

Antimetastatic effects of propolis, caffeic acid phenethyl ester and caffeic acid on mammary carcinoma of CBA mouse

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SUMMARY

We have studied the antimetastatic efficacy of water-soluble derivative of Brazilian or Croatian propolis (WSDP) and compared it with antimetastatic effect of caffeic acid phenethyl ester (CAPE) or with caffeic acid (CA). *In vitro* cytotoxicity of these compounds on tumor cells were also studied. Tumor was a transplantable mammary carcinoma (MCA). Metastases in the lung were generated by injecting 2×10^5 viable tumor cells *iv*; tested compounds were given *po* before or after tumor cell inoculation. In *in vitro* studies V79 cells, HeLa cells or MCA cells were used. treatment of mice significantly reduced the number of tumor nodules in the lung. In *in vitro* studies WSDP did not affect the growth of cells, while CAPE and CA expressed strong cytotoxic effect. Antitumor effect of WSDP was in part due to apoptosis of MCA cells.

INTRODUCTION

Propolis from honey beehives contains various chemical constituents that exhibit a broad spectrum of activities including antibacterial, antifungal, antineoplastic, cytostatic, and anti-inflammatory properties (Jeddar et al 1985; Hladon et al 1980; Koshihara et al 1984). Propolis also contains a variety of compounds including caffeic and benzoic acid and their esters, substituted phenolic acids and their esters, flavonoid glycones, and beeswax (Greenaway et al 1987). Some of the above mentioned biological activities of propolis may be due to its chemical constituents (Koshihara et al 1984; Grunberger et al 1988). Several naturally occurring compounds in fruits, vegetables and propolis, such as phenols, indoles, aromatic isothiocyanates and dithiolethiones, have been shown to inhibit several types cancer including the cancer of the colon (Wattenberg 1985). It was also demonstrated that dietary administration of hydroxycinnamates which are constituents of propolis, significantly inhibited benzo(a)pyrene-induced neoplasia in the forestomach of mice (Wattenberg et al. 1985).

Caffeic acid (3,4-dihydroxycinnamic acid) and its esters, (caffeic acid phenethyl ester-CAPE), which are present in propolis at levels of 20-25% (Greenway et al 1987) are the agents suspected of having a broad spectrum of biological activities including tumor suppression. Caffeic acid ester derivatives present in propolis are more lipophilic and thus facilitate their entry into the cells (Greenaway et al 1988). Differential cytotoxicity has been observed in normal rat/human versus transformed rat/human melanoma and breast carcinoma cell lines in the presence of CAPE.

In this studies, we describe the antimetastatic properties of the propolis obtained from Brazil and Croatia in the murine mammary carcinoma model (Bašić) and compare its antitumor efficiency with the antitumor action of CA and/or CAPE. The mode of antitumor actions of these compounds *in vivo* and *in vitro* studies are also described

MATERIALS AND METHODS

Tumor

The tumor was a transplantable mammary carcinoma (MCA), weakly immunogenic to syngeneic CBA mouse (Bašić and Varga 1979). Tumor nodules in the lung were generated by injecting 2×10^5 viable tumor cells intravenously. The mice were killed 21 days after tumor cells inoculation, and the of number of tumor nodules in the lungs was determined.

Water-soluble derivative (WSD) of propolis

The water-soluble derivative (WSD) of propolis was prepared by method described elsewhere (Nikolov et al 1987). Briefly, Brazilian (CONAP, Belo Horizonte, Minas Gerais, Brazil) or Croatian (surroundings of Zagreb) propolis was extracted with 96% ethanol, filtered and evaporated to dryness in 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 WSD, a yellow-brown powder. The WSD was stored under sterile conditions at 4°C. Before use, the

WSD was dissolved in distilled water. It was given to mice per os (*po*) at doses of 50 or 150 mg/kg.

CAPE-Caffeic acid phenethyl ester

CAPE was obtained by modified method of Grunberger et al (1988). In short, esterification of caffeic acid with phenethyl alcohol (molar ratios 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 ($60^{\circ}\text{C} < 0.1 \text{ mm Hg}$) to give pure CAPE. mp $126-128^{\circ}\text{C}$. needles (benzene or H_2O), 40% yield. All properties of natural and synthetic CAPE were identical (Grunberger et al 1988).

