide	2.0×10^{-8}	
	Arsenate	1.0×10^{-22}	

Notes
(i) Silver nitrate, sulphate and acetate are much more water-soluble than silver nitrite, sulphite, carbonate or oxalate.
(ii) Stability constants for Ag-L bonding are recorded by Hogfeldt (1982) where L is inorganic and by Perrin (1979) for some organic ligands. These indicate strength of the bonds i.e. dissociation (affinity) constants for Ag-L [Solubility data only provide some indications of bond formation but do not quantify the strength of this bond.]

Appendix B: Reproducible Production of Nano Ag^0 for Medicinal Use

(also see Laroo 2013)

Some variables that should be recognised *and* carefully controlled include as follows:

- Cleanliness of glassware, etc.
- Purity of the silver metal (Ag^0) or silver salts (Ag(I)) which should always be of the highest grade available (>99 %). DIY products using cheaper grades of metallic silver, coinage, silver buckles, etc. will generate *toxic* products—either containing lead and cadmium (from incomplete refining) or the metals added as hardeners, e.g. copper, nickel, etc.
- Purity of water or other solvents, including content of oxygen and carbon dioxide. Removing chloride ions is essential.
- Physical procedures involved in manufacture. If using
  - Radiant energy (light, gamma rays), define bandwidth and intensity of radiation, duration of exposure, etc.;
  - Microwave energy, define wattage and duration;

- Heat, define temperature and conditions for cooling;
- Sonic energy, define key variables;
- Electro-chemical procedures, define current, voltage, purity of DC supply, temperature control and degree of illumination.

- For chemical methods producing NPS from Ag(I) salts, also define
  - Purities of essential reagents used as reductants, dispersants and stabilisers
  - Methods to remove by-products and/or excess reductants: some of these may be physiologically unacceptable e.g. borate or formate or oxalate after using borohydride or formaldehyde or ethylene glycol respectively as the reducing agents. [The much cited Carey-Lea method includes ferrous ions in the mix (Frens and Overboek 1969.]
  - Qualities of essential 'extras' e.g. stabilisers/preservatives
  - Operational procedures for any further physical manipulations to separate impurities, enhance potency or lengthen shelf-life (e.g. ultra centrifugation, dialysis, etc.)

- Identify optimal storage conditions: specifying temperature, light sensitivity, nature of containers (plastic, borosilicate or soda glass, etc.)

Note Many methodologies described in the literature focus on NPS production for industrial uses *not* medicinal purposes. They should be adopted only *after* recognising the more stringent purities and stability needed for approved medications.

Appendix C: Quality Controls for NPS Products

> 'The important thing about knowledge is discrimination, not quantity'.
> Peskett (2014)

Note These quality controls can be just as important for understanding toxicities of nano-products. (Warheit et al. 2008) as well as their beneficial pharmaco-activities.

I. Physico-chemical

- Analysis for total silver content and purity[6] and proportion of ionic Ag(I); measure p[Ag].
- Definition of NPS product by determining:

[6]Atomic absorption spectrometry (AAS) is not always suitable or accurate for determining silver content; sometimes being influenced by extraneous components ('matrix effect').

– Range of particle sizes
– Particle charge (volts) and polarity (+ or −) i.e. Zeta potential
– Particle shape, if transmission electron-microscopy is accessible
– Acidity/alkalinity i.e. pH
– Electrical conductivity
– Light scattering properties and light absorption (400–450 nm)
– Sorption properties e.g. using fluorescent/fluorogenic indicators
– Flocculation by anions/cations and acidity[7]
– By-products present, if not previously removed
– Additives e.g. stabilisers, anti-flocculants, pharmaceutical adjuncts
– Light sensitivity
– Shelf life before obvious deterioration
– Optimal conditions for storage e.g. dark bottles? and temperature?.

II. Biological properties in vitro (for quick testing)

- Stability in physiological media = saline, artificial gastric juice, urine
- Compatibility with plasma proteins, etc.
- Toxicity to selected microbes (bacteria,[8] yeast, protozoa), plant tissues, e.g. germinating radish seeds, to cultured animal cells and whole organisms e.g. brine shrimps[9]

Appendix D: 'Colloidal Silver' (CS): Some Facts and Fallacies

(*Note* These are items for continuing discussion, not dogmatic truths)

Fallacies

I. A CS preparation can be a universal treatment for nearly all microbial infections—but where is the detailed evidenced?
II. It is a 'good' medicine because it has traditional origins, 'mystique', etc.—but so did apricot kernels (Laetrile) for treating cancer.
III. It is cheap and easy to manufacture and therefore suitable as a home-made remedy—but so are many poisons e.g. methanol from wood, insecticidal nicotine from tobacco leaves.
IV. 'It must be good', receiving so much attention on the Web—but much of this is advertising and too little is unbiased commentary.

[7]Use acetic or sulphuric acids; avoid nitric acid (oxidant) and hydrochloric acid (precipitates Ag (I)).

[8]Caution: certain microbiological assays e.g. using agar gels (negatively charged) may alter the proportion of Ag(I) to Ag^0 and bias test results.

[9]See Cock et al. (2012).

V. If it were useless, why would the medical establishment want to 'suppress' it? [This is a tendentious matter, based more on expediency than principle.]
VI. 'It must be safe', considering the widespread use of silver coinage, cutlery, containers, etc. in our domestic environment—but only rarely are we using unvarnished bulk silver in medicine.

Facts

(i) Unless defined by composition e.g. purity, particle size, shape and charge, and stability; a CS preparation cannot be expected to successfully interact *consistently* with a chosen pathogen/other targeted biosystem.
(ii) Current silver pharmaceuticals are remarkably non-toxic, in contrast to many inorganic drugs that were once 'in-vogue'. These latter medications were notoriously toxic with quite dreadful therapeutic indices; *vide* historic use of mercurials, antimonials, arsenicals and more recently the platinum carcinostats that are all still being used in orthodox/allopathic medicine.
(iii) Cost is misleading and many qualities may be sacrificed by using (a) impure metallic silver sources, (b) haphazard control of preparative procedures, (c) inadequate checks for impurities and (d) failure to control other essential qualities e.g. stability ex vivo (light, heat, etc.).
(iv) Drug regulators and the medical fraternity are rightfully concerned to prevent abuse and harm from any antibiotics to both the consumer *and* the modern environment e.g. the essential microbial population of sewage sludge. Current regulatory procedures for CS and other silver pharmaceuticals seem particularly oppressive (prohibition, etc.); especially when contrasted with the minimal management of *proven* health risks affecting the whole community, such as alcohol, tobacco, and the over-consumption of sugar, salt, (w6)unsaturated fats; all of which are largely unregulated.
(v) The safety of (bulk) metallic silver, as in coinage and domestic silverware, is largely irrelevant when considering latent toxicities of ingestible, cationic and nano-metallic silver preparations.

