Chapter 2
Green Leafy Vegetables in Cancer Prevention

Marja Mutanen, Mikael Niku, and Seija Oikarinen

Abstract Green leafy vegetables contain a wealth of potential chemopreventive compounds. Chlorophyll and its derivatives can trap aflatoxin and other mutagens by complex formation and appear protective against carcinogens in various animal and human models. They also have antioxidative and immunomodulatory properties. Folate is essential in DNA synthesis and methylation, and is required especially by rapidly proliferating tissues. For cancer prevention, dietary folate may be preferable to the much more stable folic acid used in fortification. Of the various green vegetables, spinach and perilla have been widely studied. Spinach has high antioxidant content, and its glycolipid fractions inhibit cancer cell proliferation and suppress tumours in murine models. Luteolin, rosmarinic acid and triterpenes extracted from perilla leaves are potent antitumourigenic and anti-inflammatory agents.

Keywords Spinach · Perilla · Chlorophyll · Folate · Luteolin · Rosmarinic acid · Triterpenes · Chemoprevention

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2.1 Introduction

Green leafy vegetables are good sources of for example chlorophyll, folates, flavonoids, polyphenols, glycolipids, and antioxidants, all of which are potential chemopreventive agents. The experimental work with different types of vegetables indicate that certain vegetables may either as such or through their specific pattern of compounds possess anticarcinogenic effects in humans and different animal models. The anticarcinogenic mechanisms have also been established in vitro for some of these compounds. The epidemiological studies have not been able to answer the question how different types of fruits and vegetables or their constituents modulate the risk of different cancers. Vegetables (sometimes with fruits) are often used as a single dietary constituent in epidemiological study approach and thus the possibility for specific vegetables to be detected as cancer preventive substance is not possible. In addition, genetic variation in genes such as cytochrome P450 family that are involved in metabolism of vegetable components potentially modify relationships between vegetable intake and cancer risk. In this chapter we concentrate on two main chemopreventive compound found in green leafy vegetables, chlorophyll and folates. In addition, two leafy vegetables commonly used in different parts of the world, spinach and perilla leaves and their constituents are discussed.

2.2 Green Leafy Vegetables as Sources of Chemopreventive Compounds

2.2.1 Chlorophylls and Chlorophyllins

Chlorophylls and chlorophyllins (Fig. 2.1) are ubiquitous pigments with reported chemopreventive properties. Chlorophylls are the principal photoreceptors in green plants, responsible for their colour. Common green vegetables contain 20–2,000 micrograms of chlorophylls per gram of fresh tissue (Khachik et al. 1986). Chlorophyllins are semi-synthetic water-soluble chlorophyll derivatives commonly used as food additives. For practical reasons, commercially available chlorophyllins are used in most experimental studies. Food processing and digestion produce a multitude of further chlorophyll derivatives, the absorption and bioavailability of which is incompletely understood (for a review, see Ferruzzi and Blakeslee 2007).

Chlorophyll derivatives have shown potential chemopreventive activity in various in vitro and in vivo models. Best documented is their ability to trap mutagens

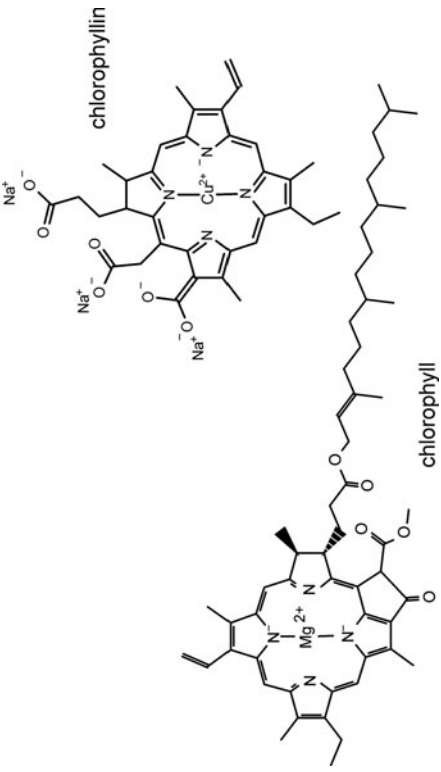

Fig. 2.1 Chemical structures of chlorophyll and clorophyllin. Source: The PubChem: http://www.ncbi.nlm.nih.gov/About/disclaimer.html

