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REVIEW ARTICLE

A review of propolis antitumour action *in vivo* and *in vitro*

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Summary

Epidemiologic findings strongly suggest that cancer rates are influenced by environmental factors that can be mitigated to a great extent, for example by a diet rich in polyphenolic/flavonoid compounds. Among natural products, honeybee propolis has been applied for centuries in traditional medicine as well as in diets and supplementary nutrition. Honeybee propolis and its polyphenolic/flavonoid compounds have been known to exhibit biological activity including immunopotentiation, chemopreventive and antitumour effects. In this review we consider the inhibition of tumour growth by honeybee propolis and their polyphenolic/flavonoid compounds as well as the mechanisms involved based on *in vivo* and *in vitro* studies. Results have shown that propolis and its polyphenolic compounds exerted an anti-metastatic and antitumour effect in mice and rats and considerable cytotoxicity without cross-resistance in both wild-type and chemoresistant human tumour cell lines. These findings suggest that propolis and their polyphenolic/flavonoid components may serve as a potent adjunct to chemotherapy and radiotherapy in the treatment of cancers. However, further in-depth studies including clinical trials are needed to fully evaluate the value of flavonoids in combination with chemotherapeutic agents for the treatment of human cancers.

Keywords: propolis, polyphenolic/flavonoid compounds, antitumour mechanisms, eoigenetic and genetic mechanisms, chemotherapy, radiotherapy.

Introduction

There has been a revival of interest in the medical properties of honey bee propolis because of the indications that it exhibits a broad spectrum of activities including antibacterial, antifungal, cytostatic, wound healing, antitumour effects, anti-allergic and anti-inflammatory properties (Oršolić and Bašić 2008a,b,c; Bašić *et al.,* 2008; Oršolić *et al*, 2008b,c; Kosalec *et al.,* 2008).

The target of recent research effort has been the discovery of natural and synthetic compounds that can be used in prevention and/or in treatment of cancer. Many plants have been shown to possess various biological activities such as immunostimulating and antitumour activity. Bee products serve both as nutritious food and as medicinal products in apitherapy. Honeybee products and their flavonoid components are of the most promising antitumour (Oršolić *et al.,* 2003a,b,c; Oršolić *et al.,* 2005a,b,c,d,e; Oršolić *et al.*,2007c; Oršolić and Bašić 2007a; Oršolić *et al.,* 2008a,b), immunomodulatory (Šver *et al.,* 1996; Kimoto *et al.,* 1998; Oršolić and Bašić 2003bc; Oršolić and Bašić 2005b; Oršolić and Bašić 2007a; Oršolić *et al*, 2003a,b; 2004b; Sforcin, 2007) and radioprotective (Oršolić *et al.* 2004c; 2006a; 2007a,b; 2008a; Benković *et al.,* 2008a,b) agents.

The enhancement of host immune response has been recognized as a possible method of inhibiting tumour growth, without harming the host. Natural antioxidants present in food rich with flavonoids, may be responsible for such activity. Flavonoids are known to affect proliferation, differentiation and apoptosis in cancer cells and may play an important role in cancer chemoprevention. Chemoprevention via nontoxic agents could be one approach for decreasing the incidence of cancer and its growth. Chemoprevention of tumour with natural components, including honeybee propolis and their related polyphenolic/flavonoid compounds has recently drawn attention as a promising antitumor approach. Growing *in vitro* and *in vivo* data have shown that chemopreventive agents such as flavonoids enhance the efficacy of chemotherapy and radiotherapy in various cancers through the regulation of Akt, NF-κB, c-Myc, cyclooxygenase (COX)-2, apoptotic, and other pathways, suggesting a novel and multitargeted therapeutic strategy against cancer (Kamsteeg *et al.,* 2003; Banerjee *et al.,* 2005; Li *et al.,* 2005, 2006; Oršolić *et al.,* 2008a,b). This strategy opens a new avenue from cancer prevention to cancer treatment.

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Propolis

Propolis (bee glue) is the generic name for the resinous substance collected by honeybees from the buds of various plant sources and it is used by bees to seal holes in their honeycombs, smooth out the internal walls, and protect the entrance of bee hive against intruders. It is rich in polyphenols, flavonoid aglycones, phenolic acid and their esters, phenolic aldehydes and ketones, terpenes, sterols, vitamins and amino acids, (Marcucci, 1995). The healing properties of propolis have been known in folk medicine in ancient times. More recently, it has been shown that propolis and its constituents have strong antimicrobial effects, acting on viruses, bacteria and fungi (Sforcin, 2007). It was also demonstrated that propolis and some of its active substances have a pronounced cytostatic (Oršolić *et al.,* 2004a; 2006; Bašić *et al.,* 2008) anticarcinogenic and antitumour effect in both *in vitro* and *in vivo* tumour models (Kimoto 1998; Oršolić and Bašić, 2007a; Sforcin, 2007). Honeybee propolis and its components [caffeic acid (CA), caffeic acid phenethyl ester (CAPE), artepilin C, quercetin, naringenin, resveratrol, galangin, genistein, plukenetione A and others] are the most promising of the antitumour agents (Marcucci 1995; Galati *et al.*, 2000; Femia *et al.*, 2001; Lou *et al.*, 2001; Kimoto *et al.*, 1998; Akao *et al.*, 2003; Oršolić *et al.*, 2001; 2003ab; 2004b, 2005b; Díaz-Carballo *et al.* 2008).

Regulation of cell proliferation and death by propolis and its polyphenolic/flavonoid compounds

Anti-proliferative activities of propolis and its polyphenolic/ flavonoid compounds

Dietary flavonoids are known to affect proliferation, differentiation, and apoptosis in cancer cells and may play an important role in cancer chemoprevention, especially for cancers of the gastrointestinal tract, because of their direct contact with food (Kuo, 1996). Direct cytotoxic effect of propolis and polyphenolic/flavonoid compounds was confirmed as an important factor on mammary carcinoma cells (Oršolić *et al.,* 2006b) and primary cultures of human papillary urothelial carcinoma cells (Bašić *et al.*, 2008). We and others have demonstrated that treatment of cancer cells with propolis and its flavonoids inhibited cell proliferation by inducing cytotoxicity and apoptosis (Scheller *et al.,* 1989, Kimoto 1998; Kanno *et al.,* 2006; Oršolić and Bašić, 2007a; Sforcin, 2007; Josipović and Oršolić, 2008). Flavonoids have been shown to inhibit the proliferation of different cancer and leukemic cells *in vitro* (Raynal *et al.,* 2008; Sánchez *et al.,* 2008; Shim *et al.,* 2008; Tolomeo *et al.,* 2008; Lugli *et al.,* 2009). Reynal *et al.,* (2008) showed that genistein induced a time and dose dependent effect on both myeloid and lymphoid leukemic cell lines. Plasma concentrations of geinsten that can be obtained from a soy-enriched diet (1–20 μM) led to a loss of clonogenicity by leukemic cells. In the *in vivo* model, the leukemic mice fed with a 0.5% genistein-enriched diet showed a significant increase in survival time as compared to control mice on a normal diet. The authors suggested that the moderate *in vivo* antileukemic effect of genistein was probably due to its very rapid inactivation (15 min) in mice; genistein did not exceed 1 μM in the blood of the mice as reported by others in mice fed with 0.6% soy extract supplement (Hewitt and Singletary, 2003). Moiseeva *et al.,* (2007) investigated the antitumor activity of curcumin, 3,3′-diindolylmethane (DIM), epigallocatechin gallate (EGCG), genistein, or indole-3-carbinol (I3C) in breast cancer MDA-MB-231 cells, exposed in long-term culture to low concentrations that are achievable *in vivo*. Curcumin and EGCG increased cell doubling time. Curcumin, EGCG, and I3C inhibited clonogenic growth by 55% to 60% and induced 1.5- to 2-fold higher levels of the basal caspase-3/7 activity. Unfortunately, no anticancer effects of genistein were found in this study. Similarly, animal studies did not show any inhibition of tumour growth in the MDA-MB-231 cell xenograft by genistein in serum concentration ~1 mM (Santell *et al.,* 2000). This correlates with the absence of a protective effect of genistein in oestrogen receptor-α–negative tumours (Linseisen *et al.,* 2004).

The mechanisms involved in inhibition of cell proliferation may be: (i) inhibition the activity of tyrosine-specific protein kinase (Williams *et al.*, 2004; Sánchez *et al.,* 2008) such as inhibition of protein kinase C, an enzyme with a key role in the regulation of cellular proliferation and tumour growth; (ii) reduction in cell proliferation and potently-induced differentiation and apoptosis in carcinoma cells (Csokay *et al.*, 1997; Hail and Lotan, 2009); (iii) changes in the mRNA levels of cell cycle- and apoptosis-related genes including nuclear transcription factor-κB (NF-κB), bcl-X(L) and COX-2; (iv) binding to the oestrogen receptor; (v) induction of apoptosis in tumour cells, (vi) downregulation of expression of mutated H-ras and p53 oncogene (Avila *et al.* 1996), (vii) modulation of gene methylation and reexpression of tumour suppressor or other genes, which were silenced by aberrant DNA methylation (Fang *et al.* 2005; Wang *et al.* 2007; Raynal *et al.* 2008), (viii) inhibition of pro-oxidative enzymes xanthine oxidase, cyclooxygenase (COX) or lipooxygenase (LOX) (Williams *et al.,* 2004, Ramos 2007, Lev-ari *et al* 2006).

