Chapter 11
Challenges in Extrapolating In Vitro Findings to In Vivo Evaluation of Plant Resources

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Introduction

Conflicting evidence exists regarding findings on the biological activity of natural products when extrapolated from the laboratory to the animal. The first approach to testing for the biological activity of a chemical – i.e., against parasites, or to favour rumen function – is to conduct screening procedures in vitro. This is because of the substantial advantages of this methodology such as low cost and rapid turnover, which allows for screening of a large number of compounds in a relatively short period of time. Nevertheless, the advantage of bioprospecting through in vitro testing comes with a cost: A positive in an in vitro test does not necessarily warrant biological activity when the chemical or product is subsequently tested in the animal. Using the same logic, it is also possible that lack of activity by a natural product in an in vitro test does not strictly mean lack of biological activity in vivo.

There are several reasons for the conflicting evidence found frequently between in vitro and in vivo testing in natural plant products or plant secondary metabolites. The main point to keep in mind is that there is a reason for laboratory bioprospecting tests to be called "in vitro:" There are several assumptions and deviations from processes typically occurring in the animal; from the physiology and kinetics of digestive processes, to the pharmacokinetics of natural plant products to the ingestive behaviour of herbivores. The present chapter is an attempt to explain how these assumptions and deviations from processes occurring in the animal influence and bias in vitro testing. Using this framework we attempt to understand discrepancies between in vitro and in vivo testing and to propose some potential solutions to the problem. We hope that this effort will aid in the development of more reliable techniques for testing the biological activity of plant resources.

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Simple vs. Complex Environments: Interactions of Bioactives with Other Chemicals

Testing purified compounds allows their activity to be quantified reliably without the interference of other plant components or nutrients [1]. Nevertheless, such a controlled environment comes with a cost when attempting to extrapolate results to the whole animal: in vitro studies normally do not deal with the interference of the complex environment present in the realm of the gastrointestinal tract.

In vitro assays designed to test for antiparasitic activity typically involve the extraction and purification of the natural compound (e.g., tannins) and subsequently the preparation of test solutions with the compound and the parasite larvae (e.g., [31, 32]). Such solutions are devoid of the chemical and biological complexity of the gastrointestinal tract. Chemicals that normally occur in the rumen fluid, digesta and faeces such as nutrients and other plant secondary metabolites have the potential to interact with bioactives creating complexes that may either depress or enhance activity significantly.

Screening for natural plant products that influence rumen function, generally use rumen fluid in the testing medium, which offers a chemical and biological dimension closer to an in vivo situation, particularly when the diets fed to the donor animals are similar to those that will be tested in vivo [6, 7]. Likewise, parasite viability in response to antiparasitic bioactives has been tested in faecal samples [18], and thus biological and chemical complexity of the media is kept during in vitro testing.

The different effects of plant actives when tested in vitro using a simple chemical and biological environment, versus when tested in vivo – under a complex chemical and biological environment – is illustrated by the strong activity of condensed tannins against abomasal nematodes of sheep in vitro [2], but not in vivo [2]. Condensed tannins are polyphenolic compounds with high affinity for proteins. The majority of condensed tannins present in the abomasums of sheep fed tannin-rich diets have been found in complexes with proteins and consequently are unavailable for action against abomasal parasites [1].

Tannins not only complex with proteins; these chemicals are highly reactive compounds which form complexes with saponins [15], alkaloids [36], and terpenes [34]. Tannins, terpenes and alkaloids can co-occur in the digesta of animals consuming diverse diets and thus the biological activity of plant secondary metabolites has the potential to be reduced significantly after the formation of such complexes.

Incubation of Plant Bioactives with Rumen Fluid: Static vs. Dynamic Fermentation Systems

The low cost and high numbers of samples that can be screened per unit of time makes the in vitro systems – similar to those originally developed by Tilley and Terry [43] – the first choice for investigating the potential impact of natural products on rumen function. Nevertheless, the in vitro system is a surrogate for the "reference" method, which involves the in vivo measurement of food digestibility.

