Nutrition and Cancer (2003) 47(2), 156 - 163

NUTRITION AND CANCER, *47*(2), 156–163
Copyright © 2003, Lawrence Erlbaum Associates, Inc.

Inhibitory Effect of Water-Soluble Derivative of Propolis and Its Polyphenolic Compounds on Tumor Growth and Metastasizing Ability: A Possible Mode of Antitumor Action

Nada Oršolić, Lidija Šver, Svjetlana Terzić, Zoran Tadić, and Ivan Bašić

Abstract: *Polyphenolic compounds are widely distributed in the plant kingdom and display a variety of biological activities, including chemoprevention and tumor growth inhibition. Propolis is made up of a variety of polyphenolic compounds. We compared how the routes of administration of polyphenolic compounds deriving from propolis and of propolis itself affect the growth and metastatic potential of a transplantable mammary carcinoma (MCa) of the CBA mouse. The influence of tested compounds on local tumor growth was also studied. Metastases in the lung were generated by 2 × 10⁵ tumor cells injected intravenously (IV). A water-soluble derivative of propolis (WSDP) and polyphenolic compounds (caffeic acid, CA, and CA phenethyl ester, CAPE) were given to mice per os (PO) or intraperitoneally (IP) before or after tumor cell inoculation. Tested compounds significantly decreased the number of lung colonies. When mice were inoculated with 10⁵ MCa cells in the exact site of subcutaneous injection of different doses of WSDP, CA, or CAPE, tumor growth was inhibited, and survival of treated mice was prolonged. Antitumor activity, according to the results obtained, is mostly related to the immunomodulatory properties of the compounds and their capacity to induce apoptosis and necrosis. In conclusion, results presented here indicate that WSDP, CA, and CAPE could be potential useful tools in the control of tumor growth in experimental tumor models when administrated PO; because PO administration is the easiest way of introducing a compound used for prevention and/or cure of any disease, it is likely that this article has reached the goal of the investigation.*

Introduction

Propolis is alleged to exhibit broad-spectrum activities, including antibiotic (1), antiinflammatory (2), antioxidant (3), antiviral, and tumor cell arrest (4–7). A report on immunomodulatory activities of aqueous extracts of propolis (8) showed that a water-soluble extract of propolis (WSDP) increased the protection from gram-negative infections probably via macrophage activation. From the literature it seems clear that antibiotic activities, immune modulatory properties as well as antiinflammatory, wound healing, and antitumor effects may be due to different components of the individual ethanolic or aqueous extracts of propolis. At least 200 different constituents of propolis are defined as terpenes, various phenylpropane derivatives such as caffeic acid (CA) esters, flavonoids, amino acids, or a large number of aldehydes and ketones (9,10). We have demonstrated that WSDP given intraperitoneally (IP) to mice suppressed the growth of a mammary carcinoma (MCa) in mice (11).

CA phenethyl ester (CAPE) exhibits differential toxicity to cancer cells versus normal cells (12,13). CAPE was reported to be a lipoxygenase inhibitor with antioxidant properties (14,15). Inhibition of the tumor promoter–mediated oxidative processes by CAPE has also been reported (16). Recent studies demonstrated that CAPE and several additional CA esters inhibited azoxymethane-induced colonic preneoplastic lesions and enzyme activities, including ornithine decarboxylase, tyrosine kinase, and lipoxygenase, associated with colon carcinogenesis (13,17–19). Inhibitory effect CA on tumor promotion in mouse skin has been shown (20). However, until now no studies on local antitumor activity of CA have been done.

The aim of this investigation was to compare how the routes (oral and/or systemic) of administration of polyphenolic compounds deriving from propolis and of propolis itself influence the growth and metastatic potential of a transplantable MCa of CBA mouse and to study their effect on modulation of immune reaction and apoptosis and necrosis of MCa cells, respectively.

Materials and Methods

Animal Studies

Animal studies were carried out according to the guidelines in force in the Republic of Croatia (Law on the Welfare

N. Oršolić, Z. Tadić, and I. Bašić are affiliated with the Department of Animal Physiology, Faculty of Science, University of Zagreb, Rooseveltov trg 6, 10000 Zagreb, Croatia. L. Šver is affiliated with the Department of Biology, Veterinary Faculty, University of Zagreb, Heinzelova 55, 10000 Zagreb, Croatia. S. Terzić is affiliated with the Croatian Veterinary Institute, Savska c. 143, Zagreb, Croatia.

of Animals, N. N. #19, 1999) and in compliance with the Guide for the Care and Use of Laboratory Animals, DHHS Publ. (NIH) 86-23. Male and female CBA inbred mice from our conventional mouse colony were used. In every experiment, mice were of the same sex and were approximately 3 mo old at the initiation of each study. The animals were kept not more than five to a cage and were maintained on a commercial rodent pellet diet and water ad libitum. Experimental groups were composed of 7–10 mice each.

Tumor

A transplantable MCa of spontaneous origin in CBA mouse was used. It arose spontaneously in an old multiparous mouse (21). The tumor was maintained by serial passages in syngeneic mice, and it was in its 110th isotransplant generation when used for experiments. Tumor is weakly immunogenic for syngeneic recipients as shown by different methods in vivo (21) and in vitro (22), respectively.