Flavonoids exhibit a range of activities which modulate signal transduction. These include the downregulation of growth factors (EGF and VEGF), alterations to survival signaling (ERK, JNK, AP-1, and NF-κB), cell cycle regulators (cyclinD1, cdk4/6, p21, and p53) and

apoptosis regulators (PARP, ceramide, and caspases (Davis and Milner, 2007; Oršolić and Bašić, 2007a; Howells *et al.,* 2007).

Cell cycle progression is a sequential process that directs dividing mammalian cells through G1, S, G2 and M phases. Transitions between G1-S or G2-M phases function as checkpoints to halt cell division if necessary. Because the balance of interactions among cyclins, cyclin-dependent kinases (CDK) and CDK inhibitors (CDI) governs the progression of the cell cycle (Weinstein, 2000), perturbation of any of the cell-cycle-specific proteins by dietary components can potentially affect and block the continuous proliferation of neoplastic cells and may serve as effect biomarkers. Dietary components that modulate cell proliferation include phenolic compounds, such as genistein, quercetin, kaempferol, myricetin, luteolin, chrysin and apigenin, and epigallocat-echin-3-gallate, which elicit cell-cycle arrest through the induction of CDI (p21 and p27) and the inhibition of CDK4, CDK2, cyclin D1 and cyclin E (Agarwal, 2000, Davis and Milner, 2007; Zang *et al.* 2009). Isothiocyanates can also induce p21 expression and inhibit cell proliferation at the G2-M checkpoint (Visanji *et al.,* 2004). Allyl sulfur compounds from foods have been reported to block the cell cycle in the G2/M phase presumably by inhibiting p34 (cdc2) kinase activity through changes in cyclin complex formation and hyperphosphorylation (Knowles and Milner, 2000). In a recent study (Zang *et al.*, 2008), it has been shown that flavones (luteolin, chrysin and apigenin) and flavonols (quercetin, kaempferol and myricetin) can induce cytotoxicity to OE33 cells (a human oesophageal adenocarcinoma cell line) by causing G_2/M arrest and inducing apoptosis.

Propolis, flavonoid galangin and other flavonoids derived from propolis are a potent COX-2 inhibitor among several natural products tested (Kang *et al.*, 2000; Shimoi *et al.*, 2000; Raso *et al.,* 2001; Hu *et al.,* 2005). Flavonoids and caffeic acid analogues are particularly effective at inhibiting the pro-oxidant enzymes xanthine oxidase (Chang *et al.,* 1993; Chan *et al.,* 1995), COX or LOX (Markovits *et al.* 1989). There is considerable evidence suggesting that angiogenesis and chronic inflammation are co-dependent. Blockage of angiogenesis results in an anti-inflammatory effect. Ethanolic extract of propolis (EEP), components of propolis as CAPE, quercetin, reservatrol, and genistein have shown an anti-inflammatory and anti-angiogenic activities in urothelial cancer (Hayashi *et al.,* 2000; Yang *et al.,* 2003; Liu *et al.*, 2003; Mousa *et al.,* 2005; Su *et al.*, 2005).

Some flavonoids such as galangin, genistein baicalein, hesperetin, naringenin and quercetin apparently exerted their anti-proliferative activity on the proliferation of an oestrogen receptor-positive human breast cancer cell line, MCF-7 (King-Batoon *et al.,* 2008; Qin *et al.,* 2009). In breast carcinoma cells, antiproliferative effects of genistein were shown to be mediated through the formation of an intracellular metabolite that inhibits cell cycle progression. In addition, genistein produces pleiotropic effects against cancer cells that can influence cell proliferation, cell cycle progression and apoptosis.

Induction of cell death by propolis and its polyphenolic/ flavonoid compounds

In general, the growth rate of pre-neoplastic or neoplastic cells outpaces that of normal cells because of malfunctioning or dysregulation of their cell-growth and cell-death machineries (Jacks and Weinberg, 2002). Apoptosis is one of the most potent defences against cancer because this process eliminates potentially deleterious, mutated cells. Many dietary cancer preventive compounds, including selenium, epigallocatechin-3-gallate, phenylethyl isothiocyanate, retinoic acid, sulforaphane, curcumin, apigenin, quercetin and resveratrol, induce apoptosis (Hu and Kong, 2004; Martin, 2006). Moreover, the experimental data indicate the existence of a direct relationship between the amount of glutathione in the tumour cells, cytotoxicity and apoptosis (Hail, 2005; Hail and Lotan, 2009.

Results of studies (Ramos 2007; Josipović and Oršolić, 2008) showed that cytotoxicity of flavonoids depends on their chemical nature, concentration and the type of leukemic or cancer cells in culture. These data point out that concentration of flavonoids is a key factor that induces proliferation and/or cell death by apoptosis and/or necrosis. It seems that the crucial parameter in the activation of the proliferation and/or apoptotic pathway is the amount of free radicals. Ramos (2007) considers that low quercetin concentrations can activate MAPK (mitogen-activated protein kinase) pathway that causes the expression of survival gene (c-fos and c-jun) and defence genes (glutathion-s-transferase) that start the survival and defence mechanism. On the contrary, high concentrations of quercetin activate the caspase pathway which leads directly into apoptosis. On the other hand, flavonoids can interfere with the metabolism of cells and induce a different effect in the same conditions. Ferraresi *et al.*, (2005) have presented interesting findings suggesting that short-term treatment with quercetin was anti-oxidative and anti-apoptotic, whilst long-term treatment resulted in pro-oxidative and pro-apoptotic effects. Intriguingly, apoptosis was correlated with decreased levels of reduced glutathione (GSH), suggesting that the presence or absence of GSH is one of the factors determining the pro- or anti-oxidative nature of this flavonoid. Therefore, the effects of quercetin on apoptosis depend on experimental conditions, including its concentration in the cell culture medium (Robaszkiewicz, *et al.* 2007). It is interesting that flavonoids cause apoptosis in transformed cells but not in normal cells; sensitivity of tumour cells to anti-tumour substances is due to their lack of capability to synthesize glutathione as an answer to oxidative stress (Lee *et al* 1989; Meister 1994). An ever-growing list of putative cancer chemopreventive agents, including many that are derived from dietary constituents (e.g., polyphenols, vanilloids, isothiocyanates, and selenium) and their synthetic derivatives, has been shown to trigger apoptosis in tumour cells *in vivo* and/or *in vitro*, which implies that the reinforcement of

cell death in transformed cells is coupled with the anticancer effects of these agents. Moreover, the vast majority of these agents initiate mitochondria-mediated apoptosis via their pro-oxidant effects on transformed cells. The resulting reactive oxygen species (ROS)-induced mitochondrial membrane permeabilization (MMP, e.g., the loss of mitochondrial inner transmembrane potential, cytochrome c release from the mitochondria, and/or mitochondrial swelling) occurs predominantely via the induction of the mitochondrial permeability transition (MPT) and/or the possible redox-regulated activity of pro-apoptotic Bcl-2 family members (i.e., Bax and Bak) (reviewed in Hail, 2005; Hail and Lotan, 2009). Thus, potential "effect" biomarkers for the cytotoxic effect of dietary components on cells may be assessed by measuring their influence on mitochondrial caspases and other apoptosis-related proteins.

Modulation of gene methylation by polyphenols

Several natural dietary and nutritional compounds have been reported to alter the epigenetic and subsequent gene expression status of tumour suppressors or other cancer relevant genes. It is known that specific enzymes and methylated DNA binding proteins play a major role in causing reduced expression of tumor suppressor genes, resulting in tumour formation and its progression. Genes involving cell cycle regulation, DNA repair, angiogenesis and apoptosis are all inactivated by the hypermethylation of their respective 5'CpG islands. Key regulatory genes including E-cadherin, pi-class glutathione S-transferase, the tumour suppressors cyclin-dependent kinases (CDKN2) phosphatase gene (PTEN) and insulin-like growth factor (IGF-II) targeted histone acetylation and deacetylation are influenced by DNA hypermethylation. Although folate intake is recognized to influence DNA methylation patterns, other nutrients, such as selenium, can also have an impact (Davis and Uthus, 2003). One approach to inhibiting the inactivation of tumour suppressor genes is to use chemical (5-azacytidine) or natural agents (for example, genistein, several isothiocyanates, cathechin, epigallocatechin-3-gallate, anthocyanins, lycopen, 3,3'-diindolylmethane, indole-3-carbinol) to prevent the hypermethylation of DNA. Accoprding data Reynal *et al.* (2008) and Fang *et al.* (2005) genistein could reactivated the expression of several tumour suppressor genes, which were silenced by aberrant DNA methylation. So, genistein (1–20 μM) induced a re-expression of two silenced tumour suppressor genes, $p57^{KIP2}$ in a human cell lines (Hl-60, MOLT-r, Raji and KG1a) and $p15^{CDKN2B}$ in a mouse cell line (L1210). The variable cDNA amplification of these genes after genistein exposure may have been due to the pleiotropic action of this isoflavone on various targets. At the higher concentrations of genistein, the expression appeared to decrease, which was probably due to the perturbation of cellular function. The reactivation of tumour suppressor genes by genistein and its demethylation effect on one of these genes suggest that part of the antileukemic activity of this agent is related to its epigenetic action.

