

**RESEARCH ARTICLE**

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**PROPHYLACTIC EFFECT OF PROPOLIS AGAINST TUMOR GROWTH IN MICE THROUGH STIMULATION OF THE IMMUNE SYSTEM****ABSTRACT**

Propolis (bee glue), a natural product derived from plant resins and collected by honeybees, has numerous biological activities including antibiotic, anti-microbial, anti-inflammatory and immunomodulatory properties. The purpose of the present study is to examine the effect of propolis on tumor in mice induced by Ehrlich ascites tumor (EAT) cell line and its possible anti-tumor mechanism of action. Peroral treatment of propolis (0.1, 1 or 10 mg/ 100 g BW) every other day for 4 weeks before the intraperitoneal inoculation of  $1 \times 10^6$  EAT cells increased the number of total peritoneal exudate cells (PECs), as well as the absolute number of both macrophages and lymphocytes. The phagocytic function, as determined with carbon clearance assay, was significantly increased. When acquired immune response was evaluated by rosette-forming and plaque-forming assays, a dose-dependent increase in both T and B cell activities was observed in propolis-pretreated mice as compared with the tumor-bearing control mice. *In vitro* studies revealed that propolis with doses (0.01, 0.1 and 1.0 mg/ ml) significantly increased the proliferation of spleen cells in the presence of concanavalin A mitogen. Furthermore, propolis pretreatment effectively decreased the proliferation of EAT cells in the peritoneal fluid, and decreased the viability of EAT cells *in vitro*. The size of solid Ehrlich tumor was significantly decreased, as measured morphologically and examined histologically. In conclusion, the present findings imply that propolis has a strong inhibitory activity against tumors in mice. The anti-tumor activity of propolis may enhance the host resistance in EAT model through increasing the activities of macrophages, T cells and B cells.

**KEY WORDS:**

Propolis, Ehrlich ascites tumor, Macrophages, Lymphocytes, T-cells, B-cells.

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**INTRODUCTION**

Cancer is considered one of the most common causes of morbidity and mortality worldwide. The target of much research has been on the discovery of natural and synthetic compounds that can be used in the prevention and/or treatment of cancer. Natural products of either plant or animal origin that exhibited antitumor activity has been discovered (Pezzuto, 1997). Several types of immunopotentiators have been developed recently and are being studied for possible use in the treatment of patients suffering from malignant diseases (Block and Mead, 2003; Sunila and Kuttan, 2004). The increased interest in new approaches to the immunotherapy of cancer, and a considerable demand for therapeutic agents which can modulate the several forms of immunodeficiency have encouraged studies on the immunomodulatory mechanism of natural and synthetic substances (Mirandola *et al.*, 2002; Valadares *et al.*, 2003).

Propolis (bee glue) is the generic name for the resinous substance collected by honeybee (*Apis mellifera* L.) from various plant sources and used by bees to seal holes in their honeycombs and protect the entrance against intruders (Park *et al.*, 2002). Propolis is one of the few natural remedies that has maintained its popularity over a long period of time. Chemical analysis of propolis have revealed that it contains a variety of flavonoids, phenols, alcohols, terpenes, sterols, vitamins, amino acids, etc (Walker and Crane, 1987). The pharmacologically active molecules in the propolis are flavonoids, phenolic acids and their esters (Castaldo and Capasso, 2002).

Propolis and its constituents have attracted the attention of many investigators because of their antibacterial (Bankova *et al.*,

1995), antiviral (Harish *et al.*, 1997), anti-hyperalgesic (Rafael *et al.* 1998), antifungal (Ota *et al.*, 2001), anti-inflammatory (Borrelli *et al.*, 2002b), radioprotective (Orsolich *et al.*, 2004b), anti-oxidant (Padmavathi *et al.*, 2006a) and anti-protozoal (Dantas *et al.*, 2006) properties. A pilot investigation was carried out by Bratter *et al.* (1999) to show the evidence of the prophylactic immunostimulatory effectiveness caused by propolis. The prophylactic application of propolis led to a time-dependent enhanced immune reactivity without undesired side effects. Ansorge *et al.* (2003) studied the effects of different propolis extracts on basic human immune cell functions. They found that propolis has a direct regulatory effect on basic functional properties of immune cells by suppressing phytohaemagglutinin-induced DNA synthesis of peripheral blood mononuclear cells and T cells, and suppressing of cytokines produced by monocytes/macrophages, by Th1 type as well as Th2 type, and by increasing the production of T cell growth factor-beta1 by T regulator cells. Sforcin *et al.* (2005) reported that propolis stimulates antibody production in bovine serum albumin-immunized rats. Recently, Fischer *et al.* (2007a&b) reported that the effect of propolis on the humoral and cellular immune responses can be exploited in the development of effective vaccines.

