

Synergistic anti-tumour effect of propolis against Ehrlich carcinoma

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Abstract: Two hundred and fifty female Swiss Albino mice were used to study synergistic anti-tumour activity of propolis to enhance methotrexate activity on mice bearing Ehrlich ascites carcinoma (EAC). They equal divided into 5 groups: 1st kept as negative control, 2nd was implanted intraperitoneally with 2.5×10^6 EAC and kept as positive control and, 3rd implanted intraperitoneally with 2.5×10^6 EAC and treated with propolis by dose (50 mg/kg body weight), were given by gastric intubations 2 hours prior to the intraperitoneal injection of EAC, 4th implanted intraperitoneally with 2.5×10^6 EAC and treated with methotrexate by dose (0.4 mg/kg body weight) and 5th implanted with the same count of the EAC cells and treated with combination of propolis and methotrexate (50 mg/kg body weight and 0.4 mg/kg body weight, respectively) for eleven successive days. Increasing mean survival time (MST), increasing life span (ILS %) and treated vs. positive control (T/C %) in the all treated groups with increased of the body weight, volume of ascitic fluid, total number of EAC cells, viable % cells and decreased of dead% cells in second group while in groups 3,4 and 5 which treated by trials of propolis, methotrexate and combination of the two compounds respectively, revealed decreasing in body weight, volume of the ascitic fluid, total number of EAC cells and the percentage of life cells. Histopathology revealed that least degree of malignancy was in combination group where malignant happens.

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1. Introduction

Malignant cancer diseases are responsible for the death of about one fifth of the population. 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. Many plants and animal extracts have shown various biological activities like immunopotentiating and anti-tumour activities. The role of the immune system in the prevention, control and destruction of tumour is less well understood. Developed tumors are generally not efficiently recognized and eliminated by the immune system. Part of the etiology of impaired immune responsiveness is due to tumor development of a number of mechanisms to avoid reduce or eliminate reactivity (Stephen *et al.*, 2001). Propolis (bee glue) is the generic name for the resinous substance collected by honey bees from various plant sources and used by bees to seal holes in their honeycombs, smooth out the internal walls, and protect the entrance against intruders (Ghisalberti, 1979). Honey bee propolis and its components are of the most promising as anti-tumour agent (Akao *et al.*, 2003). Methotrexate (MTX) is widely used as a cytotoxic chemotherapeutic agent for treatment of leukemia's and other malignancies. In addition, it has been used for the treatment of various inflammatory diseases such as

psoriasis and rheumatoid arthritis. However, the efficacy of this agent in high doses has been associated with hepatotoxicity (Jahovic *et al.*, 2003).

The present work is aimed to study some anti-tumour activity and immunological changes after treatment of Ehrlich ascites carcinoma bearing mice using natural products (Egyptian propolis) or synthetic products (MTX) and effect of combination against EAC.

2-Materials and Methods**2.1. Experimental animals**

A total of 250 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*.

2.2. Ehrlich ascites carcinoma cells

The parent line of Ehrlich ascites carcinoma cells was kindly supplied by the National Cancer Institute of Cairo University, Egypt. The tumour line was maintained by serial intraperitoneal transplantation of Ehrlich ascites carcinoma 2.5×10^6 tumour cells/0.2 ml in female Swiss albino mice (Salem *et al.*, 2011).

2.3. Antineoplastic agents

2.3.1. Propolis

Obtained from an Egyptian honey bee keeper, Propolis bulk was cut into small pieces, mixed with deionised water and shaken at 95°C for 2 hrs. It was cooled to room temperature and centrifuged at 1500 rpm for 10 min to obtain the supernatant which was kept in a dark place until used.

2.3.2. Trexan

Methotrexate (MTX) 2.5 mg Tablets. Orion Corporation Finland.