CA-Caffeic acid -3,4 dihydroxycinnamic acid

CA was purchased from Aldrich-chemie, Milwaukee, WI, USA. CAPE and CA were given to mice per os (*po*) at doses of 50 or 150 mg/kg.

Cell lines

In *in vitro* experiments, we used human cervical carcinoma cells (HeLa) and Chinese hamster lung V79 fibroblasts. The average doubling time in log phase was about 20 h for HeLa cells and 12 h for V78 cells. HeLa and V79 cells were grown in monolayer cultures in plastic disposable Petri dishes (Falcon) in Minimal Essential Medium (MEM) with 1% non-essential amino acids and 10% fetal calf serum. Cell cultures were incubated at 37°C in humid atmosphere containing 5% CO_2 in air.

Cell counting

Cells were counted using Coulter Counter (Model B, Coulter Electronics, Dunstable, England). Petri dishes with different concentrations of the studied compounds were usually incubated for 72 h. Then, the cells were counted in triplicates. Prior to cell number determination medium was removed, cells were rinsed with phosphate-buffered saline (PBS) trypsinized, diluted and counted (Maysinger et al 1987).

Long-term experiments

Long-term experiments were performed with proliferating cultures. Hundred cells were seeded into the Petri dishes. After 24 h, different concentrations of studied compounds were added and cells were cultured on 37°C for 10 days in air with 5% CO_2 . The medium was then removed and colonies were stained with 10% Giemsa. Colonies containing 50 cells were counted and results were expressed as the percent of colonies of untreated cells. The plating efficiency of untreated control culture was 54%.

Dose-response experiments

Exponential or plateau phase cells were treated with different concentrations of compounds for 24 h. The drugs were then removed and Petri dishes were rinsed three times with PBS. The cells were trypsinized, counted and seeded into Petri dishes (100 cells/dish). After 10 days, the medium was removed and cells were stained as described above. The plating efficiencies of untreated control of exponential cultures were 84 and 75%, respectively. Mean values, standard errors statistical evaluation by the t-test were calculated for each dose or time point.

Apoptosis analysis

Apoptosis was determined by techniques described by Tellford et al (1992). Briefly, bivariate flow cytometry was performed on cells grown in the presence or absence of WSD of propolis, CA and CAPE for various times (3 and 15 hours). The cells were washed in cold PBS twice and resuspended in a small volume of 1X binding buffer. Fluorescein-labeled annexin V and propidium iodide were added to the cells. They were then analysed by flow cytometry.

DNA content of the tested cells was determined by staining with propidium iodide (PI). The cells were incubated in 100 µl of fixing solution for 15 min at 4°C, washed in PBS, resuspended in permeabilizing solution in the presence of 10 µl of PI and incubated at 4°C for 15 min. The cells were then washed with PBS and immediately analyzed by flow cytometry.

RESULTS AND CONCLUSIONS

In this work, we show that WSD of Brazilian and/or Croatian propolis, CA, and CAPE prevent the growth of tumor nodules (artificial metastases) of MCA in mice. The effect of WSD of propolis, CA and CAPE on the metastatic ability of tumor cells was tested in lung colony assay. Metastasis in the lung were generated by injecting 2×10^5 viable tumor cells intravenously. Tasted compounds were given per os before or after tumor cell inoculation: the dose comprises 50mg/kg or 150mg/kg of each compound. Mice were killed 21 days after the treatment and the number of tumor nodules in the lungs was determined. **Table 1** shows that in mice receiving tested compounds, the number of tumor nodules in the lung was significantly lower ($p < 0.05$) than in untreated mice. The anti-metastatic effectiveness of WSD or propolis was well pronounced; anti-tumor effect of WSD was of higher degree than that achieved by either CA or CAPE.

These data are in agreement with our previous findings on the effectiveness of WSD of propolis and CA or CAPE on the subcutaneous growth of MCA (Bašić et al. 1995). It should be stressed out that these compounds also influenced the survival of treated mice.