Tumor Cell Suspension

Single cell suspension was prepared by digestion of tumor tissue with trypsin. Digested tissue contained no visible regions of necrosis or hemorrhage (23). Each suspension was passed through a stainless steel mesh (200 wires per inch), centrifuged three times at 24 *g* for 5 min in saline, and then resuspended in RPMI 1640 medium (Institute of Immunology, Zagreb) supplemented with 5% serum from normal syngeneic mice. Viability of cells was determined using a hemocytometer by observing the ability of intact cells to exclude Trypan blue dye and by phase contrast microscopy. Viability was found to be >95%.

In Vivo Studies

For survival analysis, CBA mice were inoculated with 10^5 MCa cells in the exact site of subcutaneous injection of different doses of WSDP and its polyphenolic compounds, and the endpoint was determined by spontaneous death of the animals. Results are expressed as percent of mean survival time of treated animals over mean survival time of the control group (treated vs. control, T/C%) and increased life span (ILS%; mean survival time of treated animals minus that of control animals over the mean survival time of the control group). By NCI criteria, T/C exceeding 125% and ILS exceeding 25% indicate that the drug has significant antitumor activity (24).

Production of Tumor Nodules (Metastases, Colonies) in Lungs

Metastases in the lung were generated by injecting 2×10^5 viable tumor cells suspended in 0.5 ml of medium RPMI 1640 supplemented with 5% syngeneic mouse serum into the tail vein of mice. Twenty-one days later, mice were killed and their lungs removed. The lobes were separated and fixed in Bouin's solution. Colonies of tumor cells were seen as white, round nodules on the surface of the yellowish lung and were counted with the naked eye. This method of counting omitted any small colonies that may have been developed deep inside the pulmonary lobes.

Water-Soluble Derivative of Propolis Treatment

WSDP was prepared by the method described elsewhere (25). According to Ref. 25, it contains CA 6.7%, γ,γ-dimethylallyl ferulate 1.2%, isopentyl-2-enyl-caffeate 7.4%, pentenyl caffeate 2.2%, γ,γ-dimethylallyl caffeate 8.5%, pinobanksin 2.3%, pinocembrin 9.2%, pinobanksin-3-acetate 13.6%, benzyl caffeate 0.4, galangin 7.8%, β-phenyl ethyl caffeate 1.2%, flavonoids 32.9%, and esters of phenyl acid 20.9%.

Briefly, Brazilian (CONAP, Belo Horizonte, Minas Gerais, Brazil) or Croatian (surroundings of Zagreb, Croatia) propolis was extracted with 96% ethanol, which was filtered and evaporated in a vacuum evaporator. The resultant resinous product was added to a stirred solution of 8% l-lysine (Sigma Chemie, Deisenhofen, Germany) and freeze-dried to yield WSDP, a yellow-brown powder. WSDP was stored under sterile conditions at 4°C. Before use WSDP was dissolved in distilled water and given to mice per os (PO) or IP at doses of 50 or 150 mg/kg; the background for selection of doses of WSDP was Ref. 8.

Polyphenolic Compounds

CA (3,4-dihydroxycinnamic acid) was purchased from Aldrich–chemie, Milwaukee, WI.

CA phenethyl ester (CAPE) was obtained by esterification of CA with phenethyl alcohol (molar ratio 1:15) in benzene (refluxing, 3–4 days, water removed by Dean-Stark trap). Following workup, excess phenethyl alcohol was removed by Kugelröhr distillation (60°C, <0.1 mm Hg) to give pure CAPE, melting point 126–128°C, needles (benzene or H_2O), 40% yield. All properties of natural and synthetic CAPE were identical (26).

CA and CAPE were dissolved in ethanol, and further dilutions were made in water. The final concentration of ethanol was ≤0.1%. Ethanol (0.1%) was used in the control group. Each experimental group was given PO CA or CAPE at doses of 50 or 150 mg/kg. Dose selection of CA and CAPE was based on our studies on cytotoxicity of these compounds on peritoneal macrophages from mice treated with either compound; we found that macrophages from mice treated with CA were more cytotoxic to tumor cells in vitro than macrophages from mice treated with CAPE (27). Furthermore, support for CA or CAPE doses was found in studies by Ploemen et al. (28) and Fitzpatrick et al. (29), who demonstrated that much higher doses than those used by us were not toxic to experimental animals.

Weight and Cellularity of Spleen and Femur, and Leukocyte Count

A dose containing 50 mg/kg of WSDP, CA, or CAPE was given PO via gastric cannula on Days 15, 10, and 5 before the measurements. Spleens from five to seven normal or WSDP-, CA-, or CAPE-treated mice were removed and weighed. Each spleen was minced and passed through a stainless steel mesh to detect cellularity. Bone marrow from a 1-cm-long shaft of femur of each mouse was washed out with a 20-gauge needle. The suspensions of spleen cells and bone marrow were then dispersed by gentle aspiration in and out of a syringe, suspended in 10 ml of saline, and counted in a hemocytometer. Samples of blood from normal or treated mice were obtained from tail vein and counted in a hemocytometer.