2.4. Experimental design

Two hundred and fifty five female Swiss Albino mice were equally divided randomly into five groups (50 mice per group). 1st kept as negative control, 2nd were implanted intraperitoneally with 2.5×10^6 EAC and kept as positive control and, 3rd implanted intraperitoneally with 2.5×10^6 EAC and treated with propolis by dose (50 mg/kg body weight) were given by gastric intubations 2 hours prior to the intraperitoneal injection of EAC, 4th implanted intraperitoneally with 2.5×10^6 EAC and treated with MTX by dose (0.4 mg/kg body weight) and 5th implanted with the same count of the EAC cells and treated with combination of propolis and MTX (50 mg/kg body weight and 0.4 mg/kg body weight) respectively by gastric intubations then daily for eleven successive days as in (Table I). Endpoint of experiment was determined by spontaneous death of animals.

2.5. Survival analysis

Five mice from each group were kept under daily observation for survival analysis. Endpoint of experiment was determined by spontaneous death of animals. Results are expressed as percent of mean survival time of treated animals over mean survival time of the control group (treated vs. positive control, T/C %). The percentage of increased life span (ILS) was calculated according the formula: $ILS \% = (T-C)/C \times 100$ where T represents mean survival time of treated animals; C represents mean survival time of the positive control group. By NCI criteria, T/C exceeding 125% and ILS exceeding 25% indicate that the drug has a significant anti-tumour activity (Plowman *et al.*, 1995).

2.6. Viability test and counting of EAC cells.

After mice were euthanized, the peritoneal cavity was opened carefully and all ascitic fluid was aspirated and examined for total number of cells. The tumour cell count was done using a Neubauer hemocytometer, erythrocytic pipette and trypan blue stain 1% (Cabral *et al.*, 2001). The ability of the

living cell to exclude trypan blue was used in viability test (Boyse *et al.*, 1964) to determine the viable, unstained, tumour cells. Stained cells were dead.

2.7. Blood sampling

Blood samples were collected from the retro-orbital venous plexus after they have been anaesthetised were taken in a sterile heparinised test tube for immunological analysis.

2.8. Immunological studies

Lymphocytic transformation assay using [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl 2H tetrazolium bromide], 2348-71-2 is a methyl tetrazolium dye (MTT) staining procedure (Bounous *et al.*, 1992), using Rosewell Park Memorial Institute 1640 "RPMI 1640" tissue medium and lymphocyte separation medium, blood collected in heparinised tubes and used to prepare leucocytes for bacterial phagocytic activity and killing power (Woldehiwet and Rowan, 1990).

2.9. Histopathology

Specimens from the peritoneum were fixed in 10% neutral buffered formalin. Paraffin sections of 5 μ thickness were prepared from all specimens and were stained by haematoxylin and eosin (H & E) and examined microscopically (Bancroft *et al.*, 1996).

2.10. Statistical analysis

The data obtained from this investigation were statistically analysed using F test (Tamhane and Dunlop, 2000). Means at the same column followed by different letters were significantly different and the highest value was represented with the letter (a).

3-Results and Discussion

In EAC bearing mice, a regular rapid increase in ascites tumour volume is seen. Ascitic fluid is the direct nutritional source for tumour cell and so a rapid increase of this fluid is very necessary factor for tumour growth and nutrition. An anticancer drug is considered reliable if it can prolong the life span of mice implanted with EAC cells (Clarkson and Burchenal, 1965).

From survival analysis results (Tables II,III,IV), shows that mean survival time (MST) and increasing life span (ILS%) was reduced with increased of the body weight, volume of ascitic fluid, total number of EAC cells, viable %cells and decreased of dead %cells in group 2 bearing EAC alone without treatment, which may be attributed to a higher mitosis and fewer cell dying which could be attributed to the decrease rate of the natural death mechanisms that occur in the tumour (Cabral *et al.*, 2001). The accumulation of ascitic

