Propolis Protects Against Methotrexate Induced Hepatorenal Dysfunctions during Treatment of Ehrlich Carcinoma

Mohamed O. T. Badr*; Nariman M.M Edrees; Amany A.M Abdallah; Mohamed A. Hashem ; Nasr A.M.N. El-Deen; Ahmed N F. Neamat-Allah and Hager T.H Ismail

Department of Clinical Pathology, Faculty of Veterinary Medicine, Zagazig University, 1 Alzeraa Street Postal Code 44511, Zagazig City, Sharkia Province, Egypt. *drosamabadr@yahoo.com

Abstract: Two hundred and fifty female Swiss albino mice were used to study the ability of Egyptian propolis to protect methotrexate induced dysfunction to liver and kidneys of mice bearing Ehrlich ascites carcinoma (EAC). They equal divided into 5 groups: 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), was 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 gastric intubations .Antioxidant analysis revealed a decrease in superoxide dismutase (SOD), reduced glutathione (GSH) and catalase (CAT) and an increase in malondialdhyde (MAD) in second and forth groups, the opposite in third group, while fifth group showed reverse in antioxidant level toward the normal control group. Biochemical analysis of serum showed that implantation of EAC in Swiss mice without treatment revealed a significant decrease in total protein and albumin levels without change in globulin level and a significant increase in creatinine level and ALT, AST activities, while the third group that received propolis revealed an improvement in these biochemical parameters compared to the normal control group. Forth group revealed a significant increase in ALT, AST activities and creatinine level and decrease in total proteins, albumin and globulin while fifth group revealed amelioration of these parameters and confirmed with histopathological examination of liver and kidneys.

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1. Introduction

Cancer is considered one of the most common causes of mortality worldwide. Progress made in cancer therapy has not been sufficient to a significantly lower annual death rate from most tumour types, and there is an urgent need for new strategies in cancer control (Lahouel *et al.,* 1987). The basis of cancer chemotherapy lies in an understanding of biochemical abnormalities during metabolism of malignant cell. Exploitation of metabolic differences between tumour and host tissue has become one way 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 rodent has provided a useful tool for basic cancer research, Ehrlich ascites tumour is such a tumour that provides a reasonably homogenous sample of malignant tissue; it is available in large quantities and grows at a fairly predictable rate (Goldie, 1956). Methotrexate (MTX), is a known antineoplastic agent, has been a useful agent for the treatment of human cancer and other diseases, like psoriasis vulgaris or rheumatoid arthritis. Theodore *et al.,* 1998, reported that MTX induced liver damage that was well characterized by fatty changes in hepatocytes and sinusoidal lining cells, mild necrosis and inflammation (Hemeida and Mohafez, 2008). It has been suggested that the therapeutic activities of propolis depend mainly on the presence of flavonoids which have been reported to induce the immune system and activity as oxygen radical scavengers (Havsteen, 1983). It has been postulated that the primary mechanism of chemoprevention of tumour by antioxidant is through the reduction of DNA damaging free radicals (Choi *et al.,* 1999). In addition to their antimutagenic properties, studies have suggested that antioxidants can directly induce apoptosis in tumour cells (Nomura *et al.,* 2001). Numerous studies showed that different antioxidants such as flavonoids reduce the adverse effects of the same chemotherapeutic agents on normal cells (Borek, 2004).

The present work is aimed to study some antioxidant, biochemical, histopathological effects of

propolis during treatment of Ehrlich ascites carcinoma bearing mice using synthetic products (MTX) and study if have protective effect on liver and kidneys.

2-Materials and Methods

2.1. Experimental animals

A total of 250 adult female Swiss albino mice (average 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.

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). The tumour cell count was done using a Neubauer hemocytometer, erythrocytic pipette and trypan blue stain 1% (Cabrales *et al.,* 2001).The ability of the living cell to exclude trypan blue was used in viability test (Boyse *et al.,* 1964).