Response of Spleen Lymphocyte to Polyclonal Mitogens

Blastogenic response of spleen lymphocyte to mitogens was assayed 7 days after intravenous (IV) inoculation of the WSDP (50 mg/kg) or CA (50 mg/kg). Routine cultures were done in triplicate in sterile microtiter plates (Falcon, England). Each well contained 2.5×10^5 spleen lymphocytes in 0.25 ml culture medium (RPMI 1640 supplemented with 10% heat-inactivated pooled fetal bovine serum). Phytohemagglutinin (PHA), concanavalin A (ConA), pokeweed mitogen (PWM), and lipopolysaccharide (LPS; all from Sigma Chemie) were used for stimulation. Final concentration of PHA and ConA was 5 μg/ml, whereas 10 μg/ml of PWM and LPS per culture was added. Cultures were incubated 72 h in humidified atmosphere containing 5% CO_2. Microcultures were labeled with 1 μCi of ^{3}H-thymidine (specific activity 5 Ci/mM; Sigma Chemie) 18 h before harvesting. The samples were counted in a liquid scintillation counter (LKB, Wallac, Sweden). The results were recorded as counts per minute (cpm) and expressed as the transformation ratio.

Apoptosis/Necrosis Analysis

Apoptosis was determined by techniques described by Telford et al. (30). Briefly, bivariant flow cytometry was performed on primary culture of MCa cells grown in the presence or absence of tested compounds in culture medium (RPMI 1640 supplemented with 10% heat-inactivated pooled fetal bovine serum) for various times (3 and 15 h). After treatment of the tested compounds, 5×10^5 to 10^6 cells were washed in cold PBS twice and resuspended in 100 μl of binding buffer (HEPES containing 2.5 mM $CaCl_2$). Fluorescein-labeled annexin V and propidium iodide (PI; "Apoptosis Detection Kit"; R &D System, Weisbaden, Germany) were added to cells. After 15-min incubation at room temperature, cells were analyzed by a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA). At least 5,000 events were collected and analyzed using CellQuest software. Annexin V binds to those cells that express phosphatidylserine on the outer layer of the cell membrane, and PI stains the cellular DNA of those that have a compromised cell membrane. This allows for the discrimination of live cells (unstained with either fluorochrome) from apoptotic cells (stained only with annexin V) and necrotic cells (stained with both annexin V and PI).

Statistics

Results are expressed as means ± SE obtained from two or three experiments. The significance of differences between means of the groups was tested by the Mann-Whitney *U*-test with the significance level >95% or with the Student's *t*-test.

Results

Antitumor and Antimetastatic Activity of WSDP and Related Polyphenolic Compounds

The effect of WSDP, CA, and CAPE on metastases formation in the lung was studied in mice injected IV with MCa cells. Tested compounds were given PO or IP; the dose comprised 50 or 150 mg/kg for WSDP, CA, and CAPE, respectively. Mice were killed 21 days after the treatment, and the number of metastases on the lung surface was determined.

Tables 1 and 2 show that, in mice treated either preventively or curatively, the number of tumor nodules in the lung was significantly lower than in untreated mice. The antimetastatic effectiveness of WSDP was of higher degree than that achieved by either CA or CAPE. Tables 3 and 4 summarize whether the presence of WSDP, CA, or CAPE in the tissue influenced tumor formation and survival rate of CBA mice. Mice in each group were given subcutaneous injection of either tested compounds (50 or 150 mg/kg). Immediately after treatment mice received injection containing 10^5 viable tumor cells into the exact site of tested compound inoculation. The growth of tumor at the site of tumor cell inoculation was checked every day thereafter. Table 3 illustrates that tumor formation was delayed in mice treated with WSDP as well as with polyphenolic compounds and was dependent on the dose applied; however, the polyphenolic compounds were more effective than either sample and dose of WSDP. Although all mice in the control group developed tumor within 22 days after tumor cell inoculation and died between 28 and 40 days thereafter (Table 4), the development of tumors in treated mice and their survival time were delayed (except for 150 mg/kg of Croation WSDP); the presence of WSDP, CA, or CAPE in the tissue of tumor cell inoculation inhibited tumor growth and ILS of mice for 18.1% to 51.74%.