Some recent publications also indicate strong antitumor effect achieved by flavonoids, the constituents of propolis, in murine tumor models (Verma et al 1988, Gassady et al 1988, Edwards et al 1979, Bissery et al 1988, Corbett et al 1986, Plowman et al 1986, Zachakko et al 1986, Scheller et al 1989).

It was also demonstrated that CAPE and several additional caffeic acid esters inhibited azoxymethane-induced colon preneoplastic lesions and enzyme activities, including ornithine decarboxylase, tyrosine protein kinases and lipoxygenase, associated with colon carcinogenesis (Rao et al 1993; Frenkel et al 1993).

In previous studies we showed that the antitumor activity of WSD of propolis is related to its immunostimulatory properties (Bašić et al 1995). Two modes of antitumor action in the lungs were proposed: a modulation of immune reaction of recipients or induction of apoptosis during the formation of metastatic nodules. Changes in several immunological parameters such as response of lymphocytes to polyclonal mi-

Table 1. The effect of WSD of propolis on MCA lung nodule formation in CBA mice. Animals were treated with 50 or 150 mg/kg of tested compounds before or after tumor cell inoculation

TREATMENT	DOSE mg/kg	TIME OF TREATMENT *	No OF TUMOR NODULES / LUNG (mean \pm SE) ^c	RANGE	
NO TREATMENT	/	/	144,30 \pm 11,75	122 - 196	
CROATIAN PROPOLIS	50	15, 10 and 5 DAYS BEFORE TUMOR CELL INOCULATION ^b	5,37 \pm 2,27	0 - 7	
	150		13,77 \pm 2,21	4 - 23	
BRAZILIAN PROPOLIS	50		7,75 \pm 1,35	0 - 13	
	150		9,55 \pm 2,16	1 - 19	
CAFFEIC ACID	50		15,00 \pm 3,55	4 - 38	
	150		15,44 \pm 2,07	7 - 29	
CAPE	50		10,62 \pm 1,94	0 - 19	
	150		15,5 \pm 4,81	5 - 38	
CROATIAN PROPOLIS	50		2, 7 and 12 DAYS AFTER TUMOR CELL INOCULATION ^b	15,66 \pm 2,97	5 - 29
	150			17,42 \pm 3,19	3 - 27
BRAZILIAN PROPOLIS	50			30,20 \pm 3,70	17 - 40
	150			16,11 \pm 2,16	8 - 26
CA ^d CA	50	47,00 \pm 6,71		33 - 68	
	150	70,00 \pm 12,74		50 - 113	
CAPE ^e CAPE	50	36,66 \pm 7,27		20 - 71	
	150	53,50 \pm 5,03		40 - 67	

- a Tested compounds were given *per os* (*po*) before and 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.
- c Groups comprised 7 - 9 mice each (mean \pm standard error)
- d Caffeic acid
- e Caffeic acid phenethyl ester

togens *in vitro*, production of IL-1 by peritoneal macrophages, rosette formation of lymphoid cells with SRBC and plaque forming ability of splenocytes to SRBC, respectively, correlated well with the antimetastatic properties, inhibitory effect on tumor growth, and tumor take, of tested compounds. These results are in line with previous results from other laboratory, suggesting that the stimulatory activity of WSD of propolis may be associated with activation of macrophages, which leads to an increase of their phagocytic capacity (Dimov et al 1992; 1991; Ivanovska 1993; 1995; Tatefuji et al 1996; Mirzoeva 1996). Increased level of IL-1 activity produced by these activated macrophage correlated directly with tumor cytotoxicity (unpublished). Activation of macrophages is important for the immunogenic property to the extract, as it leads to the production of factors regulating the functions of B and T-cells (Kurland et al 1977). The activated macrophage is a major component of host defence against infectious and neoplastic disease (Maltzer et al 1977). Example of this activity was demonstrated *in vitro*: ethanolic extract of

