Veterinary Research Communications, 29 (2005) 575–593

Peroral Application of Water-soluble Derivative of Propolis (WSDP) and Its Related Polyphenolic Compounds and Their Influence on Immunological and Antitumour Activity

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Oršolic, N. Šver, L., Terzić, S. and Bašić, I., 2005. Peroral application of water-soluble derivative of propolis (WSDP) and its related polyphenolic compounds and their influence on immunological and antitumour activity. *Veterinary Research Communications*, **29(7)**, 575–593

ABSTRACT

Polyphenolic compounds are widely distributed in the plant kingdom and display a variety of biological activities, including chemoprevention and growth inhibition of tumours. Propolis contains a conglomerate of polyphenolic compounds. We investigated the effect of propolis and polyphenolic compounds, components of propolis, on the growth and metastatic potential of a transplantable mammary carcinoma (MCa) of the mouse. Metastases in the lung were generated by 2×10^5 tumour cells injected intravenously (i.v.). A water-soluble derivative of propolis (WSDP) and the polyphenolic compounds (caffeic acid (CA) and caffeic acid phenethyl ester (CAPE)) were given to mice perorally before or after tumour cell inoculation. WSDP, CA and CAPE reduced the number of metastases in the lung. This implies that the antitumour activities of the compounds used in these studies are mostly related to the immunomodulatory properties of the compounds, their cytotoxicity to tumour cells, and their ability to induce apoptosis and/or necrosis.

Keywords: apoptosis, immunomodulation, macrophages, metastases, nitric oxide, polyphenolic compounds, propolis, tumour

Abbreviations: CA, caffeic acid; CAPE, caffeic acid phenethyl ester; CD, cluster differentiation; Con A, Concanavalin A; FITC, fluorescein isothiocyanate; LAF, lymphocyte activation factor; LPS, lipopolysaccharide; MCa, mammary carcinoma; NO, nitric oxid; PBS, phosphate-buffered saline; PE, phycoerythrin; PFC, plaque-forming cell; PHA, phytohaemagglutinin; PI, propidium iodide; PWM, pokeweed mitogen; SRBC, sheep red blood cells; WSDP, water-soluble derivative of proplis

INTRODUCTION

Propolis is alleged to exhibit a broad spectrum of activities including antibiotic (Grange and Davey 1990), anti-inflammatory (Wang *et al.*, 1993), antioxidant (Galati *et al.*, 2000; Femia *et al.*, 2001), antiviral activity and tumour cell arrest activities (Kimoto *et al.*, 1998; Kawabe *et al.*, 2000; Lou *et al.*, 2001). During the last 12 years several reports on immunomodulatory activities of aqueous extracts of propolis have been published (Dimov *et al.*, 1992; Orsi *et al.*, 2000). Dimov and colleagues (1992)

showed that a water-soluble extract of propolis (WSDP) increased the protection againt Gram-negative infections in mice; this was mediated by macrophage activation.

The literature suggests that antibiotic activity, immunomodulatory properties as well as anti-inflammatory, wound healing and antitumour effects are exhibited by various components of ethanolic or aqueous extracts of propolis. At least 200 constituents of propolis are defined as terpenes, various phenylpropane derivatives such as caffeic acid esters, flavonoids, amino acids or various aldehydes and ketones (Greenaway *et al.*, 1991; Bankova *et al.*, 1994); propolis contains around 25% caffeic acid (Ca) and caffeic acid phenethyl ester (CAPE).

CAPE exhibits differential toxicity to cancer cells compared with normal cells (Su *et al.*, 1995). It was shown that CAPE and several other caffeic acid esters inhibited enzyme activities, including ornithine decarboxylase, tyrosine kinase and lipoxygenase, associated with colon carcinogenesis (Frenkel *et al.*, 1993).

We investigated the effect of propolis and some major polyphenolic compounds, constituents of propolis, on the growth and metastatic potential of a transplantable mammary carcinoma (MCa) of the CBA mouse and possible modes of antitumour activity.

MATERIALS AND METHODS

Mice

Animal studies were carried out according to the guidelines in force in the Republic of Croatia (Law on the Welfare of Animals, N. N. # 19, 1999) and in compliance with the Guide for the Care and Use of Laboratory Animals, DHHS Publ. # (NIH) 86-123. Male and female CBA inbred mice from our conventional mouse colony were used. In any experiment, mice were of the same sex and were approximately 3 months old at the initiation of each study. The animals were kept not more than five to a cage and were maintained on a pellet diet and water *ad libitum*. Experimental groups comprised 9–13 mice each.

Tumour

A transplantable mammary carcinoma (MCa) of spontaneous origin in the CBA mouse was used. The tumour is weakly immunogenic for syngeneic recipients, as shown by skin grafting (Bašić and Varga, 1979).

Tumour-cell suspension

Single-cell suspensions were prepared by digestion of tumour tissue with trypsin (Oršolić and Bašić, 2003). Each suspension was passed through a stainless-steel mesh (80 wires/cm), centrifuged three times at 24*g* for 5 min in saline, and then resuspended

in RPMI 1640 medium (Institute of Immunology, Zagreb, Croatia) supplemented with 5% serum from normal syngeneic mice. Viability of cells was determined in a haemocytometer by observing the ability of intact cells to exclude Trypan blue dye and by phase-contrast microscopy; viability was over 95%.

Production of tumour nodules (metastases, colonies) in the lung

Metastases in the lung were generated by injecting 2×10^5 viable tumour cells suspended in 0.5 ml RPMI 1640 medium supplemented with 5% syngeneic mouse serum into the tail vein of mice. Twenty-one days later, the mice were killed and their lungs were removed. The lobes were separated and fixed in Bouin's solution. Colonies of tumour 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 developed deep inside the pulmonary lobes.