Appendix E: A Summation About NPS: The Good, the Bad, and the Ugly

Good

- Long-term stability without refrigeration
- Potential to adhere to/penetrate bacterial biofilms
- Adjunct to use with planktonic antibiotics, either for synergy or 'back-up' therapy
- Cheap. Possible to prepare near/on-site for appropriate infection control
- Restricted diffusion, for maximal topical efficacy (e.g. on skin/gastric ulcers)

- Slow-release source of pharmaco-active Ag(I) = selective toxin?
- Potential for using NPS particles as carriers/delivery systems for other pharmaca (attached covalently or by Coulombic interactions)

Not So Good, often quite Bad in fact

- Inadequate quality controls for safety, purity, stability, antibiotic activities, etc. and risk of adverse reactions.
- Flood of unproven claims on the WWW for pharmaco-efficacy; often misleading and some being nonsense
- Very limited funding for research in an area with poor prospects for high financial returns e.g. from exclusive patents
- Lack of vision by health authorities to help side-step this impasse and also support more studies of silver pharmaceuticals for controlling antibiotic-resistant microbes

Ugly

- Claims that very small silver particles (<2 nm) penetrate the brain, causing firing of the neurones
- Poorly controlled targeting for systemic use
- Ill-founded arguments about environmental toxicities (with much propaganda but too little science)
- Political interference and suppressive tactics to suit vested/misguided interests, rather than patient's welfare
- Polemics: regulation favouring *status quo* (basically 'ban silver') to the detriment of *independent* trials to (i) ascertain possible long-term medicinal benefits and (ii) understand safe limits for both individuals *and* the environment. [Certainly not good science and rather a discredit to Modern Medicine, manipulated or otherwise.]

Note This table is far from complete

Appendix F: Antibiotic Potency, Stability and/or Bio-transformation of Nanoparticulate Silver (NPS): Some Facts and Speculations

Facts

1. "One size fits all" is clearly untrue in describing antibiotic activities of NPS products, however carefully prepared to minimise heterogeneity (size, charge density, shape, etc.). So for example, the type of preparations that may be anti-protozoal may have little value as an antiviral.

2. NPS (Ag^0) prepared by chemical procedures, particularly reduction of soluble Ag(I) salts or complexes, such as $[Ag(NH_3)_2]^+$, will have their anionic/cationic character largely determined by the preparative conditions.

   Eg $AgNO_3$ and tribasic citrate salts produce anionic NPS hydrosols, readily aggregated by lanthanum (III) ions i.e. their 'superficial' charge is negative.

3. By contrast NPS preparations derived without added chemicals e.g. by photo-induced reduction of electrolytically generated Ag(I) cations (Laroo 2013) will most likely be cationic i.e. not aggregated by La(III).
4. The charge densities of NPS particles would largely determine their stability ex vivo, i.e. shelf-life. In theory, the smaller the particles, the greater their ionic character relative to total mass: consequently they will be less likely to spontaneously aggregate. But their shelf life may be no indication of their utility for combating infections in vivo, if they need first to be transformed/bioactivated in vivo (see Item iii below).
5. Many reports about the antibiotic potencies of NPS preparations provide few details of how disperse (i.e. size variation) and stable they are. For example, NPS-citrate preparations are intrinsically unstable. Commercial NPS samples, stabilised with citrate and used as reference 'standards' for sizing, can aggregate over time with distinctive changes in colour, opacity—and very often biopotency ex vivo. Such deterioration may not always be a negative feature, if it reflects spontaneous transformations that might be accelerated in vivo when used medicinally or ex vivo within the environment after excretion.

Speculations

(i) Should we be constructing a working concept of NPS 'metabolism', considering not only these spontaneous transformations in vitro (see item 5 above) but *also* the effects of the bio-environment upon an administered polysilver-X NPS complex? This perspective questions the tacit assumption that silver pharmacology is primarily all about the effects of Ag^0/Ag(I) *upon* targeted bio-systems; while usually ignoring the reverse interaction i.e. how the internal environment may act upon an NPS preparation to determine pharmaco-activity.

(ii) If so, the nature of factor(s) X may profoundly influence not only the physico-chemical *stability* of an NPS preparation ex vivo but also its potential *instability* (and pharmaceutical value) within the various biosystems it may encounter.

(iii) Concerning the nature of this factor X: if it is either a potentiator or an inactivator may then determine the medicinal value of an NPS preparation. For example:

- If X is oxygen, will this oxide/dioxygen 'film' be diminished by 'scrubbing' with acids in vivo or amplified by H_2O_2 and other bio-oxidants?
- If X is citrate, will this dissociate and be catabolised in vivo, generating a silver particle more likely to spontaneously aggregate (after losing a

particle-stabilising negative charge); perhaps providing a less efficient 'pay load' of bio-reactive Ag(I)?

- If X is ionic Ag(I), will a mobile NPS that is cationic in vitro now be neutralised, even aggregated, by chloride or phosphate ions in vivo?
- If X is an argentophilic bioligand (Table 4) captured in vivo, will this always immobilise or destroy the value of an NPS product? In some contexts it might possibly increase the hydrophilicity of an NPS preparation, altering its bio-distribution, so changing its efficacy.
- If X is a particle 'stabiliser', may this protective coating be so stable as to isolate a reactive NPS from its targeted biosubstrate? Generalisations about the inefficacy of NPS preparations as an antibiotic in vitro e.g. where X equals PEG or PVP (Xiu et al. 2012) or oleate, may be true in their context but not necessarily valid where X is readily dissociated or metabolised in vivo.

iv. So how might the nature of X further determine antibiotic activity? As an illustration of this question, here are two examples:

(a) The *acidic* environment of the stomach may determine the anti-*Helicobacter pylori* activity of an NPS-oxide preparation, to help control gastric ulceration; but the anti-ulcerant effect may not be the same in a different compartment such as within the more *alkaline* environment of the proximal duodenum. [Even though *Helicobacter* provokes both stomach and duodenal ulceration.]

(b) Some ligands such as esterified ethenoid (unsaturated) fatty acids might reversibly trap some types of NPS at strategic loci, so enhancing their local potency in vivo e.g. within lipid membranes.

v. Statements that NPS particles are less effective antibiotics for Gram-positive (GP) bacteria than Gram-negative (GN) species—as determined from standard ex vivo bioassays—sometimes may have little meaning for understanding how they might behave in vivo. For example, cationic NPS species might be expected to bind to the anionic polyphosphate teichoic acids within the outer cell wall of GP bacteria. But several reports describe the lesser microbial activity of NPS preparations upon these teichoate-coated bacteria ex vivo. This would not be surprising if the NPS preparations being tested ex vivo were themselves anionic e.g. citrate-coated. But to extrapolate from this hard data to predicting that all NPS preparations would be less useful for treating infections by GP bacteria—without thoroughly evaluating the potency of *cationic* NPS preparations—may perpetuate a grave error in our perceptions of what are the more useful formulations with which to conduct clinical trials.

vi. By contrast, GN bacteria carrying a relatively high content of lipids within their cell coating, may either preferentially take up and bond with hydrophobic NPS particles (with low charge density) or 'capture' cationic NPS particles that may disrupt the stabilising effects of their anionic membrane phospholipids.

In Conclusion

Beware of simplistic generalisations about potencies of antimicrobial NPS preparations which implicitly assume that they are always 'naked' silver particles. In fact they are more likely to be complex entities, represented here as NPS-X. So in the absence of essential details about (a) their bio-evaluation ex vivo (e.g. with or without using agar plates) and (b) their physical stability, chemical constitution (noting what factor X might be) and natural lability—we may be dealing with 'rogue products'. This is certainly no basis for a scientifically grounded antibiotic revolution, as yet.