Interestingly, demethylating activities have been reported for certain tea catechins and soybean isoflavones in breast and oesophageal squamous cell lines (Fang *et al.,* 2003; 2005). For example, the tea catechin epigallocatechin gallate (EGCG) has been shown to directly inhibit DNA methyltransferase DNMT1 and reverses the hypermethylation status of p16 in human esophageal carcinoma KYSE 510 cells (Fang *et al.*, 2007). In MCF-7 and MDA-MB-231 breast cancer cells, re-expression of the RARb gene by tea catechin was observed to be accompanied by reduced methylation of the promoter of that gene (Lee *et al.*, 2005). Sulforaphane, an isothiocyanate in broccoli, is an epigenetic modifier that inhibits histone deacetylase (HDAC) resulting in increased p21 expression with coordinate changes in histone H3 and H4 acetylation in HCT116 human HNPCC colon cancer cells and in prostate epithelial cell lines, BPH-1, LnCaP, and PC-3 (Myzak *et al.*, 2006; 2007). Genistein has also been observed to modulate DNA methylation patterns *in vivo* and *in vitro*. For example, genistein alters DNA methylation patterns in anonymous novel CpG islands in mouse prostate DNA (Day *et al.*, 2002), and when given in the diet has transgenerational epi-genetic effects on coat colour in Agouti mice *in vivo* (Dolinoy *et al.*, 2006). Genistein (2–20 μM, 48 hr resupplementation for 1 week) can specifically demethylate the p16, RARb, and MGMT genes in human oesophageal carcinoma KYSE 510 cells and in human LNCaP and PC3 prostate tumour cells (Fang *et al.*, 2007). Similarly, King-Batoon *et al.* (2008) showed that repeated dosing with 3.125 μM genistein partially demethylates the promoter of the glutathione S-transferase gene (GSTP1) and increases its expression in MDA-MB-468 breast cancer cells.

Qin *et al.* (2009) demonstrated that the use of 40-120 mg of soy isoflavones daily through one menstrual cycle have an antioestrogenic effect and alters mammary promoter hypermethylation in healthy premenopausal women. Isoflavones induced dose-specific changes in RARbeta2 and CCND2 gene methylation, which correlated with genistein levels while Fini *et al.* (2007) showed that apple polyphenols (APE) have potent demethylating activity through the inhibition of DNA methyltransferase in colorectal cancers. APE treatment strongly reduced DNA methylation in the promoters of hMLH1, $p14^{ARF}$, and $p16^{INK4a}$ with consequent restoration of normal expression.

According to Wang *et al.* (2007) phenethyl isothiocyanate (PEITC) may be a mediator in the intersection between the DNA and chromatin in demethylating and reactivating GSTP1 genes, which are critically inactivated in prostate carcinogenesis. Prostate carcinoma is characterized by the silencing of pi-class glutathione S-transferase gene, which encodes a detoxifying enzyme. Exposure of prostate cancer LNCaP cells to PEITC inhibited the activity and level of histone deacetylases (HDACs), and induced selective histone acetylation and methylation for chromatin unfolding. Concurrently PEITC demethylated the promoter and restored the unmethylated GSTP1 in both androgen-dependent and -independent LNCaP cancer cells to the

level found in normal prostatic cells. The dual action of PEITC on both the DNA and chromatin was more effective than 5'-Aza-2'-deoxycytidine, sodium butyrate, or trichostatin A, and may derepress the methyl-binding domain (MBD) on gene transcription.

Lycopene (10 μM, 48 hr), an antioxidant carotenoid found in tomatoes and other red fruits, can modulate the expression of numerous genes relevant to cell cycle control, DNA repair, and apoptosis in MCF-7 and MDA-MB-231 breast cancer cells and in MCF10A cells. King Batoon *et al.* (2008) demonstrated that dietary relevant, low doses of lycopene (2 μM) can alter DNA methylation of the GSTP1 gene in MDA-MB-468 breast cancer cells, and that this change is coordinated with increased gene expression as suggested by RT-PCR. Some authors showed that the RARb2 and HIN-1 genes that are methylated in immortalized but nontumorogenic MCF10A breast cells can be partially demethylated by lycopene, and to a lesser extent by genistein.

Propolis and its polyphenolic / flavonoid compounds; immunomodulation and antitumour activity *in vivo*

Antitumoural activity of propolis and its polyphenolic / flavonoid compounds

Propolis and flavonoids have been found to inhibit the development of chemically-induced cancers in animal models of lung, oral, oesophageal, stomach, colon, skin, prostate and mammary (breast) cancer (see review Galati *et al.,* 2000; Oršolić and Bašić, 2007; Oršolić *et al.,* 2008b,c). It was shown that experimental animals treated with the immunostimulants resisted, in various degrees, subsequent inoculation of tumour cells as evidenced by the reduced "tumour take", slowed growth of the tumour, and prolonged survival of recipients (Scheller *et al.,* 1989; Matsuno 1995; Kimoto *et al.,* 1998, Hayashi *et al.,* 2000). Scheler *et al.* (1989) reported that the ethanolic extract of propolis was capable of increasing survival of mice-bearing Ehrlich carcinoma and suggested that immunostimulatory activity of propolis may be associated with macrophage activation and enhancement of their phagocytic activity. Matsuno (1995) reported that various components of propolis possessed potent anti-inflammatory and antitumour activity. In addition Hayashi *et al.* (2000) showed that quercetin chalcone and modified citrus pectin reduced the growth of solid colon-25 primary tumour when given to mice. The oral administration of proanthocyanidin, compound from grapes, has also been found to decrease the tumour progression and the size of cutaneous carcinomas in an animal study (Mittal *et al.,* 2003). Another murine study showed that the administration of grape seed extract significantly reduced metastatic melanoma pulmonary nodules (Martinez *et al.,* 2005).

Green tea polyphenols inhibited the growth of 4T1 breast cancer cells and their metastasis to lungs in BALB/c mice. A reduction in tumour weight, increased survival time, and later tumour appearance were observed. The ratio of Bax/Bcl2 was altered in favour of apoptosis, along with a decrease in proliferating cell nuclear antigen and the activation of caspase-3 (Baliga *et al.,* 2005). Moreover, the topical application of EGCG to SKH-1 hairless mice that had been pretreated twice weekly with UVB light decreased the multiplicity of skin tumours by 44% -72% and increased the apoptotic index by 56% - 92%, again measured by increased caspase-3 activity (Lu *et al.,* 2002).

In a rat model, dietary curcumin significantly increased the apoptotic index in azoxymethane-induced colonic tumors (Samaha *et al.,* 1997). Rao *et al.* (1995) demonstrated the effect of a curcumin containing diet on azoxymethane-induced rat carcinogenesis. Curcumin significantly reduced tumour volume, as well as colonic mucosa and tumour prostaglandin $(PGE)_2$ expression by over 38%. In rats, a gavage administration of curcumin (200 or 600 mg kg^{-1}) inhibited diethylnitrosamine (DEN)-induced hepatic hyperplasia and inflammation. Specifically, the increased expression of p21ras and p53 in the liver was prevented. The decreased expression of proliferating cell nuclear antigen, cyclin E, and cdc2 was also observed, along with the inhibition of DEN-induced NF-κB activation (Chuang *et al.,* 2000).
In rats, azoxymethane treatment caused the formation of aberrant crypt foci, the number of which was significantly reduced in the presence of resveratrol (200 μg/kg per day for 100 d), with decreased bax and increased p21 expression in the crypts (Tessitore *et al.,* 2000). The treatment of dimethylhydrazine-induced aberrant crypt foci with resveratrol (8 mg / kg per day) resulted in a marked reduction in tumour incidence and degree of histological lesions (Sengottuvelan *et al.,* 2006) as well as rats fed a diet containing 15% grape extract (Kweon e*t al*., 2003). MDA-MB231 xenografts in nude mice exhibited an increase in the apoptotic index and decreased angiogenesis when treated daily with 25 mg / kg resveratrol for 3 weeks (Garvin *et al.,* 2006). Conversely, when B16M tumor cells were inoculated into mice, 20 mg / kg resveratrol did not affect tumor growth (Asensi *et al.,* 2002).