Treatment with a water-soluble derivative of propolis (WSDP)

A water-soluble derivative of propolis (WSDP) was prepared by a method described elsewhere (Nikolov *et al.*, 1987). Briefly, samples from Croatian (Cr) propolis from beehives kept at the outskirts of Zagreb, Croatia or Brazilian (Br) propolis (CONAP, Belo Horizonte, Minas Gerais, Brazil) were extracted with 96% ethanol, and extracts were filtered and evaporated to dryness 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-brownish powder. WSDP was stored under sterile conditions at 4°C. Before use, the WSDP was dissolved in distilled water and was given *per os* (p.o.) to mice at doses of 50 or 150 mg/kg. According to Nikolov and colleagues (1987), WSDP contains m/v caffeic acid 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-phenylethyl caffeate 1.2%, flavonoids 32.9%, esters of phenylic acid 20.9%.

Polyphenolic compounds

Caffeic acid (CA; 3,4-dihydroxycinnamic acid) was purchased from Aldrich Chemie, Milwaukee, WI, USA. CA was dissolved in ethanol and further dilutions were prepared with water; the final concentration of ethanol was less than or equal to 0.1%. CA was given p.o. mice at doses of 50 or 150 mg/kg.

Caffeic acid phenethyl ester (CAPE) was synthesized by esterification of caffeic acid with phenethyl alcohol (molar ratio 1:15) in benzene (refluxing, 3–4 days, water removed by Dean–Stark trap). Following work-up, excess phenethyl alcohol was removed by Kugelröhr distillation (60°C, <0.1 mmHg) to give pure CAPE, m.p.

126–128°C, needles (benzene or H_2O), 40% yield. It has been shown that properties of natural and synthetic CAPE are identical (Grunberger *et al.*, 1988). CaPE was dissolved in ethanol and further dilutions were made in water; the final concentration of ethanol was less than or equal to 0.1%. CAPE was given p.o. to mice at doses of 50 or 150 mg/kg.

Cell lines

Experiments were performed on human cervical carcinoma cells (HeLa). The average doubling time of cells in log phase was about 20 h. Cells were grown in monolayer cultures in plastic Petri dishes (Falcon, Nunc, Denmark) containing Minimal Essential Medium (MEM; Imunološki zavod, Zagreb, Croatia) with 10% fetal calf serum. Cultures were incubated at 37°C in humidified atmosphere containing 5% CO_2 in air.

Macrophage–HeLa cell co-culture

At the 5th, 10th, 15th and 20th days after treatment with WSDP, CA, or CAPE (50 mg/kg, once a day, for 3 consecutive days), peritoneal macrophages were collected by a single washing of the peritoneal cavity with 5 ml of tissue medium 199 supplemented with 100 μg/ml streptomycin and 100 U/ml penicillin (Flow Laboratories, Irvine, UK). Cell suspensions at concentrations of 1×10^6/ml were plated on 35 mm diameter wells (Falcon). After culture for 24 h at 37°C in 5% CO_2, macrophage supernatants were collected and stored at –20°C, for measurement of nitric oxide. Macrophage monolayers were than washed twice with medium 199 and 5×10^4 tumour cells well were seeded for 24 h. After 6 h, 2.5 μCi [^{3}H]thymidine (specific activity 5 Ci/mmol/L; Sigma, Germany) was added to cultures for 16–18 h. Cells were collected by trypsinization and the radioactivity was determined in a β-scintillation counter (LKB, Wallac, Sweden).

Nitrite and nitrate determination

Nitrite (NO_2^-) concentrations in the cell-free macrophage supernatants were measured by colorimetric Griess reaction as described by Stuehr and Nathan (1989), after the conversion of nitrate to NO_2^- by the enzyme nitrate reductase, as an indicator of nitric oxide (NO) production. Briefly, 50 μl sample aliquots, diluted if needed, were mixed with an equal volume of Griess reagent (1% sulphanilamide, 0.1% naphthylethylene-diamine dihydrochloride in 2% H3PO4) and incubated at room temperature for 10 min. The absorbance at 550 nm was measured in a microplate reader (Bio-Tek Instruments, Burlington, VT, USA). NO_2^- was determined using $NaNO_2$ as a standard and double-distilled water as a blank. Background NO_2^- values of buffers or media were determined in each case and subtracted from the experimental values.

Macrophage cultures and lymphocyte activation factor (LAF) activity assay

At days 3, 5, 7, 10 and 13 after treatment with the WSDP (50 mg/kg, once a day, for 3 consecutive days), mice were killed and peritoneal macrophages were collected by a single washing of the peritoneal cavity with 5 ml of RPMI 1640 medium supplemented with 20 mmol/L Hepes, 100 μg/ml streptomycin and 100 U/ml penicillin (Flow Laboratories). Cell suspensions containing 2×10^6/ml peritoneal cells were plated into 35 mm diameter wells (Falcon). After 24 h, macrophage supernatants were collected and LAF levels were determined by augmentation of [^{3}H]thymidine incorporation in mouse thymocytes (Meltzer and Oppenheim, 1977). Briefly, fresh single-cell suspensions of the thymus of CBA mouse (1×10^6 cells in 0.1 ml) were cultured with 0.1 ml of the macrophage supernatants at 1:2 dilution for 40 h in a 96-well microtissue culture plate. The culture medium contained a submitogenic concentration (1 μg/ml) of phytohaemagglutinin (PHA) (Sigma, St Louis, MO, USA) and 2.5×10^{-5} mol/L 2-mercaptoethanol (Merck, Darmstadt, Germany). Thymic cell cultures were pulsed for 6 h with 1 μCi of [^{3}H]thymidine (specific activity 5 Ci/mmol/L; Sigma, Germany).