fluid in the peritoneal cavity was either due to: (i) a reduced lymphatic recovery system, which is associated with the obstruction of the lymphatic by tumour cells. (ii) angiogenesis, which detected in ascites tumour bearing peritoneal wall, (iii) micro vessels hyperpermeability of the peritoneal cavity (Funasaka *et al.*, 2002). On the contrary in groups 3,4 and 5 which treated by trials of Egyptian propolis, Trexan and combination of the two preparations, respectively, revealed increasing of MST, ILS percentage, dead percentage cells with a reduced in body weight, volume of the ascitic fluid, total number of EAC cells and the percentage of life cells this could be due to interfere with the growth of EAC cells directly during early phase of treatment leading to a considerable elimination of these cells (Orsolich *et al.*,2005) and also may be due to animals treated with the immune-stimulants resist in various degrees subsequent inoculation of tumour cells as evidenced by the reduced “tumour take”, slowed growth of the tumours ,and prolonged survival of recipients (Hayashi *et al.*, 2000). While in the forth group may be as a result of inhibition of EAC proliferation (Alonso *et al.*, 2005) but the fifth group which revealed the best result in survival analysis, body weight and EAC cells count, that could be due to improve cellular immune response (Ma *et al.*, 2011) and antioxidant system (Miguel *et al.*, 2010) that maximize their anti-tumour activity when using water soluble of propolis combined with chemotherapeutic agents (Orsolich and Basic, 2005). These results are confirmed with examination of peritoneal wash for EAC either by Giemsa or trypan blue stains which revealed that Ehrlich ascites carcinoma film stained with Giemsa stain, numerous tumour cells with nuclear enlargement and mitosis in second group (Fig.1). Varying degrees of eosinophilic shrunken bodies with condensed and fragmented nuclei of EAC were seen in groups (3,4 and5) and were pronounced in fifth group that treated by combination of the two preparations (Figs. 2-4). On the other hand, film stained with trypan blue stain revealed numerous life cells not stained blue in second group (Fig.5) while varying number of dead EAC cells stained blue were seen in groups (3,4 and 5) and were abundant in fifth group (Figs. 6-8). In spite of reduction in total number of EAC cells in fourth group than third group, better survival time was in the third group. This may be due to methotrexate have severe toxicity including liver and acute kidney injury (Vilay *et al.*, 2010) while propolis have marked hepatorenal protective potential because of its composition of minerals and flavonoids (Bhadauria *et al.*, 2007).

This previous explanation is confirmed with the immunological results of third group received propolis

which revealed an increased of lymphocyte transformation rate (LTR) and phagocytic activity and killing percentage tests. Increased lymphocyte proliferation leads to enhanced macrophage activation and thus to an amplification of the general immunological responses (Stuehr and Nathan,1989), That consider one of the possible mechanisms of anti-tumour influence of propolis which include immunomodulatory activity of cytotoxic activity to tumour cells and their capability to induce apoptosis and / or necrosis. Thus test components may have direct and / or indirect action on tumour cells by stimulating the host cells (Orsolich *et al.*, 2006). But second group, in spite of non significant change in the (LTR) there are a significant reductions in phagocytic activity and killing % tests this may be due to development of EAC cells caused immune suppression with a reduction of lymphocyte viability (Mandal and Poddar,2007).while in forth group that received methotrexate showed a significant reduction in the LTR, phagocytic activity and killing percentage tests that could be attributed to suppress in the immune response that is the major side effect during cancer chemotherapy (Oldham and Dillman,2009) .On the other side fifth group that received combination of the propolis and methotrexate revealed an improvement in theses parameters due to immunostimulant effect of propolis (Orsatti and Sforcin,2011) (Table V).

All of the above results are confirmed with histopathological examination of the liver; kidneys, spleen and lung in the different groups which revealed varying degree of malignancy were seen. The least degree was in fifth group which received combination of propolis and methotrexate where malignant cells became smaller and showed less degree of malignancy and apoptosis. This could be due to direct cytotoxicity of methotrexate on tumour cells (Colleoni *et al.*, 2002), and propolis may be beneficial in maximizing antitumor activity of anticancer chemotherapy (Benkovic *et al.*,2007) by immunostimulant and antioxidant effect of propolis (Orsolich and Basic,2003 , Padmavathi *et al.*, 2006). This result was in agreement with the histopathological examination, serous surface of peritoneum and the hepatic and renal capsules showed stuck EAC cells. This cells were numerous in gp.(2) forming large tumour mass represented by clusters or sheets from large polymorphic cells with large vesicular hyperchromatic nuclei with mitotic activities and distinct cytoplasm replacing the omental fat .Neoplastic cells invade the adjacent renal, hepatic and splenic parenchyma (Figs.9-12).

