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Egyptian Propolis 10: It's Effect on Hematological Changes and Bacterial Load in Mice-Bearing Ehrlich Ascites Carcinoma and Concurrently Infected with *Staphylococcus aureus*

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Abstract: The influence of ethanolic extract of Egyptian propolis (EEP) on hematological changes was investigated in mature mice bearing Ehrlich ascites carcinoma (EAC) and concurrently infected with *Staphylococcus aureus*. The results revealed that the administration of propolis in a daily dose of 100 mg/kg/mouse through intraperitoneal (i.p.) injection was effective in keeping the hematological profile near the normal range as well as decreasing the bacterial load in the blood of infected mice. The combined action of propolis as antitumor and antibacterial as well as its ability to stabilize the hematological parameters in cancer bearing mice suggests the possibility of using propolis as a natural alternative to chemotherapy to avoid the side effects.

Key words: Propolis · Ehrlich Ascites Carcinoma · Hematological Parameters · *Staphylococcus aureus*

INTRODUCTION

Propolis is a natural product produced by honeybees from substances collected from parts of plants, buds and exudates [1-3]. Propolis has been used since ancient times as a medicine [2, 4] and it has grabbed a great attention of many researchers today due to its chemical composition of more than 300 compounds [5-8] as well as its biological properties including antimicrobial [1, 7], antifungal [9], antiprotozoal [10], antiparasitic [11] and antiviral [1, 12-15]. It has been also used in the treatment of hemorrhage [16-18], platelet aggregation [19], erythrocyte aggregation [20, 21]. It has been also valued due to its antioxidant activity [22-24], hepatoprotective effect [25], immunostimulating properties [7, 26-28] and cytostatic activity [8, 29].

Cancer is one of the main causes of mortality worldwide. Ehrlich Ascites Carcinoma (EAC) is a very rapidly growing carcinoma with very aggressive behavior [30]. It is able to grow in almost all strains of mice. EAC implantation induces a local inflammatory reaction, with increasing vascular permeability, which results in an intense edema formation, cellular migration and a progressive ascitic fluid formation and accumulation [31].

Propolis and its polyphenolic compounds exerted an anti-metastatic and antitumor effect in mice and rats and also exerted considerable cytotoxicity, without cross-resistance, in both wild-type and chemo resistant human tumor cell lines [32].

Malignant diseases may cause predisposition of the host to bacterial infection. The immunosuppressive effect of different types of cancer including EAC was documented earlier [33]. The antimicrobial activity of propolis due to the presence of some active compounds such as polyphenols (flavonoids, phenolic acids and their esters), terpenoids, steroids and amino acids [34] may be advantageous to prevent the bacterial infection in mice which become vulnerable to infection due to EAC.

The aim of the present study was to investigate the antitumor activity of the Egyptian propolis as well as its antibacterial activity against concurrent infection with *S. aureus* in mice bearing Ehrlich ascites carcinoma with correlation to the hematological changes.

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MATERIALS AND METHODS

Animals: The current study was carried out using a total of 200 male healthy Swiss albino mice (20 ± 2 g) obtained from Animal House of National Research Center (NRC), Giza, Egypt. The animals were kept in separate cages with sawdust bedding and maintained under standard laboratory conditions. Standard pellet diet and water were given ad libitum. The mice were acclimatized to laboratory condition for one week before commencement of experiment. The experiments were performed based on animal ethics guidelines of NRC Animals Ethics Committee.

Propolis: Propolis material was collected from apiary farm near El-Mansoura City, Dakahlia Province. The resinous materials were kept in dark bag in the refrigerator till being extracted with ethyl alcohol.

Extraction and Sample Preparation: Propolis sample was cut into small pieces and extracted at room temperature with 50 mL of 70% ethanol (twice after 24 hours) according to [11]. The alcoholic extract was evaporated under vacuum at 50°C until dryness. The percentage of extracted matter was 0.8 gm/dry weight. The dried propolis was used to prepare a solution in normal saline under aseptic conditions.