Weight and cellularity of spleen and femur, and leukocyte count

WSDP, CA, or CAPE (50 mg/kg) were given p.o. on days 15, 10 and 5 before the measurements. Spleens from contol or the WSDP-, CA-, or CAPE-treated mice were removed and weighed. The spleen was minced and passed through a stainless-steel mesh and single-cell suspensions were made. The 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 suction in and out of a syringe, suspended in 10 ml of saline and counted in a haemocytometer. Samples of blood from control or treated mice were obtained from the tail vein and the leucocyte count was determined in a haemocytometer.

Response of spleen lymphocytes to polyclonal mitogens

Blastogenic responses of spleen lymphocytes to mitogens were assayed on day 7 after i.v. inoculation of WSDP (50 mg/kg) or CA (50 mg/kg). Routine cultures were executed in triplicate in sterile microtitre plates (Falcon). Each well contained 2.5×10^5 spleen lymphocytes in 0.25 ml culture medium (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum). Phytohaemagglutinin (PHA), concanavalin A (Con A), pokeweed mitogen (PWM), and lipopolysaccharide (LPS) (all from Sigma) were used for stimulation. Final concentrations of PHA and Con A were 5 μg/ml, while 10 μg/ml of PWM and LPS were added to each culture. Cultures were incubated for 72 h in a humidified atmosphere containing 5% CO_2. Microcultures were labelled with 1 μCi of [^{3}H]thymidine (specific activity 5 Ci/mmol/L; Sigma, Germany) 18 h prior to 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.

Plaque-forming cells (PFC) assay

A haemolytic assay for the assessment of antibody-producing cells among splenocytes from either normal or WSDP-treated mice (50 mg/kg) was performed as described by Jerne and Nordin (1963). The suspension of splenocytes (0.025 ml) was mixed with 0.5 ml of agar and 0.5 ml of sheep red blood cells (SRBC), and placed onto agar on a glass slide. After 1 h incubation at 37°C, guinea-pig complement was added and slides were additionally incubated for 2 h at 37°C. Complement was then removed by vacuum pump and the PFC were counted on at least three slides per group using a light microscope.

Serum protein determination

Blood samples were obtained from mice by puncture of the axillary vein. Total serum protein levels were determined by the biuret method (Kenneth and Chavin 1975) using a spectrophotometer (UV-160, Shimadzu, Kyoto, Japan) operating at a wavelength of 546 nm. Estimates of total serum immunoglobulins were performed by semi-micro-electrophoresis on gelatinized cellulose acetate strips (Cellogel, Chemetron, Milan, Italy) as described in detail elsewhere (Stato and Kasai, 1965; Kohn, 1968).

Lymphocyte phenotyping

Spleen lymphocytes were obtained from spleens disrupted in phosphate-buffered saline, Ca^{2+}- and Mg^{2+}-free (PBS). Cell suspensions were filtered through a double layer of sterile gauze, then on a double-layer column (wetted with PBS) and centrifuged at 400*g* at 4°C for 5 min. The pellet was washed and the recovered cells were layered on a Ficol–Histopaque gradient (Sigma, St Louis, MO) and centrifuged at 700*g* for 20 min at 18°C. The ring at the interface was washed three times and the resulting pellet was diluted in PBS containing 0.5% BSA and 0.1% NaN_3. Labelling with anti-mouse monoclonal antibodies was done in the dark on ice, and lasted 30 min. To aliquots containing 10^6 viable cells, anti-mouse mAb, CD4 (0.5 μg) or CD8 (0.1 μg) (Pharmingen, San Diego, CA, USA) were added. Controls were prepared with 10^6 cells labelled with non-specific $IgG_{2a,k}$ FITC (0.5 μg) or $IgG_{2b,k}$ phycoerythrin (PE) (0.1 μg); a sample containing only cells was used for autofluorescence determination. After washing, cells were resuspended in 0.5 ml of medium mixed with 10% of a solution of 35% formaldehyde. Each analysis consisted of 10 000 events counted.

Apoptosis/necrosis analysis

Apoptosis was determined by techniques described by Telford and colleagues (1994). 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 fetal bovine serum) for various times (3 and 15 h). After treatment, 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 mmol/L $CaCl_2$). Fluorescein-labelled annexin V and propidium iodide (PI) (Apoptosis Detection Kit; R&D Systems, Weisbaden, Germany) were added to the cells. After 15 min incubation at room temperature, cells were analysed by a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). A minimum of 5000 events were collected and analysed using CellQuest software. This method 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 $\pm$ SE obtained from 2 or 3 experiments. Statistical significance was evaluated using Student's *t*-test.

RESULTS

Antimetastatic effect of WSDP and its compounds

The effect of WSDP, CA or CAPE on metastasis formation in the lung was studied in mice injected i.v. with tumour cells. All test compounds were given p.o.; the doses applied were 50 mg/kg or 150 mg/kg of WSDP, CA and CAPE, respectively. WSDP, CA and CAPE were given to mice 15, 10 and 5 days prior to tumour cell inoculation, for study of the preventive effect on lung metastasis formation, while in a curative test compounds were given to mice 2, 7 and 12 days after tumour cell inoculation. Mice were killed 21 days after tumour cell inoculation and the numbers of metastases in their lung were determined.

Table I shows that the number of tumour nodules in the lung of mice treated with WSDP, CA and/or CAPE was significantly lower ($p < 0.01$ or $p < 0.001$) than in untreated mice.