by complex formation. Chlorophyllin binds mutagens such as acridine orange, quinacrine mustard, doxorubicin, dibenzopyrene and aflatoxin, and appears to decrease mutagen binding to DNA in vitro (Pietrzak et al. 2006, 2008; Simonich et al. 2007, 2008). In animal models, chlorophyllins reduce aflatoxin intestinal absorption, tissue concentration and hepatic carcinogenesis if given together with the carcinogen (Breinholt et al. 1995, 1999; Hayashi et al. 1999). Chlorophyll protects rats from heme-induced mucosal damage; interestingly, chrolophyllin is not protective (de Vogel et al. 2005a). An epidemiological study suggest that high dietary heme/chlorophyll ratio may elevate colon cancer risk (Balder et al. 2006). A clinical intervention study with humans exposed to dietary aflatoxins showed that chlorophyllin (100 mg three times daily) can effectively reduce urinary concentrations of an excreted DNA adduct biomarker (Egner et al. 2001).

Other chemopreventive mechanisms are possible, as chlorophyllin metabolites actually are absorbed from the intestine (Egner et al. 2000). Chlorophyllin is known as a potent antioxidant, and it has been shown to protect against oxidative damage in vitro and ex vivo, after injection or dietary administration in mice (Kamat et al. 2000; Kumar et al. 2004; Kwang Kyun Park et al. 2003). It also induces phase II detoxification activity in liver (Dingley et al. 2003; Fahey et al. 2005). Immunomodulatory effects have been reported with murine macrophages in vitro (Cho et al. 2000; Yun et al. 2005, 2006). In various cancer cell lines, chlorophyllin reduces ERK activation, cyclin D1 and β-catenin and induces differentiation and apoptosis (Chiu et al. 2005; Díaz et al. 2003; Carter et al. 2004). In vivo, effects on colonic carcinogenesis appear complex and depend on dosage and the use of natural chrolophyll versus chlorophyllins (Blum et al. 2003).

2.2.2 Folate

Vitamins such as B2, B6, B12 and folate that are related to one carbon metabolism are of interest in carcinogenesis since they take part in DNA syntheses and methylation reactions in the cell. Folate is in the centre of these metabolic reactions and has been in a focus of cancer prevention during the last decade. Since green leafy vegetables and plant kingdom in general are considerable sources of folate in human diet, the evidence behind folate and carcinogenesis is discussed in this chapter.

From the green leafy vegetables especially nettle, parsley and kale are good sources of folate (190–120 μg/100 g). In addition to green leafy vegetables, also some beans contain much: brown and white beans 390 μg/100 g, soybean 370 μg/100 g, and green beans 145 μg/100 g. Similarly asparagus, beet, broccoli, Brussels sprout, cauliflower, and kohlrabi are good sources of folate (180–80 μg/100 g).

Folate is a generic name for a food-based group of B vitamins containing an aromatic pteridine ring linked to *p*-aminobenzoic acid and a glutamate residude. Humans get folate either as this natural form or in supplements or through food fortification as pharmaceutical totally oxidized form of folate, i.e folic acid (FA). Food folate is very unstable and rapidly loses its activity during storage and food

preparation. FA, on the contrary, is highly stable over months. Both folate and FA are absorbed in the intestine as carrier mediated processes, and bioavailability of FA is twice as high than natural folates. During absorption folate undergo hydrolyses to methyltetrahydrofolate (methylTHF), which is a predominant form of folate in circulation. Also FA is converted to methylTHF to some extent. It has been shown that the process becomes saturated at doses of 270 μg and at higher levels FA is transported to circulation as FA. From the carcinogenesis point of view the role of free FA has raised some concern (see below). Commonly used amount of FA in supplement is 400 μg meaning that a regular use of FA as supplement produces a sustain level of FA in plasma.

There are several extensive reviews on folate metabolism and reader is referred to them (Kim 2007) and only schematic representation of folate metabolism is illustrated here. Figure 2.2 (Hubner and Houlston 2009) shows the essential role of methylTHF for the synthesis of nucleotides and for the provision of methyl groups for the maintenance of DNA methylation in dividing cells.