Ehrlich Ascites Carcinoma: Ehrlich ascites carcinoma cells were obtained from Cancer Biology Section, National Cancer Institute, Cairo, Egypt. The EAC cells were maintained in Swiss albino mice, by intraperitoneal (i.p.) transplantation on every 9 days [35]. The ascitic fluid was collected by syringe and the tumor cell count was performed in a Neubauer hemocytometer. A total of 2×10^7 cells/mL was obtained by dilution with normal saline [36]. Tumor cell suspension showing more than 90 % viability (checked by trypan blue dye (0.4%) exclusion assay) was used for transplantation.

Standard Anticancer Drug: 5-Fluorouracil (5-FU), Calbiochem, USA, was used as a standard anticancer drug. 5-FU was injected intraperitoneally to mice at a dose of 20 mg/kg body weight [37, 38].

***Staphylococcus aureus* Strain:** *Staphylococcus aureus* (ATCC 25923) strain was used in this study and it was enriched on polymyxin agar [39], as a selective media for 24 hours at 37°C. The bacterial suspension was prepared and adjusted by comparison against 0.5 Mc-Farland turbidity standard ($5x10^7$ organisms/mL) tubes. It was further diluted to obtain a final of $5X10^6$ organisms/mL. A volume of 50 µl of the resulting broth was injected to each mouse as a single dose. All culture media used in this study were obtained from Difco Laboratories.

Table 1: Experimental groups

		Treatment				
Group	Group Name	Saline	Propolis	EAC	5-FU	S.
1	Normal control	+	-	-	-	-
2	Propolis control	-	+	-	-	-
3	*S. aureus* control	+	-	-	-	+
4	EAC control	+	-	+	-	-
5	5-FU control	-	-	-	+	-
6	Propolis+*S. aureus*	-	+	-	-	+
7	EAC+Propolis	-	+	+	-	-
8	EAC+5-FU	-	-	+	+	-
9	EAC+ *S. aureus*	+	-	+	-	+
10	EAC+*S. aureus*+Propolis	-	+	+	-	+

Determination of the Number of Bacteria in Blood : Mice were infected with *S. aureus* through a single i.v. injection with 50µl ($5X10^6$ organisms/mL). Mice infected with *S. aureus* were killed after 14 days of the beginning of experiment. Blood was diluted in sterile water containing 0.5% Triton X-100. Bacterial growth was determined by sub-culturing on trypticase soy broth after inoculated by the 0.5 mL of blood sample. The tubes were incubated at 37°C for 24 hr [40]. The growth of bacteria was measured by spectrophotometric assay as optical density (OD) at 420 nm wave length. The mean value of inhibition was calculated from triple reading in each test.

Experimental Design: Two hundred healthy Swiss albino male mice were divided into ten groups (n=20) as shown in Table 1. Both EAC cell line and *S. aureus* were injected at the beginning of the experiment (Day 0). Propolis and reference drug (5-FU) treatment were continued for subsequent 14 days starting from the second day (Day 1).

The first group (normal control) received a daily dose of 50 µl/mouse normal saline through i.p. injection for 14 days. The second group (propolis control) received a daily dose of 100 mg/kg/mouse propolis through i.p. injection for 14 days. The third group (*S. aureus* control) received a single dose of 50 µl ($5X10^6$ organisms/mL) *S. aureus* through i.v. injection followed by a daily injection of normal saline like the first group. The fourth group (EAC control) was inoculated once intraperitoneally with EAC cell line (2×10^6 cells/mouse) followed by a daily injection of normal saline like the first group. The fifth group (5-flurouracil control) received a daily dose of

20 mg/kg/mouse of 5-flurouracil as standard anticancer drug for 14 days. The sixth group (Propolis+*S. aureus*) received the same treatment of propolis like the second group following *S. aureus* infection like the third group. While the seventh group (EAC+Propolis) received the same treatment of propolis like the second group following the inoculation of EAC. The eighth group (EAC+5-FU) received the same treatment with 5-FU like the fifth group following the inoculation with EAC like the fourth group. The ninth group was infected with *S. aureus* like the third group following the inoculation of EAC like the fourth group and this was also followed by a daily injection of normal saline like the first group. The tenth group was treated with propolis the same like the second group following the inoculation of EAC like the fourth group as well as the infection with *S. aureus* like the third group.