TABLE I
Protection against MCa metastases in the lung by WSDP, caffeic acid or CAPE in preventive and curative treatments

Treatment	Dose (mg/kg; p.o.)	Preventive effect[a] Lung metastases[c] (mean no. /lung ± SE)	Range	Significance (Student's *t*-test)	Curative effect[b] Lung metastases[c] (mean no./ lung ± SE)	Range	Significance (Student's *t*-test)
Control	–	62.71 ± 6.3	38–86		62.71 ± 6.3	38–86	$p < 0.001$
Croatian WSDP	50	16.00 ± 2.35	11–21	$p < 0.001$	15.66 ± 2.97	5–29	$p < 0.001$
	150	22.5 ± 3.75	13–41	$p < 0.001$	17.42 ± 3.19	3–27	$p < 0.001$
Brazilian WSDP	50	23.7 ± 2.3	10–41	$p < 0.001$	30.20 ± 3.7	17-40	$p < 0.001$
	150	17.3 ± 3.5	11–35	$p < 0.001$	16.11 ± 2.16	8–26	$p < 0.001$
Caffeic acid	50	24.66 ± 6.26	9–55	$p < 0.01$	39.00 ± 5.3	23–57	$p < 0.01$
	150	36.83 ± 3.87	27–55	$p < 0.01$	47.00 ± 6.7	23–60	$p < 0.01$
CAPE	50	22.00 ± 2.84	11–30	$p < 0.001$	33.66 ± 7.2	20–31	$p < 0.01$
	150	32.37 ± 2.97	22–44	$p < 0.001$	43.50 ± 5.03	30–57	$p < 0.01$

[a]WSDP, caffeic acid or CAPE was given p.o. 5, 10 and 15 days before tumour cell inoculation

[b]WSDP, caffeic acid or CAPE was given p.o. 2, 7 and 12 days after tumour cell inoculation

[c]2×10^5 tumour cells/mouse were injected i.v.; the number of tumour metastases in the lung was determined 21 days after tumour cell inoculation. Groups comprised 7–9 mice each; values are mean ± standard error

Influence of WSDP, CA or CAPE on weight and cellularity of spleen and femur, and on blood leukocyte count

WSDP and related polyphenolic compounds caused profound changes in spleen cellularity and less pronounced changes in cellularity of bone marrow, while no effect on blood leukocyte count was detected (Table II).

TABLE II
The effect of WSDP (50 mg/kg), CA (50 mg/kg) and CAPE (50 mg/kg) on haematological parameters in CBA mice. Animals were treated p.o. on the 15th, 10th and 5th days before determination of the parameters listed

Treatment (p.o.)	Leukocytes ($\times 10^3 \pm$ (mean $\pm$ SE)	Spleen weight (mg) (mean $\pm$ SE)	Spleen cellularity ($\times 10^6$) (mean $\pm$ SE)	Femur bone marrow cellularity ($\times 10^6$) (mean $\pm$ SE)
–	6.789 ± 0.424	69.5 ± 5.6	104.817 ± 4.269	12.695 ± 0.746
WSDP	7.927 ± 0.589	72.7 ± 1.1	171.224 ± 10.231**	13.411 ± 0.689
Caffeic acid	7.109 ± 0.766	74.5 ± 1.75	164.062 ± 8.131**	13.541 ± 0.567
CAPE	6.484 ± 0.295	75.25 ± 3.59	141.276 ± 11.736*	17.057 ± 1.242*

Groups comprised 7–10 mice each

Asterisks indicate values that are significantly higher (*$p < 0.05$; **$p < 0.01$) than those of corresponding untreated mice

Influence of WSDP on macrophage activity and production of lymphocyte activation factor (LAF)

Adherent peritoneal cells (approximately 95% macrophages) from the WSDP-treated mice when cultured 24 h *in vitro* produced lymphocyte activation factor (LAF), which was detected by *in vitro* incorporation of [*methyl*-^{3}H]thymidine ([^{3}H]TdR) into mouse thymocytes. It was shown that supernatants of macrophages from WSDP (50 or 150 mg/kg) treated mice added to cultures of thymocytes elevated incorporation of [^{3}H]TdR 2.6-fold or 3.4-fold as compared to controls (Figure 1).

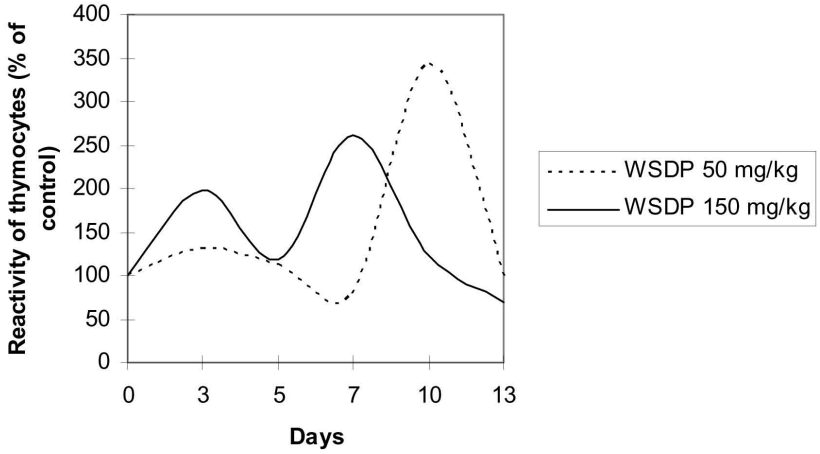

Figure 1. *In vitro* LAF activity of peritoneal macrophages after treatment of CBA mice with WSDP. Mice (n = 6) were treated i.p. with WSDP (50 mg/kg, once a day, for 3 consecutive days) and macrophages were collected at various times. Macrophages (2×10^6/ml) were plated into 35 mm diameter wells and the level of LAF in the 24 h macrophage supernatants was determined by [^{3}H]thymidine incorporation in mouse thymocytes