Different studies revealed that propolis and its active ingredients have anti-tumor properties. Luo *et al.* (2001) demonstrated that the compound PM-3, isolated from Brazilian propolis, markedly inhibits the growth of MCF-7 human breast cancer cells. Propolis treatment has also a protective affect against dimethyl benza(a)anthracene-induced breast cancer (Padmavathi *et al.*, 2006b). Banskota *et al.* (2002) reported that the Netherlands propolis showed promising anti-proliferative activity toward murine 26-L5-colon carcinoma. Furthermore, Borrelli *et al.* (2002a) reported that caffeic acid phenethyl ester (CAPE), present in propolis is a potent inhibitor of human colon tumor cell growth. Xiang *et al.* (2006) reported that CAPE induced growth arrest and apoptosis of colon cancer cells. Moreover, Orsolich *et al.* (2005b) reported that caffeic acid (CA) and CAPE derivatives of propolis suppressed human HeLa cervical carcinoma cell proliferation *in vitro*. Orsolich *et al.* (2004a; 2005a) found that propolis and the polyphenolic compounds (CA and CAPE) reduced the number of metastases in the lung of mice. Recently, Weng *et al.* (2007) isolated propolin H from Taiwanese propolis and found that, it significantly inhibited the proliferation of human lung carcinoma cell lines in MTT assays.

Crude Egyptian propolis has a strong inhibitory action against Ehrlich ascites tumor (EAT) growth. The anti-tumor mechanism may

be mediated by preventing oxidative damage and induction of apoptosis (El-Khawaga *et al.*, 2003). Propolis and polyphenolic compounds also enhance host resistance in the EAT model by increasing macrophage activity. The local presence of CA and CAPE in the tissue causes a significant delay in tumor formation and increases life span *in vivo* (Orsolich and Basic, 2003; 2005; Orsolich *et al.*, 2005c). Clinical trials using a propolis preparation combined with the anticancer drug, irinotecan, may be beneficial in maximizing antitumor activity and minimizing post-chemotherapeutic reactions to the cytostatic drug (Benkovic *et al.*, 2007).

Considering the immunostimulatory activities of propolis and the importance of both innate and acquired immunity in antitumor actions, it was hypothesized that propolis by modulating immune reaction in mice may influence host resistance to tumors. To delineate these effects, propolis was perorally administered to mice and a series of immune reactions assays were performed, and evaluation of its activity on host resistance to tumor cells was detected.

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## MATERIALS AND METHODS

### Animals:

A total of 180 female Swiss albino mice (8-10 weeks old, weighing about 20 g each) were used in this study. Mice were obtained from Helwan Research Animal Center, Cairo, Egypt. Animals were maintained in a quite room at 28°C. Mice received laboratory chow and water *ad libitum* and were allowed a period of 10 days, prior to the initiation of experiments, to acclimatize to the laboratory conditions.

### Experimental design and treatment regime:

Propolis (CC Pollen Co., Indian School Rd., AZ, USA) was dissolved in distilled water, and doses of 0.1, 1 or 10 mg/100 g body weight were orally administered to mice (0.2 ml/mouse) every other day for consecutive 4 weeks. Control mice were orally administered with 0.2 ml of distilled water only.

A line of Ehrlich ascites tumor was supplied through the courtesy of Dr. G. Klien, Amsterdam, Holland. The tumor line was maintained in The Cancer Institute (Cairo, Egypt) in female Swiss albino mice by weekly intraperitoneal transplantation of viable  $2 \times 10^6$  cells/ animal. Tumor cell suspensions were prepared in balanced salt solution at pH 7.4 to a final concentration of  $5 \times 10^6$  viable cells/ ml (Saha and Mondal, 2000). All experimental animals were inoculated with EAT cells intraperitoneally (i.p.) in a volume of 0.2 ml ( $1 \times 10^6$  cells) 24 hour after the last injection of propolis. One week later, all mice were sacrificed and lymphoid cells as well as tumor cells were obtained for immunological, carcinogenic, and histological examination.

**Harvesting of peritoneal exudates cells (PEC):**

To obtain inflammatory peritoneal phagocytes, normal, EAT-bearing mice and EAT-bearing mice pre-treated with propolis were i.p. injected with 2 ml of starch suspension (1 % starch in saline). Three days later, mice were sacrificed and the peritoneal exudates cells (PEC) were obtained by peritoneal lavage with 5 ml of HBSS. Cells were washed three times by centrifugation at 1200 r.p.m. for 10 min and resuspended in HBSS. Total and differential counts of PEC were determined using haemocytometer, by the uptake of 1 % W/V neutral red in saline (Hudson and Hay, 1989).

**Carbon clearance assay:**

The phagocytic activity of PEC was measured by using Pelikan special biological ink (Pelikan-Werke, Hannover, Germany). The original suspension was diluted 1:1 with 0.9 % NaCl solution, and 0.2 ml of the diluted ink was i.p. injected into normal, EAT-bearing mice and EAT-bearing mice pre-treated with propolis after stimulation with 2 ml of starch suspension, which was i.p. injected three days earlier. Carbon challenged animals were sacrificed 15, 30, 45 and 60 minutes after carbon injection. Five ml of 0.1% EDTA-saline solution was i.p. injected, and the peritoneal lavage was collected and centrifuged (700 r.p.m. for 5 min). The resultant supernatant was decanted into another tube, and the precipitated cells were resuspended in 1 ml of equal volumes of gelatin (2 % gelatin in saline) and ethanol potassium saline (5% KOH in 70 % ethanol), and incubated overnight at 37°C. Optical densities of both supernatant and digested cells were measured using a spectrophotometer (Spectronic 20, Bausch and Lomb Inc., Rochester, NY, USA).