The recommendation of folate intake is 400 μg/d and in several populations this is not achieved. Strong evidence behind low folate status and neural tube defect has in several countries lead to recommendations of FA supplements to women of

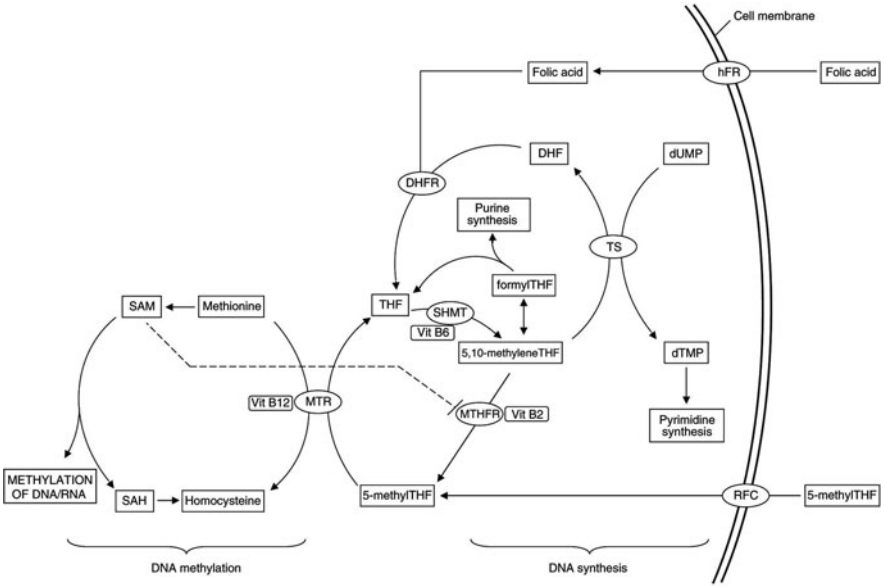

Fig. 2.2 Schematic representation of folate metabolism illustrating the entry of natural folates and folic acid into the pathway, and flow of methyl group towards either DNA synthesis or DNA methylation. RFC, reduced folate carrier; hFR, human folate receptor; MTR, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; SHMT, serine hydroxymethlytransferase; TS, thymidylate synthase; THF, tetrahydrofolate; DHF, dihydrofolate; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate. (Hubner and Houlston 2009)

reproductive age. In addition, food fortification with FA to increase folate status of the population is used in the US since 1998 and some other countries. The purpose of these fortifications has been to provide an average an additional 100 μg FA/d. Since epidemiological evidence has for long shown inverse relationship between the consumption of vegetables and fruits and the incidence of several cancers, several secondary prevention trials in humans with FA has been carried out lately. The outcome of these trials has shown the complexity in folate in cancer prevention and supported the evidence, which was got from the animal experiments, notably that the efficacy of folate in cancer prevention is time and dose dependent and supplemental FA may not always by beneficial. These aspects of folate and FA are discussed below.

Low folate status has been associated with increased risk of cancers of the colon, esophageal, gastric, pancreatic, and breast and the evidence has been recently discussed in the following articles: Kim (2007), Larsson et al. (2006), and Ulrich (2007). In his recent comprehensive review on folate and colorectal cancer (CRC) Kim (2007) comes to the conclusion that even if the overall evidence from epidemiologic, animal, and intervention studies supports the inverse association between folate status and the risk of colorectal cancer the effect of folate is bimodal. Rapidly proliferating tissues, including tumour tissue, have greater requirement for folate. Tumor tissues over express folate receptors to meet their increased need for increased demand for DNA synthesis and proliferation. In precancerous colon tissue and already established tumours folate deficiency below requirements seem to have inhibitory effect whereas folate supplementation above requirements promotes the process by supporting cell divisions. This may explain the disappointing results, which have been obtained lately in human supplementation trials. All of them were secondary preventions trials, with subjects with established primary colon cancer. In addition, FA was used in supplements indicating that there was free FA present in circulation. Since tumor tissue has much higher affinity for FA than methylTHF the demand for methylation reactions of the transformed tissue is easily achieved. In addition, unmetabolized FA in plasma has been shown to be associated with reduced natural killer cell cytotoxicity in humans (Troen et al. 2006). Natural killer cells are effector lymphocytes of the innate immune system that control several types of tumors and microbiological infection (Vivier et al. 2008). The situation is different for normal mucosa tissue. Then folate deficiency appears to predispose the tissue to neoplastic transformation and folate supplementation within physiological range prevents transformation. Also in this situation pharmacological supplemental doses of FA enhance the transformation of normal colon mucosa to precancerous tissue. The dual modulatory role of folate in carcinogenesis is illustrated in Fig. 2.3 (Kim 2007).

