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Anti-tumour effects of Egyptian propolis on Ehrlich ascites carcinoma

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Summary

A total of 150 female Swiss mice were used to study the ability of water soluble propolis derivatives (WSPD) of Egyptian propolis to inhibit the proliferation and growth of Ehrlich ascites carcinoma (EAC) cells in mice. The mice were divided equally into three groups: the first was kept as a negative control group, the second received an intraperitoneal injection of 2.5×10^6 EAC and was kept as a positive control group and the third an intraperitoneal injection of 2.5×10^6 EAC and treated with propolis (50 mg/kg body weight) administered by gastric intubations 2 h prior to the intraperitoneal injection of EAC. The propolis was administered daily for 11 successive days. An examination of EAC cells revealed a reduction in the volume, total cell count, viable percentage and increase in the percentage of dead cells in the treated group with an increasing mean survival time (MST), increasing life span (ILS) percentage and treated vs positive control (T/C) percentage. Immunological studies revealed a significant increase in the lymphocyte transformation rate (LTR), phagocytic activity and killing power in the group treated with propolis. A haematological study of the parameters revealed leucocytosis in cancer-bearing mice and propolis-treated groups with granulocytosis and monocytosis. The erythrogram revealed a significant reduction in red blood cell (RBC) count in group 2. The result showed that the implantation of EAC in Swiss mice without treatment resulted in a significant decrease in total protein and albumin levels without a change in globulin level and a significant increase in creatinine level, while the third group that received propolis showed an improvement in these biochemical parameters compared to the normal control group.

Keywords

Biochemical, Cancer, Ehrlich ascites carcinoma, Egypt, Haematology, Immunology, Lymphocyte transformation rate, Propolis, Water soluble propolis derivative.

Effetti antitumorali della propoli egiziana sul carcinoma ascitico di Ehrlich

Riassunto

Sono state studiate 150 femmine di topo Swiss per verificare se il derivato solubile in acqua della propoli egiziana fosse capace di inibire la proliferazione e la crescita delle cellule del carcinoma ascitico di Ehrlich (EAC) nei topi. Gli animali sono stati divisi in tre gruppi uguali: il primo gruppo è stato utilizzato come controllo negativo, il secondo ha ricevuto un'iniezione intraperitoneale di $2,5 \times 10^6$ di cellule EAC ed è stato utilizzato come controllo positivo, il terzo ha ricevuto un'iniezione intraperitoneale di $2,5 \times 10^6$ EAC ed è stato trattato con propoli (50 mg/kg di peso corporeo) somministrata per intubazione gastrica 2 ore prima dell'iniezione intraperitoneale di EAC. La propoli è stata somministrata su base giornaliera per 11 giorni consecutivi. L'esame delle

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cellule EAC ha evidenziato la riduzione del volume, un calo della conta cellulare totale e delle cellule vitali, con un aumento delle cellule morte nel gruppo trattato con propoli, con un aumento del tempo medio di sopravvivenza (MST), della durata di vita (ILS) nel gruppo trattato vs. i controlli. Gli esami immunologici hanno rivelato un aumento significativo della velocità di trasformazione dei linfociti (LTR), dell'attività dei fagociti e della capacità di soppressione nel gruppo trattato con propoli. Uno studio dei parametri ematologici ha documentato la presenza di leucocitosi con granulocitosi e monocitosi nei topi inoculati con le cellule cancerogene e nel gruppo trattato con propoli. All'eritrogramma è emersa una riduzione significativa della conta eritrocitaria nel gruppo 2. I risultati delle analisi hanno dimostrato che l'inoculazione di cellule EAC in topi Swiss non trattati ha determinato un calo significativo dei livelli di albumina e proteine totali, senza alterare la concentrazione di globuline, nonché un aumento significativo del livello di creatinina. Nel terzo gruppo trattato con propoli è stato osservato un miglioramento dei parametri biochimici citati rispetto al gruppo di controllo normale.