**Determination of E-rosette-forming cells (RFC):**

The procedure was performed as described by Hsu *et al.* (1975). Seven days before they were sacrificed, the mice received an i.p. injection of  $1 \times 10^8$  SRBC in 0.2 ml saline. Spleens from normal, EAT-bearing mice and EAT-bearing mice pre-treated with propolis were excised and cleaned. Single cell suspensions were prepared, washed twice by centrifugation at 1200 r.p.m. for 10 min and resuspended in HBSS to a concentration of  $2 \times 10^6$ . A volume of 0.2 ml of spleen cell suspension was mixed with an equal volume of 0.5 % SRBC in a glass tube and incubated for 2-4 hours at 37°C. The tubes containing mixture were gently shaken to resuspend the cells in the pellet. The rosettes were counted in haemocytometer and calculated per million mononuclear cells. The cells surrounded by three or more SRBC were counted as E-rosette-forming T-cells.

**Detection of the plaque-forming cells (PFC):**

The procedure was performed as described by Brousseau *et al.* (1999). Primary humoral immune responses against sheep red blood cells (SRBC) were measured after one i.p. injection of  $1 \times 10^8$  SRBC in 0.2 ml saline. Five days later, normal, EAT-bearing mice and EAT-bearing mice pre-treated with propolis were sacrificed and spleens were excised and cleaned. Single cell suspensions were prepared, washed twice by centrifugation at 1200 r.p.m. for 10 min and resuspended in HBSS to a concentration of  $2 \times 10^6$ /ml. The assay mixture was prepared by adding 50  $\mu$ l of 25 % SRBC and 50  $\mu$ l of guinea pig complement to 100  $\mu$ l of spleen cell suspension. The assay mixture was plated onto a glass slide and incubated for 30-45 min at 37°C. The plaques were scored microscopically and calculated per million mononuclear cells.

**T-cell mitogenesis assay:**

Normal and EAT-bearing mice were sacrificed, and the spleens were aseptically removed and pooled. Spleens were dispersed gently by using two sharp forceps in serum free RPMI-1640 medium. The splenocytes were then seeded into 96-well culture plates (Falcon, Oxnard, CA) at a density of  $1.5 \times 10^5$  splenocytes/well in RPMI-1640 medium supplemented with 5 % fetal calf serum (Gibco, Grand Island, N.Y.), 50  $\mu$ M  $\beta$ -mercaptoethanol (Sigma Chemical Co.) and antibiotics. The cells were stimulated with 0.04 and 0.2  $\mu$ g/well concanavalin A (Con A) in the presence of propolis (0.01, 0.1 and 1 mg/ml), and subsequently incubated in a humidified 5 % CO<sub>2</sub> environment. Three days later, 150  $\mu$ l of the medium was removed from each well. The extent of spleen cell proliferation was determined using the tetrazolium salt MTT (3 [4, 5- dimethylthiazol-2- yl] -2, 5-diphenyl tetrazolium bromide, Sigma Chemical Co.), which adheres to active mitochondria to form a dark blue formazan product (Mosmann, 1983). MTT (5  $\mu$ l of 20 mg/ml) was added to each well and incubated at 37°C for 4 hours. The dark blue crystals were dissolved by the addition of 150  $\mu$ l of 0.04 M HCl/ isopropanol. After an overnight incubation in the dark, the plates were inserted into a Dynatech MR580 microelisa spectrophotometer and optical densities were obtained using a test wavelength of 570 nm and a reference wavelength of 630 nm.

**Determination of EAT cell count:**

Control mice, as well as mice pre-treated with propolis were i.p. inoculated with  $1 \times 10^6$  EAT cells/ mouse. One week later, normal, EAT-bearing mice, as well as EAT-bearing mice pre-treated with propolis were sacrificed. EAT cells were obtained by peritoneal lavage with 5 ml of HBSS. Cells were washed three times by centrifugation at

160 r.p.m. for 10 min, resuspended in HBSS, and counted with a haemocytometer.

#### Viability of Ehrlich ascites tumor (EAT) cells:

EAT cells ( $1 \times 10^5$ ,  $5 \times 10^5$ ,  $1 \times 10^6$ ,  $5 \times 10^6$  and  $1 \times 10^7$  cells/well) were seeded into 96-well culture plates (Falcon, Oxnard, CA) in RPMI-1640 medium (100  $\mu$ l/well). Propolis (0.01, 0.1, and 1 mg/ml) was added to each EAT cell concentration in a volume of 100  $\mu$ l/well and incubated over night at 37°C. The respective cell suspensions were washed three times, and resuspended in RPMI-1640 medium. The viable cells were counted with a haemocytometer using trypan blue dye. The viability % was calculated according to the following formula:

$$\text{Viability \%} = \frac{\text{No. of viable cells}}{\text{Total No. of cells}} \times 100$$

#### Measurement of solid tumor:

EAT cells were suspended in normal saline and adjusted to a concentration of  $20 \times 10^6$  cells/ml. 0.2 ml of the cell suspension ( $4 \times 10^6$  cells) was inoculated s.c. in the right thigh of control mice and in mice pre-treated with honeybee products. Palpable tumors were measured after one week using Vernier calipers (Tricle Brand, Shanghai, China). Tumor volume was calculated so as to monitor the response to treatment. According to Papadopoulos *et al.* (1989), the formula used for this calculation was:

$$\text{Tumor volume (mm)} = \frac{4 \pi (A/2)^2 (B/2)}{3}$$

Where A is the tumor diameter in the minor axis and B is the tumor diameter in the major axis.