Meta-analysis for case-control and cohort studies on folate and esophageal, gastric, and pancreatic cancer risk showed inverse relation of dietary folate intake and risk of for esophageal and pancreatic cancers. The results for gastric cancer were inconsistent. In most studies the MTHFR 677TT genotype, which is associated with reduced enzyme activity (and lower conversion of dietary folate into methylTHF) was associated into higher risk of all three cancers (Larsson et al. 2006). It was

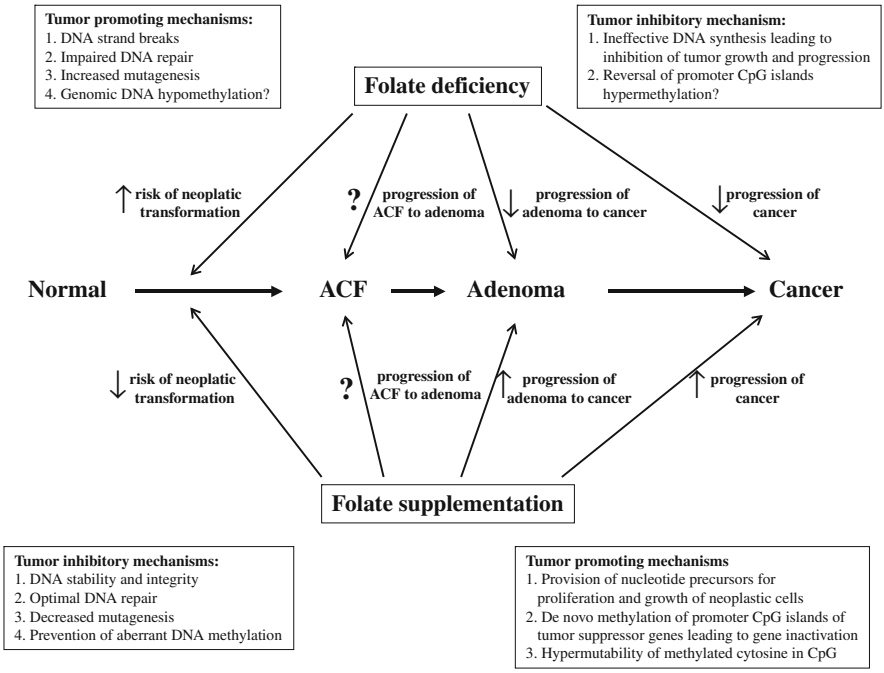

Fig. 2.3 Dual modulatory role of folate in carcinogenesis: cancer develops over decades, if not lifetime, through different stages of premalignant lesions in the target organ. Folate deficiency in normal tissues predispose them to neoplastic transformation, and modest supplemental levels suppress, whereas supraphysiologic doses of supplementation enhances, the development of tumors in normal tissues. In contrast, folate deficiency has an inhibitory effect whereas folate supplementation has a promoting effect on the progression of established neoplasms. It is unknown at present the effect of folate deficiency and supplementation on the progression of early precursor or preneoplastic lesions of CRC (e. g., aberrant crypt foci, ACF) to adenoma and to frank cancer. The mechanisms by which folate exerts dual modulatory effects on carcinogenesis depending on the timing and dose of folate intervention relate to its essential role in one-carbon transfer reactions involved in DNA synthesis and biological methylation reactions (Kim 2007)

suggested that aberrant DNA methylation may play a role in the development of these cancers. DNA methylation has been shown to be significantly lower in individuals with the TT genotype than those carrying CC genotype and TT genotype is directly correlated with folate status (Friso et al. 2002). Three prospective studies on supplemental FA and risk of pancreatic cancer support the picture that was noticed with colon cancer. An inverse association was found between dietary folate intake and pancreatic cancer risk, but none indicated an inverse relation with supplemental FA intake.