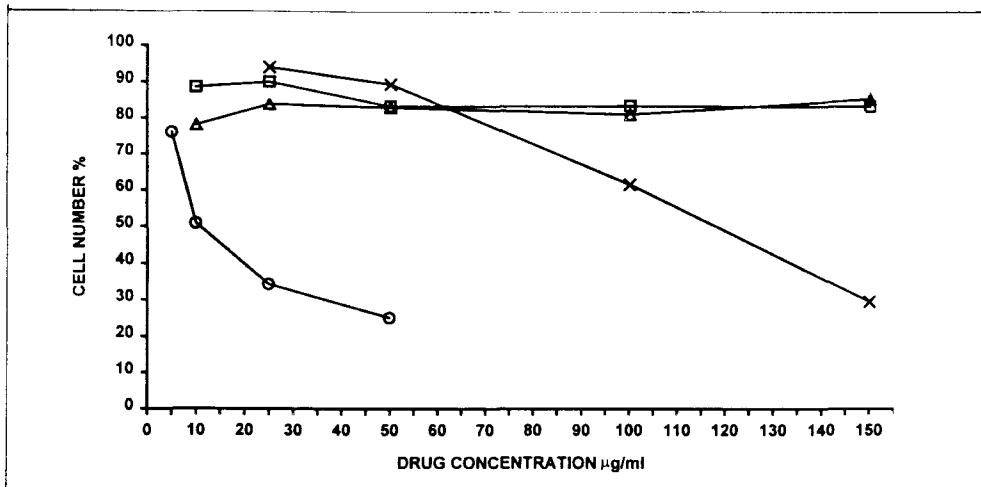


Figure 1. Growth curves of HeLa cells treated with WSD of Croatian propolis (□), WSD of Brazilian propolis (Δ), caffeic acid (×), CAPE (○). Cells were seeded 1×10^5 per Petri dish) and incubated 24 h, then drug was added. Cells were exposed to the action of the drug for next 72 h, counted and expressed as % of number of untreated cells.

propolis (EEP) increased the cytotoxicity of NK cells, inhibited the development of HeLa (cervix) and KB (nasopharynx) carcinoma cells *in vitro* and exerted cytotoxic activity on Ehrlich carcinoma cells (Scheller et al 1989; Hladon et al 1980).

To study whether the antitumor effect of tested compounds achieved *in*

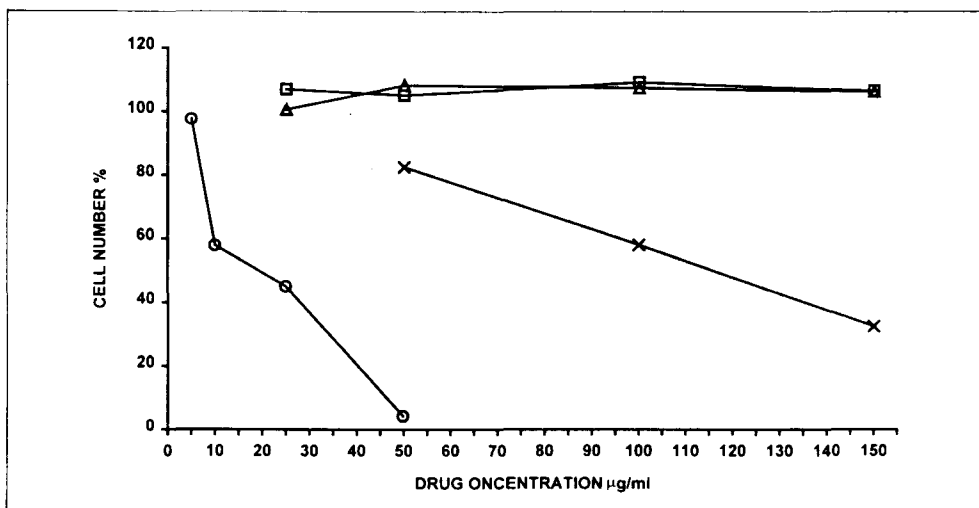


Figure 2. Growth curves of V79 cells treated with WSD of Croatian propolis (□), WSD of Brazilian propolis (Δ), caffeic acid (×), CAPE (○). Cells were seeded 1×10^5 per Petri dish) and incubated 24 h, then drug was added. Cells were exposed to the action of the drug for next 72 h, counted and expressed as % of number of untreated cells.

in vivo was due to the direct cytotoxicity to tumor cells or through other ways of action, we tested their influence on the growth of HeLa and V79 cells *in vitro*. Various concentrations of the compounds were used. Their influence was studied during 72 h incubation time. The cells were seeded in Petri dishes and 24 h thereafter the different concentration of compounds were added to the cultures. **Figure 1.** and **Figure 2.** show the influence of the compounds on the growth of the tested cells in 72 h incubation time.