Five mice were sacrificed from each group and blood samples were collected at 7, 14 and 21 days intervals post inoculation with EAC cells. Blood samples were used for the determination of hemoglobin concentration [41], total blood count [42], differentials leucocytic count [43] and resolution of *S. aureus* for bacterial count [44].

Statistical Analysis: Data are shown as the Mean ± SE (stander error) when a significant interaction between major factors was identified by ANOVA SPSS version 11.5. Duncan's new multiple range test was used post-ANOVA to identify significant differences between mean values at probability level of ($p<0.05$).

RESULTS

The results of hematological picture of mice bearing carcinoma infected with *S. aureus* were illustrated in Tables (2-5). Both the level of hemoglobin and RBCs count were subjected to decrease in all groups except in propolis control group which was comparable to the normal control group. This decrease was obvious in 21st day post inoculation with hemoglobin levels of 10.9±0.29, 11.0±0.47 and 11.8±0.46 g/dl for *S. aureus* control, EAC control and 5-FU control respectively compared with 13.4±0.29 g/dl for normal control. There was also a significant decrease in the RBCs count due to both EAC inoculation and *S. aureus* infection especially in 21st day post inoculation with RBCs count of 6.68±0.28 and 6.64±0.12 ($x10^6/mm^3$) for EAC control and *S. aureus* control groups respectively as compared with 8.5±0.37 ($x10^6/mm^3$) for normal control group. It was obvious that the treatment of mice- bearing EAC with

Table 2: Effect of propolis on hemoglobin (g/dl) and red blood count ($x10^6/mm^3$) in mice- bearing EAC and concurrently infected with *S. aureus*

		Days post inoculation		
Groups	Parameter	7	14	21
Normal control	HB	13.2±0.41	12.9±0.4	13.4±0.29
	RBCs	8.00±0.69	8.44±0.31	8.5±0.37
Propolis control	HB	13.4±0.29	13.7±0.2	13.2±0.3
	RBCs	8.1±0.48	8.52±0.42	8.2±0.5
S. aureus control	HB	13.3±0.34	11.7±0.37	10.9±0.29
	RBCs	7.46±0.31	6.6±0.22	6.64±0.12
EAC control	HB	13.3±0.25	12.7±0.25	11.0±0.47
	RBCs	6.92±0.31	7.46±0.43	6.68±0.28
5-FU control	HB	13.1±0.33	12.3±0.25	11.8±0.46
	RBCs	7.5±0.61	7.76±0.28	7.22±0.27
Propolis+*S. aureus*	HB	13.1±0.33	11.5±0.27	11.4±0.43
	RBCs	7.7±0.48	6.62±0.3	7.62±0.35
EAC+Propolis	HB	12.9±0.33	11±0.47	11.6±0.29
	RBCs	7.68±0.34	6.68±0.28	7.24±0.31
EAC+5-FU	HB	12.5±0.16	11.8±0.25	11.8±0.25
	RBCs	7.24±0.37	7.38±0.33	7.38±0.33
EAC+ *S. aureus*	HB	12.1±0.29	11.6±0.29	10.6±0.29
	RBCs	7.68±0.65	7.64±0.39	6.42±0.14
EAC+*S. aureus*+Propolis	HB	11.9±0.16	11.4±0.47	11.6±0.47
	RBCs	8.54±0.37	7.68±0.28	7.68±0.28

Table 3: Effect of propolis on WBCs count ($x10^3/mm^3$) and lymphocyte percentage in mice-bearing EAC and concurrently infected with *S. aureus*

