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Inhibitory effects of Rosemary (*Rosmarinus officinalis L.)* on Ehrlich ascites carcinoma in mice

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KEYWORDS

Rosemary (*Rosmarinus officinalis* L.), EAC cell lines, anti oxidants, super oxide dismutase.

A B S T R A C T

Rosemary *(Rosmarinus officinalis L.)* is considered as a rich source of active phenolic compounds and terpenes, responsible for various desirable physiological activities. The present study aimed to investigate the hepatopreventive and anticancer effects of rosemary methanolic extract against Ehrlich ascites carcinoma in mice. Animals were randomly assigned to four groups of equal number and weight. Group I, kept as a normal control group; Group II, Positive control group infected with Ehrlich ascites carcinoma cell line by intraperitoneal injection $1X10^6$ cells / mouse, Groups III, Pre-treatment group, mice were pretreated with rosemary volatile oil for 8 days(320 mg/kg BW), then at the 9th day, they were infected with Ehrlich ascites carcinoma cell line(1x106 cells/ mouse) . Group IV, Post-treatment group, mice were infected first with Ehrlich ascites carcinoma cell line, then treatment with rosemary volatile oil with the same dose with group III. Phenolic compounds and Flavonoids were fractionated and identified by HPLC, DPPH free radical scavenging assay. The following biological parameters were investigated: Liver function tests (ALT, AST, Albumin, T-bilirubin and alkaline phosphatase), Serum MDA, GSH, TAC, and SOD, complete blood count, as well as histopathological examination for liver and kidney. Results showed that, α-pinene was the predominant phenolic compound (49.77%) in the methanolic extract. The three major phenolic constituents were Cinnamic acid, vanailic acid and ferulic acid at concentrations of 192.929, 152.607 and 76.876 mg/100g rosemary, respectively. Administration with Ehrlich's ascites carcinoma (Ehrlich group) had negatively affected the hematological parameters antioxidant activity. Rosemary extract was found to increase significantly the Hb, RBCs count and Hct and decreased WBCs count, also caused significant attenuation of liver enzymes ALT, AST, ALK with improvement of serum albumin and bilirubin in both rosemary-treated groups. Rosemary extract caused significant improvement in the redox state in the form of reduction in MDA with enhancing the total antioxidant capacity (TAC), GSH, SOD and catalase enzymes activities. In addition, pretreatment with rosemary had a powerful effect than post-treatment. The observed reduction of tumor volume, tumor cell count, was shown in Ehrlich + Rosemary group and in Rosemary + Ehrlich group in comparison with Ehrlich group. In conclusion, treatment Ehrlich group with Rosemary possessd marked improvement but not matched with the control or Rosemary-treated groups. ositive. One case attempted to miscarriage while one decided to continue the pregnancy.

Introduction

Rosemary (*Rosmarinus officinalis L.*) is a spice and medicinal herb widely used around the world. Constituents in rosemary have shown a variety of pharmacological activities for cancer chemoprevention and therapy in *in vitro* and *in vivo* model (1). Those constituents such as phenolic acids (e.g. vanillic, caffeic, chlorogenic, rosmarinic acid), phenolic diterpenes (e.g. carnosol, rosmanol, isorosmanol, carnosic acid), and pentacyclic triterpenes (e.g. ursolic, oleanolic, betulinic acid, betulin, alphaamyrin, beta-amyrin) were reported to be major compounds in rosmary extract (2,3).

According to the World Health Organization (WHO) report on cancer Estimated Incidence, Mortality and Prevalence Worldwide in 2012, there was 8.2 million cancer deaths reported (4). Cancer is caused by internal factors (tobacco, chemical, radiations, and infectious organisms), and external factors (mutations, hormones and immune conditions (5). Cancer can be treated with surgery, radiation, chemotherapy, and biological therapy. Among these plants rosemary, which have shown pharmacological activities of cancer chemoprevention and thereby and in *in vitro* and *in vivo* models (1).

Also, Rosemary has potent anti-cancer effects. These anti-cancer properties are probably associated with rosemary's antioxidant property, which is more effective than synthetic food additives such as BHT and BHA (6). Free radicals, reactive oxygen species are associated with many biological phenomena, such as inflammation, aging, and carcinogenesis. The antioxidant's activity of polar extracts of rosemary is related with the content of phenolic compounds (carnosol, carnosic acid). Constituents in rosemary have shown a variety of pharmacological activities of cancer chemoprevention and therapy in vitro and, in vivo models (1).