The recent epidemiological evidence on folate and the risk of breast cancer follows the picture already suggested in connection with colon carcinogenesis (Ulrich 2007). There exist a nonlinear relation between folate status and breast cancer risk. Higher folate status may be needed to protect postmenopausal women

with very low folate intake. However, those having adequate intake do not benefit increased intake of folate and high supplemental FA may actually increase cancer risk (Stolzenberg-Solomon et al. 2006).

Due the low stability it is not possible to do clinical prospective trials or mechanistic studies in animals with natural dietary folate. Also studies using foods with high folate concentration are complicated with accompanying compounds ingested, which also may have a role in cancer susceptible. The accumulating data, however, support natural folate over the supplemental FA in cancer prevention. Absorption and movement of folate through cell membranes differs between folate and FA and physiological consequences of unmetabolized FA in still largely unknown. The adequate folate status should be sustained throughout the lifespan and very high intakes (at least through FA) should be avoided. This is best achieved through diet that contains good folate sources.

2.3 Spinach (*Spinacia oleracea*)

Spinach is composed of various active compounds, such as flavonoids and other polyphenolic active ingredients acting synergistically as anti-inflammatory, antioxidative, and anticancer agents. Spinach leaves contain approximately 1,000 mg of total flavonoids per kilogram fresh weight, of which main are patuletin (3,5,7,3′,4′-penthahydroxy-6-methoxyflavone) and spinacetin (3,5,7, 4′-tetrahydroxy-6,3′-dimethoxyflavone). In addition, spinach contains several flavonol and flavonoid glycosides. These are glucuronides and acylated di-and triglycosides of methylated and methylene dioxide derivatives of 6-oxygenated flavonols. Glucuronides are more water-soluble than glycosides and acylated compounds (Lomnitski et al. 2003). Furthermore spinach contains several glycolipids (Murakami et al. 2003) as well as considerable amounts of chlorofyll. Spinach is one of the most important antioxidative vegetables, usually consumed after boiling either fresh or frozen leaves.

2.3.1 Glycolipids Fraction from Spinach

The major glycolipids fraction from spinach consists mainly of three glycolipids; monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), and sulfoquinovosyl diacylglycerol (SQDG) with slightly different effects in in vitro (Murakami et al. 2003). Glycolipids fraction, especially SQDG, inhibits DNA polymerase activity and cancer cell growth in vitro (Kuriyama 2005; Maeda et al. 2007). Injection of SQDG also suppressed tumour growth significantly in nude mice bearing solid tumors of HeLa cells (Maeda et al. 2007). Oral administration of three main glycolipids (20 mg/kg) in BALB/c mice in a colon-26 tumor graft study induced about 56% decrease in the solid tumor volume. This decrement was accompanied with an inhibition of angiogenesis and the expression of cell

proliferation marker proteins such as Ki-67, proliferating cell nuclear antigen (PCNA), and Cyclin E in the tumor tissue (Maeda et al. 2008). SQDG, but not other glycolipids, binds to Cdt1 and inhibits Cdt1-geminin interaction in vitro, with 50% inhibition observed at concentration of 2 μg/ml. Cdt1, a human replication initiation protein, regulates DNA replication and geminin modulates Cdt1 action by direct binding (Mizushina et al. 2008). Similarly, only SQDG fraction inhibited in vitro the double-stranded DNA (dsDNA) binding activity of human p53 DNA binding domain DBD indicating that SQDG might regulate the activity of p53 for cell division, cell cycle checkpoint and tumor suppression (Iijima et al. 2007). Both MGDG and SQDG have been shown to suppress microvessel growth in an ex vivo angiogenesis model using a rat aortic ring (Matsubara et al. 2005).