		Days post inoculation		
Groups	Parameter	7	4	21
Normal control	WBCs	11.681±0.13	12.233±0.2	12.173±0.1
	Lymphocytes	80.20±0.97	79.40±1.08	80.00±0.45
Propolis control	WBCs	11.891±0.18	13.064±0.39	13.789±0.23
	Lymphocytes	80.40±0.81	80.00±0.71	80.20±0.80
S. aureus control	WBCs	12.227±0.29	13.409±0.24	14.764±0.35
	Lymphocytes	79.80±0.66	80.40±1.33	79.80±0.37
EAC control	WBCs	12.53±0.18	13.29±0.41	15.85±0.27
	Lymphocytes	80.00±1.52	80.80±0.86	86.00±0.71
5-FU control	WBCs	12.85±0.32	12.85±0.32	14.21±0.44
	Lymphocytes	80.20±0.86	80.40±0.81	83.20±0.37
Propolis+*S. aureus*	WBCs	12.544±0.23	13.218±0.47	14.867±0.45
	Lymphocytes	80.40±1.21	81.00±0.95	84.80±1.16
EAC+Propolis	WBCs	13.29±0.41	13.409±0.24	12.761±0.29
	Lymphocytes	80.80±1.83	82.40±1.12	80.80±1.16
EAC+5-FU	WBCs	12.85±0.32	13.14±0.2	12.728±0.29
	Lymphocytes	80.40±0.60	80.20±0.37	80.20±0.37
EAC+ *S. aureus*	WBCs	13.29±0.41	14.12±0.21	11.82±0.24
	Lymphocytes	81.00±1.14	81.20±0.73	77.40±0.81
EAC+*S. aureus*+Propolis	WBCs	13.19±0.24	11.90±0.2	13.218±0.47
	Lymphocytes	79.20±0.73	79.40±0.51	80.20±0.37

Table 4: Effect of propolis on monocytes and neutrophils percentages in mice- bearing EAC and concurrently infected with *S. aureus*

		Days post inoculation		
Groups	Parameter	7	14	21
Normal control	Monocytes	0.80±0.20	1.00±0.32	1.00±0.32
	Neutrophils	16.80±0.37	16.60±0.51	16.80±0.20
Propolis control	Monocytes	0.60±0.40	1.40±0.51	1.20±0.37
	Neutrophils	16.80±0.58	15.80±0.58	16.60±0.51
S. aureus control	Monocytes	1.40±0.24	0.80±0.37	0.60±0.40
	Neutrophils	16.40±0.51	16.00±0.71	18.00±0.63
EAC control	Monocytes	1.00±0.45	1.00±0.32	0.60±0.24
	Neutrophils	15.80±0.58	15.60±0.51	11.80±0.37
5-FU control	Monocytes	1.40±0.24	1.40±0.24	1.00±0.32
	Neutrophils	16.20±0.49	15.20±0.58	13.00±0.32
Propolis+*S. aureus*	Monocytes	1.00±0.45	1.00±0.32	0.80±0.37
	Neutrophils	16.20±0.49	15.40±0.51	12.80±0.58
EAC+Propolis	Monocytes	0.80±0.37	0.60±0.24	1.40±0.24
	Neutrophils	15.80±0.97	15.00±0.55	15.80±0.49
EAC+5-FU	Monocytes	0.80±0.20	1.20±0.37	1.00±0.45
	Neutrophils	16.20±0.58	15.80±0.49	16.00±1.45
EAC+ *S. aureus*	Monocytes	1.00±0.32	1.00±0.32	1.20±0.37
	Neutrophils	15.80±0.66	15.80±0.37	19.00±0.55
EAC+*S. aureus*+Propolis	Monocytes	1.40±0.40	1.00±0.45	1.00±0.00
	Neutrophils	15.60±0.75	15.80±0.37	17.00±0.45

Table 5: Effect of propolis on basophil and eosinophil percentages in mice- bearing EAC and concurrently infected with *S. aureus*

		Days post inoculation		
Groups	Parameter	7	14	21
Normal control	Basophil	0.80±0.37	1.00±0.32	0.80±0.37
	Eosinophil	1.40±0.60	2.00±0.55	1.40±0.60
Propolis control	Basophil	0.80±0.37	0.60±0.24	1.00±0.45
	Eosinophil	1.40±0.24	2.20±0.20	1.00±0.45
S. aureus control	Basophil	0.60±0.24	1.00±0.45	0.20±0.20
	Eosinophil	1.80±0.49	1.80±0.58	1.40±0.24
EAC control	Basophil	1.00±0.45	1.00±0.32	1.00±0.45
	Eosinophil	2.20.± 0.37	1.60±0.40	0.60±0.24
5-FU control	Basophil	0.40±0.24	0.80±0.37	1.20±0.58
	Eosinophil	1.80±0.49	2.20±0.37	1.60±0.24
Propolis+*S. aureus*	Basophil	0.80±0.20	0.60±0.24	0.60±0.40
	Eosinophil	1.60±0.51	2.00±0.45	1.00±0.32
EAC+Propolis	Basophil	0.60±0.40	0.80±0.37	0.60±0.24
	Eosinophil	2.00±0.32	1.20±0.58	1.40±0.68
EAC+5-FU	Basophil	0.80±0.20	0.60±0.24	0.80±0.37
	Eosinophil	1.80±0.20	2.20±0.49	2.00±0.32
EAC+ *S. aureus*	Basophil	0.60±0.24	0.80±0.37	1.00±0.45
	Eosinophil	1.60±0.51	1.20±0.58	1.40±0.51
EAC+*S. aureus*+Propolis	Basophil	1.60±0.24	1.40±0.24	0.60±0.40
	Eosinophil	2.20±0.37	2.40±0.40	1.20±0.37