Parole chiave

Biochimica, Cancro, Carcinoma ascitico di Ehrlich, Egitto, Ematologia, Immunologia, Linfociti, Propoli.

Introduction

Cancer is considered one of the most common causes of mortality worldwide. The target of many research studies has been 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 have exhibited antitumor activity have been discovered (19). The basis of cancer chemotherapy lies in an understanding of biochemical abnormalities during the metabolism of malignant cells. The exploitation of metabolic differences between tumour and host tissue has become a method of treating tumours effectively. Rodent tumours are a case in point where the genetic and biochemical characteristics can be studied and they have become the basis of most cancer chemotherapy-screening operations. The transplantability of certain tumours in rodents has provided a useful tool for basic cancer research. Ehrlich ascites carcinoma (EAC) is a tumour which provides a reasonably homogenous sample of malignant tissue; it is available in large quantities and grows at a fairly predictable rate (10).

Propolis is a mixture of compounds obtained from beehives that has a strong characteristic smell and taste. The mixture contains a number of plant products, including flavonoids, and is deposited in beehives by bees where is thought to prevent microbial infection within the hives. Propolis has attracted the interest of many investigators because of its antiviral (25), antibacterial (26), immunostimulatory (17) and anti-tumour effects (16, 18). It has been suggested that the therapeutic activities of propolis depend mainly on the presence of flavonoids (12). Flavonoids have also been reported to enhance the immune system (27).

Our work aimed to study the anti-tumour activity, immunological, haematological and biochemical changes after treatment of EAC-bearing mice using natural products (crude Egyptian propolis) and study its ability to reduce the side-effects of EAC proliferation if present.

Materials and methods

Experimental animals

A total of 150 adult female Swiss albino mice (average 18-20 g in weight) were obtained from the laboratory animal farm of veterinary medicine at Zagazig University in Egypt. All mice were reared under strict standard hygienic conditions and were fed a balanced diet. Water was available *ad libitum.* Experiments were conducted in accordance with the guidelines set by Animals Health Research Ethics Training Initiative, Egypt, and experimental protocols were approved by the institutional animal ethics committee.

Ehrlich ascites carcinoma cells

The parent line of EAC cells was kindly supplied by the National Cancer Institute of Cairo University. The tumour line was

maintained by serial intraperitoneal transplantation of EAC 2.5 × 10^6 tumour cells/0.2 ml in female Swiss albino mice.

Propolis

Crude propolis was obtained from an Egyptian honey beekeeper; it was purified manually from impurities, such as wood, straw, fragments and insects. Propolis bulk was cut into small pieces, mixed with deionised water and shaken at 95°C for 2 h. It was cooled to room temperature and centrifuged at 1 500 rpm for 5 min to obtain the supernatant which was stored in a dark bottle until use.

Experimental design

A total of 150 female Swiss mice were equally divided into three groups at random (50 mice per group). Group 1 was kept as the negative control group, group 2 mice were injected intraperitoneally with 2.5 × 10^6 EAC cells and kept as the positive control group and group 3 was treated orally with water soluble propolis derivatives (WSPD) of Egyptian propolis (50 mg/kg body weight) 2 h prior to the transplantation of EAC using a bent stainless steel stomach tube then dosed daily for eleven successive days as shown in Table I. The endpoint of the experiment was determined by the spontaneous death of animals.

Survival analysis

Five mice from each group were kept under daily observation for survival analysis. The endpoint of experiment was determined by the spontaneous death of animals. Results are expressed as a percentage of mean survival time (MST) of treated animals over the MST of the control group (treated vs positive control, T/C %). The percentage of increased life span (ILS) was calculated in accordance with the following formula: ILS % = (T-C)/C × 100 where T represents the MST of treated animals; C represents MST of the positive control group. In accordance with the criteria of the National Cancer Institute, a T/C result that exceeded 125% and an ILS result that exceeded 25% indicated that the drug presented significant anti-tumour activity (20).