Appendix G: Political Considerations

This ought not to be part of any scientific enquiry into the merits or hazards of any drug, new or old. The facts, the theories, the clinical evaluations, etc. should all be dispassionately considered—without interference or censorship from either vested interests or poorly informed government regulators. At present, silver therapeutics is not allowed this freedom. There are several reasons for this situation. Here are three:

1. Governmental regulations concerning use of, and claims for, medicinal silver are presented in the following e-publications.
   http://www.fda.gov/ohrms/dockets/98fr/081799a.txt
   http://www.gpo.gov/fdsys/pkg/FR-1999-08-17/pdf/99-21253.pdf
   http://nccam.nih.gov/health/silver and for Australia https://www.tga.gov.au/colloidal-silver-related-products
   They are dogmatic, deny medicinal claims and even punitive if you disagree. This is not a good state of affairs for progressing the healing arts, especially those with traceable histories of their benefits exceeding possible harm *and* supported by unambiguous scientific observations. But wisely considered, these current regulations concerning the medical use of silver should catalyse a genuine debate about how to unshackle laboratory and clinical research on silver pharmaceuticals—to move beyond a negative risk-averse mindset (i.e. 'thou shalt not….'). This needs to be replaced by a climate of open *and* honest enquiries about what may be beneficial to humankind (and animals) and about what may not be so (especially for the environment).
   Recently, we have seen other traditional therapies decriminalised e.g. cannabis for cancer pain. So why not silver therapeutics, especially if they might save lives from infections that are currently antibiotic-resistant? [But how will we ever know this under the present suppressant regulations?]
2. The power of industrial lobbyists (Angell 2004; Goldacre 2012).
   The influence of pharmaceutical companies upon the 'education' of physicians and operations of various drug-regulatory agencies has inverted some of the

functions of these agencies [They were originally established to prevent harm to the public, not to seemingly protect monopolistic interests at the expense of public wellbeing.]
We are left with the unsatisfactory status of alternative/traditional therapies, like those using silver and without patent protection. They are the subject of considerable misinformation and rarely funded to provide the evidence demanded by these lobbyists, insisting they be treated as 'new drugs'—even when historically shown to be much more acceptable and rather cheaper than many licensed/patented alternates. This is another aspect of the 'rhetoric versus reality' (Angell 2004) as practised by Big Pharma to destroy its competition.

3. 'Consumer protection'
In theory, this is a very good thing but so often it is captured by vested interests and debased in practise.[10] It is naive to believe official dogmas that such alternate/traditional therapies are nearly always either unproven(!) (but see Bone and Mills 2013) or too dangerous to be licensed—even when Quality Assured. Agencies minding our health need to do their own homework; if necessary sponsor and publish their own research and (for a change) listen to non-industrial advocates sometimes.

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Recommended Further Reading

(i) A short overview of colloidal nanosilver—production, properties, standards and bio-efficacy (Laroo 2013). [This is an open access article, distributed under the Creative Commons.]
(ii) An extensive review of 'Nanobiosilver ' and its uses in medicine with over 600 references (Eckhardt et al. 2013)
(iii) A comprehensive survey entitled "Nanosilver", sponsored by the US Environmental Protection Agency, with over 500 references (Varner et al. 2010). [Ironically, this arm of the US government considers it to be 'safe', in contrast to the FDA which does not seem to agree with this conclusion.]
(iv) A valuable review of silver disposition, toxicity and utility for preventing infection, with over 160 references (Lansdown 2010) [This is also an open access article, distributed under the Creative Commons.]
(v) A valuable bibliography particularly relating to the healing potential of silver colloids and electrically generated silver ions, with over 180 references (Flick 2009)

[10]An astonishing development in some parts of Europe is that sales of vitamins, formerly freely available OTC, are being regulated—apparently being deemed unsafe without a prescription. The beneficiaries seem to be those pharmaceutical companies which have taken over former suppliers or created new subsidiaries to provide the now licensed nutritional supplements. Not surprisingly, costs to consumers have risen quite considerably, a corollary of over-ruling a free market.

(vi) A short review covering silver's history and silver nanoparticles as antimicrobials (Rai et al 2009)
(vii) A timely survey of silver nanotechnologies and the environment, with over 100 references (Luoma, 2008)
(viii) Challenging insight from Material Scientists about the complexities of aqueous nanosilver systems—including altered states of water and oxide 'skins' being formed around the nanoparticles (Roy et al 2007)
(ix) The phenomenon/problem of microbial silver resistance, reviewed by (Simon) Silver* (2003) (*Not a typographical error: this is the author's surname.)
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Index

A
Aβ accumulation, 149
A-beta formation, 149
A-beta levels, 149
A-CSE, 144
Abdominal pain, 8
Aboriginals, 164
Abortifactant, 197
Accelerate cartilage destruction, 44
Acemannan, 214, 218, 221
Acetaminophen, 136
2-acetonyl-7-hydroxy-8-(2-furanonyl)-7-hydroxy-5-methylchromone, 207
2-acetonyl-7-hydroxy-8-(3-hydroxyacetonyl)-5-methylchromone, 208
2-acetonyl-8-(2′,caffeoyl)-glucopyranosyl-7-hydroxy-5-methylchromone, 208
2-acetonyl-8-(2′,cinnamoyl)-glucopyranosyl-7-hydroxy-5-methylchromone, 208
2-acetonyl-8-(2′,6′-di-O,O-coumaroyl)-glucopyranosyl-7-hydroxy-5-methylchromone, 208
2-acetonyl-8-(2-furoylmethyl)-7-hydroxy-5-methylchromone, 208
Acoustic trauma, 41
Activates macrophages, 215
Adjuvant-induced arthritis rats, 115
Adjuvants, 168
Adjuvant treatment, 43
Adrenal cortex, 40
Adrenal glands, 36
Advanced kidney failure, 19
Adverse effects, 107
Adverse events, 56, 68
Adversely affected GIT microbiome, 8
Ae. gardnerii, 148
Ae. lineatopennis, 148
Africa, 155
Ag^0, 244
Age-related endocrine involution, 40
Ageing, 40
Agilent Bioanalyzer, 38
Aging, 149
Ajmoda, 134
Ajumu, 134
AKT kinase, 150
Alcoholic extract, 133
Algal blooms, 107
Alkaloids, 179, 210
Allergic disease, 6, 16
Allergic rhinitis, 16
Allergies to shellfish, 107
Allopathic medicines, 183
Aloctin A, 220
Aloe, 179
Aloe africana Miller, 197
Aloe alkaloids, 210
Aloe anthrones, 206
Aloe arborescens, 179, 183, 197
Aloe barbendol, 204, 206
Aloe barberae Dyer, 198
Aloe benzene, 211
Aloe broomii Schönland, 197
Aloe chabaudii Schönland, 197
Aloe christianii Reynolds, 197
Aloe chromones, 207, 208
Aloechrysone, 202, 203
Aloe ciliaris Harvey, 198
Aloe coumarins, 209
Aloe deserti Berger, 198
Aloe emodin-10-C-rhamnoside, 204, 206
Aloe emodin, 199–201, 203
Aloe-emodinanthrone, 204, 206
Aloe-emodin-11-O-rhamnoside, 203
Aloe ferox, 179, 197