Table I: Experimental design

Groups	No. of mice	Design	IP EAC 2.5×10^6 cells	
			IP EAC 2.5×10^6 cells	Treatments
1	50	Normal control	-	-
2	50	EAC	+	-
3	50	EAC+WSPD	+	50 mg/kg body weight
4	50	EAC+MTX	+	0.4 mg/kg body weight
5	50	EAC+WSPD+MTX	+	50 & 0.4 mg/kg body weight

EAC Ehrlich ascites carcinoma
IP Intraperitoneally
WSPD Water soluble propolis derivatives
MTX Methotrexate

Table II: Effect of WSPD propolis and methotrexate (50 mg/kg body weight, 0.4mg/kg body weight) on MST, ILS% and T/C% in Ehrlich ascites carcinoma bearing mice.

Groups	Range of survival time	Parameters		
		MST	ILS (%)	T/C (%)
2(Mice bearing EAC)	11-14	12.5	-	-
3(Propolis treated group)	17-24	20.5	64	164
4(Methotrexate treated group)	15-23	19	52	152
5(Combination treated group)	22-31	26.5	112	212

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

Table III: Effect of WSPD propolis and methotrexate (50 mg/kg body weight, 0.4mg/kg body weight) respectively on body weight and volume of ascitic fluid (mean values \pm SE)

Group	Parameters	
	Body weight (gm)	Volume of ascites fluid (ml)
1(Control)	20.39 ^c \pm 0.17	-
2(Mice bearing EAC)	28.49 ^a \pm 0.70	6.23 ^a \pm 0.18
3(Propolis-treated group)	24.83 ^b \pm 0.25	2.89 ^b \pm 0.12
4(Methotrexate treated group)	24.03 ^b \pm 0.19	1.87 ^c \pm 0.10
5(Combination treated group)	21.35 ^c \pm 0.10	0.75 ^d \pm 0.08
F test	**	**
LSD	1.06	0.34

** Highly significant difference at $p \leq 0.01$
EAC Ehrlich ascites carcinoma
LSD least significant difference

Table IV: Effect of WSPD propolis and methotrexate (50 mg/kg body weight, 0.4mg/kg body weight) respectively on total, life% and dead % of EAC cells (mean values \pm SE)

Groups	Total EAC cells ($N \times 10^5$)	Parameters	
		Life %	Dead %
1(Control)	-	-	-
2(Mice bearing EAC)	973.8 ^a \pm 28.35	98.82 ^a \pm 0.10	1.18 ^d \pm 0.10
3(Propolis-treated group)	651.8 ^b \pm 10.25	96.66 ^b \pm 0.12	3.34 ^c \pm 0.12
4(Methotrexate treated group)	446.0 ^c \pm 19.78	91.83 ^c \pm 0.29	8.17 ^b \pm 0.29
5(Combination treated group)	118.0 ^d \pm 1.41	88.04 ^d \pm 0.39	11.96 ^a \pm 0.39
F test	**	**	**
LSD	47.80	0.67	0.67

** Highly significant difference at $p \leq 0.01$
EAC Ehrlich ascites carcinoma
LSD least significant difference

Table V: Effect of WSDP propolis and methotrexate (50 mg/kg body weight, 0.4mg/kg body weight) respectively on LTR, phagocytic activity and killing % (mean values \pm SE)

Groups	Parameters		
	LTR	Phagocytic activity	Killing %
1(Control)	1.433 ^b \pm 0.04	82.21 ^b \pm 0.20	80.41 ^b \pm 0.24
2(Mice bearing EAC)	1.379 ^b \pm 0.04	80.21 ^c \pm 0.37	78.00 ^c \pm 0.70
3(Propolis-treated group)	1.715 ^a \pm 0.01	87.21 ^a \pm 0.20	84.61 ^a \pm 0.24
4(Methotrexate treated group)	1.237 ^c \pm 0.01	75.61 ^d \pm 0.24	73.21 ^d \pm 0.58
5(Combination treated group)	1.414 ^b \pm 0.01	79.41 ^c \pm 0.50	76.61 ^c \pm 0.60
F test	**	**	**
LSD	0.07	0.97	1.51