Kimoto *et al.* (1998) reported that artepilin C (a component of propolis) possessed cytostatic and cytotoxic effect onto various malignant tumour cells *in vitro* and *in vivo* and that it activated the immune system, especially increasing the number of macrophages and their phagocytic activity, causing lymphocytosis in peripheral blood and exerting direct antitumour activity. We demonstrated that macrophage activation by propolis and related polyphenolic compounds was likely to be the most important effector mechanism of antitumour activity of test compounds *in vivo* (Oršolić and Bašić, 2003b,c; Oršolić *et al.,* 2005a,b,e; 2006b). These findings suggested that propolis and some of its components given intraperitoneally (*ip*) or per orally (*po)* at doses of 50 or 150 mg / kg stimulated

macrophages and reduced the number of mammary carcinoma (MCa) metastases in CBA mouse (Oršolić *et al.,* 2003a,b; Oršolić and Bašić 2005a). Results also demonstrated a reduction of tumour volume and increase in life span (ILS) for 14.89 to 40.76% when test compounds (50 or 150 mg / kg) were given before tumour cell inoculation (Oršolić *et al.,* 2005d).

Preventive treatment with WSDP given *po* at doses of 50 or 150 mg / kg or with caffeic acid (CA) was very effective in inhibiting tumour growth; antitumour activity was mediated by stimulated macrophages acting directly on tumour cells. Indirect antitumour activity is likely to be mediated by the products from stimulated macrophages such as NO (Oršolić *et al.,* 2006b) as was also described by others (Elgert *et al* 1998; Orsi *et al.,* 2000).

To test both direct and indirect effects on tumour growth, we injected WSDP or CA or caffeic acid phenethyl ester (CAPE), subcutaneously (*sc*) at doses of 50 or 150 mg / kg and immediately thereafter at the spot of their introduction inoculated tumour cells. Results (Oršolić *et al.,* 2005c) indicated that the presence of CA or CAPE in the tissue of tumour cells inhibited tumour growth and increased life span (ILS) of treated animals (29.30% to 51.74%), while WSDP was less effective (Oršolić *et al.,* 2005d). These findings suggested that CA or CAPE suppressed mammary carcinoma growth via other mechanism(s) different from those mediated by WSDP. These include the ability of CA and CAPE to inhibit DNA synthesis in tumour cell cultures (Oršolić *et al* 2004b), and their capability to induce apoptosis of tumour cells (Oršolić *et al.,* 2003b; 2004a). Furthermore, the ability of CA and CAPE to induce apoptosis suggested their potential use in preclinical and clinical trials as anticancer therapeutic agents.

We showed that WSDP and/or its related polyphenolic (caffeic acid, naringenin, quercetin, chrysin) compounds (50 or 150 mg / kg *po* or *ip* during 7 constitutive days) were effective at reducing tumour volume as measured by the total number of cells in peritoneal cavity in mice-bearing Ehrlich ascites tumour (EAT) (Oršolić *et al.,* 2005a). The analysis of cells present in peritoneal cavity of mice revealed that all experimental groups inoculated with tumour cells in the presence of WSDP or related polyphenolic components, exhibited a significant reduction of tumour cells. The survival rates of EAT-bearing mice were increased after treatment with test components (Oršolić and Bašić 2005a). These findings suggested that test components might have interfered with the growth of Ehrlich ascites tumour cells directly during the early phase of treatment leading to a considerable elimination of these cells, as showed in studies *in vitro* (Oršolić *et al.* 2005a).

Another possible antitumour mechanism of WSDP and related polyphenolic compounds may involve macrophage activation in conjunction with the production of many different soluble factors by them which may exert direct or indirect effect on tumour cells (Kimoto *et al.,* 1998; Oršolić *et al.,* 2006b,c; Bašić *et al.,* 2008).

***Macrophage activation by propolis and related polyphenolic compounds is an important effector mechanism of antitumour activity* in vivo**

A malignant tumour often causes both the decrease of immune function and the abnormality of peripheral blood, and a leukemic reaction is even observed in tumour patients during their later stages of disease. In fact, the abnormal increase of leukocyte in tumour patients is usually due to an increase of neutrophils (Satomi *et al.,* 1995). The immune system plays an important role in maintaining body homeostasis by eliminating endogenously formed mutated cells such as virus-infected or tumour cells as well as exogenous invasion by microbial organisms. Immunomodulation through natural or synthetic substances may be considered an alternative for the prevention and cure of infectious diseases (Azuma and Jolles, 1987) and of neoplastic diseases (Oršolić and Bašić, 2000; 2003a,b,c; 2005a,b; 2006c; 2007a; Oršolić *et al.,* 2001a,b; 2003a; 2004b; 2005ac,d,e).

Immunomodulatory effects of propolis are well known (Dimov *et al.,* 1992; Kimoto *et al.,* 1998; Oršolić and Bašić 2003b,c; Oršolić *et al.,* 2005 a,b,e). Studies done by us (Oršolić and Bašić 2003b,c; Oršolić *et al.,* 2005 a,b,e) and others (Dimov *et al.,* 1992; Kimoto *et al.,* 1998; Sforcin 2007) suggested that water-soluble derivative of propolis (WSDP) stimulated macrophages and influenced specific and non-specific immune mechanism(s), through the release of migration inhibitory factor, macrophage phagocytosis, elevation of the number of rosette-forming and antibody producing cells, respectively. Activated macrophages were shown to be a major component of host defence against neoplastic growth in experimental tumour systems (Oršolić and Bašić 2000; Oršolić and Bašić 2003b,c). It was demonstrated that increased levels of IL-1 and TNF (Dimov *et al.,* 1992) produced by activated macrophages correlated directly with two other criteria for macrophage activation: enhanced *in vitro* responsiveness to chemotactic stimuli and macrophage-induced tumour cytotoxicity (Oršolić and Bašić 2003b; 2007a; Oršolić *et al.,* 2006b). Among the best characterized lytic factors produced by activated macrophages are hydrogen peroxide (H_2O_2) and other reactive oxygen intermediates, TNF-a, nitric oxide (NO) and reactive nitrogen intermediates (Oršolić and Bašić 2003b,c; 2006b; 2007a). Macrophages were capable of destroying microorganisms and tumour cells by their products such as H_2O_2 including oxygen radicals and NO. Orsi *et al.* (2000), carried out a study to evaluate macrophage activation and oxygen intermediate metabolites concentrations after administered 10% hydro alcoholic solution to mice. It was observed hat propolis (5, 10, and 20 µg / mL) induced an increase in H_2O_2 production but did not induce significant alternations in the production of NO.

The spreading ability of macrophages is an important marker of their activation, and it was shown that its capacity was associated with an increase in adherence and phagocytic activities of these cells.

Results of our studies on macrophage spreading revealed that the treatment with WSDP or its polyphenolic components significantly increased the spreading ability of cells compared to untreated control (Oršolić and Bašić, 2005b). The findings implied an enhanced ability of macrophages to phagocyte; thus, the increase of macrophage spreading might have been responsible for the slower growth of tumour cells. It is well known that mononuclear cells (MN), mainly macrophages, are the major cells involved in tumour destruction (Kimoto 1998; Oršolić and Bašić 2003b,c; 2005b; Sforcin 2007). Since in comparison with other treatments the strongest antitumour effect was achieved by WSDP treatment, it is likely that the antitumour activity of WSDP was the consequence of synergistic activities of polyphenolic compounds present in WSDP. Stimulation of macrophages might induce production and release of several cytokines such as IL-1, IL-6, IL-8, TNF-a (Dimov *et al.,* 1992; Oršolić and Bašić 2003b) and NO (Oršolić and Bašić 2003b,c; Oršolić *et al.,* 2006b). Some of these cytokines may express direct cytotoxic effects on tumour cells while others act on NK cells and cytotoxic T lymphocytes stimulating their activities (Oršolić *et al.,* 2005e). Findings (Oršolić and Bašić, 2003b,c) suggested that WSDP possesses the property to activate macrophage to produce factors capable to regulate the function of B- and T-cells, respectively. The elevation of both $CD4^+$ and $CD8^+$ T-cell subsets in tumour-bearing mice after treatment with WSDP showed a dose-dependent effect of WSDP that leads to progressive reduction of the $CD4^+/CD8^+$ ratio in favour of $CD8^+$ cells. It thus appears that, the antimetastatic activity of WSDP was, at least in part, the consequence of immunomodulation of the host's immune system. These results are consistent with the observations by Kimoto *et al.* (1998) who reported that the artepilin C from Brazilian propolis suppressed tumour growth after intratumour injection of 500 mg; in contrast results of these studies showed that increased ratio of CD4/CD8 was in the favour of CD4 cells. In addition, these cytokines might stimulate production of antibody, C-reactive protein and complement factor C3 that could act as opsonin to tumour cells (Oršolić and Bašić 2005b; 2007a; Oršolić *et al.,* 2005e) and as such activate antibody-dependent cellular toxicity (ADCC). Combination of these effects might impede tumour growth and lead to elimination of tumour cells.