The anti-proliferative and antioxidative properties of crude extracts of rosemary (Rosmarinus officinalis.) waere reported in several cancer cell lines on human leukemia and breast carcinoma cells (7). Furthermore, rosemary components have a protective effect against cancer by reducing the enzymes converting pro-carcinogens to more portent carcinogens and stimulating the enzymes catalyzing the inhibition reaction of carcinogens (8,9). Previous studies (10s), showed that administration with 200 mg/kg of *Rosmaritzus officinalis* extract prevented alterations of biochemical and histological parameters induced by CCL_4 in the rat.

In the present study, we aimed to evaluate the *in vivo* antitumour activity of methanol extract of Rosemary against Ehrlich ascites carcinoma (EAC) in mice. As well as antioxidant, activities of methanolic rosemary leaf extracts were evaluated.

Materials and Methods

Rosemary leaves (*Rosmarinus officinalis L.*) were obtained from a local Market in El-Mansoura city, Egypt. Forty male Albino mice 12 weighting 25± 2 g were obtained from animal house, Research center, Faculty of pharmacy, Mansoura University, housed under proper conditions (temp 24 ± 2C° and 12 hr light- dark cycle). Mice were allowed free access to food and water.

Extraction of Rosemary leaves

300 ml of methanol were added to 50 gm of rosemary leaves powder for 10 – 12 hrs. Then filtered, and the solvent was evaporated using rotary evaporator. The resultant extract was dehydrated in an oven at 50 C° for 24 hours and kept at 4 C° (**11**).

Separation and identification of Rosemary volatile oil

Volatile compounds in extracted rosemary oil were identified using gas chromatography (GC) technique at the Central laboratory of National Research Center, Dokki, Giza, Egypt.

Extraction and identification of phenolic compounds

Extraction of phenolic compounds from rosemary leaves was carried out according

to the method reported (12,13)Total phenolic compounds of rosemary leaves methanolic extract were determined (14,15)using the Folin Ciocalteu reagent. And calculated as mg gallic acid / gm of dry weight materials. Phenolic compounds in methanolic extract of rosemary leaves were identified using high performance liquid chromatography (HPLC), Hewelt Packard (Model 1100) (16). The determinations were taken place in Central laboratory of Agriculture Research center, Giza.

Determination of total phenolic content (TFC)

The level of total phenols in the crude extract of rosemary as mg of Gallic acid equivalent was determined according to (17).

Identification of phenolic compounds

Phenolic compounds were fractionated and identified by HPLC according to the method of (18). The sample of 5 g was mixed with methanol and centrifuged at 10.000 rpm for 10 min under cooling temp. (Harrier Sanyo; 18/80; Uinted Kingdom). The supernatant was filtered through a 0.2 um Millipore membrane filter then; 1-3 ml was collected in a vial for injection into HPLC Hewllet Packared (series 1050) equipped with auto-sampling injector; solvent degasser; ultraviolet (UV) detector set at 280 nm and quarter HP Pump (series 1050). The column temperature was maintained at 35°C. Gradient separation was carried out with methanol and acetonitrile as a mobile phase at flow rate 1ml/min. Phenolic acids standard from Sigma Co. were dissolved in a mobile phase and injected into HPLC. Retention time and peak area were used to calculation of phenolic compounds concentration by the data analysis of Hewllet Packard software.

Identification of Flavonoids compounds

Flavonoids compounds were determined by HPLC according to the method of (19) as described above, except the detector was set at 330 nm.

Determination of antioxidant activity

DPPH free radical scavenging assay carried out according to (20) and (21) at the Central laboratory of Agricultural Research Center.

Biochemical Investigations

The following Liver function parameters were measured, Alanine transaminase (ALT) and aspartate transaminase (AST) were determined in serum according to (22) Serum albumin was determined calorimetrically according to (23). Total bilirubin was determined according to (24). Alkaline phosphatase was determined according to (25).

Malondialdehyde was determined according to the method of (26). While, Serum reduced glutathione was determined according to the method of (27). (CAT) was determined by the method of (28). Total antioxidant capacity was determined according to (29). Superoxide Dismutase was determined by the method of (30). All these determinations were carried out using the specific commercially available kit (Biodiagnostic, Egypt).White blood cells count (WBCs), red blood cell count (RBCs), hemoglobin concentration (Hb) were determined according to (31).

Histopathological Studies

Specimens of liver and kidney of sacrificed rats were fixed in 10 % phosphate buffered formalin. Embedded in paraffin, Serial 5-µm histological sections were cut and stained

with hematoxylin and eosin and examined under bright-field light microscopy (32).