** Highly significant difference at $p \leq 0.01$ EAC Ehrlich ascites carcinoma LTR lymphocyte transformation rate

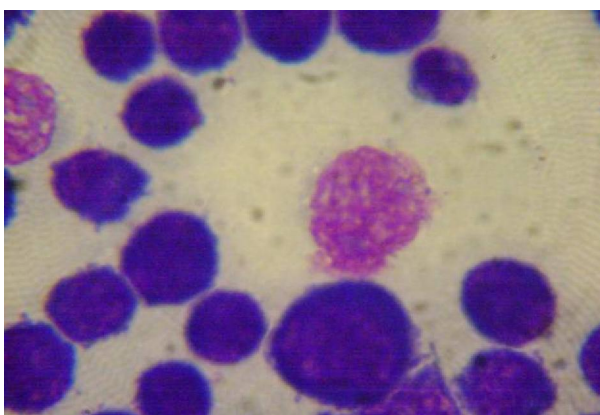


Figure 1
Gp.(2), Ehrlich ascites cells smear showing nuclear enlargement and mitosis, Giemsa stain, X400.

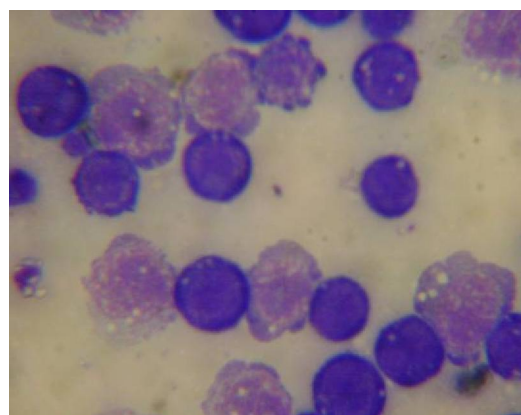


Figure 2
Gp.(3), Ehrlich ascites cells smear showing eosinophilic shrunken bodies with condensed and fragmented nuclei, Giemsa stain, X400.

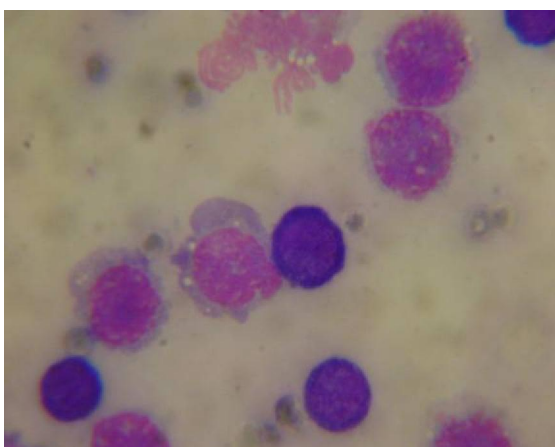


Figure 3
Gp.(4), Ehrlich ascites cells smear showing numerous eosinophilic shrunken bodies with condensed and fragmented nuclei, Giemsa stain, X400.

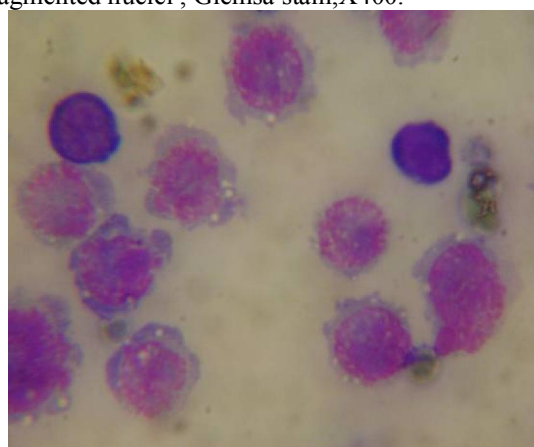


Figure 4
Gp.(5), Ehrlich ascites cells smear showing numerous eosinophilic shrunken bodies with condensed and fragmented nuclei without mitosis and nuclear enlargement, Giemsa stain, X400.