Effect of CAPE on the immune system in Balb/c mice, after oral application for 14 days, was evaluated by assessment of body and organ weight, lymphocyte blastogenesis, plaque-forming cells assay, lymphocyte subpopulation by flow cytometry and cytokine production (Park *et al.* 2004). The change of body weight was not observed in the CAPE-treated group, thymus weight and/or cellularity of thymus and spleen were decreased, while CAPE had no effect on B lymphocyte proliferation induced by lipopolysacharide (LPS) but increased T lymphocyte blastogenesis induced by concanavalin A (Con A). Moreover, $CD4^+$ T cells subset was significantly increased after exposure to CAPE as well as the antibody responses to T lymphocyte dependent antigen, sheep red blood cell and keyhole limpet hemocyanin (KLH). Likewise, the cytokine, IL-2, IL-4 and INF-γ were significantly increased in CAPE-treated mice.

Guo *et al.* (2001) provided evidence that genistein could modulate the immune system in adult B6C3F1 mice. Consistent with the chemopreventive effect of genistein, exposure to this compound significantly increased host resistance to B16F10 tumor as reflected by decrease in the number of lung tumour nodules after tumour injection. Inhibition of B16F10 tumour might be related to the increase in the activities of cytotoxic T cells and NK cells. Furthermore, *in vitro* interleukin IL-2-stimulated NK cell activity was significantly enhanced in the high (20 mg / kg) genistein dose group. Exposure to genistein did not alter the activity of the mononuclear phagocytic system and the cytotoxyc/cytostatic function of thioglycollate-recruited peritonel cells on B16F10 tumor cells as well as genistein did not produce changes in spleen immunoglobulin IgM and IgG antibody-forming cells responses.

According to mentioned data, it is likely that propolis and its polyphenolic compounds can trigger various host defence mechanisms. Destruction of tumour cells by the polyphenolic components may be the result of one or more mechanisms described above. Immunomodulatory mechanisms of propolis and flavonoids was described in detail in review papers (Oršolić and Bašić, 2007; Sforcin, 2007; Fischer and Vidor, 2008).

Summary of antitumour mechanisms of propolis and its polyphenolic / flavonoid compounds

Collectively, overwhelming evidence demonstrates that a variety of polyphenolic/ flavonoid compounds can influence a number of key intracellular targets that are associated with the cancer process. A fundamental action of several bioactive food components is that they serve as regulators of gene expression and/or modulate gene products. It has been suggested that the therapeutic activities of propolis depend mainly on the presence of flavonoids (Oršolić and Bašić 2007a; Oršolić *et al.,* 2005a,c; 2007c). Flavonoids have also been reported to induce the immune system (Oršolić and Bašić, 2003b; Oršolić *et al.,* 2005b,e), and to act as strong oxygen radical scavengers (Oršolić and Bašić 2005a; Oršolić *et al.,* 2007a,b, 2008a,b; Benković *et al.,* 2007; Kosalec *et al.,* 2008). Dietary intake of antioxidants has been associated with a diminished risk of cancer at various anatomical sites (Oršolić *et al.,* 2005a,c,d; 2006b; Oršolić and Bašić 2007a).

According to our results, immunomodulation with propolis and its polyphenolic/flavonoid components is an important factor in antitumour and antimetastatic activities. Preventing the expansion of new blood vessel networks results in reduced tumour size and metastasis and is another mechanism whereby dietary components

inhibit tumour growth. Dietary components that inhibit angiogenesis include polyunsaturated fatty acids (Rose and Connolly, 2000) flaxseed, (Bergman Jungestrom *et al.,* 2007) and polyphenols such as epigallocate-chin-3-gallate, resveratrol, curcumin and genistein (Cao *et al.,* 2002; Dulak *et al.,* 2005; Oak *et al.,* 2005). Additionally, flavonoids can delay or block the development of cancer cells *in vitro* and *in vivo* by protection from carcinogenic stimuli, by inhibition of tumour cell proliferation, by induction of cell cycle arrest and by induction of apoptosis via intrinsic and extrinsic signaling pathways. With respect to its relationship with molecular targets relevant to cancer prevention, quercetin aglycone has been shown to interact with some receptors, particularly an aryl hydrocarbon receptor, which is involved in the development of cancers induced by certain chemicals (Murakami *et al*. 2008). Murakami *et al*. (2008) suggest multitarget cancer prevention by quercetin. Some of the target sites and mechanisms of the possible effects of propolis and flavonoids on tumours and other diseases have been reviewed (Oršolić and Bašić, 2007a).

Flavonoids exhibit powerful antioxidant activities *in vitro*, being able to scavenge a wide range of reactive oxygen, nitrogen, and chlorine species, such as superoxide, hydroxyl radical, peroxyl radicals, hypochlorous acid, and peroxynitrous acid. They can also chelate metal ions, often decreasing their pro-oxidant activity (Paya *et al.,* 1992; Pannala *et al.,* 1997; Boersma *et al.,* 1999; Halliwell, 2000; Ketsawatsakul *et al.,* 2000; Silva *et al*., 2002; Mira *et al.,* 2002). First, flavonoids as effective scavengers of free radicals may decrease the levels of oxidative DNA damage *in vivo*. Phenols might exert direct antioxidative effects within the gastrointestinal tract; these effects could include binding of pro-oxidant iron, scavenging of reactive nitrogen, chlorine, and oxygen species, and perhaps inhibition of cyclooxygenases and lipoxygenases. Second, flavonoids and other phenols are complex molecules and are likely to have multiple potential biological activities, such as inhibiting telomerase (Naasani *et al.,* 2003), affecting signal transduction pathways (Wiseman *et al*., 2001; Rosenkranz *et al.,* 2002; Levites *et al.,* 2002), inhibiting COX and LOX (Laughton *et al.,* 1991; Schewe *et al*., 2001; Sadik *et al.,* 2003), decreasing xanthine oxidase (Van Hoorn *et al.,* 2002), matrix metalloproteinase (Isemura *et al*., 1999), angiotensin-converting enzyme (Actis-Goretta *et al*., 2003), sulfotransferase (Marchetti *et al.,* 2001) activities and interacting with sirtuins (Howitz *et al.,* 2003). Flavonoids may also interact with cellular drug transport systems (Boumendjel *et al*., 2002), compete with glucose for transmembrane transport (Vera *et al.,* 1996), interfere with cyclin-dependent regulation of the cell cycle (Gupta *et al*., 2002) and affect platelet function (Murphy *et al*., 2003).

Third, isoflavones and lignans are phytooestrogens and have been demonstrated to modulate hormone-dependent carcinogenesis in animals. Fourth, flavonoids are essentially xenobiotics, as indicated by their patterns of metabolism, and cytotoxic effects have been observed *in vitro* and *in vivo* (Laughton *et al*., 1989; Skibola *et al*., 2000; Strick *et al.,* 2000, Awad *et al.,* 2001; Sakamoto *et al.,* 2001). Modulation of different oncogenes, tumour suppressor genes, and signal transduction pathways, leading to inhibition of cell proliferation, transformation, and angiogenesis as well as to the induction of apoptosis, has been proposed by many investigators as mechanisms for the chemopreventive activities of many polyphenolic compounds.

Benefits of the use of propolis and related polyphenols against the toxicity of chemotherapeutic agents

Propropolis and its polyphenolic/flavonoid compounds enhance anti-cancer effect of chemotherapeutics and reduce their toxicity to healthy cells

It is known that standard cancer therapy produces acute toxicity during treatment and that toxicity can be severe enough to cause the discontinuation of therapeutic agents. Agents which could reduce the toxicity of standard therapy on normal cells and/or which could increase the response of tumour cells to standard therapy, could markedly improve the current management of human cancer. Moreover, maintaining the peripheral blood of normal tumour patients is an essential condition to all kinds of treatment.