#### Histological architecture of tumor mass:

Tumor-bearing mice, as well as tumor-bearing mice pre-treated with propolis were sacrificed one week after inoculation of EAT ( $4 \times 10^6$  cells/mouse) in the right thigh of each mouse. The solid tumors were excised and fixed in 10 % neutral buffered formalin. The specimens were then dehydrated in ascending grades of ethyl alcohol, cleared in terpinol, washed in benzene, embedded in paraffin wax, sectioned at 5  $\mu$ , and stained with haematoxylin and eosin (Delafield, 1984). The stained sections were then examined under light microscopy for assessment of the tumor cell growth using X 160 microscopic magnification.

#### Statistical analysis:

All *in vivo* results are expressed as the mean  $\pm$  SD of groups consisting of 6 mice. The *in vitro* data are also expressed as the mean  $\pm$  SD of groups consisting of four wells. Each experiment was performed independently at least three times. All data were analysed for significance using Student's t-test. (# significantly different from

control group at  $P < 0.05$ , ## significantly different from control group at  $P < 0.01$ ; \* significantly different from tumor-bearing group at  $P < 0.05$  and \*\* significantly different from tumor-bearing group at  $P < 0.01$ ).

## RESULTS

#### Effect on peritoneal exudate cell (PEC) count:

As shown in table 1, the total number of PEC of EAT-bearing mice was significantly increased when compared to that of normal mice. Pre-treatment of EAT-bearing mice with propolis (0.1, 1 or 10 mg/ 100 g BW, every other day for 4 weeks) caused a statistically significant decrease in PEC count with doses 0.1 ( $P < 0.05$ ), 1 and 10 mg ( $P < 0.01$ ). Table 1 also shows that the absolute number and the relative proportion of macrophages were significantly decreased ( $P < 0.01$ ), while the absolute number and the relative proportion of lymphocytes were significantly increased ( $P < 0.01$ ) in EAT-bearing mice as compared to the corresponding values of normal mice. Pre-treatment of EAT-bearing mice with propolis caused a statistically significant increase in the absolute number and the relative proportion of macrophages with the dose 10 mg ( $P < 0.01$ ), while it caused a statistically significant decrease in the absolute number and the relative proportion of lymphocytes with doses 1 and 10 mg ( $P < 0.01$ ) as compared with the corresponding values of the EAT-bearing control mice.

Table 1. Total peritoneal exudate cells (PEC) count, the absolute number and relative proportion (%) of both macrophages and lymphocytes in normal and in tumor-bearing mice pre-treated orally with vehicle (0.2 ml distilled water) or propolis (0.1, 1 or 10 mg/ 100 g BW) every other day for 4 weeks. (## at  $P < 0.01$  in comparison with the control group. \* at  $P < 0.05$ ; \*\* at  $P < 0.01$  in comparison with the tumor-bearing group)

Treatment	Total PEC count. Mean $\pm$ SD $\times 10^6$	Macrophages		Lymphocytes	
		Absolute No. Mean $\pm$ SD $\times 10^6$	%	Absolute No. Mean $\pm$ SD $\times 10^6$	%
Normal control	6.41 $\pm$ 1.03	4.32 $\pm$ 0.49	67.4	2.09 $\pm$ 0.54	32.6
Tumor-bearing + Vehicle	27.89 $\pm$ 1.49##	1.58 $\pm$ 0.32##	5.7	26.31 $\pm$ 1.40##	94.3
Tumor-bearing + Propolis(0.1mg)	20.64 $\pm$ 5.61*	1.68 $\pm$ 0.41	8.1	18.96 $\pm$ 5.46*	91.9
Tumor-bearing + Propolis (1 mg)	15.03 $\pm$ 5.07**	2.13 $\pm$ 0.66	14.2	12.90 $\pm$ 4.12**	85.8
Tumor-bearing + Propolis (10 mg)	16.43 $\pm$ 4.88**	2.75 $\pm$ 0.67**	16.7	13.68 $\pm$ 4.86**	83.3

#### Effect on the phagocytic function of peritoneal exudate cells (PECs):

Table 2 shows that carbon uptake by PECs of EAT-bearing mice was significantly decreased ( $P < 0.01$ ) when compared with that of normal mice. However, pre-treatment of EAT-bearing mice with propolis (0.1, 1 or 10 mg/ 100 g BW, every other day for 4 weeks) caused a progressive increase in the scavenger activity of PECs ( $P < 0.01$ ). On the

other hand, the carbon particles, which remained in the peritoneal fluid of EAT-bearing mice, were significantly increased as compared with those of normal mice ( $P < 0.01$ ). Pre-treatment of EAT-bearing mice with honey caused a gradual decrease carbon contents as compared with those of the EAT-bearing control mice ( $P < 0.05$ ;  $P < 0.01$ ).