Table 6: Bacterial load in blood of mice- bearing EAC and concurrently infected with S. aureus 14 days post infection as measured in (OD) at 420 nm.

Group Name	Bacterial load of *S. aureus*
S. aureus normal growth	1.559 ± 0.005*
EAC+ *S. aureus*	1.901± 0.015
Propolis+*S. aureus*	0.681 ± 0.004
EAC+S. aureus+Propolis	1.142 ± 0.002

* Growth = the growth measured by optical density (OD) at 420 nm analyzed by spectrophotometer.

propolis reduced the decrease in both hemoglobin concentration and RBCs count. The results of hemoglobin concentration and RBCs count in Propolis+*S. aureus* group were 11.4±0.43 g/dl and 7.62±0.35 ($x10^6/mm^3$) respectively compared with 10.9±0.29 g/dl and 6.64±0.12 ($x10^6/mm^3$) respectively in *S. aureus* control group. While the results of hemoglobin concentration and RBCs count in EAC+Propolis group were 11.6±0.29 g/dl and 7.24±0.31 ($x10^6/mm^3$) respectively compared with 11.0±0.47 g/dl and 6.68±0.28 ($x10^6/mm^3$) respectively in EAC control group.

EAC inoculation increased WBCs count starting from 7^{th} day post inoculation. WBCs count was 15.85±0.27 ($x10^3/mm^3$) in 21^{st} day post inoculation in EAC group as compared with 12.173±0.1 ($x10^3/mm^3$) for the normal control group (Table 3). Injection of *S. aureus* and propolis also increased WBCs count but with lower levels compared with EAC.

Differential leucocytic counts for all groups are shown in Tables (3, 4 and 5). The results showed an increase in the percentage of lymphocytes following inoculation with EAC while injection of *S. aureus* caused an increase in the percentage of neutrophils. Meanwhile injection of propolis brought both percentages to near the normal range. Monocytes, basophiles and eosinophils did not show any significant changes when compared with normal control group.

The influence of administration of propolis on the bacterial load in mice bearing EAC and concurrently infected with *S. aureus* was illustrated in Table (6). There was a significant decrease in bacterial load in blood of infected mice following the administration of propolis in both Propolis+*S. aureus* and EAC+*S. aureus*+Propolis when compared with *S. aureus* normal growth and EAC+ *S. aureus* groups respectively.

DISCUSSION

Malignant diseases may cause predisposition of the host to bacterial infection [45, 46]. The immunosuppressive effect of different types of cancer including EAC was documented earlier. In EAC, this effect was due to the presence of low molecular weight factors in the ascetic fluid that can cause an impairment of macrophages function [33].

Propolis exhibited antitumor activity in mature mice bearing Ehrlich carcinoma [47]. The antitumor activity of propolis and other bee products could be due to the presence of a variety of compounds considered to be the most promising of the antitumour agents including caffeic acid (CA), caffeic acid phenethyl ester (CAPE), artepilin C,

quercetin, naringenin, resveratrol, galangin, genistein, plukenetione A and others [48-58]. CAPE isolated from propolis was found to have strong inhibitory activity on processes essential for tumor promotion and exhibited potent chemopreventive activity when used to treat mice topically even in low doses [29]. CAPE possesses various therapeutic effects including antimicrobial, antioxidant, anti-inflammatory and cytotoxic properties [59]. Artepillin C was also isolated from Brazilian propolis which exhibited preferential cytotoxic activity against tumor cells cultured *in vitro* [51, 60].