WSD of Croatian or Brazilian propolis did not express cytostatic effect on V79 cells (normal cells) and was found to be the least effective growth inhibitor of HeLa cells (tumor cells) in comparison to CA and CAPE, respectively. Growth inhibition with CA on HeLa and V79 cells was dose-dependent. Also, the inhibitory effect of CAPE on HeLa cells (tumor cells) was more effective at much lower concentrations ($IC_{50} < 13\mu\text{g/ml}$) when compared with V79 cells ($IC_{50} > 19\mu\text{g/ml}$); inhibition concentration (IC_{50}) was determined from the corresponding growth curve indicating the drug concentration causing 50% growth inhibition.

However, in colony-forming studies, the cytotoxic efficacy of CA and CAPE on HeLa cells was highly pronounced (**Figure 3.**) as compared to

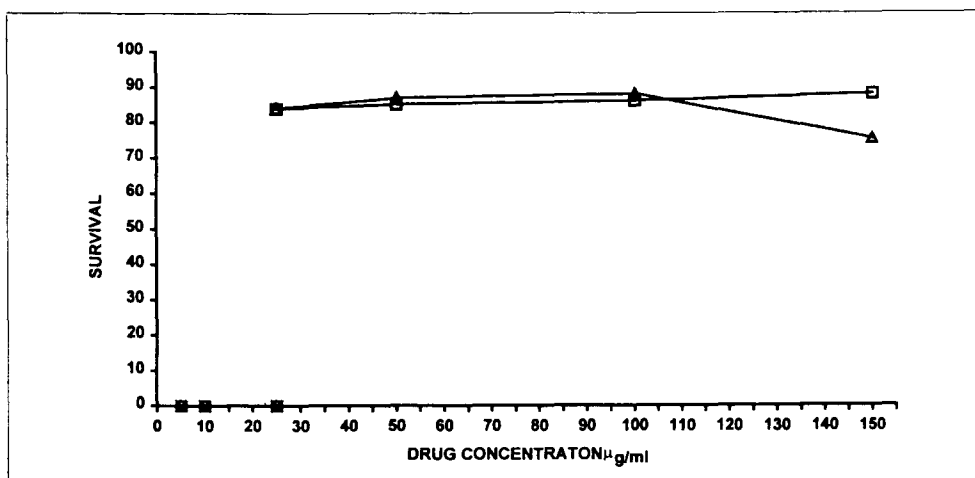


Figure 3. Survival of proliferating HeLa cells exposed to increasing concentrations of WSD of Croatian propolis (\square), WSD of Brazilian propolis (Δ), caffeic acid (\times), CAPE (o) for 10 days. Cells were seeded for colonies and supplemented with a drug. Colonies were counted and counts were expressed as % of colonies of untreated cells.

their effects achieved in growth studies. The dose of $5\mu\text{g/ml}$ was shown to be lethal for the cells. In contrast, the effect of WSD of propolis in colony-forming studies was insignificant.

Figure 4. shows killing action of WSD of propolis, CA or CAPE on V79 cells. After long term exposure, V79 cells were more sensitive to WSD of Brazilian propolis than WSD of Croatian propolis. The degree of killing action CAPE was more pronounced than CA. The concentration