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Aloe flavonoids, 212
Aloe gel, 182
Aloe harlana Reynolds, 198
Aloe kedongensis Reynolds, 198
Aloe lateritia Engl., 198
Aloe leaf gel, 200
Aloe leaf morphology, 182
Aloe macrosiphon Bak., 198
Aloe marlothii Berger, 183, 197, 204
Aloenin, 209
Aloenin A, 199
Aloenin acetal, 209
Aloenin aglycone, 209
Aloenin B, 209
Aloe nyeriensis Christian & Verd., 198
Aloe perryi, 179
Aloe plicatilis Miller, 197
Aloe-2′-p-O-coumaroyl ester, 209
Aloe polysaccharides, 220
Aloe pyrans, 209
Aloeresin A (2′-O-p-coumaroylaloesin), 207
Aloeresin B, 206
Aloeresin C, 206, 207
Aloeresin D, 206, 207
Aloeresin E, 206, 207
Aloeresin F, 206, 207
Aloe rupestris Baker, 197
Aloesaponarin, 203
Aloesaponol, 202, 203
Aloesaponol-6-O-glucoside, 203
Aloesaponol-8-O-glucoside, 203
Aloesaponol-O-methyl-4-O-glucoside, 203
Aloe secundiflora Engl., 198
Aloe secundifolia Engl., 198
Aloesin, 199–201, 206
Aloesin (aloeresin B), 207
Aloesol, 208
Aloesone, 208
Aloe species biodiversity, 181
Aloe sterols, 213
Aloe striatula Haw., 198
Aloe toxicity, 199
Aloe variegata L., 197, 198
Aloe vera, 179
Aloin, 199, 202, 204
Aloin (barbaloin), 206
Aloinoside, 206
Alpha lipoic acid, 19
Alpha-secretase, 149
Amelioration of neurological diseases, 149
Amritsar, 136
Amyloid precursor protein secretion (alpha APPs), 149
Anaerobic periodontal pathogens, 126
Anaphylactic shock, 135
Angola, 180
Anklyosing spondylitis, 249
Anthraquinone dimers, 202
Anthraquinones, 179, 182, 198, 202, 204, 220
Anthrones, 179, 182, 198, 202
Antiapoptotic protein Mcl-1, 220
Anti-arthritic and gastroprotective (GP) activities, 171
Anti-asthmatic activity, 123
Anti-bacterial, 109, 216
Antibacterial activity, 217, 249
Antibiotic potency, 264
Anticancer activity, 222
Antidiabetic activity, 223
Anti-coagulant, 109
Anti-fungal, 109, 216
Anti-fungal activity, 217
Antigenic tolerance, 2
Anti-histamine activity, 115
Anti-hypertensive, 109
Anti-inflammatory, 109
Anti-inflammatory activity, 115, 219
Anti-inflammatory cytokines, 126
Anti-inflammatory effects, 140
Antimicrobial, 162
Antimicrobial activity, 136, 216
Antimicrobial properties, 196
Anti-oxidant, 18, 19, 45, 109, 162, 167, 218, 223
Antioxidant activity, 136, 200, 210, 212, 219
Antioxidant effects, 223
Antioxidant/prooxidant activities, 204
Anti-self, 168
Antispasmodic, 135
Antiseptic, 238
Anti-thrombin, 109
Antiviral activity, 217
Apigenin, 212
Apium graveolens, 134
Apoptosis, 150, 210, 220
Appetite, 135
APP processing, 149
Arabian Peninsula, 180
Arachidonate, 160
Argyria, 248, 254
Armigeres subalbatus, 148
Arteperon®, 45
Arthritis, 94, 133, 134
Articular cartilage degradation, 15
Asian green mussel, 93
Aspergillus flavus, 145
Aspergillus fumigatus, 145
Aspergillus niger, 145

Asphodelin, 203
Asphodeloideae, 180
Aspirin, 44, 136
Astaxanthin, 165
Asthma, 16
Asthmatic children, 16
Asthmatic symptoms, 97
Atopic asthma, 97
Atopic dermatitis/eczema, 16
ATP, 21
ATP production, 217
ATP synthesis, 252
Auditory system, 41
AU4 Regeneresen®, 40
Australasia, 155
Australian Aborigines, 157
Australian Blue mussel, 94
Autoimmune arthritic diseases, 15
Autoimmune diseases, 123
Auto-oxidation, 97
Avi-pharmaceuticals, 156
Ayurvedic medicine, 134, 196

B
Bacillus clausii, 24
Bacillus subtilis, 143
Bacteria—bacteria interactions, 25
Bacterial adhesion, 22
Bacterial biofilms (BBF), 251
Bacterial/toxin translocations, 14
Bacteroides-Porphyromonas-Prevotella, 126
Bacteroidetes, 127
Barbados Aloe, 180
Barbaloin, 204
Barrier function, 25
Bass Straits islands, 164
Bazr-ul-karafs, 134
Beagle International Pty Ltd (Nerang, QLD, Australia), 136
Behavior, 15
Benzene, 179
Benzopyrones, 210
β-glucuronidase, 222
Beta-selinene, 149
β-sitosterol, 219
Bianthracene, 203
Bifidobacteria, 14
Bifidobacterium, 126
Bifidobacterium infantis, 8, 10
Bifidobacterium longum, 8, 9, 24
Big-defensins, 110
Bioactive lipid fractions, 110
Bioactive metabolites, 108
Bioactive protein, 110
Bioavailability of ingested aloe, 199
Biomembranes, 252
Bio-reactivities, 245
Biostatic/biocidal mechanisms of silver species, 244
Biotherapeutic modality, 26
Biotoxins, 95
2;3-bisphosphoglycerate, 20
Bisbenzopyran, 209
Bivalvia, 93
Bladder, 135
Bloating, 9
Blood glucose, 224
Blood lipid profiles, 15
Blood pressure, 147
Blood pressure reduction, 147
Blood purification, 135
Bone marrow, 43
Botswana, 180
Brachialgia, 43
Bradykinase, 219
Bradykinin, 219
Brain function, 36
Breast cancer, 212
Broadrange flocks, 163
Bronchoconstrictor response, 123
Bruises, 196
Burn aloe, 180
Burns, 196, 197
Burundi, 180
Bushman, 182
3-n-butyl-4,5-dihydrophthalide (sednenolide), 136
3-n-butyl phthalide, 136, 147, 148
3-n-butyl-tetrahydrophthalide, 149

C
Caco-2 cells, 25
Caco-2 human colon carcinoma cell line, 199
CAH is a dimer, 144
Calmodulin, 19
Campylobacter jejuni, 143
Cancer, 35, 200
Candida albicans, 145, 197
Candida alibicans, 143
Candida guilliermondii, 145
Candida krusei, 145
Candida parapsilosis, 145
Candida tropicalis, 145
Cape aloe, 183
2-(Carboxyethenyl)-5,7-dihydroxychromone, 208