Influence of WSDP, CA, or CAPE on Weight and Cellularity of Spleen and Femur and on Blood Leukocyte Count

WSDP, CA, or CAPE administered PO enhanced weight and cellularity of spleen (Table 5). However, neither cel-

Table 1. Protection against MCa Metastases in the Lung by WSDP-, CA-, or CAPE-Preventive Treatment

Treatment[a]	Dose (mg/kg)	Lung Metastases (mean no./lung ± SE)[b]	Range	Statistics (Mann-Whitney U-test)
None	—	62.71 ± 6.3	38–86	
Croatian WSDP	50 (IP)	18.75 ± 1.94	15–25	Z = 2.64, P = 0.008
	50 (PO)	16 ± 2.35	11–21	Z = 3.13, P = 0.001
	150 (IP)	13.8 ± 1.45	10–19	Z = 2.84, P = 0.004
	150 (PO)	22.5 ± 3.75	13–41	Z = 3.00, P = 0.002
Brazilian WSDP	50 (IP)	25.33 ± 3.76	10–37	Z = 3.00, P = 0.003
	50 (PO)	23.7 ± 2.3	10–41	Z = 3.10, P = 0.001
	150 (IP)	13 ± 1.79	6–19	Z = 3.13, P = 0.002
	150 (PO)	17.3 ± 3.5	11–35	Z = 3.18, P = 0.001
CA	50 (PO)	24.66 ± 6.26	9–55	Z = 3.13, P = 0.001
	150 (PO)	36.83 ± 3.87	27–55	Z = 2.50, P = 0.012
CAPE	50 (PO)	22 ± 2.84	11–30	Z = 3.13, P = 0.001
	150 (PO)	32.37 ± 2.97	22–44	Z = 2.62, P = 0.009

a: WSDP, CA, or CAPE was given IP or PO 5, 10, and 15 days before tumor cell inoculation.
b: 2×10^5 tumor cells per mouse injected IV; the number of tumor nodules in the lung was determined 21 days after tumor cell inoculation. Groups comprised of seven to nine mice each (mean ± SE).

Table 2. Protection against MCa Metastases in the Lung by WSDP-, CA-, or CAPE-Curative Treatment

Treatment[a]	Dose (mg/kg)	Lung Metastases (mean no./lung ± SE)[b]	Range	Statistics (Mann-Whitney U-test)
None	—	62.71 ± 6.3	38–86	
Croatian WSDP	50 (IP)	15.42 ± 1.34	5–23	Z = 2.84, P = 0.004
	50 (PO)	15.66 ± 2.97	5–29	Z = 2.88, P = 0.004
	150 (IP)	19.96 ± 1.27	9–30	Z = 3.00, P = 0.003
	150 (PO)	17.42 ± 3.19	3–27	Z = 3.00, P = 0.003
Brazilian WSDP	50 (IP)	26.4 ± 2.4	11–41	Z = 2.71, P = 0.007
	50 (PO)	30.20 ± 3.7	17–40	Z = 2.74, P = 0.006
	150 (IP)	17.5 ± 2.97	10–34	Z = 2.84, P = 0.004
	150 (PO)	16.11 ± 2.16	8–26	Z = 3.18, P = 0.001
CA	50 (PO)	39 ± 5.3	23–57	Z = 2.56, P = 0.01
	150 (PO)	47 ± 6.7	23–60	Z = 2.56, P = 0.01
CAPE	50 (PO)	33.66 ± 7.2	20–31	Z = 2.88, P = 0.004
	150 (PO)	43.50 ± 5.03	30–57	Z = 2.74, P = 0.006

a: WSDP, CA, or CAPE was given IP or PO 2, 7, and 12 days after tumor cell inoculation.
b: 2×10^5 tumor cells per mouse injected IV; the number of tumor nodules in the lung was determined 21 days after tumor cell inoculation. Groups comprised of seven to nine mice each (mean ± SE).

Table 3. Tumor Formation in CBA Mice Treated with WSDP, CA, or CAPE

	Tumor Formation (no. of days after tumor cell inoculation)			
Treatment (mg/kg)[a]	17	22	27	32
	7/9[b]	9/9[b]		
Croatian WSDP (50)	5/9	6/9	9/9	
Croatian WSDP (150)	6/8	8/8		
Brazilian WSDP (50)	6/8	6/8	8/8	
Brazilian WSDP (150)	7/9	9/9		
CA (50)	4/9	5/9	9/9	
CA (150)	3/9	4/9	6/9	9/9
CAPE (50)	2/9	6/9	9/9	
CAPE (150)	0/9	1/9	5/9	9/9

a: CBA mice were inoculated with 10^5 MCa cells in the exact site of subcutaneous injection of different doses of WSDP, CA, or CAPE. Tumor cells were introduced immediately after injection of tested compounds.
b: Number of animals with tumor per number of mice in the group.

lularity of bone marrow nor leukocyte count was changed in mice treated with WSDP or CA; CAPE increased cellularity of bone marrow.

Effect of WSDP and CA on Splenocyte Responses to Polyclonal Mitogens

Because tested compounds increased the cellularity of spleen in treated mice, we tested the effect of treatment with WSDP and CA given PO on responses of splenocytes to polyclonal mitogens. Figure 1 shows that WSDP treatment of mice increased the responses of spleen cells to all polyclonal mitogens tested (PHA, ConA, PWM, and LPS; $P < 0.01$). In contrast, the response of spleen cells of mice treated with CA was significantly suppressed ($P < 0.001$).