The problem, however, with the in vitro system is that it assumes that the conditions in the incubators remain similar to rumen content for 48 h (or more) in spite of the accumulation of end products. On the contrary, end products, and even substrates such as natural plant products typically cross the rumen wall or they flow with the digesta and thus processes in the rumen fluid do not remain static but are inherently dynamic. As an example, terpenes – an array of natural plant products found in many shrubs and trees- are small, fat-soluble molecules that are absorbed quickly through the rumen epithelium. The average terminal elimination of plasma terpene concentrations is in the order of minutes after terpenes are infused into the rumen [11]. Consequently, during in vitro testing the ruminal activity of terpenes could be overestimated because under static fermentation techniques terpenes remain in the medium during the whole incubation process, which increases the likelihood for interactions. In contrast, when animals ingest terpenes these compounds are rapidly absorbed and metabolized and thus the residence time of terpenes in the rumen is much shorter.

Due to the dynamism of ruminal processes and the static nature of in vitro testing, discrepancies have been found between in vivo and in vitro digestibility studies. Terpenes in *Artemisia tridentata* have marked antibacterial effects in the rumen and in vitro studies they have been reported to inhibit digestibility [35]. However, in a recent in vivo study we found that terpenes from *A. tridentata* caused increases rather than decreases in dry matter and fibre digestibility [48]. Digestibility depression is a function of the competition between rates of digestion and passage [44], variables that are not taken into account in static systems. Terpenes apparently increase rumen retention times, which in turn increase the extent of digestion [48].

Because of the dynamic nature of digestion processes, in vitro continuous systems are a step closer to "reality" than standard in vitro techniques because they mimic the constant digesta turnover that occurs in the animal [44]. In vitro continuous systems have been used successfully to determine the effects of natural plant extracts on ruminal fermentation [6].

A proposed approach to in vitro studies is to initiate the screening process in a traditional non-dynamic in vitro system and then test the most promising bioactives in a continuous system before the development of in vivo trials.

Concentration of Bioactives in Biological and Artificial Media

Crude or purified extracts from plants are used for in vitro testing. This procedure may not give results that are always relevant to the in vivo situation [1]. For instance, purified condensed tannins in *Cichorium intybus* have been shown in vitro to inhibit deer nematodes [33]. However, the concentration of condensed tannins in *C. intybus* is very low and thus it is highly unlikely that the concentrations found to be successful in vitro could be attained in vivo [1]. A constraint on using plant chemicals as biological active agents is the potentially large quantities of plants required to achieve meaningful doses in the herbivore [49]. Concentrations yielding promising results in vitro may never be reached in vivo.

Another constraint on using purified chemical compounds during in vitro testing is that it may be unpractical, economically unfeasible or even impossible to feed purified compounds or plant extracts to animals. When feeding plants with bioactives – instead of purified compounds – a point to consider is that the concentration of a chemical in the plant's tissue may not represent the amount, which is actually available to the animal.

A new approach for testing biological activity of plants in vitro against gastrointestinal nematodes is the use of rumen fluid taken from animals grazing pure stands of plant secondary metabolites-rich plants [1]. With this methodology researchers can test for activity in a medium that contains concentrations of chemicals in the rumen fluid that are realistic instead of theoretical. By using rumen fluid, the technique also adds chemical and biological complexity to the medium (see above), which is also a step closer to the in vivo scenario. Nevertheless, when testing for activity against intestinal nematodes, the possible lack of plant secondary metabolites activity in the rumen fluid does not necessarily mean lack of activity in the duodenum or large intestine, as plant secondary metabolites might become active in the lower parts of the gastrointestinal tract [1].

Ruminal Adaptation: Short vs. Long Term Effects

The diverse microbial populations in the foregut can perform many reactions that modify plant products and thus influence their biological activity [13]. For instance, Cardozo et al. [6] found that although some natural plant extracts have a short-term effect on ruminal microbial fermentation, ruminal microbes were adapted after 6 days and differences from controls (without plant extracts) disappeared. This led Cardozo et al. [6] to suggest that data from short-term in vitro fermentation studies may lead to erroneous conclusions.