Statistical analysis

Data were expressed as mean ± STD. All statistical analyses were carried out using SAS software Version 9.1 (SAS Institute Inc., Cary, NC, USA), using general linear models (GLM). The least significant difference (LSD) were determined at ($P < 0.05$).

Results and Discussion

Separation and identification of Rosemary volatile oil

The results of separation and identification of Rosemary volatile oil are presented in tables 1 and 2. Volatile oil was found to be the most important component of rosemary leaves and its content was about 1.7432 with a specific gravity of 0.9088 ± 0.205 gm/cm3 (Table 1). The constituents of rosemary volatile oil (RVO) was separated by gas chromatography (GC) analysis. From data illustrated in table (2), it could be noticed that 10 components were separated and identified in rosemary volatile oil. Rosemary volatile oil are characterized by the presence of large percentages of α-pinene (49.77%), while, predominant alcohol was 1,8-Cinole (16.97%), which was also the major component of volatile fractions mainly responsible for their aromatic profiles. On the other hand, Thujene and β-Caryophyllene were detected as trace compounds.

Antioxidant measurement of Rosemary extract

Table (3) presents the total phenolic compounds content of rosemary methanolic extract. Data show that total phenolic content of rosemary methanolic extract was 25.56 mg/gm dry matter as gallic acid equivalent while, the total dried methanolic extract was 13.52 ± 0.421%. Also, the ability of the rosemary methanolic extract to scavenge the DPPH was measured and results are shown in table (3). Rosemary methanolic extract inhibited almost DPPH absorption and the radical activity was found to be 94.30% at the above concentration (25.56 mg\g gallic acid)

Fractionation of Rosemary phenolic compounds

Total phenolic compounds of rosemary methanolic extract were separated and identified by HPLC and the results are shown in table (4), it could be noticed that ten phenolic compounds were separated. Cinnamic acid was the most abundant phenolic compound in rosemary leaves (192.929 mg/100g) followed by Vanaillic (152.607 mg/100g). Moreover, Ferulic acid, Caffeic, Catechol, Protocatchuic acid and Pyrogallol were also detected in medium amounts (76.8758, 68.7443, 53.9923, 46.3041 and 43.3969 mg/100g, respectively). On the other hand, the Coumarine, Synergic acid and P.OH-Benzoic acid were also detected in small amounts (29.9932, 10.9817 and 3.658 mg/100g , respectively).

Fractionation of Rosemary Flavonoids Compounds

Total flavonoids compounds of rosemary methanolic extract were separated and identified by HPLC and the results are shown in table (5). As recorded in the table, nine flavonoids compounds were separated. Rosmarinic was the most abundant flavonoids compound in rosemary leaves (77.83 mg /100 g), followed by quercitrin, rutin and hesperetin.

Effects on body weight, survival and life span in EAC-bearing mice

As shown in figure 1 A-C. Data show significant increase in body weight in Ehrlich group; Ehrlich + Rosemary group and in Rosemary + Ehrlich group in comparison with rosemary group. Also, Ehrlich+ Rosemary and Rosemary + Ehrlich groups showed significant decrease in body weight in comparison with Ehrlich group ($p< 0.001$).

The mean survival time (MST) showed significant increase with Ehrlich+ Rosemary and Rosemary + Ehrlich groups in comparison with Ehrlich group. Moreover, there was no statistical significant differences in MST between Ehrlich+ Rosemary and Rosemary + Ehrlich groups. While, the percentage of increasing life span was significantly high in both Rosemary + Ehrlich and in Ehrlich+ Rosemary group in comparison with Ehrlich group. Moreover, the percentage of increasing life span was significantly high in Rosemary+ Ehrlich in comparison with Ehrlich +Rosemary group or with Ehrlich group.

The results of hematological parameters (Hemoglobin, red blood cells, hematocrit and white blood cells) are shown in table (6). Hemoglobin (Hb) content showed significant decrease in Ehrlich group, Ehrlich + Rosemary group and in Rosemary + Ehrlich group in comparison with rosemary group. Also, Ehrlich+ Rosemary and Rosemary + Ehrlich groups showed significant increase in Hb in comparison with Ehrlich group ($p< 0.05$). Moreover, Hb showed significant increase in Rosemary + Ehrlich group in comparison with Ehrlich+ Rosemary group ($p< 0.05$).