Counting of Ehrlich ascites carcinoma cells

After the mice were euthanised, the peritoneal cavity was opened carefully and all ascitic fluid was aspirated and examined for volume and total number of cells, in addition to the calculation of the percentage of live and dead cells. The tumour cell count was performed using a Neubauer haemocytometer, erythrocytic pipette and trypan blue stain 1% (3).The ability of the living cell to exclude trypan blue was used in a viability test (2) to determine the viable, unstained, tumour cells. Dead cells were revealed by a blue stain (Fig. 1).

Blood sampling

At day 12 after the commencement of the experiment, 45 mice in each group were used for blood collection from the retro-orbital venous plexus after they had been anesthetised by ether. The first set of blood samples was taken in heparinised tubes for immunological studies. A second set of blood samples was collected in dipotassium salt of ethylene-diaminetetraaceticacid (EDTA) tubes for haematological analysis. A third set of blood samples was taken without anticoagulant in a

Table I
Experimental groups of mice, treatment and administration route of Ehrlich ascites carcinoma cells and propolis

Groups	No. of mice	Design	Treatment	
			EAC cells implanted intraperitoneally	Water soluble propolis derivatives orally
1	50	Normal control	–	–
2	50	EAC	2.5 ×10^6	–
3	50	EAC + WSPD	2.5 ×10^6	50 mg/kg body weight

EAC Ehrlich ascites carcinoma
WSPD water soluble propolis derivatives

sterile test tube for separation of serum which was used to measure biochemical parameters. The remaining mice were used for survival analysis.

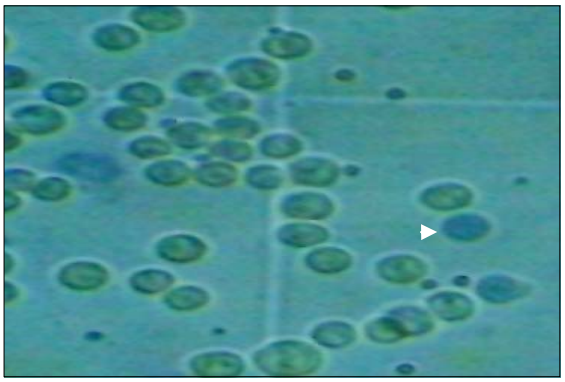

Figure 1
Viability test showing Ehrlich cell stained with trypan blue at 1%
Bluish cell is dead (arrowhead)
(×100)

Immunological studies

A lymphocytic transformation assay using [3-(4,5-dimethylthiazol-2-7l)-2,5-diphenyl 2H tetrazolium bromide] was used with 2348-71-2 which is a methyl tetrazolium dye (MTT) staining procedure (1). Blood samples were collected in heparinised tubes and used to prepare leucocytes for bacterial phagocytic activity and killing power (28).

Haematological studies

The measurement of the red blood cells (RBCs), total leucocytic and differential leucocytic counts was evaluated in an automatic cell counter (Hospitex Hemascreen 18, Florence).

Biochemical studies

The serum total protein (TP) and serum albumin levels were measured (6, 7). The serum globulin level was calculated by subtracting the albumin from the total protein (4). The serum creatinine level was also determined colorimetrically (14).

Statistical analysis

Data obtained from this investigation were analysed statistically using the F test (24). Means in the same column followed by different letters were significantly different and the highest value was represented by the letter (a).