Carcass fat, 165
Cardiovascular, 68
Cardiovascular disease (CVD), 18, 200
Carminative, 135
Carrageenan-induced paw edema, 141
Carribean region, 196
Caspase 3 activity, 220
Caspase 8, 220
Catalase, 218
8-[C-B-D-[2-O-(E)-cinnamoyl] glucopyranosyl]-2-[(R)-2-hydroxypropyl]-7-methoxy-5-methylchromone, 207
Celephthalide A-C, 136
Celerioside glucosides, 136
Celery-carrot-mugwort syndrome, 135
Celery preparations, 134
Celery seed extract (CSE), 133, 136
Celery seed oil, 136
Cell division/cell cycle, 222
Cell growth, 220
Cellular uptake, 199
Central America, 196
Central nervous system, 35
Cerebral amyloid-beta (A-beta) plaque deposition, 149
Cerebral cortex, 40
C-glycosyl anthrone isomers, 202
8-C-glycosyl-(2′-O-cinnamoyl)-7-O-methyl-aloediol, 207
8-C-glycosyl-7-O-methylaloediol, 207
8-C-glycosyl-7-O-methyl-S-aloesol, 207
8-C-glucosyl-noreugenin, 207
8-C-glycosyl-S-aloesol, 207
Changes in ankle thickness, 140
Changes in body weights of the arthritic rats, 141
Changes in paw thickness, 140
Charnley prosthetic hip, 44
Chemical composition, 135
Chemotactic, 220
Chemotactic effect, 220
Chemotaxis, 220
Chemotherapy-induced mucositis, 162
Childhood exposure to infections, 4
Chinese aloe, 180
Chinese celery, 149
Chinese Umbelliferae, 134
Chlamydia trachomatis, 126
Cholesterol, 94, 146
Cholesterol-lowering properties, 148
Chondroitin sulphate, 44
Chromones, 179, 182, 206
Chromosome instability, 222
Chronic fatigue syndrome, 40
Chronic inflammation, 136
Chronic kidney disease (CKD), 16
Chronic liver disease, 14
Chronic obstructive lung disease, 40
Chrysophanol, 203
Chrysophanolanthrone, 204, 206
Chrysophanol-8-methyl ether, 203
Cinnamic acid, 204, 217
Citric acid cycle activity, 20
Clinical assessment geriatric scale (SCAG), 42
Clinical global impressions (CGI), 55
Clinically relevant shifts, 65
Clinical therapeutic activity, 97
Clostridium, 126
Clostridium butyricum miyairir, 24
Clostridium difficile, 23, 24
Co-enzyme oxidoreductase, 21
Cognitive deficits, 149
Coiine, 210
Colds and flu, 198
Colloidal silver (CS), 247, 248, 262
Colonic damage, 108
Colonic transit time, 9
Combining prebiotics with probiotics, 17
Commensal bacteria, 23, 124
Components of celery, 136
Compound with Anti-Helicobacter Activity (CAH), 144
Connective tissue metabolism, 45
Connective tissues, 40, 45, 239
Constipation, 24
Coumaric acid, 217
Coumarins, 179, 182, 208, 210
Coumaroyl derivatives, 204
COX, 115
COX-1, 115
COX-2, 115
Crypto antigens, 168
Cryptococcus neoformans, 145
CSE as an antimicrobial agent, 143
CSE in preventing gastrointestinal irritation, 142
Culex tritaeniorhynchus, 148
Columbia University Rating Scale (CURS), 42
Culex quinquefasciatus, 148
Culex vishnui group, 148
Culpeper, Nicholas, 134
Cyanobacteria, 250
Cyclin-dependent kinase, 150
Cyclooxygenase-2, 219
Cyclooxygenases, 212
Cytochrome P450, 146

Cytokine-mediated cartilage destruction, 44
Cytokine markers, 107
Cytokine production, 219, 220
Cytokines, 218, 219
Cytolytic T lymphocytes, 218

D
Deacetylaloesin, 208
Deacetyllittoraloin, 204, 206
Deafness, 40, 41
Degenerative diseases of joints, 35
7′-deoxyaloeresin D, 208
Desmosterol/brassicasterol, 94
Desoxyerythrolaccin, 203
Dextran, 42
Diabetes, 200, 213
Diabetes mellitus, 223
Diarrhea-predominant IBS, 9
Diarrhoea, 198
Diastolic blood pressure, 147
Dietary polysaccharides, 125
Digestive ailments, 215
Digestive upsets, 135
Dihydrocoumarone (2,3-dihrdrobenzofuran), 211
Dihydroisocoumarin glycoside, 209
Dihydroisorhamnetin, 212
1,5-dihydroxy-3-hydroxymethylanthraquinone, 202, 203
Dilate blood vessels, 219
Dinoflagellates, 110
Dioscordes codes Aniciae Juliana, 182
Dioxygen, 20
Dipyrone, 44
Discomfort, 8
Disruption of the GIT, 7
Diuretic, 135
Dizziness, 41
Djibouti, 180
DNA mutations, 21
DNA synthesis, 220
Docosahaexanoic acid, 115
Dosage, 123
Dose-ranging studies, 107
Double-transgenic AD mouse model (AβPP/PS1), 149
Dromaiols, 160
Dromaius Novae Hollandiae, 157
Dropsy, 135
Dry air hyperpnea, 123
dsRNA Poly(I)-poly(CU), 40
Dysbiotic GIT, 7
Dysregulated inflammation, 25

E
Ear, 40
Ear problems, 198
Early Cape Dutch settlers, 197
Eastern African Aloe species, 198
E.coli DSM 17252, 8
Eco-toxicity, 255
Eczema, 197
Efficacy of CSE, 136
Eicosapentaenoic acid methyl ester, 109
Electron acceptors (electrophiles), 204
Electron ionization mass spectrum of CAH, 144
Elgonicardine, 203
Emmenagogue, 198
Emodin, 202, 220
Emu, 157
Emu farming, 159
Emu fat, 160
Emu oils, 157
Endogenous bio-reductants, 254
Endothelial activation, 212
Enterobacteria, 250
Enterococci, 250
Enterococcus faecalis, 143
Enterococcus faecalis DSM 16640, 8
Enterococcus faecium SF68, 24
Enzyme interaction assays, 147
Epidermophyton floccosum, 145
Epithelial barrier function, 22
Eritrea, 180
Erythematous rash, 56
Escherichia coli, 25, 143
Eskimos/Inuits, 157
Essential oil, 135
Essential oil components, 136
Ethanolic *Apium graveolens*, 148
Ethiopia, 180
Eucalyptus oils, 167
Eukaryotic cell, 2
Experimental arthritis, 171
Experimental inflammation, 162
Exploratory target criteria, 53
Extracellular matrix, 239

F
FAO/WHO Expert Consultation and Working Group on probiotics, 17
Fat depots, 160
Fatigue, 40
Fatty acid, 110, 115
Fatty acid methyl esters, 160
Fatty alcohol, 165

Fecal microbiota transplants (FMT), 23
Feralolide, 209
Feroxidin, 211
Feroxidin A, 211
Feroxidin B, 211
Fibrillar A-beta plaques, 149
Firmicutes, 127
First Aid Plant, 180
Flatulence scores, 9
Flavonoids, 179, 182, 211
Focal adhesion kinase, 23
Food additives, 17
Food-based medicines, 134
Free fatty acids (FFAs), 94
Free radicals, 218
Free radical scavenging, 210
Free radical scavenging activity, 200, 202, 208, 212
Furan compounds, 211
Furan derivatives, 179
3-furanmethanol, 211
Furoloesone, 208