Apoptosis/Necrosis of Tumor Cells

In flow cytometry studies we examined the rate at which MCa cells in the presence of WSDP, CA, or CAPE were un-

Table 4. In Vivo Antitumor Activity of WSDP, CA, and CAPE[a]

Group	Mice per Group	Dose (mg/kg)	Range of Survival Time (days)	Median Survival Time (days)	ILS%	T/C%
Control	9	—	28–40	35.66	—	—
Croatian WSDP	9	50	35–51	42.11	18.09	118.09
	8	150	30–37	33.875	–5.01	94.99
Brazilian WSDP	8	50	31–48	42.375	18.83	118.83
	9	150	40–46	43.44	21.817	121.817
CA	9	50	37–55	46.11	29.30	129.30
	9	150	34–59	46.55	30.538	130.538
CAPE	9	50	42–50	46.66	30.846	130.846
	9	150	36–80	54.11	51.738	151.738

a: CBA mice were injected subcutaneously with 10^5 MCa cells in the exact site of subcutaneous injection of different doses of WSDP, CA, or CAPE immediately after injection of tested compounds. ILS% = (T - C)/C × 100; T/C, treated vs. control; T, mean survival days of treated group; C, mean survival days of control group.

Table 5. The effect of WSDP, CA, and CAPE on Hematological Parameters in CBA Mice

Treatment[a,b] (PO)	Leukocytes × 10^3 (X ± SE)	Spleen Weight (mg; X ± SE)	Spleen Cellularity (× 10^6; X ± SE)	Femur Bone Marrow Cellularity (× 10^6; X ± SE)
—	6.789 ± 0.424	69.5 ± 5.6	104.817 ± 4.269	12.695 ± 0.746
Croation WSDP	7.927 ± 0.589	72.7 ± 1.1	171.224 ± 10.231[c]	13.411 ± 0.689
CA	7.109 ± 0.766	74.5 ± 1.75	164.062 ± 8.131[c]	13.541 ± 0.567
CAPE	6.484 ± 0.295	75.25 ± 3.59	141.276 ± 11.736[d]	17.057 ± 1.242[d]

a: Groups comprised 7–10 mice each.
b: Animals were treated PO on Days 15, 10, and 5 before determination of the hematological parameters; doses were 50 mg/kg.
c: Significantly higher ($P < 0.01$) than in untreated mice.
d: Significantly higher ($P < 0.05$) than in untreated mice.

dergoing apoptosis or necrosis. Cells were exposed to tested compound for 3 or 15 h, and their effects were analyzed by bivariant flow cytometry (image analysis of annexin V- and PI-labeled cells). The percentage of apoptotic MCa cells was 0.56–31.24% after 3 h, whereas it was 0.3–26.43% after the incubation with tested compound for 15 h (Fig. 2). The most pronounced necrosis of MCa cells was found in the presence of CAPE (32.19%).

Discussion

The term chemoprevention describes the blocking effects of natural or synthetic compounds in the processes of mutagenesis and carcinogenesis as well as processes of proliferation and progression of tumor cells (15). Polyphenolic compounds that occur naturally in food of plant origin are present in propolis and have been considered to be possible che-

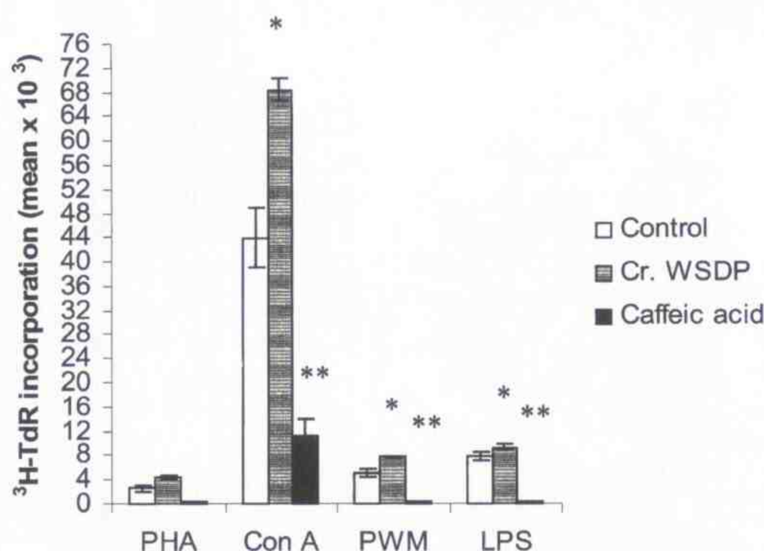

Figure 1. The effect of WSDP and CA on splenocyte responses to PHA, ConA, PWM, and LPS in CBA mice. Five mice per group received IV 50 mg/kg WSDP or CA, and their spleen cells were tested 7 days after the treatment. Incorporation of ^{3}H-thymidine (^{3}H-TdR) by splenocytes expressed as mean cpm ± SE of 15 samples (5 mice in triplicate). Significantly (* $P < 0.01$; ** $P < 0.001$) different from untreated controls.