There is evidence suggesting rumen microbes have a direct impact on transforming and inactivating natural plant products. Gradual exposure to increasing levels of oxalic acid to ruminants leads to a change in the composition of the rumen microbial population, which results in the breakdown of oxalic acid [10]. Chronic exposure to terpenes in sheep increases their ability to consume terpenes in *A. tridentata* [45]. Rumen microbes adapt to monoterpenes [11, 21], and ruminal microbes in goats modify diterpene diesters present in *Euphorbia esula* [23]. Deconjugation of phytoestrogens [4] and metabolism of mycotoxins [26] by the gut microflora has been reported in livestock.

Ingestive Behavior

Food Aversions and Willingness to Consume Bioactives

A problem with validating in vitro results for biological activity is the assumption that animals will be willing to consume concentrations of natural plant

products, which parallel the amounts that yield biological activity in artificial systems. However, many of the chemicals in plants with biological activity are secondary compounds, which at certain doses can adversely affect mammals through their negative actions on cellular and metabolic processes [8, 9]. Herbivores feed to avoid exceeding a threshold dose of particular plant secondary metabolites [28, 40, 41] such that ingestion of plant bioactives would not exceed their capacity to biotransform and eliminate these compounds.

A mechanism used by herbivores to prevent toxicosis is the stimulation of the emetic system and the development of food aversions [40]. Food aversion learning is the process by which after eating or drinking a specific food, a physiological event or physiochemical agent causes nausea [17]. Thus, aversions result from the stimulation of the emetic system of the midbrain and brain stem [30]. This system can be stimulated by toxins in the cardiovascular system and cerebrospinal fluid and through vagal and splanchnic afferents from the gastrointestinal tract [5]. After ingesting a food containing a plant secondary metabolite, afferent impulses to the central nervous system cause malaise, which in turn causes the animal to decrease intake of food. In turn, efferent impulses from the central nervous system to the gut cause a decrease in motility and a decrease in absorption of the plant secondary metabolites [42]. Consistent with this mechanism, antiemetic drugs attenuated food aversions in sheep caused by the toxicant LiCl [39]. Likewise, administration of a selective antiemetic (ondansetron), an antagonist of 5-hydroxy tryptamine (5HT3) serotonin receptors, increased intake by marsupials of diets containing secondary metabolites present in *Eucalyptus* [25]. Collectively, the information presented suggests that there is a limit on how much plant bioactive an herbivore is willing to ingest. In certain circumstances, small amounts of plant secondary metabolites will stimulate the emetic system causing strong food aversions that will prevent animals from consuming the therapeutic or active doses found during in vitro studies.

A possible course of action to enhance the animal's willingness to consume plants with bioactives is to provide an adequate level of nutrition with the basal diet. When animals ingest adequate amounts of energy and protein, they can eat more foods that contain plant secondary metabolites. This is because rates of detoxification are influenced by the nutrient status of an animal. The general mechanism of detoxification involves converting more toxic lipophilic compounds to less toxic water-soluble compounds that can be excreted in the urine [8, 9]. Biotransformation of toxins is carried out largely in the liver and usually occurs in two steps. The first step (Phase I) introduces a reactive group – such as OH, NH_2, COOH, or SH – into the structure of the toxin; those interactions typically produce a less toxic compound. During the second step (Phase II), the newly formed compound is conjugated with a small molecule such as glucuronic acid, amino acids (e.g., glycine), sulphates, acetates, or methyl groups [37]. Importantly, these transformations require nutrients such as protein and energy in the form of glucose [19, 20]. Thus, detoxification processes reduce the protein and energy that otherwise would be available for maintenance and production [14, 20]. Lambs can ingest more of the toxin LiCl as the energy content of their diet increases [50]. Likewise, sheep offered terpene-containing diets with increasing concentrations of energy or protein

consume terpenes in a graded fashion with a direct relationship between energy or protein available and terpene intake [47]. Supplemental energy and protein increase the ability of sheep and goats to eat foods that contain plant secondary metabolites such as terpenes [45], tannins [46], and saponins [27]. Conversely, herbivores restrict ingestion of higher amounts of plant secondary metabolites when levels of nutrients such as sodium are low [16].