G
Galactosamino–glycuronylglycan sulphate, 44
γ-coniceine, 210
Gamma-irradiated, 218
Gamma-selinene, 149
Gas chromatography coupled with mass spectrometry, 149
Gastric ulcers, 216
Gastrointestinal function, 196
Gastrointestinal protection, 124
Gastroprotection, 143
Gastroprotective activity, 108
Gastroprotective effects of celery extracts, 142
General Side effect profile array, 147
Geriatric conditions, 40
German commission E monograph, 135
Germany, 48
Gingival fibroblast proliferation, 221
GIT autoimmune diseases, 6
GIT metabolic dysfunction, 15
GIT microbiome, 7, 14
GIT microbiota, 3, 15, 125, 126
Glial activation, 149
Global assessment of efficacy, 55
Global health state, 55
Glucomannans, 179
Glucosamine/chondroitin sulphate, 44
Glucosamine, 44, 127
Glucose homeostasis, 224
Glucose levels, 123
Glucose uptake, 217
4′-O-glucosyl-isoaloeresin DI, 208
4′-O-glucosyl-isoaloeresin DII, 208
Glutathione, 22, 254
Glutathione-S-transferase, 218
Glycogen, 109
Glycogen synthase kinase 3β, 150
Glycosylated dimer, 202
Goanna oil, 166, 171
Goannas, 166
Gonorrhoea, 197
Gout, 196
Gram-negative, 126, 266
Gram-positive, 266
Gram-positive organisms, 249
Growth factors, 221
Guna-Teekshna, 134
Gut bacterial cohort, 25
Gut barrier, 23
Gut epithelial barrier, 6
Gut lesions, 108
Gut-liver axis, 10
Gut microbiota in brain development, 15
Gut permeability, 126

H
Haemolymph, 110
Heart, 40
Heart rate, 147
Heavy metals, 95
Helicobacter pylori, 133, 143, 266
Helminth infection, 6
Helminthosporin, 202, 203
Hemoglobin, 20
Hepatic encephalopathy, 14
Hepatobiliary disorders, 135
Hepatocellular protection, 135
Herbal preparation, 136
Herbal remedies, 134
Herpes genitalis, 40
Hexane-extracted *Apium graveolens*, 148
Historical uses of Celery, 134
History of silver medication/disinfection, 241
HIV/AIDS, 198
Homonataloin, 204, 206
Homonataloside, 204, 206
Hoof fat, 167
Host-gut microbiome, 18
Hottentot, 182
HSG1 cell, 210
Human leukaemia, 210
Hyaluronic acid, 45
Hydrogen peroxide, 252

Hydroperoxides, 218
5-hydroxyaloin A, 204, 206
5-hydroxyaloin A-6'-O-acetate, 204, 206
7-hydroxy-2,5-dimethylchromone, 208
7-hydroxyaloe emodin, 202, 203
7-hydroxyaloin, 204, 206
7-hydroxyaloin-4′,6′-O- diacetate, 204, 206
7-hydroxyaloin-6′-O- monoacetate, 204, 206
10-hydroxyaloin, 206
10-hydroxyaloin-6-O-acetate, 204
10-hydroxyaloin B, 204
10-hydroxyaloin B 6′-O-acetate, 206
Hygiene hypothesis, 4, 6
Hyperchlosterolemic, 148
Hyperlipidemia, 148
Hypersensitivity reactions, 41
Hypertensive patients, 147
Hypothalamic–pituitary axis, 15
Hypothalamus, 40
Hypoxia-inducible factor-alpha (HIF**α**), 20

I

Ibuprofen, 52
IL-1β, 97
IL-10, 10
IL-12, 10
IL-2, 220
IL-2 mRNA expression, 219
Immune deficiencies, 40
Immune modulatory effects, 218
Immune reactions of the skin, 16
Immune response, 36
Immune stimulation, 218
Immune suppression, 218
Immune system surveillance, 2
Immune tissue maturation, 2
Immuno-inflammatory reactions, 45
Immunomodulation, 217
Immunomodulatory potential, 217
Immunostimulant, 217
Immunosuppressant activities, 217
Incidences of adverse events, 69
India, 133, 136
Indian celery seed (A-CSE), 136
Indomethacin, 44
Infections, 216, 249
Infective diseases, 198
Infective enteritis, 162
Inflammation, 198
Inflammatory bowel disease, 4, 24, 108, 124, 162
Inflammatory conditions, 133
Inflammatory diseases, 38
Innate immune system, 6
Innate immunodefense, 110
Inner leaf pulp, 182
Insect repellency, 148
Interferon induction, 36
Intestinal absorption, 199
Intestinal absorption of aloin, 199
Intestinal antiseptic, 135
Intestinal barrier function, 14
Intestinal irritation, 199
Intestinal microbiota, 124
Intestinal motility, 198
Intestinal mucosa, 22
Intragluteal injection, 47
Ionic Ag(I), 244
Ischemia-reperfusion, 22
Isoaloesin, 208
Iso-aloeresin A, 206, 207
Iso-aloeresin D, 206
Isoaloeresin D, 207
Isoeleutherol, 211
Isoeleuthrol-5-O-glucoside, 211
Isorabaichromone, 207
Isovitexin, 212
Isoxanthorin, 202, 203

J

Japan aloe, 183
Jaundice, 135
Joint degenerative processes, 45
Joint diseases, 15

K

Karoo region, 197
Kenya, 180
Khoi-San, 197
Kidney, 37, 40
Kidney complaints, 135

L

Laboratory findings, 72
Laccaic acid D methyl ester, 202, 203
Lactobacilli, 14, 23, 24, 126
Lactobacillus acidophilus, 8, 24
Lactobacillus bulgaricus, 9
Lactobacillus casei, 15, 16, 25
Lactobacillus lactis, 8
Lactobacillus plantarum, 8
Lactobacillus reuteri ATCC 55730, 10
Lactobacillus rhamnosus, 9
Lactobacillus rhamnosus GG, 16, 24
Lactobacillus salivarius, 10
Lactoperoxidase (LPO), 252
Laxative, 202
Laxative/purgative, 198, 199

Laxative/purgative effects, 215
L-3-n-butyl-phthalide, 149, 150
LDL cholesterol, 212
Learning, 36, 149
Leg pain, 198
Lesotho, 180
Lethargy, 40
Leukocyte, 219
Leukotriene, 115
Ligand binding, 147
Lignan glucosides, 136
Limonene, 136
Linguistic forms of WOMAC, 49
Linoleate, 160
Lipid peroxidation, 202, 218
Littoraloin, 204, 206
Littoraloside, 204, 206
Liver, 36, 40, 135
Liver glycogen, 224
Long-term spatial memory, 149
Low density lipoprotein cholesterol, 148
Lower blood pressure, 196
LTB4, 115
Lungs, 37
Lymphocytes, 220