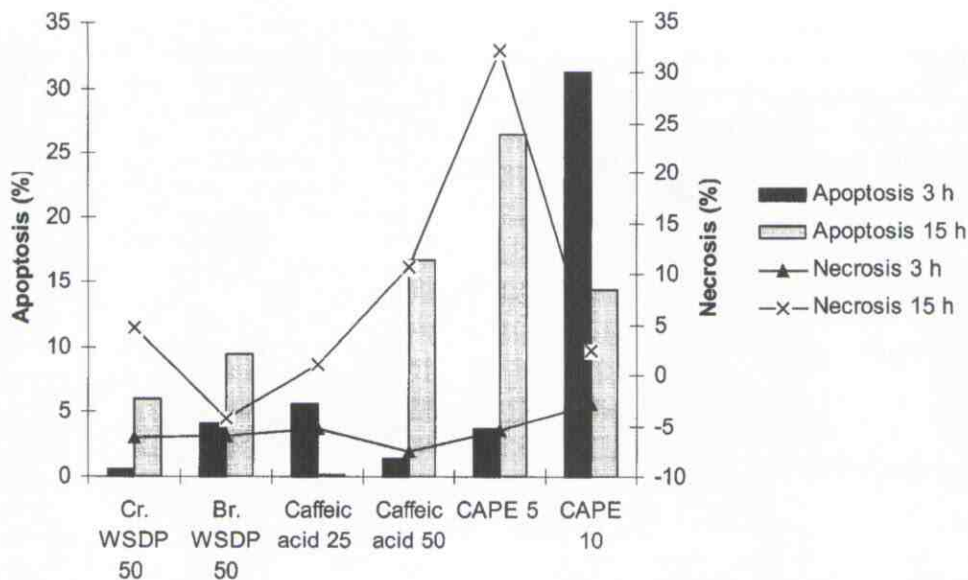

Figure 2. Effects of WSDP, CA, or CAPE on induction of apoptosis and necrosis in MCa cells. Cells were cultured in the absence or the presence of WSDP (50 μg/ml), CA (25 or 50 μg/ml), or CAPE (5 or 10 μg/ml) for 3 or 15 h. After incubation cells were washed and stained with fluorescein-labeled annexin V and PI and then analyzed by flow cytometry. The result is the percentage of apoptosis and/or necrosis obtained by substruction of control from experimental values.

mopreventive cancer agents. Because our studies (11,27) and those by others (4,8) have shown that WSDP, CA, and CAPE possess strong immunomodulatory properties and because immunomodulation is known to be of importance to control tumor growth and its spread (11,23), we have chosen the scheme of preventive and curative administration of propolis (WSDP) and its polyphenolic components to study their antitumor and antimetastatic properties due to immunomodulation and other models of their activity (apoptosis and/or necrosis).

This investigation clearly demonstrated the inhibitory effects of WSDP, CA, or CAPE on metastasis formation of MCa in CBA mice treated either preventively or curatively (Tables 1 and 2). These compounds not only reduced the number of experimental metastases but also delayed tumor formation and increased survival of treated animals. To test the direct effect on tumor growth we injected tested compounds locally and immediately thereafter at the spot of their introduction where tumor cells were inoculated. Data shown in Tables 3 and 4 indicate that the presence of CA or CAPE in the tissue of tumor cell inoculation inhibited tumor growth and ILS of treated animals (29.30–51.74%), whereas WSDP was less effective. There are findings indicating strong antitumor or anticarcinogenic effects achieved by polyphenolic compounds, the constituents of propolis, in murine tumor models (5,17,18,31–33). Reports also described the potential use of synthetic flavonoids, such as flavone acetic acid (NSC 347512), in protection of mice against the growth of solid tumors (34,35).

Considering the possible mode of antitumor action of the tested compounds, it is likely that it could be mediated by their immunomodulatory activity. Our previous data suggested that propolis and its components stimulate antitumor activity of macrophages (11), cells that could play the central role in the control of metastasis formation. Activated macrophages produce increased levels of reactive oxygen species, including H_2O_2, which are known to modulate cellular functions including those of lymphocytes (36) as shown in Fig. 1. Results of these studies support described findings because the response of spleen cells to polyclonal mitogens was suppressed in mice treated with CA, whereas WSDP exerted the opposite effect (Fig. 1); both immunostimulation and immunosuppression have been known to be the consequence of macrophage activation (37). These pro-proliferative effects may predominate when macrophages and lymphocytes interact in optimal quantitative ratios (37). We also showed that WSDP increased IL-1 production (11), which might be associated with enhanced T- and B-cell proliferation (Fig. 1). In addition, our results confirmed the different modulating effects of tested compounds on the hematological parameters (Table 5). PO treatment of mice with these compounds caused changes in the cellularity of spleen (Table 5). However, neither cellularity of bone marrow nor leukocyte count was changed in mice treated with WSDP or CA; CAPE, however, increased cellularity of bone marrow. Kimoto et al. (33) reported that artepillin C (a component of propolis) has cytostatic and cytotoxic effects on various malignant tumor cells in vitro and in vivo, and immunomodulation was the reason for increasing the number and function of lymphocytes.

Our results related to CA and CAPE suggested the other possible mechanism(s) of inhibition of tumor development, which include apoptosis and/or necrosis (Fig. 2). These mechanism(s) also include the ability of CA and CAPE to inhibit DNA synthesis in tumor cell cultures (unpublished). Furthermore, the ability of CA and CAPE to induce apoptosis suggests their potential use in preclinical trials as anticancer therapeutic agents.