2.3. Antineoplastic agents

2.3.1.Propolis

Obtained from an Egyptian honey bee keeper and 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 5 min to obtain the supernatant which was kept in a dark bottle until used.

2.3.2. Trexan

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

2.4. Experimental design

Two hundred and fifty female Swiss mice were equally divided randomly into fives groups (50 mice per group). 1st kept as negative control, 2nd were implanted intraperitoneally with 2.5×106 EAC and kept as positive control and, 3rd implanted intraperitoneally with 2.5×106 EAC and treated with propolis by dose (50 mg/kg body weight) given by gastric intubations 2 hours prior to the intraperitoneal injection of EAC ,4th implanted intraperitoneally with 2.5×106 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) given by gastric intubations daily for eleven successive days as in (Table I). Endpoint of experiment was determined by spontaneous death of animals.

2.5. Blood sampling

Blood samples were collected from the retro-orbital venous plexus without anticoagulant in a sterile test tube for separation of serum which was used to measure biochemical parameters.

2.6. Antioxidant enzymatic activities in liver tissue homogenate.

The supernatant obtained after centrifugation of liver homogenates was used for the determination of enzyme activities. Catalase activity CAT (Aebi, 1984) ,lipid peroxidase activity expressed by Malondialdhyde MAD (Satoh,1978), reduced Glutathione GSH (Beutler *et al.,* 1963) and superoxide dismutase activity SOD (Nishikimi *et al.,* 1972) were determined colorimetrically in tissue liver homogenate.

2.7. Biochemical studies

The serum total protein and serum albumin levels were measured (Doumas *et al.,* 1981; Drupt, 1974). Serum globulin level was calculated by subtracting the albumin from the total protein (Coles. 1986). The serum creatinine level was also determined colorimetrically (Husdan and Rapoport, 1968). Serum activities of alanine aminotransferase ALT and aspartate aminotransferase AST were determined (Reitman and Frankel, 1957).

2.8. Histopathology

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

2.9. 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

Result of antioxidant (Tables II) revealed a significant increase in MAD level in liver homogenate of EAC bearing mice which could be

due to cancer is as a multifactor disease, where oxidative stress may be involved in both initiation and promotion of multi step carcinogenesis, reactive oxygen species can accelerate DNA damage, stimulate pro-carcinogenesis, initiate lipid peroxidation (MAD), inactivate antioxidant enzyme systems and thus can modulate the expression of genes related to tumour promotion (Fenninger and Mider,1954) and malondialdehyde (MDA) the end product of lipid peroxidation, are seen to be higher in cancer tissues than in non diseased organ (Meister, 1988) ,while decreased in other antioxidant levels SOD,GSH and CAT may be as a result of tumour growth and emergence of the malignancy (Marklund *et al.,* 1982 ; Yagi, 1987). where in forth group treated only with MTX revealed the same result but a higher elevation in MAD or diminution in SOD, GSH and CAT that may be as a result of significantly altered the oxidant/antioxidant balance, oxidative stress or oxidative cellular damage with its dual of free radical generation and profound lipid peroxidation are hallmarks of MTX toxicity (Jahovic *et al.,* 2004). Actually, the decrease in liver GSH content promoted by MTX represents an alteration in the cellular redox state, suggesting that the cells could be more sensitive to reactive oxygen metabolites (Fiocchi,2004) and leads to a reduction in the effectiveness of the antioxidant enzyme defense system (Babiak,1998). While in propolis treated groups showed more increase SOD, GSH, CAT and decrease MAD activities than positive and negative control and MTX treated groups that perhaps due to propolis, protects tissues from reactive oxygene species mediated oxidative stress in toxic injuries (Oktem *et al.,* 2005) and improvement of antioxidant system one of suggested role of propolis against cancer is preventing oxidative damage (El-khawaga *et al.,* 2003).