Table 2. The phagocytic activity of peritoneal exudate cells (PEC), as determined by carbon uptake by PEC and carbon particles remained in the peritoneal fluid in normal and in tumor-bearing mice pre-treated orally with vehicle (0.2 ml distilled water) or propolis (0.1, 1 or 10 mg/ 100 g BW) every other day for 4 weeks. (## at  $P < 0.01$  in comparison with the control group. \* at  $P < 0.05$ ; \*\* at  $P < 0.01$  in comparison with the tumor-bearing group)

Treatment	Carbon uptake by PEC				Carbon particles remained in fluid			
	15 min	30 min	45 min	60 min	15 min	30 min	45 min	60 min
Normal control	0.85 ± 0.08	1.05 ± 0.07	1.11 ± 0.25	1.51 ± 0.14	1.00 ± 0.18	0.81 ± 0.04	0.47 ± 0.08	0.43 ± 0.15
Tumor-bearing + Vehicle	0.32 ± 0.06##	0.47 ± 0.11##	0.51 ± 0.12##	0.82 ± 0.11##	1.77 ± 0.15##	1.54 ± 0.23##	1.45 ± 0.20##	1.08 ± 0.10##
Tumor-bearing + Propolis (0.1 mg)	0.55 ± 0.16*	0.76 ± 0.09**	0.89 ± 0.05**	1.00 ± 0.04**	1.33 ± 0.14**	1.15 ± 0.09**	0.89 ± 0.09**	0.74 ± 0.07**
Tumor-bearing + Propolis (1 mg)	0.66 ± 0.09**	0.74 ± 0.13**	0.85 ± 0.04**	1.16 ± 0.08**	1.22 ± 0.10**	1.03 ± 0.11**	0.78 ± 0.14**	0.63 ± 0.05**
Tumor-bearing + Propolis (10 mg)	0.63 ± 0.16**	0.90 ± 0.05**	0.97 ± 0.03**	0.95 ± 0.23	1.28 ± 0.13**	0.99 ± 0.08**	1.14 ± 0.12*	0.93 ± 0.14

**Effect on rosette-forming cells (RFCs) count:**

As shown in table 3, the number of RFCs in EAT-bearing mice was significantly decreased when compared with that of normal mice ( $P < 0.01$ ). Pre-treatment of EAT-bearing mice with propolis (0.1, 1 or 10 mg/ 100 g BW, every other day for 4 weeks) caused a statistically significant increase in the number of RFCs with doses 1 and 10 mg as compared with that of the corresponding EAT-bearing control mice ( $P < 0.01$ ). The increment of this increase reached about 1.3 and 1.9 folds, respectively.

Table 3. Number of rosette-forming cells (RFCs) and plaque-forming cells (PFCs) /  $10^6$  nucleated spleen cells in normal and in tumor-bearing mice pre-treated orally with vehicle (0.2 ml distilled water) or propolis (0.1, 1 or 10 mg/ 100 g BW) every other day for 4 weeks. All mice were immunized i.p. with 0.2 ml of SRBC 4 days before sacrifice. (## at  $P < 0.01$  in comparison with the control group. \*\* at  $P < 0.01$  in comparison with the tumor-bearing group)

Treatment	No. of RFCs/ million nucleated spleen cells (Mean ± SD $\times 10^5$ )	No. of PFCs/ million nucleated spleen cells (Mean ± SD $\times 10^3$ )
Normal control	2.45 ± 0.35	1.42 ± 0.09
Tumor-bearing + Vehicle	0.87 ± 0.10##	1.10 ± 0.13##
Tumor-bearing + Propolis (0.1 mg)	1.19 ± 0.41	1.34 ± 0.26
Tumor-bearing + Propolis (1 mg)	2.06 ± 0.34**	1.48 ± 0.22**
Tumor-bearing + Propolis (10 mg)	2.51 ± 0.19**	1.68 ± 0.26**

**Effect on plaque-forming cells (PFCs) count:**

As shown in table 3, the number of PFCs in EAT-bearing mice was significantly decreased when compared with that of normal mice ( $P < 0.01$ ). Pre-treatment of EAT-bearing

mice with propolis (0.1, 1 or 10 mg/ 100 g BW, every other day for 4 weeks) caused a progressive increase in the number of PFCs as compared with that of the corresponding EAT-bearing control mice. However, this increase was statistically significant with doses 1 and 10 mg ( $P < 0.05$ ), and reached about 0.35 and 0.53 folds, respectively.

**Effect on T-lymphocyte mitogenesis *in vitro*:**

As shown in table 4, in the absence of Con A mitogen, propolis by itself elicited a gradual mitogenic effect under the cultured conditions. This effect was statistically significant with the dose 1 mg ( $P < 0.01$ ). In the presence of Con A (0.04 and 0.2  $\mu\text{g}$ / well), propolis at concentrations of 0.01, 0.1 and 1 mg/ ml significantly stimulated the proliferative response of cultured splenocytes ( $P < 0.01$ ).