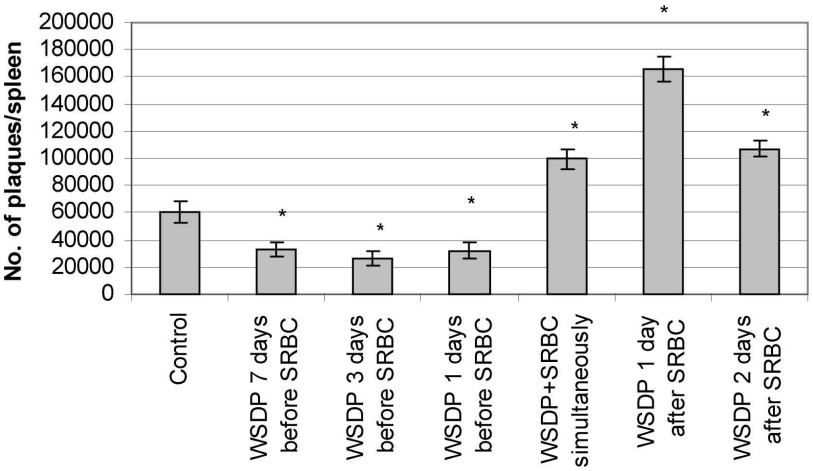

Figure 2. Mean ($\pm$SE) numbers of splenic PFC in CBA mice injected i.v. (50 mg/kg) with WSDP 7 days, 3 days and 1 day before, simultaneously with, or 1 and 2 days after immunization with 4×10^8 SRBC in 0.5 ml of RPMI 1640 medium. Animals were killed 4 days after immunization with SRBC. Values are mean ($\pm$SE) from 12–15 samples (5 mice in triplicate). *Significantly ($p < 0.001$) different from untreated controls

Effect of WSDP on the spleen plaque-forming ability

To study the effect of WSDP on the spleen plaque-forming ability, treated mice were immunized with SRBC (4×10^8, intraperitoneally) at different intervals before or after treatment with WSDP. Four days after immunization, the ability of their splenocytes to produce antibodies to SRBC was determined. Figure 2 shows that treatment with WSDP did not affect the production of PFC when given 7 days, 3 days or 1 day before SRBC as antigen. However, when WSDP was injected to mice at the time of injection of SRBC or 24 or 48 h after SRBC, a significant elevation of PFC was found; the most pronounced effect was achieved in the group of mice receiving WSDP 24 h after SRBC treatment. It is likely that the dose of WSDP plays an important role in PFC formation. When different doses of WSDP were given to mice within 24 h (at 8 h intervals) after injection of SRBC, a significant elevation of PFC production was found only in mice receiving 75 mg/kg of WSDP (Figure 3).

Effect of WSDP on specific serum protein levels

In studies on the effect of WSDP on specific serum protein levels in mice treated with WSDP before and after immunization with SRBC, we detected the concentrations of specific proteins in the serum of mice receiving WSDP (50 mg/kg i.v.) before or after immunization with antigen (Table III) Significant differences were found between the

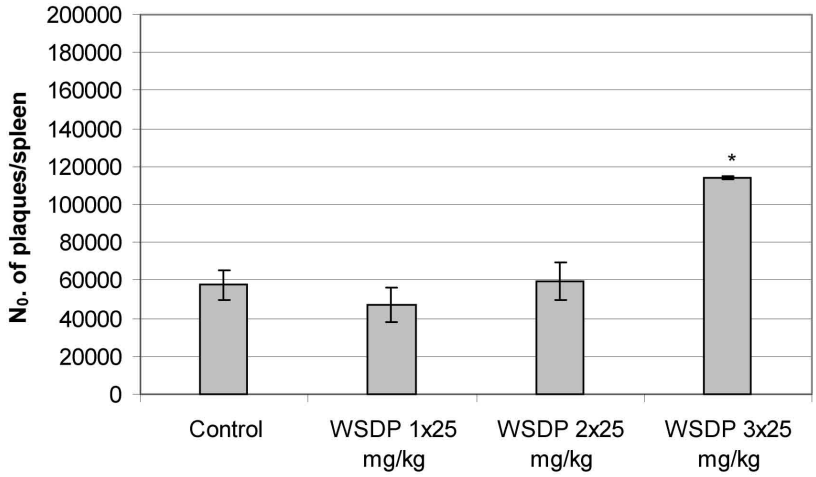

Figure 3. Mean (±SE) numbers of splenic PFC in CBA mice injected i.v. with 1–3 repeat doses (25 mg/kg) WSDP, with 8 h between doses and 24 h after immunization with 4×10^8 SRBC in 0.5 ml of RPMI 1640 medium. Animals were killed 4 days after immunization with SRBC. Values are mean (±SE) from 12–15 samples (5 mice in triplicate). *Significantly ($p < 0.01$) different from untreated controls

TABLE III
Specific serum protein levels in mice after treatment with WSDP

Treatment[a] (post-treatment day)	Specific serum proteins (g/L)[b]				
	Albumin	α_1-Globulin	α_2-Globulin	β-Globulin	γ-Globulin
–	45.73 ± 0.21	7.81 ± 0.52	4.59 ± 0.33	9.67 ± 0.20	5.37 ± 0.29
WSDP					
–7	48.44 ± 0.44**	6.20 ± 0.17*	4.03 ± 0.08	7.65 ± 0.50*	8.47 ± 0.21***
–3	45.72 ± 0.22	6.47 ± 0.30	4.13 ± 0.12	10.28 ± 0.29	7.94 ± 0.03***
–1	49.81 ± 0.36***	8.04 ± 0.07	5.42 ± 0.23	11.27 ± 0.27**	6.33 ± 0.15*
0	27.86 ± 0.23***	4.29 ± 0.12**	3.30 ± 0.11*	7.09 ± 0.12***	4.06 ± 0.12*
1	32.28 ± 0.19***	9.52 ± 0.23*	4.07 ± 0.12	12.48 ± 0.12***	4.62 ± 0.17
2	33.70 ± 0.21***	10.63 ± 0.07**	5.89 ± 0.09*	14.22 ± 0.17***	5.30 ± 0.15