We showed that WSDP preparation from natural propolis was able to prevent proliferation of tumour cells and metastasis formation in the lung (Oršolić and Bašić 2005a). WSDP expressed a strong antimetastatic effect in mice treated either preventively or curatively (Oršolić and Bašić 2005a). The combined treatment with WSDP and epirubicin profoundly inhibited metastasis formation (Oršolić and Bašić 2005a). This synergistic effect was maximised when epirubicin and WSDP were administrated after tumour cell inoculation. It is likely that the enhanced antitumour effect of epirubicin is elevated by the flavonoids present in WSDP, which through their ability to inhibit different kinases and topoisomerase II activities, reduced the growth of tumour cells (Galati *et al.,* 2000). Similar inhibitory effect of flavonoids and cisplatin on melanoma cell growth *in vivo* was reported by Caltagirone *et al.,* (2000) and Suzuki *et al.,* (2002), who demonstrated that oral administration of crude water-soluble propolis (CWSP) concurrently with 5-fluorouracil (5-FU) or mitomicin (MMC), significantly increased tumour regression, as compared with the respective chemotherapy alone, illustrating the adjuvant effect of orally administered CWSP for tumour regression when combined with chemotherapeutic agents. In addition, the same authors showed that orally administered CWSP significantly reduced the cytopenia induced by 5-FU or MMC, resulting in recovery of white as well as red blood cell counts. Our results are in consistent with the data mentioned above; WSDP prevented the epirubicininduced hematological toxicity in mice bearing metastases of the mammary carcinoma. In mice treated with WSDP and epirubicin the number of leukocytes and

erythrocytes were elevated, as compared to untreated and mice treated with epirubicin alone. Collectively, these results suggest that the antimetastatic effect of WSDP reflects its multiple mechanisms of action that include antioxidant potential, immunomodulation and inhibition of CYP1A2 enzyme activity (Oršolić and Bašić 2007a). These results are in line with previous results from this laboratory and others (Oršolić and Bašić 2005a; 2007a; Oršolić *et al.,* 2008c; Suzuki *et al.,* 2002), suggesting that flavonoids from WSDP possess a hemostimulative, antioxidative, protective and regenerative properties.

Flavonoids have biochemical and pharmacological activities beneficial to human health, including antioxidant, anticarcinogenic, anti-inflammatory, antiproliferative, anti-angiogenic, and antioestrogenic, oestrogenic effects (see review Oršolić and Bašić 2007a, Sforcin *et al.,* 2007), and that their ingestion produces no or very little toxicity (Oršolić and Bašić 2007a; 2008a,b Oršolić *et al.,* 2008a,b,c). A few *in vivo* studies support the concept that antioxidants selectively enhance the effect of standard therapy on tumour cells by increasing tumour response. The purpose of recent studies (Oršolić *et al.,* 2008a,b,c) was to analyze the possible use of propolis and related flavonoids as an adjunct to standard cancer therapy. Another part of our proposed hypothesis was that propolis and its flavonoids compounds in combination with standard tumour therapeutic agents may reduce the toxicity of chemotherapeutic agent (irinotecan, cisplatin, doxorubicin) on normal cells. Several studies using animal models also support this part of the hypothesis (Scheller *et al.,* 1989; Dimov *et al.,* 1992; Kimoto *et al.,* 1998; Caltagirone *et al.,* 2002; Suzuki *et al.,* 2002; Oršolić and Bašić 2007a).

Experimental studies and clinical trials have demonstrated the beneficial effects of chemopreventive agents, including soy isoflavone, curcumin, epigallocatechin-3-gallate (EGCG), resveratrol, indole-3-carbinol (I3C), and 3,3'-diindolyl-methane (DIM) in cancer prevention and treatment. More importantly, the published studies have shown that isoflavone genistein could potentiate the antitumour effects of chemotherapeutic agents in various cancers *in vitro* and *in vivo* in preclinical studies. Sarkar and Li (2007) have reported that *in vitro* genistein potentiated growth inhibition and apoptotic cell death caused by cisplatin, docetaxel, doxorubicin, gemcitabine, and CHOP (cyclophos-phamidine, doxorubicin, vincristine, prednisone) in lymphoma and cancers of prostate, breast, pancreas, and lung (Mohammad *et al.,* 2003; Banerjee *et al.,* 2005, Li *et al*., 2005). Dietary genistein *in vivo* could enhance the antitumour activities of gemcitabine and docetaxel in a tumour model, resulting in apoptotic cell death and the inhibition of tumour growth (Banerjee *et al.,* 2005, Li *et al.,* 2005). The synergistic action of genistein and cisplatin or carmustine (BCNU) on the growth inhibition of glioblastoma and medulloblastoma cells has also been observed (Khoshyomn *et al.,* 2000; 2002). Similar observations by other investigators have also showed that the antitumour effects of chemotherapeutics, including 5-fluorouracil (5-FU), adriamycin, and tamoxifen could be potentiated by genistein (Satoh *et al.,* 2003;Hwang *et al.,* 2005).

EGCG *in vivo* also inhibits tumour promotion and metastasis in murine melanoma (Taniguchi *et al.,* 1992). Importantly, it has been found that EGCG combined with tamoxifen significantly induced apoptosis and growth inhibition in MDA-MB-231 human breast cancer cells (Chisholm *et al.,* 2004). EGCG could also chemosensitize resistant tumour cells to doxorubicin in the human carcinoma xenograft model (Zhang *et al.,* 2004), suggesting its effects on cancer therapy in combination with chemo-therapeutics. Curcumin also potentiated the antitumour activities of cisplatin, doxorubicin, and taxol in HA22T/VGH hepatic cancer cells, HeLa cells, or CAOV3 and SKOV3 ovarian cancer cells (Chan *et al.,* 2003; Notarbartolo *et al.,* 2005; Bava *et al.,* 2005)

Our previous work (Benković *et al.,* 2007) has shown that WSDP, EEP, naringin and quercetin given *ip* at dose of 100 mg / kg for three consecutive days before tumour inoculation given in combination with chemotherapeutic agent irinotecan (50 mg / kg on days 1, 13, and 19) delayed Ehrlich ascites tumour (EAT) growth and increased the life span of EAT-bearing mice. EEP and WSDP in combined treatment with irinotecan increased median survival time (59.00 ± 9.87 days; 70.00 ± 9.22 days) as compared to control group or the group treated with irinotecan alone (39.00 ± 2.67 days). The analysis of the total number of cells present in the peritoneal cavity of mice revealed that all the experimental groups inoculated with tumour cells in the presence of WSDP or polyphenolic compounds of propolis exhibited a significantly lower number of cells in the peritoneal cavity as compared to the control. Combined treatment of test components with irinotecan showed a strong antitumour activity; the total number of cells in the peritoneal cavity of mice treated with irinotecan in combination with quercetin or naringin was reduced by 87.89% or 81.12% as compared to the control and by 28.21% or 21.44% as compared to irinotecan alone, respectively (Benković *et al.,* 2007; Oršolić *et al.,* 2008b,c). The exact mechanism(s) of action by which the test components interacted with irinotecan including the suggestions given in reference (Suzuki *et al.,* 2002) remains unknown. Some of possibilities include:

a) maintaining high circulating levels of irinotecan by WSDP or related flavonoids due to P-glycoprotein pump efflux activity,
b) the test component may act as an efficient vehicle for selective delivery of chemotherapeutic agents to tumour cells, rather than acting on tumour cells directly,
c) the test components may act on tumour cells through enhanced immunity and direct DNA damage induced by apoptotic processing,
d) the test components may have a potential to alter the metabolic activation of therapeutically administered drug,
e) synergistic action of flavonoids and chemotherapeutic on topoisomerase I and II.

We determined that the efficacy profile of propolis and related polyphenolic compounds treatment alone, or in combination with chemotherapeutic irinotecan, enhanced the activity of immunological effector cells and haematopoiesis in mice bearing tumour. In addition, propolis and its polyphenolic compounds may reduce the toxicity of irinotecan to normal cells (liver, kidney and blood) (see review Oršolić *et al.,* 2008b,c). The major new findings are that the pre-treatment of mice-bearing tumour with propolis and propolis related compound such as naringin or quercetin in combination with irinotecan resulted in (i) decreased number of total cells in peritoneal cavity, (ii) increased number of WBC, (iii) enhanced macrophage and PMN activity, (iv) protection of liver and kidney cells against irinotecan-induced toxicity, (v) decrease of the number of micronucleated cells in peripheral blood. According to this observation, it is likely that activation of the immune system is involved in *in vivo* tumour regression. Thus, in mice treated with propolis preparation or flavonoids the number of polymorphonuclear (PMN) cells in the peritoneal cavity was significantly ($p<0.05$) increased as was the number of macrophages in combined treatments. WSDP combined with irinotecan in preventive and EEP combined with irinotecan in therapeutic treatment increased the macrophage spreading activity.

It is likely that macrophage activation and their effect on other immunological effectors are responsible for the destruction of tumour cells. Consistent with these results are our findings of restored lymphocyte/polymorphonucler leukocyte ratio (L/P) activity indicating that the overall immunological activity may be significantly elevated in animals receiving propolis preparations. These results suggest that the test components used in this study might interfere with the growth of EAT cells by activation of macrophages and neutrophils. The functional activity of neutrophils and/or macrophages is related to the amount of reactive oxygen species (ROS) produced during the respiratory burst after their activation (Elgert *et al.,* 1998; Watson, 2002; Oršolić and Bašić 2003b,c; Oršolić *et al.,* 2006b). The ROS production in activated neutrophils seems to influence their lifespan which can in turn be modulated by antioxidants (Watson, 2002). Therefore, manipulation of these processes is likely to be a key strategy in the acceleration or delay of apoptotic events in the entire population of circulating neutrophils and/or macrophages before they are replaced by mature cells released from the bone marrow. However, little is known about the effects of a combined treatment on neutrophils with antioxidants and drugs, on their lifespan and functions. Many authors suggest that antioxidants used as an adjunct in chemotherapy enhance the efficacy of antineoplastic drugs and/or lower their adverse effects on surrounding normal tissue (Caltagirone *et al.,* 2000; Suzuk *et al.,* 2002; Watson, 2002; Conklin *et al.,* 2004).