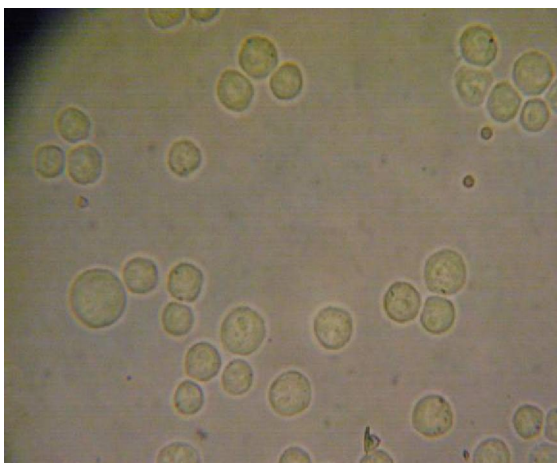


Figure 5
Gp.(2), Ehrlich ascites cells stained by trypan blue 1% showing numerous life cells not stained blue,X400.

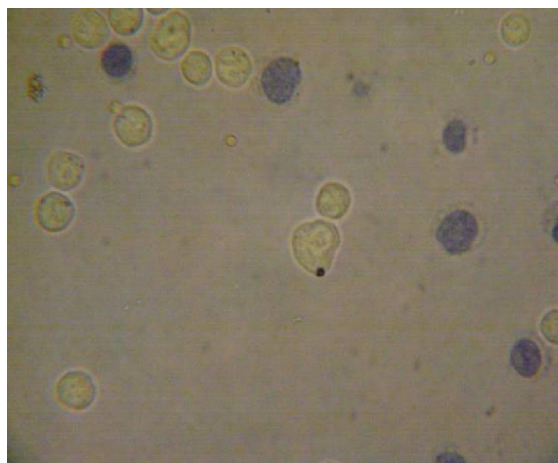


Figure 6
Gp.(3), Degenerated Ehrlich ascites cells stained blue by trypan blue 1% and other unstained life cells,X400.

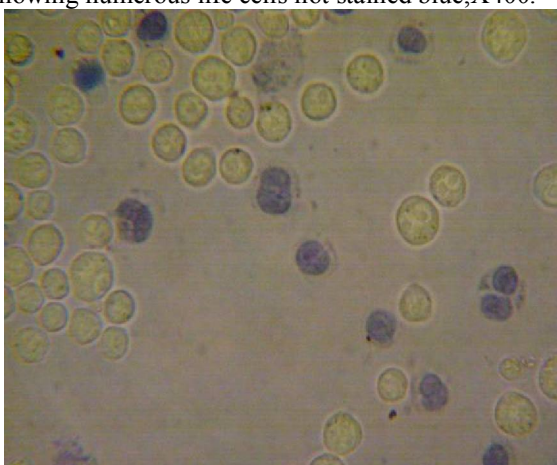


Figure 7
Gp.(4), Increased degenerated Ehrlich ascites cells stained blue by trypan blue 1% and other unstained life cells,X400.

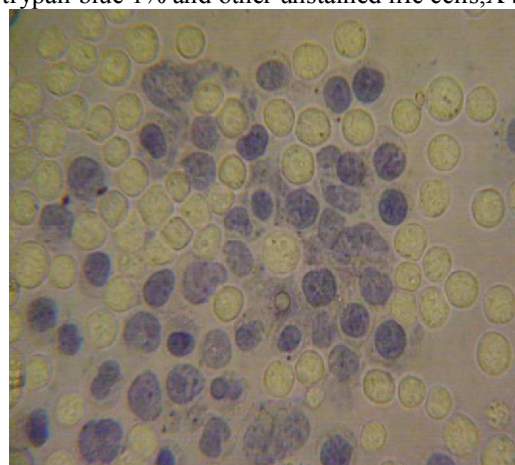


Figure 8
Gp.(5),very increased of degenerated Ehrlich ascites cells stained blue by trypan blue 1% and other unstained life cells,X400.