On the other hand the biochemical result (Table III) 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 the capillary permeability, which permit the escape of plasma proteins into the peritoneal cavity (Garrison *et al.,* 1987). Furthermore, hypoproteinemia and hypoalbuminemia may be due to excessive nephritis and also certain cases of massive ascites and also associated with liver disease (Coles, 1986) which confirmed to the result of increased ALT and AST activities with increased of creatinine level in this group which may be attributed to hepatic and renal damage as a result of cancer cells invasion (Hashem *et al.,* 2004). While in group 3 displayed amelioration of these parameters toward the normal control group levels which reflects a protective effect of propolis against organ dysfunction and cellular injury (Sforcin, 2007).where in group treated with MTX revealed dysfunction of liver and kidney more than other groups that appeared by decrease in total proteins, albumin and increase in ALT, AST and cretinine which could be due to liver damage mild necrosis and inflammation (Hemeida and Mohafez,2008) and renal damage as a result of MTX therapy (Abraham *et al.,* 2010) while in fifth group protected by propolis revealed an amelioration toward the normal control group as propolis have marked hepatoprotective potential because of its composition of minerals, flavonoids (Bhadauria *et al.,*2007) that able to restore the hepatic damage alteration in the liver (Shukla *et al.,* 2004).These previous results confirmed with histopathological examination of parenchymatous organs (liver and kidneys) in different groups which in second group, renal parenchyma showed neoplastic invasions with pressure atrophy and necrosis of the surrounding tubules (Fig.1) .Some glomeruli suffered from proliferation of their tufts with empty capillaries. Others had cellular or hyaline casts could be seen inside the lumen of some tubules with focal coagulative necrosis of the hepatic parenchyma was common. The remaining hepatic cells revealed various degenerative changes (Fig.2) varied from cloudy swelling to hydropic degeneration. Thickened hepatic capsule (peritonitis) could be seen. Numerous newly formed bile ductules could be seen in some portal area. While in third group, renal tissue adjacent to tumor showed mild degenerative changes with reduction in tumor mass without renal invasion beside lymphoid infiltration of tumor (Fig.3). Thickened glomerular basement membrane and periglomerular round cell aggregation was common with hepatic cells suffered from reversible hydropic degeneration with interstitial mononuclear cell infiltration (Fig.4). While in fourth group, renal tubules in the visinicity of neoplasm showed degenerative changes with proliferative mesengial cells in the glomeruli (Fig.5) with severe congestion, cloudy swelling and hydropic degeneration were common in liver (Fig.6). Lastly in fifth group renal parenchyma was apparently normal (Fig.7).Few tubules appeared cystic and contoured living and dead neutrophils and interstitial and portal leucocytic aggregation mainly lymphocyte with normal hepatic cells were seen (Fig.8). While decreasing in serum globulin levels in forth group could be due to immunosuppressive effect of methotrexate (Incecik *et al.,* 2009). While in third and fifth groups there is an improvement of these parameters toward the normal control group as a cause of propolis makes protective effect against organ dysfunction and cellular injury of liver and kidney (Sforcin, 2007).

Table I: Experimental design

Groups	No. of mice	Design	IP EAC Cells 2.5×10^6	Oral 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 WSPD Water soluble propolis derivatives
IP Intraperitoneally MTX Methotrexate

Table II: Effect of propolis, methotrexate and their combination (50 mg/kg body weight, 0.4mg/kg body weight) respectively on SOD, GSH, CAT and MAD activities on liver homogenate (mean values ±SE) in mice bearing EAC.