2.3.2 A Water-Soluble Natural Antioxidant (NAO)

Water-soluble extract isolated from spinach leaves, so called a natural antioxidant mixture of spinach (NAO) is an effective free-radical scavenger and inhibits the lipoxygenase enzyme activity. The main active compounds of NAO are polyphenols, which include flavonoid and *p*-coumaric acid derivatives (Bergman et al. 2001). The antioxidative activity of NAO in vitro and in vivo exceeds to that of other known antioxidants such as *N*-acetylcysteine, butylated hydroxytoluene, and vitamin E. Furthermore, NAO is stable at high temperature and it lacks toxicity (Lomnitski et al. 2003). At the level of 200 mg/kg NAO reduced plasma peroxide levels and significantly reduced hyperplasia in dorsal and lateral lobes in the TRAMP mice, a model of prostate cancer, and also dose-dependently inhibited cellular proliferation in prostatic carcinoma cell lines (Nyska et al. 2003; Bakshi et al. 2004). Topically or orally administered NAO reduced dermally induced skin papilloma multiplicity in the v-Ha-*ras* transgenic mouse model (Nyska et al. 2001).

2.3.3 Neoxanthin, a Major Carotenoid in Green Leafy Vegetables

Green leafy vegetables including spinach leaves contain carotenoids called epoxyxantophylls (epoxide-containing xanthophylls). The main epoxyxanthophyll in spinach is neoxanthin that is partially converted during digestion into neochrome-isomers. The ability of neoxanthin and (R/S)-neochrome to inhibit PC-3 human prostate cancer cells proliferation in vitro has been shown (Asai et al. 2004). To evaluate the relevance of the in vitro studies in humans, estimate the intestinal absorption of neoxanthin were evaluated by measuring the plasma concentrations of epoxyxanthophyll and their metabolites before and after 1 week of spinach intake (3.0 mg neoxanthin/d). The plasma concentrations of neoxanthin and its metabolites (neochrome stereoisomers) remained very low (about 1 nmol/l), whereas those of beta-carotene and lutein were markedly increased. These results indicated that the plasma response to dietary epoxyxanthophylls was very low in humans even after 1-week intake of epoxyxanthophyll-rich diets (Asai et al. 2008).

2.4 Perilla (*Perilla frutescens*)

2.4.1 Perilla Leaf Extracts (PLEs)

Perilla (*Perilla frutescens* (L.) Britton, Lamiaceae, also known as the mint family) is an annual herb native to Asia. Perilla leaves are used as vegetables and spicy herbs or medical purposes, and perilla seed oil, a rich source of the omega-3 fatty acid alpha-linolenic acid, is used as edible oil. Anticarcinogenic effect of PLE and its affecting components have been tested in a murine, two-stage skin carcinogenesis model where cancer is initiated by application of 7,12-dimethylbenz[*a*]anthracene (DMBA) and promoting by application of 12-tetradecanoylphorbol 13-asetate (TPA). TPA induces inflammation and is a skin tumour-promoting agent. Another mouse model for skin cancer is the Tg.AC mouse carrying the v-Ha-*ras* structural gene linked to a ζ-globulin promoter. Mice carrying this oncogene exhibit epithelial proliferation and formation of papillomas when treated with tumour promoters such as TPA.

Topical application twice a week with 1 mg of PLE to DMBA/TPA-treated mice resulted in a significant reduction in tumour incidence and multiplicity. When DMBA/TPA-treated mice ingested PLE ad libitum, no significant effect was observed in tumour incidence or average number of tumours, but treatment resulted in a significant reduction in the papilloma weight (Ueda et al. 2003). The same authors (Ueda et al. 2002) have previously shown that PLE suppressed the tumour necrosis factor-alpha (TNF-alpha) production in vivo. Mechanistically, PLE has been shown to dose-dependently induced apoptosis through the combinations of mitochondrial, death receptor-mediated, and endoplasmic reticulum pathways and suppressed the cell proliferation via p21-mediated G1 phase arrest in human leukemia HL-60 cells (Kwak et al. 2009). PLE induced also apoptosis on human hepatoma HepG2 cells (Lin et al. 2007).

Various PLEs were further fractionated to find out the affecting anti-carcinogenic components. So far, luteolin (3′,4′,5,7-tetrahydroxyflavone) (Ueda et al. 2002, 2003), rosmarinic acid (Osakabe et al. 2004; Lin et al. 2007) and triterpene acids (Banno et al. 2004) isolated from perilla has been shown to be the most promising chemopreventive molecules.