Results and discussion

Tables II and III show that the MST and ILS percentage were reduced with increased body weight, volume of ascites fluid, total number of EAC cells, viable cells percentage and decreased dead cells percentage in group 2-bearing EAC (Figs 2 and 3). This may be due to higher mitosis (Fig. 4) and fewer cells dying (Fig. 5) which could be attributed to the decreased rate of natural death mechanisms that occur in the tumour (3). The accumulation of ascites fluid in the peritoneal cavity could have been due to the following:

- A reduced lymphatic recovery system which is associated with the obstruction of the lymphatic system by tumour cells
- Angiogenesis, which was detected in the ascites tumour-bearing peritoneal wall
- The hyperpermeability of micro vessels in the peritoneal cavity (8). On the contrary,

Table II
Effect of water soluble derivatives of Egyptian propolis (50 mg/kg body weight) on mean survival time, increasing life span percentage and percentage of treated/positive controls

Group	Parameters			
	Range of survival time	MST	ILS (%)	T/C (%)
2 Mice bearing EAC	11–14	12.5	–	–
3 Propolis-treated group	17-24	20.5	64	164

MST mean survival time
ILS percentage of increasing life span (day)
T/C percentage of treated animals vs positive controls
EAC Ehrlich ascites carcinoma

Table III
Effect of water soluble derivatives of Egyptian propolis (50 mg/kg body weight) on body weight, volume of ascites fluid, total Ehrlich ascites carcinoma (EAC) cells and percentage of live and dead cells (mean values ± standard error) in mice-bearing EAC

Group	Parameters				
	Body weight (g)	Volume of ascites fluid (ml)	Total EAC cells ($n \times 10^5$)	Live cells (%)	Dead cells (%)
1 Control	20.38 ± 0.17(c)	–	–	–	–
2 Mice-bearing EAC	28.48 ± 0.70(a)	6.22 ± 0.18(a)	972.8 ± 28.35(a)	98.81±0.10(a)	1.19 ± 0.10(b)
3 Treated with propolis	24.82 ± 0.25(b)	2.88 ± 0.12(b)	650.8 ± 10.25(b)	96.65 ± 0.12(b)	3.35 ± 0.–12(a)
F test	*	*	*	*	*
LSD	1.31	0.38	51.60	0.28	0.28

a) highest value
b) intermediary value
c) lowest value

EAC Ehrlich ascites carcinoma
LSD least significant difference

* highly significant difference at $p \leq 0.01$

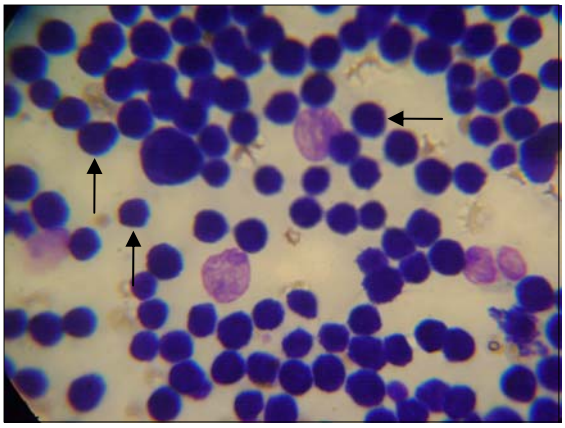

Figure 2
Ehrlich ascites cells smear showing numerous tumour cells
Giemsa stain
(×400)
(Group 2)

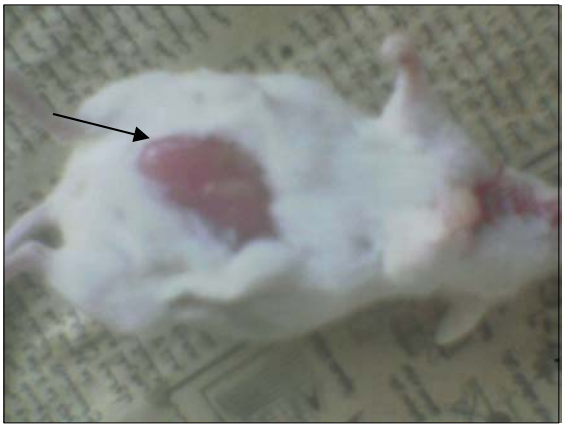

Figure 3
Ehrlich bearing mouse showing voluminous ascitic fluid
(Group 2)