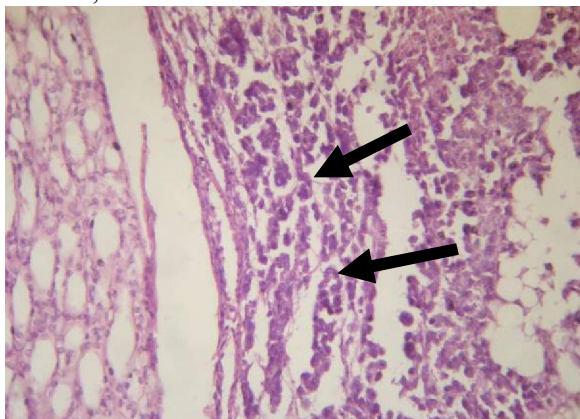


Figure 9
Gp.(2), Peritoneum of mice showing clusters or sheets from neoplastic cells with the criteria of malignancy(arrow),H & E , X 300.

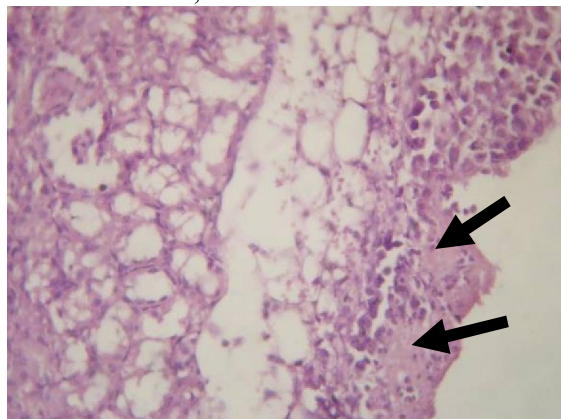


Figure 10
Gp.(3), Peritoneum of mice showing apoptosing and necrosis of neoplastic cells(arrow), H & E , X 300.

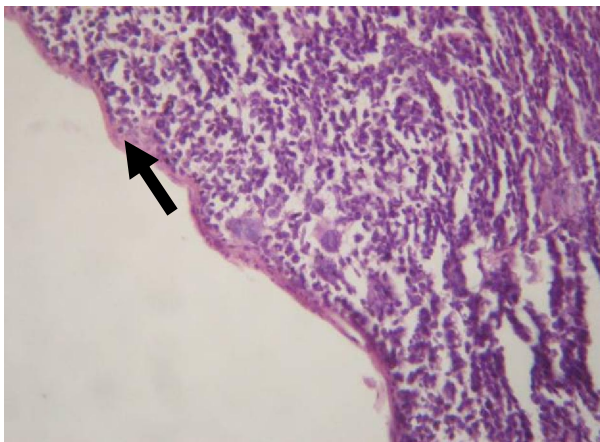


Figure 11
Gp.(4), Peritoneum of mice showing apoptosing and necrosis of neoplastic cells(arrow), H & E , X 120.

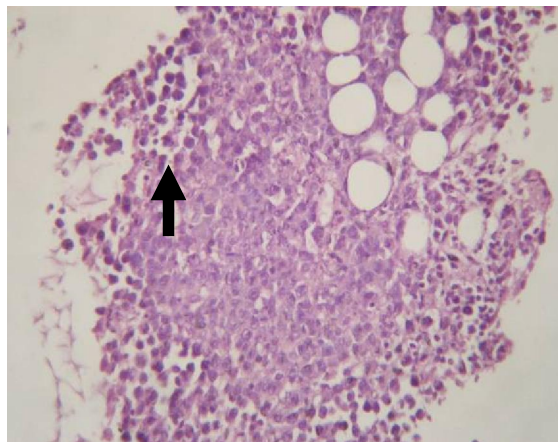


Figure 12
Gp.(5), peritoneum of mice showing tumor mass invaded by different leucocytes, H &E , X 300.

Conclusions

Treatment of Ehrlich ascites carcinoma 2.5×10^6 transplanted intraperitoneally in Swiss mice by combination of Egyptian propolis (50 mg/kg body weight) and methotrexate (0.4 mg/kg body weight) most effectiveness on EAC than each of them administrated alone, observed as increasing the mean survival time and life span.

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