It is known that standard therapy with cytostatics produces acute toxicity during treatment and that toxicity can be severe enough to cause discontinuation of therapeutic agents. Agents, which could reduce the toxicity of standard cytostatic therapy on normal cells and/or which could increase the response of tumour cells to standard therapy, may markedly improve the current management of human cancer. Usually, in cancer chemotherapy, the major problems that are being encountered are myelosuppression and anaemia (Oršolić and Bašić 2005a; Suzuki *et al.,* 2002). It is known that the major side-effects of irinotecan in clinical use are myelosuppression and diarrhoea. We showed that propolis and related flavonoids (100 mg / kg) in combined treatment with a cytostatic may protect WBC, but have no effect on the haemoglobin content and red blood cells (RBC). Moreover, propolis and related flavonoids may guard RBC in the peripheral blood from irinotecan–induced toxicity (Oršolić *et al.,* 2008c) and decreased number of micronucleated cells showed by micronucleus assay. In this experiment and a previous study (Benković *et al.,* 2007) we did not observe side effect such as either diarrhoea, or a loss of body weight in combined treatment.

These observations are in concordance with Fitzpatrick *et al.* (2001) and Ballester *et al.* (2006); they suggest that for biological modulation of undesirable effects such as diarrhoea, flavonoids may be of great utility in states of acute or chronic diarrhoea through the inhibition of intestinal secretion and motility, and may also be beneficial in the reduction of chronic inflammatory damage to the intestine, by affording protection against oxidative stress and by preserving mucosal function.

Propolis and related flavonoids reduced irinotecan-induced DNA damage to kidney, liver, and leukocytes (Brozović *et al.,* 2005; Oršolić *et al.,* 2008c), as well as chromosomal breakage in groups of mice without tumour treated with test compounds, and in all groups with tumour except the group of mice treated with quercetin combined with irinotecan. In the micronucleus assay quercetin in combination with irinotecan increased the number of micronucleated cells indicating a pro-oxidative effect of quercetin.

Furthermore, our data showed that preparations of propolis (50 mg / kg) given to mice preventively and/or curatively in combination with cisplatin (10 mg / kg) and/or doxorubicin (20 mg / kg) may increase the life span of treated mice, decrease tumour burden and side effect of chemotherapeutics in mice. Intraperitoneal application of the propolis preparation with cisplatin inhibited *ip* tumour growth whether given before tumour cells inoculation or after stabilization of tumour growth. The life span of mice treated with propolis preparations and cisplatin before tumour cells inoculation was significantly prolonged, as compared to control (ILS for WSDP+ CIS=100%; EEP +CIS= 39,42%); in curatively treated mice, ILS for both groups (WSDP+ CIS, EEP +CIS) was about 36%. EEP in combination with doxorubicin exerted better effect in curatively treated mice than that in those preventively treated mice, which is in agreement with the results found for metastases formation (Oršolić *et al.*, 2008b,c). Moreover, the propolis preparation significantly reduced

the toxicity of chemotherapeutics; an indicator of toxicity was calculated for individual animals as the maximum percentage of the animal's weight loss.

Our study (Oršolić *et al.*, 2008c) suggests that propolis and/ or flavonoids could prevent a hematological toxicity of the studied drugs such as cisplatin and doxorubicin. The data shows the absence of leukopoenia among animals that received flavonoids a few days before the administration of the chemotherapeutic agents, while mice treated with chemotherapy only showed a significant decrease in white blood cells. Moreover propolis and flavonoids also permitted the maintenance of normal levels of red blood cells, as well as haematocrit and haemoglobin. The protective role of the WSDP in combination with doxorubicin on spleen weight, cellularity spleen and bone marrow was also observed. Moreover, the administration of WSDP allowed the suppression of thrombocytopenia, which was noted in animals treated with the chemotherapeutic agent.

Chemotherapeutic drugs are often associated with some degree of toxicity, which are caused, in part by reactive metabolites generated by the biotransformation of anticancer drugs in the liver. The combination of flavonoids and chemotherapy with irinotecan showed a reduction of drug toxicity (Oršolić *et al.*, 2008c) while irinotecan alone increased the activities of ALT and AST indicating organ dysfunction and cellular injury (Sforcin, 2007). Administration of propolis and its flavonoids with a cytostatic shifted the activities of these enzymes to normal levels; the protective effect against organ dysfunction and cellular injury of liver or kidney was more expressed in preventive than in curative treatment with test components.

Padmavathi *et al.* (2006) and Sforcin (2007) reported that natural flavonoids have the ability to decrease serum transferases activity in intoxicated animals. More than 38 flavonoids have been found in propolis (Padmavathi *et al.,* 2006) and these flavonoids might be responsible for the beneficial effect of propolis on these enzymes; findings indicate a reduction in ALT and AST activities up on propolis treatment. In addition, Padmavathi *et al.,* (2006), studied the therapeutic effect of paclitaxel and propolis (etanolic extract) on lipid peroxidation and antioxidant system in 7,12 dimethylbenz(a)anthracene, DMBA-induced breast cancer in female rats. It was observed that administration of paclitaxel and propolis effectively suppressed breast cancer, decreased lipid peroxidation and increased the activities of antioxidants enzymics or non-enzymic (superoxide dismutase and vitamin C) when compared to therapy of paclitaxel or propolis alone.

Our results confirm that pre-treatment with natural antioxidant can reduce the adverse effects of the same chemotherapeutic agents on normal cells with equal or increased efficiency to tumour cells. In addition, the haematological, liver and kidney toxicity due to common drugs such as irinotecan may be avoided by the use of propolis and related flavonoids. The enhanced effects of chemotherapy by chemopreventive agents may also be related to immunopotentiating activities through the reduction of interleukin (IL)-6 (Chan *et al.,* 2003) and the enhancements of lymphocyte proliferation, NK cell cytotoxicity, the CD4+/CD8+ ratio, IL-2, and interferon (IFN)-g productions (Zhang *et al.,* 2005). These results clearly suggest that chemopreventive agents are pleiotropic and thus could be considered as multitarget agents that are likely to revolutionize our approach for the prevention and treatment of cancer.

Effect of propolis and its polyphenols on P-glycoprotein (P-gp) pump activity

A number of studies have looked at the effects of some naturally occurring flavonoids on P-gp-mediated drug efflux, but conflicting results have been reported. For example, the flavonoids quercetin and kaempferol have been shown to decrease adriamycin accumulation in HCT-15 colon cells by stimulation of P-gp (Critchfield *et al.*, 1994). However, in another study, quercetin was shown to inhibit P-gp in MCF-7/ADR cells (Scambia *et al.,* 1994). Zhang *et al.* (2003) have shown that both biochanin A and silymarin can significantly increase the 1.5-h cellular accumulation of digoxin and vinblastine (VBL) (well-known P-gp substrates) in Caco-2 cells, and their effects on digoxin accumulation were shown to be flavonoid-concentration dependent.

Using membrane vesicles from human cell lines overexpressing P-gp and MRP, it was found that both P-gp and MRP are involved in the active efflux of SN-38 (active metabolite of irinotecan) and irinotecan (Chu *et al.,* 1999). Our results suggest that propolis and flavonoids can modulate P-gp function and that they could be used in some combination as safe and potent MDR reversing agents in chemotherapy (see review Oršolić *et al.,* 2008).

An overview of different flavonoids and their effects as ABC transporter inhibitors on MDR, intracellular accumulation and bioavailability of bioactive compounds was shown by Brand *et al.* (2006). The paper by Brand *et al.* (2006) focuses on the role of flavonoids as important modulators or substrates of intestinal ABC transport proteins.

The development of resistance to the drug is a major problem in cancer chemotherapy. Studies on camptothecin, an analogue of irinotecan suggest the following general mechanisms of resistance: (i) variable levels of the enzymes involved in the conversion of irinotecan; (ii) reduced cellular accumulation from active drug efflux; (iii) reduced levels of Topo I expression; (iv) alterations in the structure of Topo I from different mutations; (v) alterations in the cellular response to camptothecin–Topo I–DNA complex formation, which involves proteasome degradation of Topo I and/or enhanced DNA repair; and (vi) activation of the transcription factor, nuclear factor kappa B, by DNA damage and subsequent suppression of apoptosis. Multiple approaches using pharmacological and biological modulation to circumvent the above mechanisms of resistance have been

incorporated into clinical trials and are expected to enhance the antitumour activity of irinotecan and reduce its systemic toxicity. A combination of drug treatment and/or natural antioxidants as an adjunct to standard cancer therapy may be one way to overcome this problem.