animals in group 3 which was protected with propolis, revealed increasing percentages of MST, ILS and dead cells, with a reduction in body weight, volume of the ascites fluid, total number of EAC cells and percentage of live cells (Figs 6, 7 and 8) which could be due to interference of propolis with the growth of EAC cells directly during the early phase of treatment and leading to a considerable elimination of these cells (18). It may also be due to animals treated with the immuno-stimulants resisting, to various degrees, to the subsequent inoculation of tumour cells as evidenced by the reduced 'tumour take', slowed growth of tumours and prolonged survival of recipients (13). This explanation is confirmed by the immunological effects of propolis which revealed an increase in the lymphocyte transformation rate (LTR) and phagocytic activity and killing percentage tests (Table IV). Increased lymphocyte prolifer-ation lead to enhanced macrophage activation and thus to an amplification of the general immunological responses (22). Group 2 showed a reduction in phagocytic activity and killing percentage which may be due to the development of EAC cells which

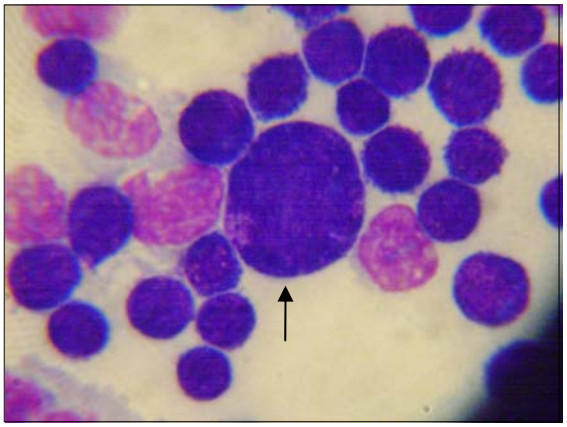

Figure 4
Ehrlich ascites cells smear showing nuclear enlargement and mitosis
Giemsa stain
(×1 000)
(Group2)

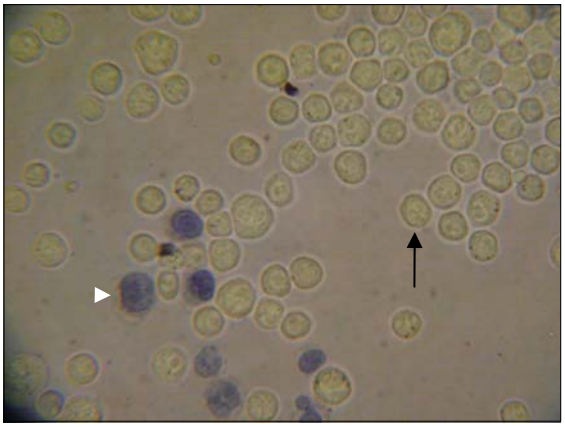

Figure 6
Degenerated Ehrlich ascites cells stained trypan blue 1% (arrowhead) and other unstained live cells (arrows)
(×400)
(Group 3)

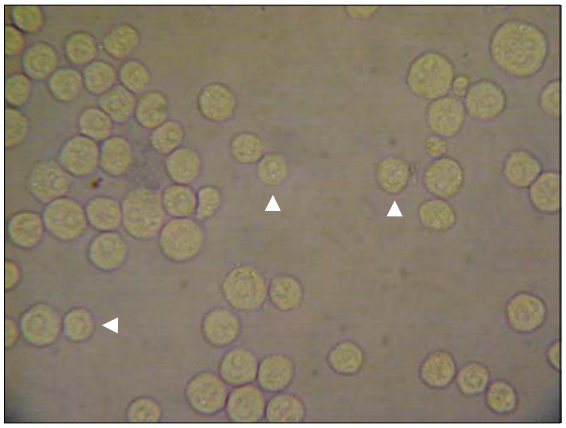

Figure 5
Ehrlich ascites cells showing numerous live cells not stained with trypan blue 1% (arrowheads)
(×400)
(Group 2)