Table 4. T cell mitogenic response *in vitro*. Cultured splenocytes ( $1.5 \times 10^5$  cells/ well) were exposed to Culture medium (Control), Con A (0.04  $\mu\text{g}$ / well) or Con A (0.2  $\mu\text{g}$ / well) in the absence or presence of propolis (0.01, 0.1 or 1 mg/ ml) for 72 hours. (# at  $P < 0.05$ ; ## at  $P < 0.01$  in comparison with the control group)

Treatment	Optical density (570 nm)		
	Control (Culture medium)	Con A (0.04 $\mu\text{g}$ / well)	Con A (0.2 $\mu\text{g}$ / well)
Vehicle	0.23 ± 0.03	0.28 ± 0.04	0.31 ± 0.05
Propolis (0.01 mg/ ml)	0.28 ± 0.04	0.64 ± 0.10##	0.60 ± 0.11##
Propolis (0.1 mg/ ml)	0.32 ± 0.06	0.62 ± 0.10##	0.65 ± 0.12##
Propolis (1 mg/ ml)	0.34 ± 0.06#	0.70 ± 0.12##	0.82 ± 0.13##

**Effect on EAT cell count:**

As shown in table 5, the number of EAT cells one week after incubation in the abdominal cavity of mice pre-treated with propolis (0.1, 1 or 10 mg/ 100 g BW, every other day for 4 weeks) was progressively decreased as compared with that of the corresponding vehicle-treated control mice. However, this decrease was statistically significant with doses 1 and 10 mg ( $P < 0.01$ ), with a percentage of decrease reached about 0.37 and 0.38 %, respectively.

Table 5. Number of tumor cells one week after incubation of  $1 \times 10^6$  tumor cells in the abdominal cavity of mice pre-treated orally with vehicle (0.2 ml distilled water) or propolis (0.1, 1 or 10 mg/ 100 g BW) every other day for 4 weeks. (## at  $P < 0.01$  in comparison with the control group)

Treatment	Number of tumor cells (Mean ± SD $\times 10^6$ )
Vehicle	24.12 ± 2.92
Propolis (0.1 mg)	19.03 ± 4.95
Propolis (1 mg)	15.26 ± 3.13##
Propolis (10 mg)	15.01 ± 4.52##

**Effect on the viability of EAT cells *in vitro*:**

As shown in table 6, *in vitro* incubation of propolis (0.01, 0.1 or 1 mg/ ml) with serial concentrations of EAT cells ( $1 \times 10^5$ ,  $5 \times 10^5$ ,  $1 \times 10^6$ ,  $5 \times 10^6$  and  $1 \times 10^7$ ) for 24 hours elicited a progressive decrease in the % of viable

tumor cells as compared with those of vehicle control.

Table 6: Viability % of tumor cells in vitro. Serial concentrations of tumor cells ( $1 \times 10^5$ - $1 \times 10^7$  cells) were exposed to vehicle (100  $\mu$ l RPMI medium) or propolis (0.01, 0.1 or 1 mg/ml) and incubated for 24 hours

Treatment	% of viable tumor cells				
	$1 \times 10^5$	$5 \times 10^5$	$1 \times 10^6$	$5 \times 10^6$	$1 \times 10^7$
Vehicle	92	81	76	86	90
Propolis (0.01 mg/ml)	83	70	74	62	82
Propolis (0.1 mg/ml)	67	60	43	65	77
Propolis (1 mg/ml)	73	77	54	62	71

### Effect on the volume of solid tumor:

As shown in table 7, the volume of solid Ehrlich tumor of mice pre-treated with propolis (0.1, 1 or 10 mg/ 100 g BW, every other day for 4 weeks) was markedly decreased when compared with that of vehicle-treated control group. The percentage of decrease reached about 43.8, 79.9 and 84.9 %, respectively.

Table 7: Percentage of solid Ehrlich carcinoma growth of mice pre-treated orally with propolis (0.1, 1 or 10 mg/ 100 g BW) every other day for 4 weeks, compared to the tumor-bearing control group

Treatment	Measurement of tumor			
	(A) Minor axis (mm)	(B) Major axis (mm)	Volume (mm <sup>3</sup> )	%
Tumor-bearing + Vehicle	2.0	2.5	5.23	
Tumor-bearing + Propolis (0.1 mg)	1.5	2.5	2.94	43.8
Tumor-bearing + Propolis (1 mg)	1.0	2.0	1.05	79.9
Tumor-bearing + Propolis (10 mg)	1.0	1.5	0.79	84.9

$$\text{Tumor volume (mm}^3\text{)} = [4 \pi (A/2)^2 \times (B/2)] / 3$$

### Histological architecture of EAT:

Administration of EAT cells within the thigh muscles of mice resulted in proliferation and growth of the tumor cells that form tumor masses infiltrating the muscle fibers (Fig. 1a). Pre-treatment of the tumor-bearing mice with propolis in a dose of (0.1 mg/ 100 g BW) led to an increase in tumor cell masses (Fig. 1b). A remarkable reduction in the size of tumor cell masses was noticed when propolis is used in a dose of 1 mg/ 100 g BW (Fig. 1c). However, increasing the dose of propolis to 10 mg/ 100 g BW did not result in a further reduction of the tumor cell masses (Fig. 1d).