2.4.2 Luteolin, a Flavonoid

Luteolin is a flavone, a subclass of flavonoids, that exists a part from perilla in vegetables and (medicinal) herbs e.g. artichoke, celery, green pepper, spinach, parsley, sage and, thyme (Anonymous 2007). Daily intake of luteolin has been estimated to be less than 1 mg/d (Seelinger et al. 2008; Somerset and Johannot 2008). The anticarcinogenic mechanism of luteolin has been widely studied in vitro and in vivo, and its anticancer property is associated with the induction of apoptosis, and inhibition of cell proliferation, metastasis and angiogenesis (Lim do et al. 2007; Lin et al. 2008; Seelinger et al. 2008). Furthermore, it suppresses cell survival pathways such

as phosphatidylinositol 3′-kinase (PI3K)/Akt, nuclear factor kappa B (NF-kappaB) and X-linked inhibitor of apoptosis protein (XIAP) pathways (Lin et al. 2008). Ueda et al. (2003) suggested that luteolin was the chemopreventive component in PLE, and luteolin treatment with a dose 1 mg/mouse/application inhibited mouse skin tumour promotion. In addition, some epidemiological studies suggest an inverse correlation between dietary flavone (including apigenin and luteolin) intake and the risk of some cancer types (see Seelinger et al. 2008).

2.4.3 Rosmarinic Acid (RA), a Polyphenol

An anticarcinogenic effect of *Perilla frutescens* leaf extracts containing rosmarinic acid (RA), caffeic acid and luteolin were tested in the DMBA/TPA-induced murine skin cancer model (Osakabe et al. 2004). All fractions reduced significantly the tumour incidence and tumour multiplicity. The fraction containing 40% (w/w) of RA was the most efficient in preventing tumour progression. Therefore, the authors prepared a new fraction from perilla that contained 68% (w/w) of RA, while the level of luteolin and caffeic acid was negligible. Short-term experiments (1–5 h to 24 h) showed that RA containing fraction or an equivalent amount of pure RA had significant anti-inflammatory properties such as ear histology of TPA-treated mice, myeloperoxidase activity, chemokine expression, eicosanoid concentration, and cyclooxygenase-2 expression. Furthermore, both the high RA fraction and RA were able to decrease the levels of oxidative stress markers and the levels of 8OH-dG adducts. Lin et al. (2007) have reported that PLE (containing RA) induced apoptosis and regulated the expression of several apoptosis-related genes in human hepatoma HepG2 in vitro. Pure RA (10 μg/ml; a dose equivalent to 105 μg/ml of PLE) had similar, but less potent effect. In human leukemia U937 cells RA inhibits TNF-alpha-induced ROS generation and NF-kappaB activation, and enhances TNF-alpha-induced apoptosis (Moon et al. 2010). In two human colon carcinoma-derived cell lines, HCT15 and CO115, RA induces apoptosis in both cell lines, whereas cell proliferation was inhibited only in HCT15. RA inhibited ERK phosphorylation in HCT15 and had no effects on Akt phosphorylation in CO115 cells (Xavier et al. 2009).

2.4.4 Tormentic Acid, a Triterpene

Nine triterpene carboxylic acids isolated from ethanol extracts of the leaves of green and red perilla and eight of them were tested for their anti-inflammatory properties on TPA-induced ear edema inflammation in mice (Banno et al. 2004). All the compounds tested inhibited TPA - induced inflammation at 0.03–0.3 mg/ear of the 50% inhibitory dose. One of these compounds, tormentic acid, exhibited the strongest inhibitory effect, quite comparable with that of a commercial drug hydrocortisone. Topical application of tormentic acid resulted in a significant reduction in tumours with DMBA/TPA-treated mice. The average number of papillomas

was 4.8 and 8.6/mouse in the control group after 10 and 18 weeks experimental period, and 1.4 and 4.5/mouse in the tormentic acid-treated group at the same time points.

2.5 Conclusions

Based on animal and in vitro cell culture studies it can be concluded that at least some type green leafy vegetables and their constituents may have stronger potential in cancer prevention than so far realized. Epidemiological evidence is difficult to obtain due to the complex nature of diet and difficulties to separate different types of green leafy vegetables consumed. Human studies with well-established biomarkers are needed to show the efficacy of certain type of green leafy vegetables or their specific compounds on cancer prevention.

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