M

3-Methylnaphto[2,3-c]furan-4,9-dione, 211
3-Methylnaphto[2,3-c]furan-4(9H)-one, 211
Macrophage, 218, 220
Macrophage stimulatory bioactivities, 210
Major histocompatibility complex class I, 123
Malawai, 180
Malnutrition, 135
Mammalian species, 38
Mansonia uniformis, 148
Maoris, 94, 164
MAP kinase pathways, 23
Marine phytoplankton, 110
Marlborough sounds, 94
Matrix laser desorption (MALDI) mass spectrum, 144
Matrix remodelling, 222
Mechanisms of action of silver pharmaceuticals, 252
Medicinal silver, 240
Membrane-Ca^{++}ATPase, 19
Membrane-Ca^{++}ATPase activation, 19
Memory, 149
Ménierè's disease, 41
Menstrual problems, 135
Metabolic diseases, 15
Metallothioneins, 254
Met hemoglobin, 20
Methionine, 19
Methionine sulfoxide, 19
Methyl-p-coumarate, 210, 211
MHC-1 gene expression, 123
Microalgae, 107
Microbial diseases, 216
Microbial environment, 6
Microbiome, 1, 3
Microdontin, 204, 206
Micro-RNA, 37, 38
Micro-RNA research, 76
Microsporum canis, 145
Microsporum gypseum, 145
Microstigmin A, 204, 206
Mild asthma, 162
Mini-mental-state scale, 42
Mitochondrial, 20
Mitochondrial oxidative metabolism, 46
Mitochondrial proteins, 20
Mitogen activated protein kinase (MAPK), 150
Monounsaturated acid, 165
Moon bird, 164
Moscow, 48
Mosquito bites, 148
Mozambique, 180
Mucosal administration, 37
Mucus production, 6
Multiple sclerosis, 249
Multispecies probiotic supplement, 14
Multistrain probiotic formulations, 24
Multistrain probiotics, 8
Muscular strength, 40
Mutagenicity, 37
Mutton bird oil, 164
Mycobacterium tuberculosis, 254
Myocardium, 37
Myrcene, 136
Mytilidae, 93
Mytilus galloprovincialis, 94

N

N-acetyl glucosaminidase, 222
NADPH oxidase-1, 22
NADPH oxidase 1 (Nox1), 23
Namibia South Africa, 180
Nandi people of Kenya, 198
Nanoparticles, 247
Nanoparticulate copper, 243
Nanoparticulate silver, 242, 264
Nanoparticulate silver dispersions, 250
Nanosilver preparations, 249, 255
Naphthalene, 211
Narcotics, 44
Naringenin, 212

Natal aloe, 183
Nataloe emodin, 202, 203
Nataloe emodin-2-O-glucoside, 203
Nataloe emodin-8-methyl ester, 202, 203
Nataloin, 204, 206
Natural RNA extracts, 38, 76
n-butylphthalide, 136, 143
Neatsfoot oil, 167
Negative ion electrospray mass spectrometry, 161
Neoaloesin A, 207
Nerve stimulant, 135
Neural degeneration, 200
Neuroblastoma cells, 150
Neutraceuticals, 134
New Zealand, 93, 164
NFATc1, 46
NF-κB, 15
Nitric oxide, 20
Nitrosylation, 20
N-Methyltryamine, 210
Noble (coinage) metal pharmacology, 239
Non-amyloidogenic pathway, 149
Non-receptor-mediated mechanism, 37
Non-steroidal anti-inflammatory drugs (NSAIDs), 44
n-pentyl-cyclohexadiene, 136
NSAID-enteropathy, 162
NSAIDs, 136
NPS-oxide preparation, 266
Nuclear factor-kappa B (NF-kB), 126
Nutritional content, 94
Nutritional supplement, 21

O
Obese individuals, 14
Obesity, 200
6′-O-cinnamoyl-5-hydroxyaloin A, 204, 206
6′-O-cinnamoyl-8-O-methyl-7-hydroxyaloin, 204, 206
6′-O-coumaroylaloesin, 208
Oedema, 210
2′-O-feruloylaloesin, 201, 208
O-glycosides, 202
5-OH-3-Methylnaphto[2,3-c]furan-4(1H)-one, 211
Oil triglycerides, 160
Oligonucleotides, 37
Omega-3 PUFA, 165
Omega-6 PUFA, 165
7-O-methylaloeresin A, 207
7-O-methylaloesin, 207
7-O-methylaloesinol, 207
8-O-methyl-7-hydroxyaloin, 204, 206
O,N-dimethyltryamine, 210
2′-O-p-coumaroylaloesin, 200, 201
6′-O-p-coumaroyl-7-hydroxyaloin, 204, 206
Oral, 37
Oral bacteria, 252
Organ-specific effects, 37
Osteoarthritis, 15, 38, 43
Osteochondrin®, 40, 43
Osteochondrin S, 50
Osteochondrosis, 43
Osteoclasts, 46
Osteoporosis, 43
Ostrich, 162
Ostrich farming, 163
Ostrich fat, 163, 164
Ostrich oils, 163
2′-O-tigloylaloesin, 208
Otitis media, 40
Ovary, 37, 40
Overgrowth of pathogenic bacteria, 7
Overweight children, 14
Oxidative damage, 19
Oxidative stress, 149, 200, 222
Oxidative stress induction, 224
Oxygen scavenging activity, 200

P
Painful injections, 47
Pain reducing effects, 115
Palmitic acid methyl ester, 109
Palmitoleic acid, 14
Paracetamol, 44
Para-pharmaceutical, 240
Paravertebral injection, 47
Parkinson’s disease, 42
Pathogenic bacteria, 3
Pathogenic commensal cohort, 8
Pentoxifylline, 42
Percutaneous absorption, 167
Perna canaliculus, 93
Pernin, 110
Peroxisome proliferator-activated receptor gamma (PPAR-g) activation, 15
Peroxynitrite, 20
Perry’s aloe, 196
Petrel, 164
Pharmacobiotics, 1, 17
Phenolic compounds, 206
Phenolic phytochemicals including alkaloids, 182
Phospholipid membranes, 199
Phospholipids, 94
Phototoxic furancoumarin, 135
3-phthalide glycosides, 136

Physical properties of silver (Ag^0), 243
Phytosterols, 179, 212
Pimples, 198
Pinene, 136
Pituitary gland, 40
PKC alpha expression, 149
Placebo responses, 74
Placenta, 40
Plaques, 149
Platelet aggregation, 212
Plicataloside, 211
Pluridone, 210, 211
Polyarthritis, 133
Polyunsaturated oils, 166
2′-p-O-methlcoumaroylaloesin, 208
P. perna (brown or rock mussel), 93
Prebiotics, 17
Prechrysophanol, 202, 203
Presenilin 1, 149
Proapoptotic mechanism, 220
Probiotic and prebiotic, 18
Probiotic bacteria, 6
Probiotic research, 26
Probiotic species/strain, 8
Probiotic supplementation, 14
Probiotic supplements, 9
Probiotics, 6
Probiotics coagulated, 8
Production of nano Ag^0, 260
Proinflammatory, 22
Pro-inflammatory mediators, 7
Proinflammatory response, 3
Prokaryote, 2
Proliferation, 220
Prooxidant, 204, 210, 221
Prooxidant effects, 223
Propionibacterium freudenreichii ssp. *shermanii*, 9
Prostacyclin (PGI_2), 219
Prostaglandin E_2(PGE_2), 219
Prostaglandins, 219
Prostate, 37
ProSymbioflor, 8
Proteasome, 21
Proteasome activity, 21
Proteasome degradation, 20
Protein amino acid residues, 19
Protein kinase C, 150
Proteolysis, 21
Proteus, 249
Protocatechuic acid, 210, 211
Proton pump inhibitor, 24
Proto oncogenes, 222
Pruritus, 56
Pseudomonas, 250, 254
Pseudomonas aeruginosa, 143
Psoriasis, 197
Puffinus Tenuirostris, 164
Punjab, 134, 136
Purgative, 202
Putrescine, 14
P. viridis, 93
Pyrans, 179
Pyrocatechol, 217
Pyrones, 179, 182, 209