In conclusion, these results indicate that WSDP, CA, and CAPE are potential useful tools in the control of tumor

growth in experimental tumor models. It is likely that the antitumor activity of WSDP is the result of synergistic activities of its polyphenolic components. There was no significant difference between Croatian and Brazilian WSDP concerning their influence on tumor growth and apoptosis and necrosis of tumor cells, suggesting that geographical origin of propolis does not influence their biological properties. It is important to emphasize that PO route of administration influences the metastatic potential of MCa cells, giving priority to oral use of tested compounds in further investigations either in experimental or clinical trials.

Acknowledgments and Notes

Address correspondence to N. Oršoli, Department of Animal Physiology, Faculty of Science, University of Zagreb, Rooseveltov trg 6, 10000 Zagreb, Croatia. Phone: 385 1 4826 266. FAX: 385 1 4826 269/280/313. E-mail: norsolic@yahoo.com.

Submitted 17 February 2003; accepted in final form 22 October 2003.

References

1. Grange JM and Davey RW: Antibacterial properties of propolis (bee glue). *J R Soc Med* **83,** 159–160, 1990.
2. Wang L, Mineshita S, Ga I, Shigematsu T, and Matsuno T: Anti-inflammatory effect of propolis. *Jpn J Pharmacol Ther* **24,** 223–224, 1993.
3. Scheller S, Wilczok T, Imielski S, Krol W, Gabrys J, et al.: Free radical scavenging by ethanol extract of propolis. *Int J Rad Biol* **57,** 461–465, 1990.
4. Scheller S, Krol W, Swiacik J, Owczarek S, Gabrys J, et al.: Antitumoral property of ethanolic extract of propolis in mice-bearing Ehrlich carcinoma, as compared to bleomycin. *Z Naturforsch* **44c,** 1063–1065, 1989.
5. Kawabe M, Lin C, Kimoto N, Sano M, Hirose M, et al.: Modifying effects of propolis on MeIQx promotion of rat hepatocarcinogenesis and in a female rat two-stage carcinogenesis model after multiple carcinogen initiation. *Nutr Cancer* **37,** 179–186, 2000.
6. Lou J, Soh JW, Xing WQ, Mao Y, Matsuno T, et al.: PM-3, a benzo-gamma-pyran derivative isolated from propolis, inhibits growth of MCF-7 human breast cancer cells. *Anticancer Res* **21,** 1665–1671, 2001.
7. Kobayashi N, Unten S, Kakuta H, Komatsu N, Fujimaki M, et al.: Diverse biological activities of healthy foods. *In Vivo* **15,** 17–23, 2001.
8. Dimov V, Ivanovska N, Bankova V, and Popov S: Immunomodulatory action of propolis. IV. Prophylactic activity against gram-negative infections and adjuvant effect of the water-soluble derivate. *Vaccine* **10,** 817–823, 1992.
9. Greenaway W, May J, Scaysbrook T, and Whatley FR: Identification by gas chromatography-mass spectrometry of 150 compounds in propolis. *Z Naturforsch* **46,** 111–121, 1991.
10. Bankova V, Christov R, and Popov S: Volatile constituents of propolis. *Z Naturforsch* **49,** 6–10, 1994.
11. Oršolić N and Bašić I: Immunomodulation by water-soluble derivative of propolis (WSDP), a factor of antitumor reactivity. *J Ethnopharmacol* **84,** 265–273, 2003.
12. Su ZZ, Lin J, Prewett M, Goldstein NI, and Fisher PB: Apoptosis mediates the selective toxicity of caffeic acid phenethyl ester (CAPE) toward oncogene-transformed rat embryo fibroblast cells. *Anticancer Res* **15,** 1841–1848, 1995.
13. Frenkel K, Wei H, Bhimani R, Ye J, Zadunaisky JA, et al.: Inhibition of tumor promoter-mediated processes in mouse skin and bovine lens by caffeic acid phenethyl ester. *Cancer Res* **53,** 1255–1261, 1993.
14. Sudlina GF, Mirzoeva OK, Pushkareva MA, Korshunova GA, Sumbatyan NV, et al.: Caffeic acid phenethyl ester as a lipoxygenase inhibitor with antioxidant properties. *FEBS Lett* **329,** 21–24, 1993.
15. Heo MY, Sohn SJ, and Au WW: Anti-genotoxicity of galangin as a cancer chemopreventive agent candidate. *Mutat Res* **488,** 35–50, 2001.
16. Bhimani RS, Troll W, Grunberger D, and Frenkel K: Inhibition of oxidative stress in HeLa cells by chemopreventive agents. *Cancer Res* **53,** 4528–4533, 1993.
17. Rao CV, Desai D, Kaul B, Amin S, and Reddy BS: Effect of caffeic acid esters on carcinogen-induced mutagenicity and human colon adenocarcinoma cell growth. *Chem Biol Interact* **84,** 277–290, 1992.