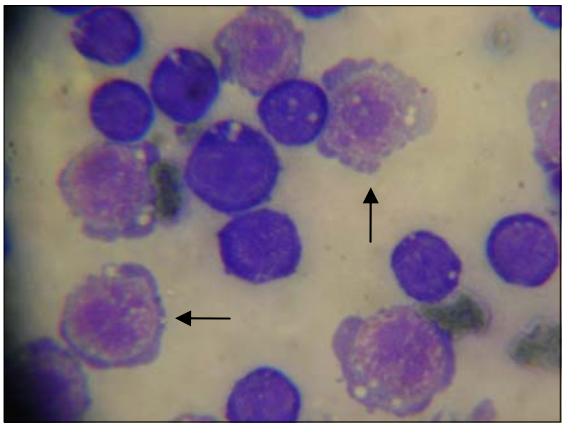

Figure 7
Ehrlich ascites cells smear showing numerous eosinophilic shrunken bodies with condensed and fragmented nuclei
Giemsa stain
(×1 000)
(Group 3)

caused immune suppression with a reduction of lymphocyte viability (15).

The results of haematological studies (Table V) revealed a significant reduction of RBCs in group 2 which may be due to suppressive effect of EAC on bone marrow erythropoiesis (5). On the other hand, the granulocytic leucocytosis that was observed might be due to the acute inflammatory response or stress due to the proliferation of Ehrlich cells (11). Granulocytosis, monocytosis and leucocytosis in group 3 treated with propolis could be interpreted as a result of the immuno-stimulating effect of propolis and the defence of the host against tumour cells (23).

The biochemical results (Table VI) revealed a decrease in the total proteins and albumin levels in group 2; this may be attributed to increased mitotic division of tumour cells with high bloody fluid withdrawal and capillary permeability, which enable the escape of plasma proteins into the peritoneal cavity (9). Furthermore, hypoproteinemia and hypo-albuminemia may be due to excessive

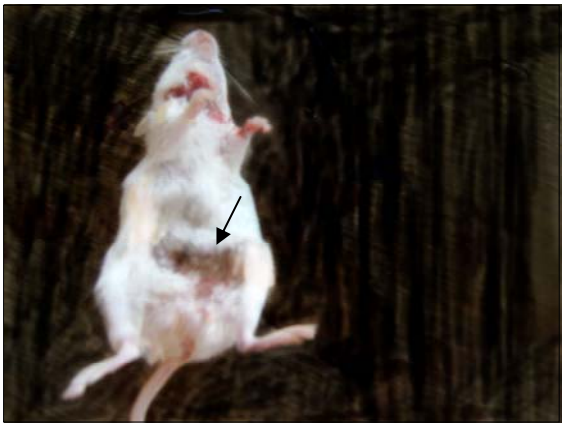

Figure 8
Mouse showing decreased quantity of ascitic fluid
(Group 3)

nephritis (4) which confirmed the result of increased creatinine levels in this group that may be attributed to renal damage as a result of cancer cell invasion (11). Group 3 displayed improvements of these parameters in comparison to the normal control group levels which indicated a protective effect of propolis against organ dysfunction and cellular injury (21).

Conclusions

The treatment of Swiss mice that had received an intraperitoneal injection of EAC 2.5×10^6 and treated with Egyptian propolis (50 mg/kg body weight) 2 h prior to transplantation of EAC and then daily for eleven successive days, resulted in an increase in MST and ILS, the percentage of dead cancer cells and a reduction in the total number and viable percentage of these cells with an increase in LTR, phagocytic activity and killing power in the propolis-treated group. The anti-tumour effects of propolis appeared to contribute to an improvement in haematological and biochemical changes when the treated group was compared to the control group.