[a]Groups comprised 5 mice each

[b]CBA mice injected i.v. (50 mg/kg) with water-soluble derivative 7, 3, 1 days before, simultaneously with, or 1 and 2 days after immunization with 4×10^8 SRBC in 0.5 ml of RPMI 1640 medium. Animals were killed 4 days after immunization with SRBC. Values are mean ± standard error

Asterisks indicate values that are significantly (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) different from corresponding untreated controls

levels of albumin and γ-globulins in treated and control mice. The levels of γ-globulin or albumin in WSDP-treated mice were increased ($p < 0.05$; $p < 0.01$; $p < 0.001$) when mice were given WSDP before immunization with antigen; γ-globulin levels were higher when the time between WSDP and antigen was longer. Treatment with WSDP at the time of antigen injection resulted in the lowest level of γ-globulins. The quantity of albumin in WSDP-treated mice was significantly decreased ($p < 0.001$) when WSDP was given after immunization with SRBC.

Influence of WSDP on T lymphocyte population

In the study of the influence of WSDP on the T lymphocyte population, clustering of splenocytes was a function of the percentage of cells showing $CD4^+$ and $CD8^+$ antigens. The study performed on mice injected i.v. with MCa tumour cells and treated with WSDP (25 or 50 mg/kg body weight, respectively) on days 2, 7 and 12 after injection of tumour cells showed that WSDP significantly increased the percentage of $CD8^+$ cells and, to a lesser extent, the percentage of $CD4^+$ cells as compared to untreated tumour-bearing mice (Figure 4). However, treatment of tumour-bearing mice with WSDP caused a reduction in the ratio between $CD4^+$ and $CD8^+$ lymphocytes: 1.4 ± 0.5 in control compared to 0.8 ± 0.3 in both groups of WSDP-treated mice.

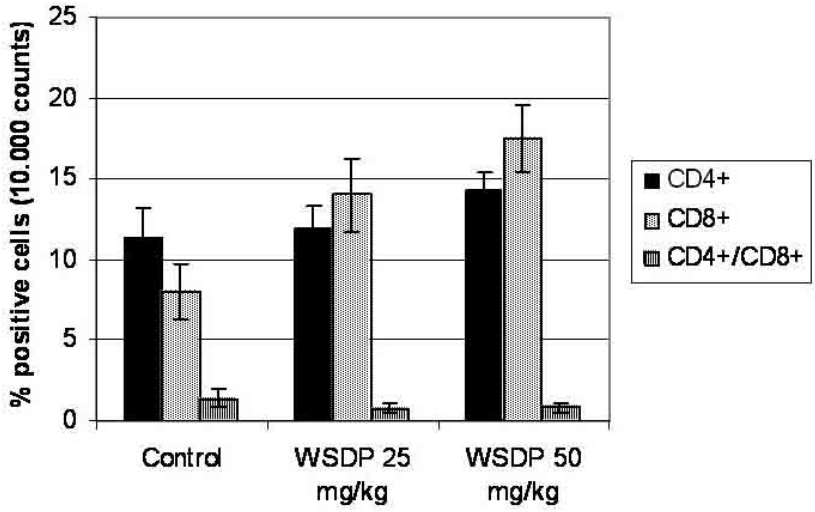

Figure 4. $CD4^+$ and $CD8^+$ expression on splenocytes of mice with MCa mammary carcinoma metastases in the lungs 18 days after i.v. injection of tumour cells (2×10^5). Mice were treated i.v. with 25 or 50 mg/kg of WSDP on days 2, 7 and 12 after injection of tumour cells

Effect of activated macrophages on DNA synthesis of HeLa cells and production of NO by activated macrophages

Table IV shows that peritoneal macrophages of mice treated with 50 mg/kg of WSDP when co-cultured with HeLa cells produced significantly higher amounts of NO ($p < 0.05$) at 10 and 15 days after treatment. At the same time, the percentage of [^{3}H]TdR incorporation into tumour cells was lower than that of control cells. Peroral treatment with CAPE also elevated the production of NO at days 5 and 10 after treatment, which declined by 15 days following treatment. Incorporation of [^{3}H]TdR into HeLa cells was strongly suppressed during elevation of NO production. On the other hand, in mice treated with CA a significant ($p < 0.01$) drop in production of NO was found throughout the observation period (Table IV). In contrast, peritoneal macrophages from mice treated with CA expressed very strong cytotoxicity to HeLa cells as compared to control.