Group	Parameters SOD U/gm	GSH mg/gm	CAT U/gm	MAD nmol/gm
1(Control)	27.93^{b}±1.07	1.09^{b}±0.09	118.28^{b}±3.93	141.40^{d}±1.50
2(Mice bearing EAC)	17.56^{d}±1.40	0.81^{c}±0.02	70.18^{d}±1.47	180.20^{b}±1.98
3(Propolis-treated group)	32.74^{a}±0.20	1.65^{a}±0.05	122.86^{a}±2.54	101.40^{e}±3.82
4(Methotrexate treated group)	10.67^{e}±0.59	0.60^{d}±0.03	54.74^{e}±3.81	207.80^{a}±6.25
5(Combination treated group)	20.34^{c}±0.33	1.10^{b}±0.03	80.85^{c}±2.31	153.60^{c}±3.15
F test	**	**	**	**
LSD	2.52	0.16	0.43	11.05

** highly significant difference at $p \leq 0.01$ EAC Ehrlich ascites carcinoma SOD Superoxide dismutase
GSH Reduced glutathione CAT Catalase MAD Malondialdhyde LSD least significant difference
Means in the same column with different superscript letters are significantly different.

Table III: Effect of propolis, methotrexate and their combination (50 mg/kg body weight,0.4mg/kg body weight) respectively on total proteins, albumin, globulins ALT, AST and creatinine (mean values ±SE) in mice bearing EAC.

Groups	Parameters Total proteins (g/dl)	Albumin (g/dl)	Globulins (g/dl)	ALT Unit/l	AST Unit/l	Creatinine (mg/dl)
1(Control)	6.98^{a}± 0.16	3.80^{a}±0.20	3.18^{a}±0.17	19.78^{e}±0.92	32.27^{c}±1.02	0.47^{d}±0.03
2(Mice bearing EAC)	4.96 ^{c}± 0.07	1.84^{c}±0.10	3.12^{a}±0.16	30.28^{b}±1.12	55.29^{b}±1.63	1.34^{b}±0.07
3(Propolis-treated group)	5.50^{b}± 0.21	2.76^{b}± 0.13	2.74^{a}±0.25	22.59^{d}±0.36	34.91^{c}±2.15	0.56^{d}±0.06
4(Methotrexate treated group)	3.50^{e}± 0.17	1.86^{c}±0.19	1.64^{b}±0.29	49.40^{a}±0.41	96.01^{a}±2.78	1.87^{a}±0.03
5(Combination treated group)	4.40^{d}± 0.12	3.00^{b}±0.00	1.40^{b}±0.12	25.37^{c}±0.21	50.15^{b}±1.00	0.85^{c}±0.02
F test	**	**	**	**	**	**
LSD	0.47	0.43	0.62	2.07	5.47	0.22

** highly significant difference at $p \leq 0.01$ EAC Ehrlich ascites carcinoma ALT alanine aminotransferase
AST aspartate aminotransferase LSD least significant difference
Means in the same column with different superscript letters are significantly different.

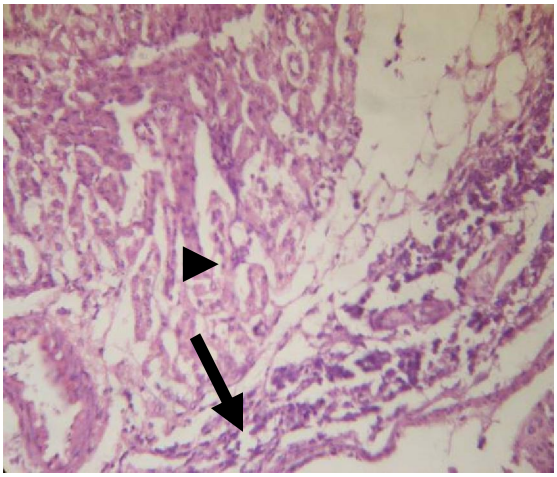

Figure 1. Gp. (2), Kidney of mice showing invasion of the renal tissue by carcinoma cells (arrow) with pressure atrophy and necrosis of the surrounding renal parenchyma (arrow head), H & E , X300.