A combined treatment with irinotecan or vincristine and propolis and its flavonoids have shown additive or synergistic antitumour effects *in vivo* and in *in vitro* on both parental sensitive Hep2 and vincristine resistant VK2 cells, respectively. These findings indicate that irinotecan therapy, as single agents, is less effective than the combined treatment. Increased survival of animals and improved antitumour effect *in vivo* than that *in vitro* showed that irinotecan is subject to extensive metabolic conversion by various enzymatic systems in the body and that the conversions of irinotecan to the more active form SN-38 requires carboxylesterase. For the antitumor effects of combined treatment with propolis and irinotecan we proposed several different mechanism: (i) enhance drug cytotoxicity or promote apoptosis; (ii) overcoming drug resistance to cancer therapy by inhibiting P-gp-mediated drug efflux; (iii) synergistic action flavonoids and chemotherapeutic on topoisomerase I and II; (iv) inhibition of protein tyrosine kinase activities; (v) downregulation of the transcription factor nuclear factor kappa B; (vi) modulation of detoxification enzymes phase I and II; vii) stimulation of immune system; (viii) modulation of steroid hormones (phytoestrogen activity); (ix) inhibition of angiogenesis; (x) inhibition of mitotic signal activation; (xi) downregulation of tumour oncogenes and expressed tumour suppressor genes; (xii) inhibition of pro-oxidative enzymes.

Díaz-Carballo *et al*. (2008) investigated a new component (plukenetione A) isolated from Cuban propolis. They found an anti-metastatic effect in mice and considerable cytotoxicity without cross-resistance in both wild-type and chemoresistant human tumour cell lines. Plukenetione A induced G0/G1 arrest and DNA fragmentation occurred in colon carcinoma cells. Furthermore, the activities of both topoisomerase I and DNA polymerase were inhibited, while the expression of topoisomerase II-beta, EGF receptor, and multidrug resistance-related protein genes was found repressed. The authors assumed that plukenetione A contributes to the anti-tumoral effect of Cuban propolis mainly by targeting topoisomerase I as well as DNA polymerase.

Interestingly, the forced expression of COX-2 caused enhancement in multiple drug resistance (MDR)1 expression and functional activity, suggesting the existence of a causal link between COX-2 activity and MDR1 expression (Sorokin, 2004). Therefore, the use of COX-2 inhibitors to decrease MDR1 function may enhance the accumulation of chemotherapy agents and decrease the resistance of tumours to chemotherapeutic drugs. Moreover, selective COX-2 inhibitors were found to enhance tumour response to radiotherapy or radiochemotherapy, suggesting that these agents can improve the response of various cancers to conventional cancer therapies (Liao *et al.,* 2003; Komaki *et al.,* 2004). Thus, proanthocyanidin has been found to enhance doxorubicin-induced antitumour effects and reverse drug resistance in doxorubicin-resistant K562/DOX cells, breast cancer cells, and mouse tumour xenograft models (Sharma *et. al.,* 2004; Zhang *et al.,* 2005). EGCG could also chemosensitize resistant tumour cells to doxorubicin in the human carcinoma xenograft model (Zhang *et al.,* 2004), suggesting its effects on cancer therapy in combination with chemo-therapeutics.

Propolis and its polyphenolic/flavonoid compounds as an adjunct to radiation therapy

Exposure to ionizing radiation leads to undesirable damage, both on cellular and on the whole organism level. Ionizing radiation, alone or in combination with other therapies, is one of the most widely used anticancer treatments. It is well know that haematopoietic toxicity and immune suppression are one of the major problems caused by the irradiation of patients. Radioprotective agents appear related to free radicals in competition with oxygen or with increased repair of radiation injury. In contrast, radiosensitizers are chemical agents that have the capacity to increase the lethal effects of radiation.

Whole body exposure of tumour-bearing animals to g-radiation (4 Gy) resulted in an increase in the comet assay parameters (such as tail length, % DNA in tail, tail moment) of blood lymphocytes as well as in tumour cells, as a results of damage to cellular DNA. Using the comet assay, we clearly demonstrated that propolis and related flavonoids applied to mice-bearing tumour at dose of 100 mg / kg before or after irradiation have different effect on normal and tumour cells; a significant decrease was observed in the comet parameters of blood lymphocytes but not in the tumour cells of irradiated animals (Oršolić *et al.,* 2008a). Our results obtained with propolis and related flavonoids are in agreement with the result obtained by Bhosle *et al.,* (2005) and Maurya *et al.,* (2004). Studies Bhosle *et al.,* (2005) on the combined effects of radiation and ellagic acid (EA) both *in vitro* and *in vivo* on normal and tumour cells have shown a generation of increasing ROS as a function of radiation dose especially after 3 Gy in human cervical cell line, HeLa. EA was found to be prooxidant *in vitro* at concentration of 100 μM in HeLa cells. Moreover, generation of ROS was found to increase with the increasing concentration of EA. Tumour transplanted mice subjected to radiation and EA treatment showed a loss in cellular viability after 1 h, which increased significantly after 24 h. The decrease in mitochondrial potential and the loss of cell viability were remarkably greater in tumour cells from mice treated with EA and radiation than with either treatment alone. Moreover, EA was found to protect against radiation-induced oxidative stress in splenic lymphocytes of tumour-transplanted mice. Measurement of antioxidant enzymes such

as superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px) and glutathione reductase (GR) in tumour cells showed decrease after treatment with EA and radiation *in vivo.* Treatment of tumour bearing mice with EA and radiation showed significant decrease in the animal's body weight suggesting reduced tumour burden. According to our results and those of Bhosle *et al.* (2005), we proposed that variability in antioxidant defence or DNA repair capability, the induction of apoptosis, as well as the above mentioned difference between cell types and growth state, could be important in determining the susceptibility of the cells to genetic destabilization, cell death or mutation.

Antioxidants may interfere with the initial mediation of apoptosis by ROS (Maurya *et al.,* 2004; Bhosle, 2005), as well as later membrane lipid peroxidation, which is characteristic of radiation-induced apoptosis (Oršolić *et al.,* 2008a). Central issues involve whether radiation-induced apoptosis can be promoted by some antioxidants in tumours but not in normal tissue, and when it is useful to protect against radiation-induced apoptosis in normal cells. Thus, experimental studies showed that antioxidant vitamins and some phytochemicals selectively induce apoptosis in cancer cells but not in normal cells and thus prevent angiogenesis and metastasis spread, suggesting a potential role for antioxidant as adjuvants in cancer therapy (Borek, 2004).

To conclude, the DNA damage caused by test components and radiation in our study (Oršolić *et al.,* 2008a) can be based on the experimental evidence of radiation and different mode of action of the test component on tumour as compared to normal cells. The mode of action may include: (i) inhibition of various enzymes involved in DNA repair; (ii) induction of reactive oxygen species (ROS) capable of inflicting DNA damage, (iii) the inability of tumour cells to use extra antioxidants in a repair capacity, (iv) the difference in cellular biochemistry or a lack of sufficient concentration of the propolis and polyphenolic compounds in tumour tissue to elicit radioprotection (v) the biodistribution of this compound in tumour and normal tissues, the hypoxic environment of the tumour and the poor vasculature in the tumour, (vi) the variations in the physiological and biochemical status of the cells of the tumour compared to normal cells at the time of irradiation and (vii) a selective protection of normal tissues from damage induced by irradiation and cytotoxicity to tumour cells.

This observations are in concordance with (Hillman *et al.,* (2001; 2004), who have demonstrated that the combination of genistein and radiation exerted enhanced inhibitory effects on DNA synthesis, cell growth, colony formation, and metastasis. Genistein also enhanced radiosensitivity in human esophageal and cervical cancer cells (Akimoto *et al.,* 2001;Yashar *et al.,* 2005) suggesting the beneficial effects of genistein in cancer radio-therapy. In cancer radiotherapy, curcumin at a low concentration also showed significant enhancement to radiation-induced clonogenic inhibition and apoptosis in PC-3 prostate cancer cells (Chendil *et al.,* 2004).

Conclusion

In conclusion, the results presented here indicate that honeybee propolis might be a potentially useful tool in the control of tumour growth in experimental tumour models. The chemopreventive activity of honeybee propolis in animal models and cell cultures are likely to be the result of their ability to inhibit DNA synthesis in tumour cells, their capability to induce apoptosis of tumour cells, and their property to activate macrophages to produce factors capable of regulating the function of B-, T- and NK-cells, respectively. Moreover, these results suggest that flavonoids from propolis play a protective role against the toxicity of the chemotherapeutic agents or radiation in mice, giving hope that they may have similar protective action in humans. The combination with an adjuvant antioxidant therapy may enhance the effectiveness of chemotherapy by ameliorating the side effect on leukocytes, liver and kidneys and consequently enabling dose escalation.

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