Effect of WSDP and CA on splenocyte responses to polyclonal mitogens

Since WSDP increased the production of NO in treated mice, and since it has been shown that NO can influence immunological reactions, we tested the effect of treatment with WSDP and CA given p.o. on responses of splenocytes to polyclonal

TABLE IV
Incorporation of [^{3}H]TdR into HeLa cells in activated macrophage/HeLa co-cultures and NO production by macrophages from untreated and WSDP-, caffeic acid- or CAPE-treated mice

Treatment	Intervals[a]	Incorporation of [^{3}H]TdR (%)[b]	NO (μmol/5×10^5 cells) (mean ± SE)[c]
–	–	100.0	16.59 ± 0.29
WSDP	5	69.3	16.50 ± 0.14
	10	32.9	18.05 ± 0.34*
	15	63.4	18.87 ± 0.85*
	20	87.7	16.75 ± 0.35
Caffeic acid	5	25.4	12.86 ± 0.51**
	10	48.1	13.76 ± 0.27**
	15	57.0	14.26 ± 0.12**
	20	78.4	11.00 ± 1.32*
CAPE	5	38.3	19.18 ± 0.45**
	10	48.4	18.23 ± 0.37*
	15	70.0	15.36 ± 0.21*
	20	80.0	14.56 ± 0.48*

[a]Days after p.o. treatment with WSDP, CA or CAPE (50 mg/kg) when macrophages were collected

[b]Values are percentage of controls

[c]Mean ± standard error ($n = 6$)

Asterisks indicate values that are significantly (*$p < 0.05$; **$p < 0.01$) different from the corresponding untreated controls

mitogens. Table V shows that WSDP treatment of mice increased ($p < 0.01$) the responses of spleen cells to all polyclonal mitogens tested (PHA, Con A, PWM and LPS). In contrast, the response of spleen cells of mice treated with CA were significantly suppressed ($p < 0.001$).

Apoptosis and/or necrosis of tumour cells

In the flow cytometry study we examined the rate at which MCa and HeLa cells in the presence of WSDP, CA or CAPE underwent apoptosis or necrosis. Cells were exposed to test compounds for 3 h or 15 h and the effect was analysed by bivariant flow cytometry (image analysis of anexin V- and PI-labelled cells). The percentage of apoptotic MCa cells was 0.56–5.67% after 3 h, while it was 6.02–26.43 after incubation

TABLE V
The effect of WSDP (50 mg/kg) and CA (50 mg/kg) given i.v. to mice CBA on splenocyte responses to phytohaemagglutinin (PHA, 10 μg/ml), concanavalin A (Con A, 10 μg/ml), pokeweed mitogen (PWM, 5 μg/ml) and lipopolysaccharide (LPS, 10 μg/ml)

	[^{3}H]Thymidine[a] incorporation (mean cpm $\times 10^3 \pm$ SE) into stimulated splenocytes[c]			
Treatment[b]	PHA	Con A	PWM	LPS
–	2.55 ± 0.37	43.85 ± 4.9	5.03 ± 0.57	7.93 ± 0.67
WSDP	4.39 ± 0.30*	68.34 ± 1.93*	7.7 ± 0.07*	9.28 ± 0.46*
Caffeic acid	0.41 ± 0.06**	11.04 ± 3.03*	0.21 ± 0.03**	0.23 ± 0.04**

[a]Three mice per group received i.v. 50 mg/kg WSDP or CA

[b]Incorporation of [^{3}H]thymidine by splenocytes expressed as mean counts per minute $\pm$ SE of 9 samples (3 mice in triplicate)

[c]Cells were taken 7 days after treatment

Asterisks indicate values that are significantly (*$p < 0.01$; **$p < 0.001$) different from the corresponding untreated controls

with the test compounds for 15 h (Figure 5). Maximum apoptosis (26.43%) was visible in MCa cultures of cells treated with CAPE (10 μg/ml) after 15 h. The percentage of necrotic cells was highest in CAPE-treated MCa cells (32.19%) compared to other compounds used (data not shown). CA and CAPE induced apoptosis in HeLa cells in a time-dependent fashion (Figure 6), whereas they had no significant effect on necrosis rate in HeLa cells (data not shown). The highest percentage of apoptosis (37.58%) was seen in HeLa cells after 15 h of treatment with 5 μg/ml of CAPE.

DISCUSSION

This investigation clearly demonstrated the inhibitory effects of WSDP, CA or CAPE on metastasis formation of mammary carcinoma (MCa) in CBA mice treated either preventively or curatively (Table I). The antimetastatic effect of WSDP was of higher degree than that achieved by either CA or CAPE. It is likely that the antitumour activities of WSDP, CA and CAPE are strongly dependent on the dose of the compounds; smaller doses exhibited stronger antitumour activity. It has been demonstrated (Frenkel *et al.*, 1993) that higher doses of CAPE interfered with cell processes such as enzyme and glutathione levels, including macrophage activities; this may also apply to both WSDP and CA.

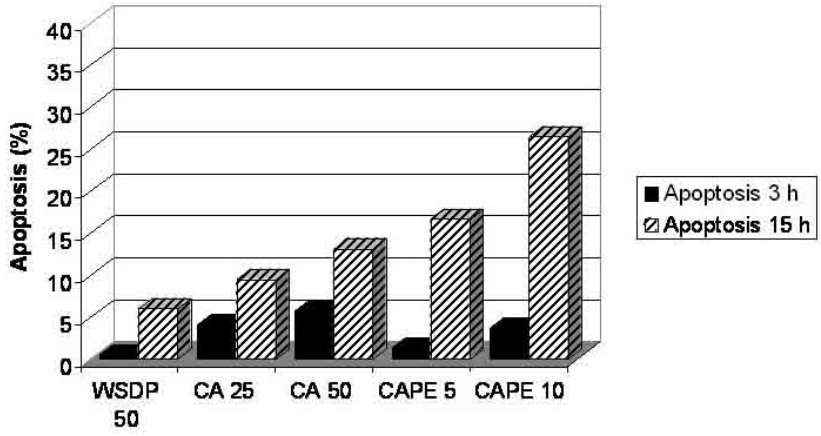

Figure 5. Effect of WSDP and related polyphenolic compounds on induction of apoptosis in MCa cells. Cells were cultured in the presence or absence of WSDP (50 μg/ml) and CA (25 or 50 μg/ml) or CAPE (5 or 10 μg/ml) for 3 or 15 h, washed, stained with fluorescein-labelled anexin V and analysed by flow cytometry. Results are the percentage of apoptosis obtained by subtraction of treated and control samples