Honeybee products in general and their flavonoid components are of the most promising antitumor natural products [32], due to their immunomodulatory activity [51, 54-57, 61-65]. Scheller *et al* [47] reported that the ethanolic extract of propolis was capable of increasing the survival of mice-bearing Ehrlich carcinoma and suggested that the immunostimulatory activity of propolis may be associated with macrophage activation and enhancement of their phagocytic activity.

Matsuno, [66] isolated and identified a new clerodane diterpenoids in Brazilian propolis capable to arrest tumor cells at S phase and killed them within 3 days. However, the compound showed little cytostatic effect on human diploid cells. In another study, Matsuno, [60] isolated several compounds from Brazilian propolis including flavonoids, caffeic acid phenethyl ester and three- clerodane diterpenoids which showed tumouricidal activity. Flavonoids, terpenoids and steroids have received considerable attention in recent years due to their diverse pharmacological properties including antioxidant and antitumor activity [67, 68]. Meanwhile, Crude Egyptian propolis exhibited strong inhibitory activity against tumors. The anti-tumor mechanism was suggested to be mediated by preventing oxidative damage and induction of apoptosis [69].

Bacterial infection is one of the major causes of death worldwide. *S. aureus* is a dangerous Gram-positive pathogen which causes a wide range of infectious diseases including abscesses of various organs, pneumonia, osteomyelitis, endocarditis, arthritis and sepsis. Treatment of these infections has become more difficult because of the emergence of multidrug-resistant strains [70]. The antibacterial activity of propolis was also documented and it was shown that propolis has the ability to inhibit the bacterial growth by preventing cell division as well as disorganizing the cytoplasm, the cytoplasmic membrane and the cell wall causing a partial bacteriolysis and it also inhibits protein synthesis [71].

In cancer chemotherapy the major problems are myelo-suppression and anemia [72, 73]. The anemia encountered in tumor bearing mice is mainly due to reduction in RBC or he-moglobin percentage and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions [74].

In this study it was obvious that the treatment of mice bearing EAC with propolis reduced the decrease of hemoglobin concentration and erythrocytes count. The decrease in both hemoglobin concentration and erythrocytes count have been reported in mice bearing mammary carcinoma as well as mice bearing solid extramedullary Ehrlich ascites tumor. This was found to be, in part, due to the suppression of erythropoiesis in the bone marrow to less than 30% [75, 76]. On the other hand tumor growth was accompanied with characteristic red cell deformation (echinocytosis) [77, 78].

The presence of flavonoids in propolis may be responsible on the improvement in the anemic status of mice as it is known that the majority of flavoniods protect against deformability of erythrocytes and they also found to improve erythrocyte osmotic fragility [79-81]. The administration of propolis and malic acid returned the values of the RBCs indices including MCV, MCH and MCHC near or above normal values [82].

Leukocytosis has been reported in mice bearing Ehrlich carcinoma [76]. This may be due to disturbances in bone marrow activity possibly as an immune defense reflex [83]. This disturbance leads to marked decrease in mature forms of granulocytes and lymphocytes which might reflect increased rate of release of these cells in peripheral blood circulation as a compensatory mechanism. The administration of propolis was found to modulate the peripheral blood mononuclear cells (PBMCs) mitogenic responses which was suggested to be due to the presence of immunoregulatory components [84].

Bee products including propolis were found to modulate the immune response against infection. This modulation may be due to the presence of artepillin C and its ability to increase the ratio of CD4/CD8 T cells [51]. Also, Hegazi et al. [85] studied the effect of some bee products on immune response of chicken infected with virulent Newcastle Disease Virus (NDV). They found that, the mortality rate was reduced in groups infected with virulent NDV and subsequently treated with propolis when compared with the infected groups only.

The ability of flavonoids isolated from propolis to prevent the toxicity of some drugs such as cisplatin and doxorubicin was documented [86]. These findings suggest that propolis can be used, not only separately as

a single therapeutic agent, but also in combination with other drugs for more efficiency and less side effects. Further studies are needed to standardize the use of propolis as potent multifunctional drug for the treatment of different diseases.

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