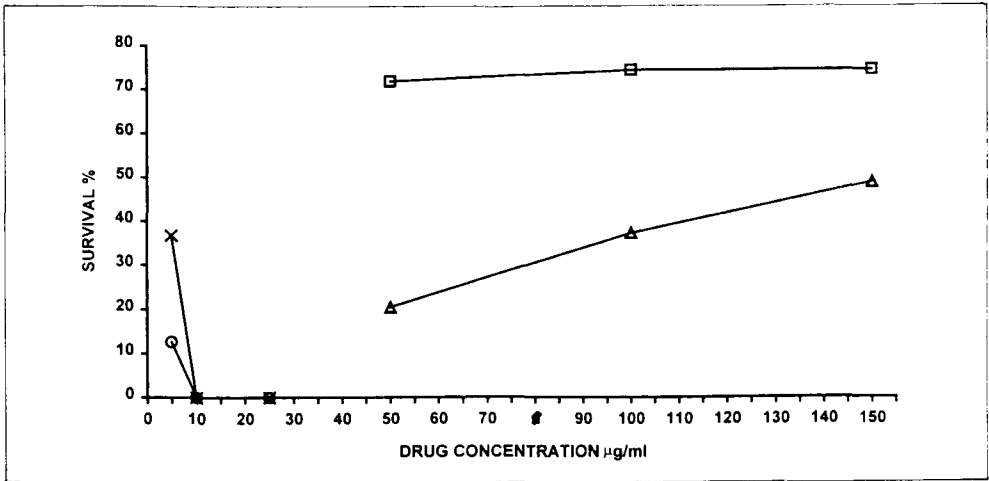


Figure 4. Survival of proliferating V79 cells exposed to increasing concentrations of WSD of Croatian propolis (□), WSD of Brazilian propolis (Δ), caffeic acid (×), CAPE (o) for 10 days. Cells were seeded for colonies and supplemented with a drug. Colonies were counted and counts were expressed as % of colonies of untreated cells.

of 10µg/ml of CA or CAPE was lethal for V79.

Since the cytotoxicity of tested compounds was observed, it was of importance to determine if there was a growth-dependent sensitivity. Cells in exponentially growing phase were treated with tested compounds for 24 h (Figure 5. and Figure 6.). Figure 5. shows that survival of HeLa

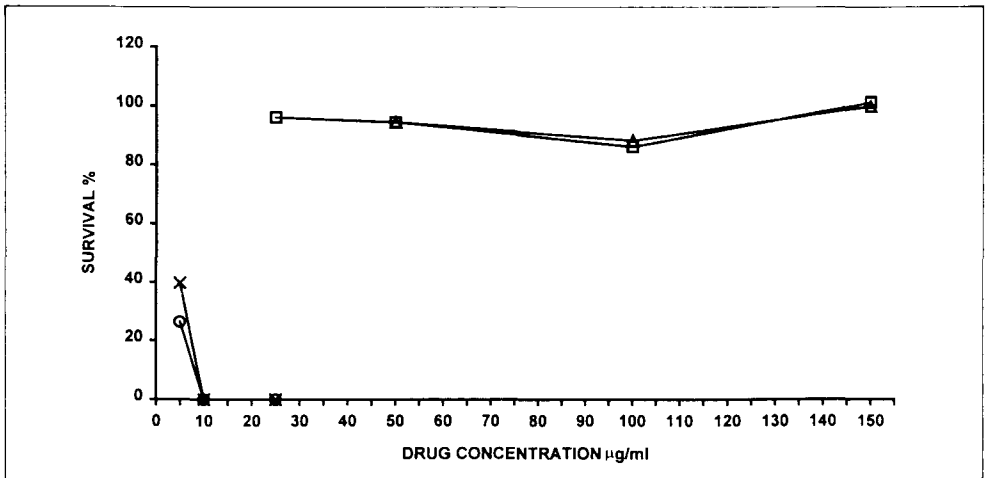


Figure 5. Survival of proliferating HeLa cells exposed to increasing concentrations of WSD of Croatian propolis (□), WSD of Brazilian propolis (Δ), caffeic acid (×), CAPE (o) for 24 hours. Cells were seeded for colonies and supplemented with a drug. Colonies were counted and counts were expressed as % of colonies of untreated cells.