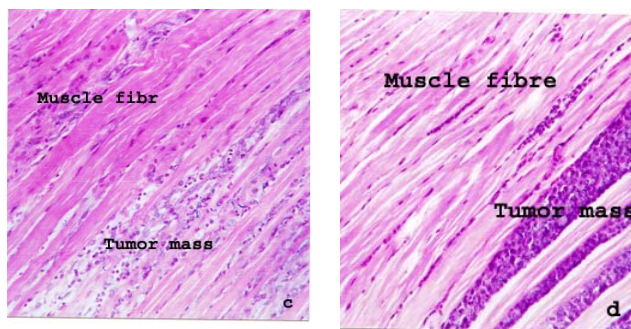
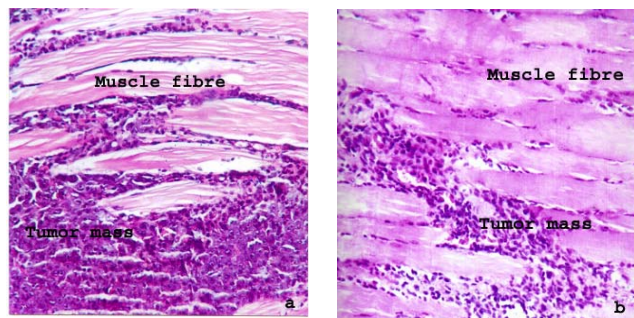


Fig. 1. a: Longitudinal sections of the thigh muscle of EAT-bearing mice showing proliferation and growth of the tumor cells. b – d: Longitudinal sections of the thigh muscles of tumor-bearing mice pre-treated with propolis (0.1, 1, and 10 mg/100g bw) showing remarkable reduction in the size of tumor cell masses. X 160)

### DISCUSSION

Ehrlich ascites tumor cells grow rapidly in almost any mouse strain (Carry *et al.*, 1972) inducing profound haematopoietic and immune dysfunction (Bincoletto *et al.*, 2005). EAT cells produce either ascitic or solid tumors, killing their host even when given in extremely small doses. EAT produces impairment in the number of granulocyte-macrophage colonies, associated with splenic haematopoiesis (Justo *et al.*, 2000; Queiroz *et al.*, 2001; Mirandola *et al.*, 2002). Earlier, Pessina *et al.* (1982) reported that EAT growth in mice seriously affects haemolymphopoietic compartment by causing anemia, thymus depletion, immunosuppression, and granulocyte-dependent leukocytosis.

The analysis of the total number of cells present in the peritoneal cavity revealed that all tumor-bearing groups pre-treated orally with propolis (0.1, 1 or 10 mg/ 100 g BW) every other day for 4 weeks exhibited significantly higher number of macrophages than that in tumor-bearing control group. Moreover, the phagocytic function of peritoneal exudate cells (PECs), as determined by carbon clearance assay, was significantly higher in all tumor-bearing groups pre-treated with propolis than the tumor-bearing control group. The increase of macrophage activity might have been responsible for the slower growth of the tumor cells. It is well known that mononuclear cells, mainly macrophages, are the major component of host defense against neoplastic growth in experimental tumor system (Kimoto *et al.*, 1998; Orsolich and Basic, 2003). These results suggest that propolis might interfere with the growth of EAT cells by activation of macrophages.

Pre-treatment of tumor-bearing mice with propolis elicited a significant increase in the number of T cells, measured by RFC assay, as compared with the tumor-bearing control mice. *In vitro* incubation of propolis with Con A, the T cell mitogen, also induced a progressive proliferative response. These results are consistent with the observations of Kimoto *et al.* (1998) who reported that the artemisin C from Brazilian propolis suppressed the tumor growth by increasing the ratio of

CD4/CD8 T cells and the number of helper T cells. The present results also confirm the results by Orsolich *et al.* (2005c) who reported an elevation of both CD4+ and CD8+ T cells subsets in tumor-bearing mice after treatment with water-soluble derivative of propolis. It is possible that, the increased lymphocyte proliferation leads to enhanced macrophage activation and thus to an amplification of the general immunological responses. The interaction of T cells with macrophages results in production of several cytokines including IL-1, IFN- $\gamma$  and TNF- $\alpha$  that have been implicated in host resistance to tumor cells and are known to play a role in macrophage activation (Dimov *et al.*, 1991, 1992; Orsolich and Basic, 2003).

The number of PFCs in tumor-bearing mice pre-treated with propolis was significantly increased in a dose-dependent manner as compared with that of the tumor-bearing control mice, when mice were immunized with SRBCs 4 days before sacrificing. These data are in line with the previous results by Scheller *et al.* (1988), Orsolich *et al.* (2005c) and Fischer *et al.* (2007a), and suggest an adjuvant effect of propolis. Although PFC is an endpoint to evaluate the humoral immune response, the response to SRBCs requires the cooperation of a number of cell populations, including B cells, helper T cells and macrophages (Orsolich *et al.*, 2005c). Findings from these experiments confirm that propolis can strongly activate the processes included in production of antibodies.

The data of the present study revealed that pre-treatment of mice with propolis (0.1, 1 or 10 mg/ 100 g BW) every other day for 4 weeks elicited a significant decrease in the number of EAT cells one week after inoculation in the abdominal cavity. Moreover, the viability of tumor cells was also decreased after incubation with propolis (0.01, 0.1 or 1 mg/ ml) *in vitro*. These results agree with the results of Banskota *et al.* (2002) and El-Khawaga *et al.* (2003) suggesting that administration of propolis before inoculation of Ehrlich ascites carcinoma could lead to arresting of tumor cells in the S-phase cell cycle preventing the proliferation of the tumor cells; and could induce the sub-G1 apoptosis process leading to the reduction of viability and the number of tumor cells.