18. Femia AP, Caderni G, Buzzigoli C, Cocca E, Salvadori M, et al.: Effect of simple phenolic compounds on azoxymethane-induced aberrant crypt foci in rat colon. *Nutr Cancer* **41,** 107–110, 2001.
19. Galati G, Teng S, Moridani MY, Chan TS, and O'Brien PJ: Cancer chemoprevention and apoptosis mechanisms induced by dietary polyphenolics. *Drug Metabol Drug Interact* **17,** 311–349, 2000.
20. Huang MT, Smart RC, Wong CQ, and Conney AH: Inhibitory effect of curcumin, chlorogenic acid, caffeic acid, and ferulic acid on tumor promotion in mouse skin by 12-O-tetradecanoylphorbol-13-acetate. *Cancer Res* **48,** 5941–5946, 1988.
21. Bašić I and Varga E: Immunogenicity of mammary carcinoma and a fibrosarcoma of CBA mice. *Period Biol* **81,** 335–337, 1979.
22. Eljuga LJ, Šamija M, Eljuga D, Košuta D, and Bašić I: Effect of rIL-2 and LAK cells on metastatic formation of an anaplastic carcinoma of the rat. *Libri Oncol* **22,** 177–181, 1993.
23. Milas L, Hunter N, and Withers RH: Corynebacterium granulosum induced against artificial pulmonary metastases of syngeneic fibrosarcoma in mice. *Cancer Res* **34,** 613–620, 1974.
24. Plowman J, Dykes DJ, Hollingshead M, Simpson-Herren L, and Alley M-C: Human tumor xenograft models in NCI drug development. In *Anticancer Drug Development Guide: Preclinical Screening, Clinical Trials, and Approval*, Teicher B (ed). Totowa, NJ: Humana, 1995, p 101.
25. Nikolov N, Marekov N, Bankova V, Popov S, Ignatova R, et al.: Method for the preparation of water-soluble derivative of propolis. *Bulg J Pat Appl* 79903/28, 05, 1987.
26. Grunberger D, Banerjee R, Eisinger K, Oltz EH, Efros L, et al.: Preferential cytotoxicity on tumor cells of caffeic acid phenethyl ester isolated from propolis. *Experientia* **44,** 230–232, 1988.
27. Oršolić N and Bašić I: Immunomodulatory action of a water-soluble derivative of propolis, caffeic acid and caffeic acid phenethyl ester: nitric oxide and macrophage function. *Mellifera* **3,** 56–64, 2003.
28. Ploemen JHTM, Vanommen B, Dehaan A, Schefferlie JG, and Vanbladeren PJ: In vitro and in vivo reversible and irreversible inhibition of rat glutathione S-transferase isoenzymes by caffeic acid and its 2-S-glutathionyl conjugate. *Food Chem Toxicol* **31,** 475–482, 1993.
29. Fitzpatrick LR, Wang J, and Le T: Caffeic acid phenethyl ester, an inhibitor of nuclear factor-kappaB, attenuates bacterial peptidoglycan polysaccharide-induced colitis in rats. *J Pharmacol Exp Ther* **299,** 915–920, 2001.
30. Telford WG, King LE, and Fraker PJ: Rapid quantitation of apoptosis in pure and heterogeneous cell populations using flow cytometry. *J Immunol Methods* **172,** 1–16, 1994.
31. Rao CV, Desai D, Rivenson A, Simi B, Amin S, et al.: Chemoprevention of colon carcinogenesis by phenylethyl-3-methylcaffeate. *Cancer Res* **55,** 2310–2315, 1995.
32. Kimoto T, Koya-Miyata S, Hino K, Micallef MJ, Hanaya T, et al.: Pulmonary carcinogenesis induced by ferric nitrilotriacetate in mice and protection from it by Brazilian propolis and artepillin C. *Virchows Arch* **438,** 259–270, 2001.
33. Kimoto T, Arai S, Kohguchi M, Aga M, Nomura Y, et al.: Apoptosis and suppression of tumor growth by artepillin C extracted from Brazilian propolis. *Cancer Detect Prev* **22,** 506–515, 1998.

34. Zaharko DS, Grieshaber CK, Plowman J, and Cradock JC: Therapeutic and pharmacokinetic relationship of flavone acetic acid: an agent with activity against solid tumors. *Cancer Treat Rev* **70,** 1415–1421, 1986.
35. Bissery MC, Valeriote FA, Chabot GG, Crissman JD, Yost C, et al.: Flavone acetic acid (NSC-34 7512)-induced DNA damage in Glasgow osteogenic sarcoma in vivo. *Cancer Res* **48,** 1279–1285, 1988.
36. Kono K, Salazaronfray F, Petersson M, Hansson J, Masucci G, et al.: Hydrogen peroxide secreted by tumor-derived macrophages down-modulates signal-transducing zeta molecules and inhibits tumor-specific T cell- and natural killer cell-mediated cytotoxicity. *Eur J Immunol* **26,** 1308–1313, 1996.
37. MacMicking J, Xie Q-W, and Nathan C: Nitric oxide and macrophage function. *Annu Rev Immunol* **15,** 323–350, 1997.

Copyright of Nutrition & Cancer is the property of Lawrence Erlbaum Associates and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.