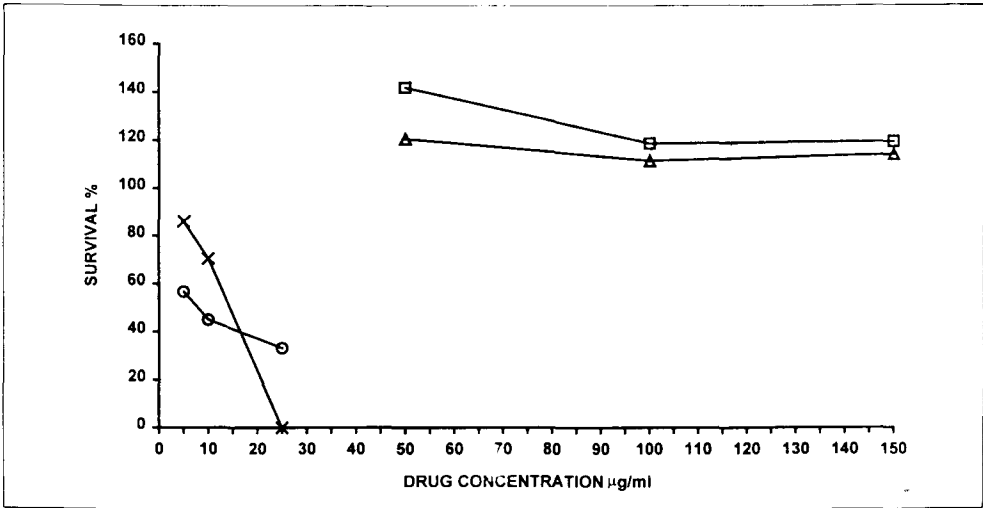


Figure 6. Survival of proliferating V79 cells exposed to increasing concentrations of WSD of Croatian propolis (□), WSD of Brazilian propolis (Δ), caffeic acid (×), CAPE (○) for 24 hours. Cells were seeded for colonies and supplemented with a drug. Colonies were counted and counts were expressed as % of colonies of untreated cells.

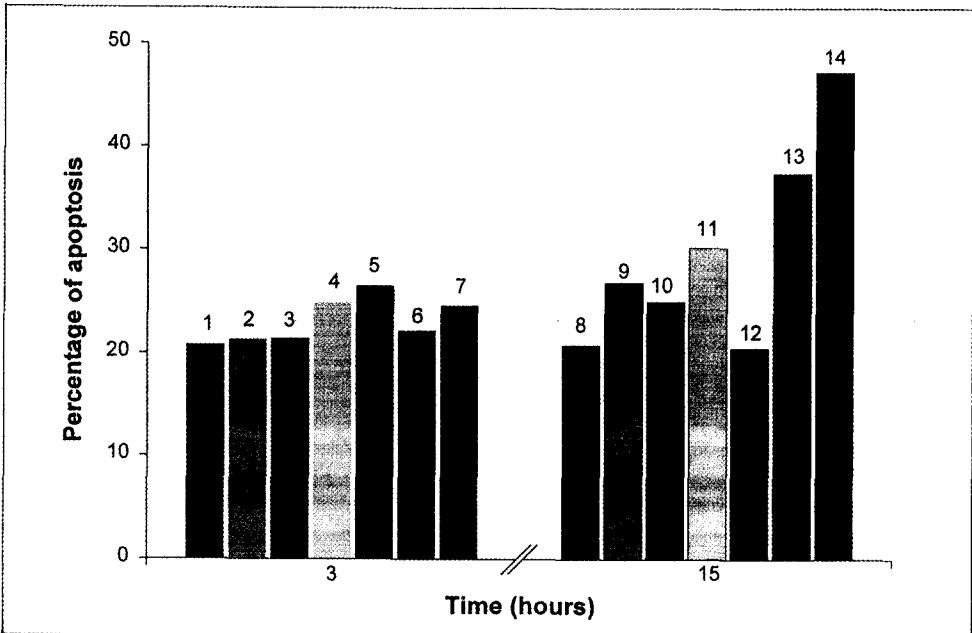


Figure 7. The effect of WSD of propolis, CA and CAPE on induction of apoptosis in MCA cells. The cells were analyzed by flow cytometry. 1-control, 2-Croatian propolis (50 µg/ml), 3-Brazilian propolis (50 µg/ml), 4-CA (25 µg/ml), 5-CA (50 µg/ml), 6-CAPE (5 µg/ml), 7-CAPE (10 µg/ml), 8-control, 9-Croatian propolis (50 µg/ml), 10-Brazilian propolis (50 µg/ml), 11-CA (25 µg/ml), 12-CA (50 µg/ml), 13-CAPE (5 µg/ml), 14-CAPE (10 µg/ml)

cells was not affected by the presence WSD of Croatian or Brazilian propolis, while CA and CAPE in concentration of 10 μ g/ml killed all the cells in culture.