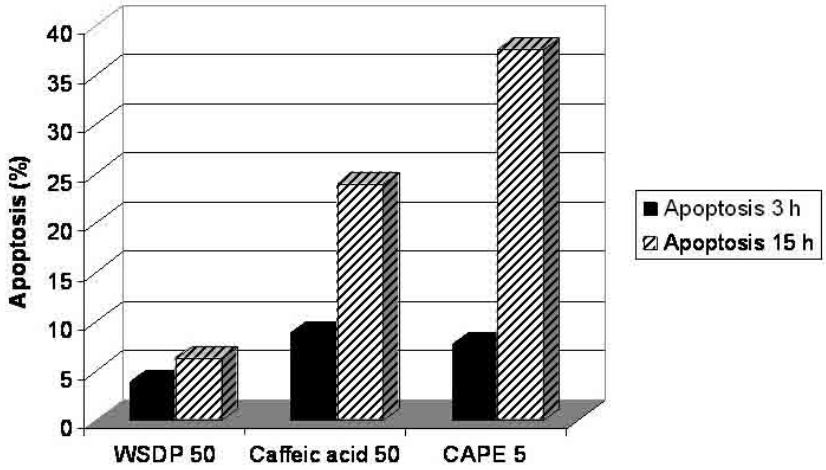

Figure 6. Effect of WSDP and related polyphenolic compounds on induction of apoptosis in HeLa cells. Cells were cultured in the presence or absence of WSDP (50 μg/ml) and CA (50 μg/ml) or CAPE (5 μg/ml) for 3 or 15 h, washed, stained with fluorescein-labelled anexin V and analysed by flow cytometry. Results are the percentage of apoptosis obtained by subtraction of treated and control samples

Since immunomodulation is known to be of importance in controlling tumour growth and spread, we studied the effect of WSDP and its polyphenolic components on haematological and immunological parameters. We demonstrated that the mitogenic effect of supernatant of macrophages from mice treated with WSDP exerted a strong activity of LAF that influenced incorporation of [^{3}H]thymidine in primary cultures of syngeneic mouse thymocytes (Figure 1). Increased levels of LAF activity produced by the WSDP-activated macrophages correlated directly with the reduction of metastases in the lung of treated mice (Table I) and *in vitro* with tumour cytotoxicity (Oršolić *et al.*, 2001; Oršolić and Bašić, 2003). These findings also suggest that WSDP is able to activate macrophages to produce factors regulating the function of B and T cells (Tables II–V; Figures 2–4).

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 is at least in part due to the immunomodulation of the host's interaction with tumour cells. Other possibilities for antitumour activity include the ability of test components to induce apoptosis of tumour cells (Figures 5 and 6).

The findings from these experiments confirm that WSDP is a strong activator of the processes involved in the production of antibodies; it remains to be clarified whether the activation of antibody production is connected with macrophage activation (Orsi *et al.*, 2000) by WSDP or whether other phenomena of immunological reactivity are involved. These findings, however, confirmed that the dose of WSDP is an important factor for activation of the mechanisms involved in antibody production, as well as the time intervals between antigen introduction and treatment with WSDP. Thus, these findings suggest that a continuous presence of WSDP is necessery to ensure activation of mechanisms involved in antibody production, such as macrophage activation and production of factors regulating the functions of B and T lymphocytes (Kurland *et al.*, 1997).

Since interferon gamma (INF-γ), tumour necrosis factor alpha (TNF-α) and interleukin 2 (IL-2), produced by T_H1 lymphocytes as promoters of host defence, induce the synthesis of NO by macrophages (MacMicking *et al.*, 1997; Elgert *et al.*, 1998), it was of interest to check whether WSDP and related polyphenolic compounds (CAPE, CA) influence synthesis of NO. Our studies revealed that peritoneal macrophages of mice treated with 50 mg/kg of WSDP or CAPE, when co-cultured with HeLa cells, produced significantly higher amounts of NO ($p < 0.05$) after treatment (Table IV). At the same time, the percentage [^{3}H]TdR incorporation into tumour cells was lower than in controls (Table IV). In contrast, peritoneal macrophages from mice treated with CA expressed very strong cytotoxicity to HeLa cells as compared to controls (Table IV), suggesting that other mechanisms than that of WSDP and CAPE should be considered. It was shown by Ivanovska and colleagues (1993) and Orsi and colleagues (2000) that treatment with CA elevated the production of H_2O_2 by macrophages of treated mice. Chan and colleagues (1995) and Monks and Lau (1997) demonstrated that CA can act as a pro-oxidant and an effective irreversible inhibitor of glutathione *S*-transferases that causes a decrease in the generation of NO by activated

macrophages. Activated macrophages produce increased level of reactive oxygen species, including H_2O_2, which are known to modulate cellular functions, including those of lymphocytes (Kono *et al.*, 1996). Our results support these findings since the response of spleen cells to polyclonal mitogens was suppressed in mice treated with CA, while WSDP exerted an opposite effect (Table V). These pro-proliferative effects may predominate since WSDP as shown (Figure 1) increased LAF activity, which may be associated with enhanced T and B cell proliferation.

Antitumour activity, according to the results obtained in this study together with a previous one (Bašić *et al.*, 1998), is mostly related to the immunomodulatory properties of the compounds, their cytotoxic activity to tumour cells, and their capacity to induce apoptosis.

In conclusion, these results suggest that WSDP, ca and CAPE could be useful tools in the control of tumour growth in experimental tumour models. It is likely that the antitumour activity of WSDP is the result of synergistic activities of its polyphenolic compounds.

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(Accepted: 1 July 2004)