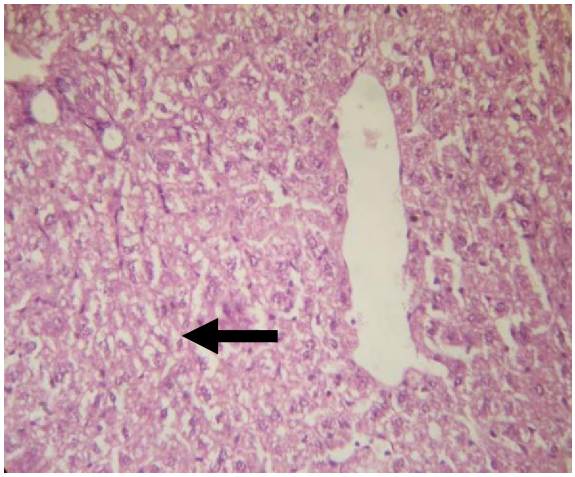

Figure 2. Gp. (2), Liver of mice showing various degenerative changes in the hepatic cells (arrow), H & E, X 300.

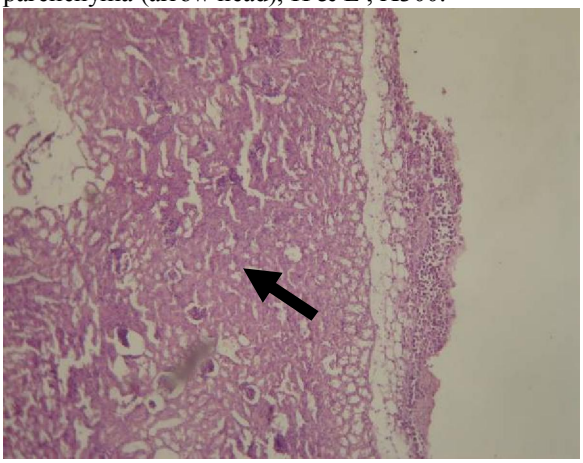

Figure 3. Gp.(3), Kidney of mice showing reduction in tumor mass without renal invasion beside lymphoid infiltration of tumor(arrow), H&E , X 120

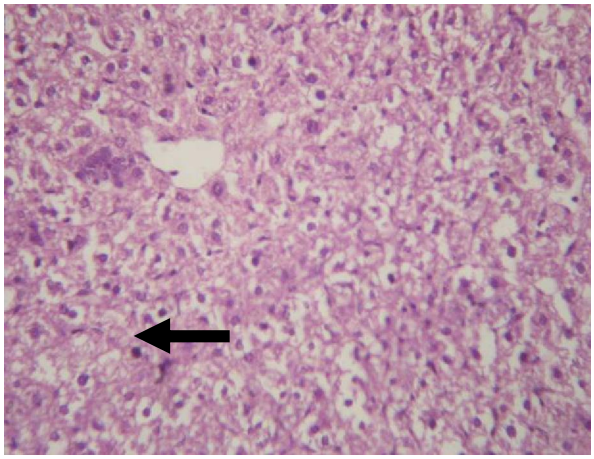

Figure 4. Gp. (3), Liver of mice showing hydropic degeneration of the hepatic cells and mild mononuclear cell infiltration beside hyperplastic Kuffer's cells (arrow), H & E, X 300.

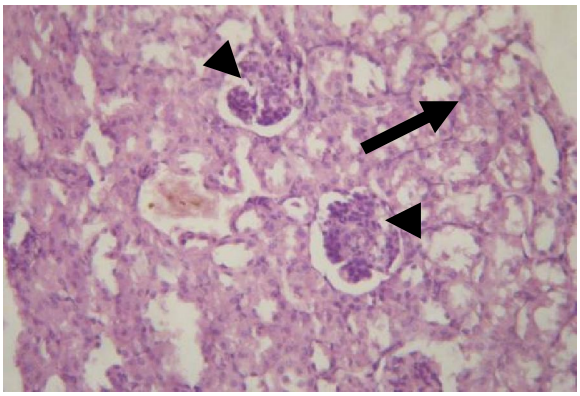

Figure 5. Gp. (4), Kidney of mice showing degenerative changes in renal tubules (arrow) and proliferative mesangial cells in glomeruli (arrow head), H &E , X 300.