The present results showed that propolis pre-treatment decreased the size of solid Ehrlich tumor in the thigh muscle of

mice, as measured morphologically and examined histologically. These results are consistent with the results by Mishima *et al.* (2005) who found that baccharin and drupamin, cinnamic acid derivatives of propolis, possess *in vivo* tumoricidal activity in mice bearing sarcoma S-180 cells. These compounds may induce tumor cell death, with less genotoxic to normal hematopoietic cells than anti-cancer drugs. Kimoto *et al.* (1998) and Xiang *et al.* (2006) found that artepillin C, an extract from Brazilian propolis, exhibited a cytotoxic effect and inhibited the growth of both human and murine malignant tumor cells *in vitro* and *in vivo*. Lee *et al.* (2000) found that caffeic acid phenethyl ester (CAPE) and its ethyl analogues showed a significant cytotoxicity on oral submucosal fibroblast, neck metastasis of gingival carcinoma, and tongue squamous cell carcinoma. They suggest that CAPE-like compounds may be potential chemotherapy agent against oral cancer. Luo *et al.* (2001) found that PM-3 from Brazilian propolis markedly inhibits the growth of human breast cancer cells. This effect was associated with inhibition of cell cycle progression and induction of apoptosis. Recently, Li *et al.* (2007) demonstrated that Brazilian propolis extracts have significant inhibitory effect on proliferation of human prostate cancer cells. Inhibition was achieved through regulation of protein expression of cyclin D1, B1 and cyclin-dependent kinase.

Chia-Nana *et al.* (2004) and Chen *et al.* (2007) found that propolin A and propolin B from Taiwanese propolis could induce apoptosis in human melanoma cells and significantly inhibit xanthine oxidase activity. They also found that propolin C effectively induced cytotoxic effect on human melanoma cells. Moreover, propolin C was capable of releasing cytochrome C from mitochondria to cytosol. These findings suggest that propolin C may activate a mitochondria-mediated apoptosis pathway. On the other hand, propolin C showed a strong capability to scavenge free radicals and inhibit xanthine oxidase activity. Moreover, Woo *et al.* (2005) found that chrysin, a biologically active compound extracted from propolis, possesses potent anti-inflammatory, anti-cancer and anti-oxidation properties. Chrysin significantly suppressed the lipopolysaccharide-induced cyclooxygenase-2 (COX-2) protein and mRNA expression in a dose dependent manner. Nuclear factor for IL-6 was identified as responsible for the chrysin-mediated COX-2 downregulation.

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### تأثيرات التأثير الوقائي لصمغ النحل ضد نمو الورم في الفئران من خلال تنشيط جهاز المناعة

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للورم والتي سبق حقنها بصمغ النحل مقارنة بالمجموعات الحاملة للورم. وقد أظهرت الدراسة في المختبرات الزجاجية (*In vitro*) أن إضافة صمغ النحل بتركيزات 0.01 و 0.1 و 1.0 مجم/مل قد أدى إلى زيادة معدل انقسام خلايا الطحال للفئران وذلك في وجود محفز الانقسام كونيكتافالين-أ. أما عند قياس نشاط الورم، فإن المعالجة بصمغ النحل قد قللت من تكاثر خلايا إيرلش الورمية داخل الجسم، كما قللت من حيوية هذه الخلايا خارج الجسم. بالإضافة إلى ذلك، فقد نقص حجم الورم الصلب نقصاً معنوياً وذلك عند قياسه مورفولوجياً وفحصه هستولوجياً. وتخلص هذه الدراسة إلى أن صمغ النحل له نشاط مثبط لنمو الورم في الفئران، وقد يرجع هذا النشاط المضاد للورم إلى قدرة صمغ النحل في زيادة المناعة الطبيعية الممثلة بالخلايا البلعمية والمناعة المكتسبة الممثلة بالخلايا التائية والخلايا البائية.

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صمغ النحل هو مادة راتنجية يتم جمعها من الأشجار بواسطة نحل العسل. ولصمغ النحل العديد من الأنشطة الحيوية، فهو يستخدم كمضاد حيوي، مضاد للميكروبات، مضاد للالتهاب، بالإضافة إلى خصائصه المعدلة للجهاز المناعي. وتهدف الدراسة المقدمة إلى معرفة التأثير الوقائي للمعالجة بصمغ النحل على نمو الورم المستحدث في الفئران باستخدام خلايا إيرلش الورمية، هذا بالإضافة إلى ميكانيكية العمل المحتملة لهذا التأثير. وقد أظهرت هذه الدراسة أن معالجة الفئران بصمغ النحل بجرعات (0.1، 1، أو 10 مجم/100 جم من وزن الجسم) يوماً بعد يوم لمدة 4 أسابيع وذلك قبل حقن خلايا إيرلش الورمية (1×610 خلية) في التجويف البريتوني للجسم قد أصلح من المناعة الطبيعية ممثلة بالعدد الكلي لخلايا الرشيش البريتوني وكذلك العدد النسبي لكل من الخلايا البلعمية والخلايا الليمفاوية، بالإضافة إلى ذلك، فقد تحسنت الوظيفة البلعمية لهذه الخلايا. وعند قياس المناعة المكتسبة، فقد ظهرت زيادة تدريجية في نشاط كل من الخلايا البائية المنتجة للأجسام المضادة باستخدام اختبار PFC والخلايا التائية باستخدام اختبار RFC في المجموعات الحاملة