Pulse-Delivery of Bioactives to the Digestive Tract

Even if we assume animals are willing to consume concentrations of plant secondary metabolites that yield biological activity, the rate at which those compounds enter the digestive tract is not continuous but in pulses. Ingestion of plant bioactives in nature is not continuous because, as mentioned before, plant secondary metabolites at certain concentrations have negative impacts on cells and metabolic processes. Thus, animals consume plant secondary metabolites in small amounts during discrete feeding bouts distributed throughout the day. At critical thresholds, toxins satiate the detoxification capabilities of herbivores [13]. At these levels, animals quit feeding, and they resume eating only after plant secondary metabolites concentrations in the body decline due to detoxification and elimination [11, 12, 38]. These processes cause cyclic patterns of intakes of particular foods with peak intakes at the lowest concentration of plant secondary metabolites in the body [13, 38]. During these cycles, pulses of substrate are sent down the gastrointestinal tract. Pulsative feeding could create cyclic perturbations of the microbial populations through shifts in the relative proportions of such populations [44]. Consequently, pulse-like feeding behaviour could create conditions in vivo that deviate substantially from the continuous or static conditions normally present in artificial fermentation or incubation systems. Experience from the administration of antibiotics to patients has clearly demonstrated the advantages of continuous dosage over intermittent administration of antibiotics [22].

Voluntary Intake and Sequence of Feeding Patterns

The sequence in which “medicinal” and other components of the diet are ingested may be another reason findings in vitro diverge from those obtained in vivo. The temporal order at which foods enter the rumen may influence the likelihood of interactions among different dietary chemicals. For instance, lambs offered plant secondary metabolites in the sequence of tannins followed by terpenes consume twice as much food as lambs offered a meal of terpenes followed by a meal of tannins [34]. Tannins are large molecules that interact with other compounds as they move slowly through the gastrointestinal tract [24, 29]. Consumption of tannins first increases the likelihood of interaction, and possible deactivation, of terpenes fed subsequently in a meal. In contrast, terpenes are small non-polar molecules, highly

Table 11.1 Some causes of discrepancy between in vitro and in vivo studies and a proposed course of action to reduce such differences

Cause of discrepancy	Action
• In Vitro: Chemically simple testing media In Vivo: Chemically and biologically complex media with more likelihood for chemical interactions and inactivation	Testing medium should be as close as possible to the chemical and biological conditions where bioactives are expected to act
• In Vitro: Static systems (e.g., in vitro digestibility) In Vivo: Dynamic system	Use static systems as a first screening approach, followed by a continuous system such as artificial rumen, Rusitec, etc.
• In Vitro: Purified extracts, concentrations may never reach the amounts used in vivo. Reduced bioavailability	Use lyophilized plant material. Use rumen fluid taken from animals grazing pure stands of plant secondary metabolites-rich-plants. It might not be always possible
• Ruminal adaptation due to changes in microbial populations	Conduct screening studies for periods longer than 1 week
• Animals may develop aversions to the bioactives and thus they will not consume the doses of bioactives tested in vitro	Offer bioactives along with a nutritious food such that the likelihood of a food aversion declines
• Pulsative feeding on bioactives creates gastrointestinal disturbances	Deliver bioactives in pulses in continuous in vitro systems
• Sequential feeding patterns may influence the likelihood of interactions among bioactives and other chemicals in the digesta	In a continuous system try to mimic the feeding patterns which occurs in vivo

soluble in membranes; they are absorbed readily through the gastro-intestinal tract walls [11]; if they are fed first in the sequence the likelihood for interaction with tannins decreases. Likewise, the sequential supply of a tanniferous shrub (*Acacia cyanophylla*) followed by protein rich feed substantially increases the chances of protein interacting with tannins, which in turn reduces ammonia formation and increases protein retention in sheep and goats [3]. Collectively, the information presented suggests that the biological activity of plant secondary metabolites will depend on the sequence at which plant secondary metabolites-containing plants are fed relative to the remaining components of the diet (Table 11.1).

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