Q
Quality controls for NPS, 261

R
Rabaichromone, 207
Racehorses, 165
Radiation, 37
Rasa-katu, 134
Rashes, 198
Rawanda, 180
Reactive oxygen species (ROS), 1, 20
Red carotenoid pigments, 165
Redox, 22
Redox character, 204
Redox state, 179
Reduce gastric irritation, 133
Reduced glutathione, 218
Reducing power, 208
Regeneration, 37
Regenerative effects, 37
Regeneresen®, 38, 40, 41
Regulated inflammation, 4
Regulatory B cells, 6
Rescue pain medication, 97
Respiratory allergies, 16
Responses to therapy, 49
Retinitis pigmentosa, 40
Rhamnose, 204
Rhea, 162
Rheumatism, 134, 198
Rheumatoid arthritis, 135, 162, 196, 249
Ribonucleic acids (RNAs), 35, 36
Ribonucleinates, 35
Ringworm, 198
RN13, 40
RNA, 36, 40
RNA regenerative therapy, 36, 39
ROS-stimulating bacteria, 23
ROS generation, 23
Rumalon®, 45

S

Saccharomyces boulardii, 24
Safety, 56
Safety of CSE, 145
Scavenge hydroxyl radicals, 200
Sedanenolide, 136, 143
Sedanolide, 136
Sedanonine anhydride, 136
Sedenolide, 143
Seleno-enzymes, 254
Selinene, 136
Seminal vesicle, 37
Sensory organs, 35
Serine protease inhibitor, 110
Serious adverse events, 75
Serum globulin, 146
Serum total cholesterol, 148
Serum triglycerides, 146
Sexually transmitted infections, 197
SFE lipid-rich fraction, 123
Shewanella oneidensis, 250
Short-chain fatty acids (SCFA), 125
Short-tailed shearwater, 164
Side effects, 136
Signal transduction, 23
Silencing RNA, 37
Silver chemistry, 243
Silver nitrate, 248
Silver pharmaceuticals, 257
Silver pharmacology, 249
Sinusitis, 16
Skeletal muscle, 37
Skin ailments, 135
Skin cancers, 197
Skin diseases, 198
Skin irritations, 196, 197
Smallage, 134
Small RNA, 38
SOCs, 69
Solubilities of some silver salts/complexes, 259
Somalia, 180
Some silver bioligands, 246
South Africa, 197
South African rock paintings, 182
South African-Zimbabwe ostrich oils, 164
South Kenya, 198
South Sudan, 180
Species (ROS), 1
Spleen, 40, 135
Splenocytes, 115
Spondylosis, 43
Spreading factor, 22
Squamous cell carcinoma, 220
Stabilised powder extract, 109
Standardized celery preparation, 133
Staphylococci, 126, 249
Staphylococcus aureus, 143
Staphylococcos epidermidis, 254
Staphylococcus thermophilus, 9
State food and drug administration of China, 150
Sterol esters, 94
Stomach, 216
Stomach and digestive complaints, 196
Stomach oil, 165
Streptococci, 249
Streptococcus pneumoniae hyaluronate lyase, 22
Streptococcus thermophilus, 8, 17
Streptozotocin-induced diabetic, 218
Streptozotocin-induced diabetic rats, 224
Structures of phthalide compounds, 145
Struthio camelus, 163
Struthiols™, 164
Supercritical extracts of CSE (S-CSE), 136
Supercritical fluid extraction, 94
Supercritical fluid extraction (CO_2-SFE), 97
Superoxide, 20
Superoxide anion/hydrogen peroxide oxidation, 19
Superoxide dismutase, 218
Swaziland, 180
Symbiotic microorganisms, 4
Symptom scores in patients, 8
Synbiotics, 18
Synergistic benefits, 26
Synthetic, 38
Syphilis, 197
Systemic immune, 6
Systemic toxicity, 146
Systolic blood pressure, 147

T

Tangles, 149
Tannins, 213
Tanzania, 180
Tapetoretinal dystrophia, 40
Tartaric acid, 96
Tasmanian herbal preparation of celery, 136
Tau phosphorylation, 149
T-cell responses, 46
Tenderness pain, 54
Teratogenetic effects, 37
Terpenoids, 162
Testis, 36, 40
T lymphocytes, 219
Thalamus, 40
Th1 and Th2 antigen-driven, 6

Therapeutic adjunct, 250
Therapeutic guide to herbal medicines, 135
Thiocyanate (SCN^-), 252
Thiol-depleting xenobiotics, 254
Thioredoxin, 22
Thioredoxin reductases (TrxR), 254
Thrombosis, 212
Thromboxane A_2, 219
Thymus, 37
Thyroid, 36
Tight junctions, 25
Tinnitus, 40
TNF-α, 97
Tolerability, 69
Toll-like receptors (TLRs), 6
Tonic, 135
Topical treatment, 197
Toxicity, 107
Toxicity of silver pharmaceuticals, 253
Toxicology studies, 133
Trace element, 246
Traditional Mexican ethnomedicinal, 196
Transcription factors, 221
Transmembrane transport, 199
Traumatic brain injury, 15
Treat wounds and skin conditions, 198
Trichophyton mentagrophytes, 145
Trichophyton mentagrophytes var. interdigitale, 145
Trichophyton rubrum, 145
Trichophyton verrucosum, 145
Triglyceride, 94, 115, 164
Triglyceride nutritional oils, 157
Triple-transgenic Alzheimer's disease (AD) mouse model, 149
True aloe, 180
Tukhm-e-karafs, 134
Tumour growth, 220
Tumour suppressor genes, 222
Tumour therapy, 223
Typhoid, 198
Tyrosine phosphatases, 23

U
Uganda, 180
Ulcerative colitis, 24
Ultra violet damaged skin, 16
UMBELLIFERAE, 134
Uremic toxins, 17
Uteri, 37

V
Vanillin, 149
Vascular dementia, 150
Vasoactive amines, 219
Vasodilation, 219
Veerya-ushna, 134
Vipaka-katu, 134
Virus-related dysplasia, 40
Visual field, 40
Vital signs, 68
Vitamin C, 19, 21, 135, 200
Vitamin E, 200
Volatile oil, 135

W
Warts, 198
Western Australian, 159
Western Ontario and McMaster Universities OsteoArthritis (WOMAC) Index, 49
Wild celery, 134
Wound healing, 215, 216, 220, 221, 238
Wounds, 197

Y
Yeast, 40, 45
Yeast RNA, 40
Yemeni, 196
Yogurts, 6
Yolla, 164
Yolla oils, 166

Z
Zambia, 180
Zimbabwe, 180
Zoopharmaca, 156
Zooplankton, 110
ZUNG depression scale, 42