Figure 6. Shows that the survival of V79 cells was higher by the presence of WSD of Brazilian and Croatian propolis than in the control group. CA and CAPE killed cells in a dose-dependent fashion. CA in concentrations required to kill 50% of cells in the exponential phase of growth (IC₅₀) ranged from 12 to 15 μ g/ml, while CAPE killing action IC₅₀ ranged from 5 μ g/ml to 7 μ g/ml.

Studies on apoptotic or necrotic death of MCA cells (**Figure 7.** and **8.**) show that the fragmentation of tumor cell DNA starts 3 hours after the treatment of MCA cells with either tested compound. After 15 hours of incubation of MCA cells, the percentage of apoptotic death was 5-23%. The most pronounced DNA fragmentation was induced by CAPE. At some time, the similar rate of MCA cell necrosis was obtained. This suggests that WSD of Brazilian or Croatian propolis as well as CA and CAPE kill certain proportion of tumor cells by inducing apoptosis. The results of these studies could be summarized as follows:

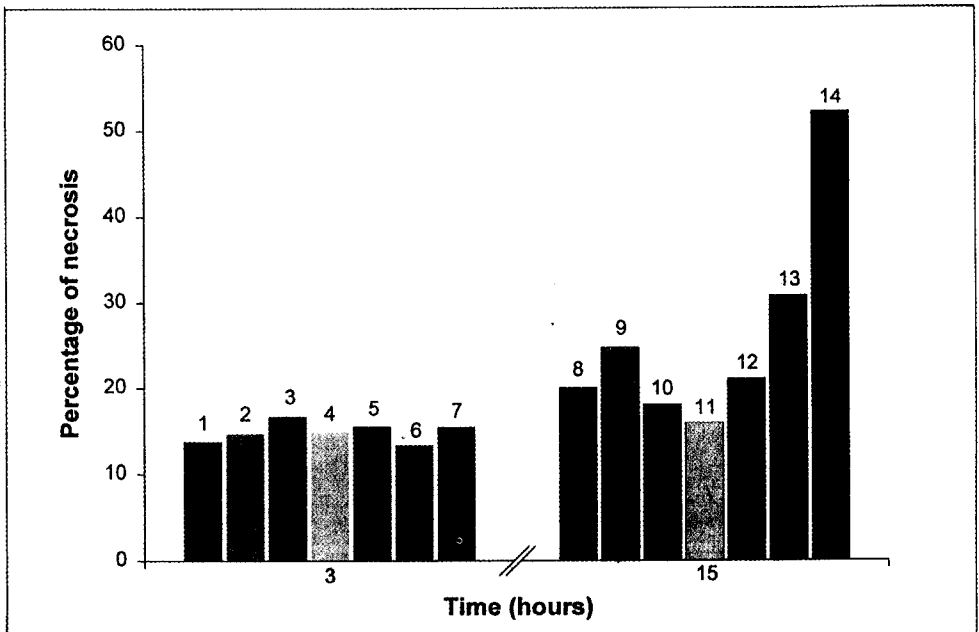


Figure 8. The effect of WSD of propolis, CA and CAPE on induction of necrosis in MCA cells. The cells were analyzed by flow cytometry. 1-control, 2-Croatian propolis (50 μ g/ml), 3-Brazilian propolis (50 μ g/ml), 4-CA (25 μ g/ml), 5-CA (50 μ g/ml), 6-CAPE (5 μ g/ml), 7-CAPE (10 μ g/ml), 8-control, 9-Croatian propolis (50 μ g/ml), 10-Brazilian propolis (50 μ g/ml), 11-CA (25 μ g/ml), 12-CA (50 μ g/ml), 13-CAPE (5 μ g/ml), 14-CAPE (10 μ g/ml)

a) WSD of Brazilian and Croatian propolis expressed strong antitumor activity against tumor nodules of MCA in CBA mice.

b) The antimetastatic activity of CAPE and CA was much less pronounced as compared to the effects of the WSD of propolis.

c) The antitumor activity of propolis could be partially related to its apoptotic and immunomodulatory activities.

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