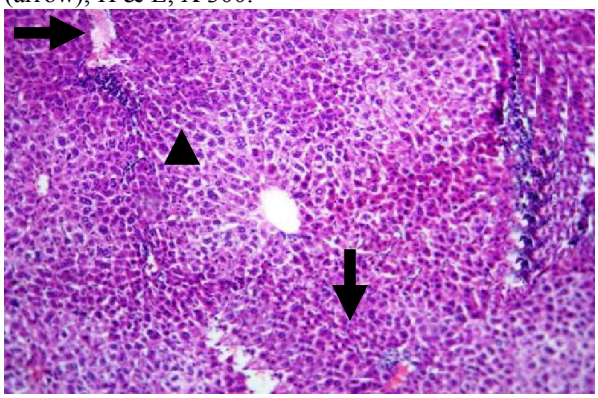

Figure 6. Gp. (4), Liver of mice showing severe congestion(arrow), cloudy swelling and hydropic degeneration (arrow head), H &E, X 300.

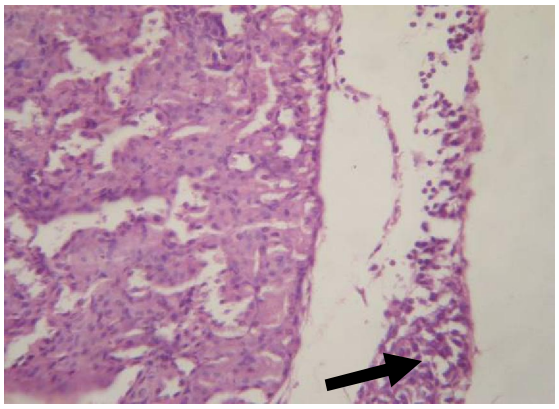

Figure 7. Gp.(5), Kidney of mice showing a little neoplastic cells in the peritoneum infiltrated with lymphocytes without renal invasion and normal parenchyma(arrow), H & E , X 300.

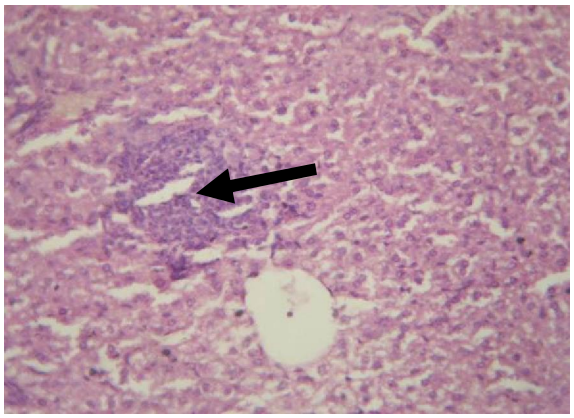

Figure 8. Gp.(5), Liver of mice showing interstitial leucocytic aggregation (arrow) and normal hepatic cells, H & E , X 300.

Conclusions

Treatment of Ehrlich ascites carcinoma 2.5×10^6 transplanted intraperitoneally in Swiss albino mice by combination of Egyptian propolis (50 mg/kg body weight) and methotrexate (0.4 mg/kg body weight) ameliorate alteration in antioxidant state and biochemical analysis of the implanted mice toward normal control which confirmed with histopathological examination that revealed slight alterations in liver and kidneys in propolis treated groups.

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Corresponding author

Prof. Dr. Mohamed O. T. Badr

Prof. of Clinical Pathology, Faculty of Veterinary Medicine, Zagazig University, 1 Alzeraa Street Postal Code 44511, Zagazig City, Sharkia Province, Egypt.

drosamabadr@yahoo.com

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