Acknowledgments

The authors would like to thank members of Clinical Pathology and Virology Departments of the Faculty of Veterinary Medicine at Zagazig University in Egypt for their valuable help and support. Access to their facilities meant our work could be conducted in optimum conditions.

Table IV
Effect of water soluble derivatives of Egyptian propolis (50 mg/kg body weight) on lymphocyte transformation rate, phagocytic percentage and killing percentage (mean values ± standard error) in mice-bearing Ehrlich ascites carcinoma

Group	Parameters		
	LTR	Phagocytic percentage	Killing percentage
1 Control	1.432 ±0.06[b]	82.20 ±0.20[b]	80.40 ±0.24[b]
2 Mice bearing EAC	1.378 ±0.01[b]	80.20 ±0.37[c]	78.00±0.70[c]
3 Treated with propolis	1.714 ±0.05[a]	87.20 ±0.20[a]	84.60 ±0.24[a]
F test	*	*	*
LSD	0.105	0.80	1.34

a) highest value
b) intermediary value
c) lowest value

LTR lymphocyte transformation rate
EAC Ehrlich ascites carcinoma
LSD least significant difference

* highly significant difference at $p \leq 0.01$

Table V
Effect of water soluble derivatives of Egyptian propolis (50 mg/kg body weight) on haematological parameters (mean values ± standard error) in mice-bearing Ehrlich ascites carcinoma

Group	Parameters				
	RBC count $\times 10^6$/µl	WBC count $\times 10^3$/µl	LYM $\times 10^3$/µl	MID $\times 10^3$/µl	GRA $\times 10^3$/µ
1 Control	7.796 ± 0.35[a]	8.32 ± 0.57[c]	6.16 ± 0.50[b]	1.40 ± 0.19[c]	0.72 ± 0.15[c]
2 mice bearing EAC	6.676 ± 0.10[b]	12.10 ± 1.03[b]	6.92 ± 0.55[a, b]	3.08 ± 0.32[b]	2.08 ± 0.22[b]
3 Treated with propolis	7.494 ± 0.32[a, b]	14.94 ± 0.17[a]	7.67 ± 0.15[a]	4.14 ± 0.10[a]	3.11 ± 0.15[a]
F test	*	**	*	**	**
LSD	0.84	2.04	1.30	0.66	0.52

a) highest value
b) intermediary value
c) lowest value

RBC total erythrocytic count
WBC total leucocytic count
LYM lymphocytes
MID monocytes and some eosinophils
LSD least significant difference
GRA neutrophils, eosinophils and basophils
EAC Ehrlich ascites carcinoma

* significant difference at p≤0.05
** highly significant difference at *p*≤0.01

Table VI
Effect of water soluble derivatives of Egyptian propolis (50 mg/kg body weight) on total proteins, albumin, globulins and creatinine (mean values ± standard error) in mice bearing Ehrlich ascites carcinoma

Group	Parameters			
	Total proteins (g/dl)	Albumin (g/dl)	Globulins (g/dl)	Creatinine (mg/dl)
1 Control	6.98 ± 0.16[a]	3.80 ± 0.20[a]	3.18 ±0.16[a]	0.47 ±0.03[b]
2 Mice bearing EAC	4.96 ± 0.07[c]	1.84 ± 0.10[c]	3.12 ± 0.16[a]	1.34 ± 0.07[a]
3 Treated with propolis	5.50 ± 0.21[b]	2.76 ± 0.12[b]	2.74 ± 0.25[a]	0.56 ± 0.06[b]
F test	*	*	NS	*
LSD	0.48	0.44	–	0.11

a) highest value
b) intermediary value
c) lowest value

EAC Ehrlich ascites carcinoma
NS not significant
LSD least significant difference

* highly significant difference at *p*≤0.01

Ethical issues

Our experiments were conducted in accordance with the guidelines set by Animals Health Research Ethics Training Initiative in Egypt and experimental protocols were approved by the Institutional Animal Ethics Committee.

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