Red blood cells (RBCs) count showed significant decrease in Ehrlich group, Ehrlich + Rosemary group and in Rosemary + Ehrlich group (5.16 ± 0.29) in comparison with rosemary group. Also, Rosemary + Ehrlich group showed significant increase in RBCs in comparison with Ehrlich + Rosemary group ($p< 0.05$). Hematocrit also showed significant decrease in Ehrlich group (37.40 ± 2.10), Ehrlich + Rosemary group (41.60 ± 2.41) and in Rosemary + Ehrlich group (47.80 ± 2.95) in comparison with rosemary group (55.00 ± 5.24) ($p< 0.05$).Moreover , Rosemary + Ehrlich group showed significant increase in hematocrit in comparison with Ehrlich + Rosemary group ($p< 0.05$). Significant increase in WBC in Ehrlich group (19.84 ± 1.92), Ehrlich + Rosemary group (13.40 ± 1.994) and in Rosemary + Ehrlich group (10.84 ± 1.27) in comparison with rosemary group (5.78 ± 1.18) ($p< 0.05$). Also, Ehrlich+ Rosemary and Rosemary + Ehrlich groups showed significant decrease in WBCs in comparison with Ehrlich group ($p< 0.05$). Moreover, WBCs showed significant decrease in Rosemary + Ehrlich group in comparison with Ehrlich+ Rosemary group ($p< 0.05$).

Effects on tumor volume and cell count in EAC-bearing mice

As shown in table (7), tumor volume (ml), a significant decrease in Ehrlich + Rosemary group and in Rosemary + Ehrlich group is observed in comparison with Ehrlich group Also, Rosemary + Ehrlich group showed significant decrease in tumor volume in comparison with Ehrlich + Rosemary group.

Tumor cell count (10^6/ ml) showed significant decrease in Ehrlich + Rosemary group and in Rosemary + Ehrlich group in comparison with Ehrlich group. Also, Rosemary + Ehrlich group showed significant decrease in tumor cell count in comparison with Ehrlich + Rosemary group ($p< 0.05$).

Effects on liver function in EAC-bearing mice

The results of liver functions tests (ALT, AST, ALK, total bilirubin and albumin) are shown in table (8). Serum alkaline phosphatase (ALK) showed significant increase in Ehrlich group, Ehrlich + Rosemary group and in Rosemary + Ehrlich group in comparison with rosemary group. The highest increase was recorded in Ehrlich group in comparison with all other groups.

Concerning Serum aspartate transaminase (AST) and Serum alanine transaminase (ALT), it showed similar trends in significant increase in Ehrlich group, Ehrlich + Rosemary group and in Rosemary + Ehrlich group in comparison with rosemary group. Moreover, AST and ALT showed significant decrease in Rosemary + Ehrlich group in comparison with Ehrlich+ Rosemary group.

Total bilirubin (mg/dl) showed significant increase in Ehrlich group; Ehrlich + Rosemary group and in Rosemary + Ehrlich group in comparison with rosemary group. Also, Rosemary +Ehrlich group showed significant decrease total bilirubin in comparison with Ehrlich group.

Finally, serum albumin (g/dl) showed significant decrease in Ehrlich group; Ehrlich + Rosemary group and in Rosemary + Ehrlich group in comparison with rosemary group. Also, Rosemary + Ehrlich group showed significant decrease in serum albumin in comparison with Ehrlich group. Moreover, serum albumin showed non-significant change between Rosemary+ Ehrlich group and Ehrlich+ Rosemary group.

Effect of rosemary extract on redox state parameters (MDA, CAT and SOD, reduced glutathione and total antioxidant capacity) of Ehrlich ascites carcinoma (EAC) - bearing mice

The results of redox state parameters are shown in table (9). Serum superoxide dismutase (SOD) showed significant decrease in Ehrlich group, Ehrlich + Rosemary group and in Rosemary + Ehrlich treatment in comparison with rosemary group. However, SOD showed no significant change between Rosemary + Ehrlich and Ehrlich+ Rosemary groups. Serum catalase showed significant decrease in Ehrlich group, Ehrlich + Rosemary group and in Rosemary + Ehrlich group in comparison with rosemary group. Also, Rosemary + Ehrlich group showed significant increase in GSH in comparison with Ehrlich group. Moreover, GSH showed significant increase in Rosemary + Ehrlich group in comparison with Ehrlich+ Rosemary group.

Serum reduced glutathione (GSH) showed significant decrease in Ehrlich group; Ehrlich + Rosemary group and in Rosemary + Ehrlich group in comparison with rosemary group. Moreover, GSH showed significant increase in Rosemary + Ehrlich group in comparison with Ehrlich+ Rosemary group.

Serum malondialdehyde (MDA) showed significant increase in Ehrlich group Ehrlich + Rosemary group and in Rosemary + Ehrlich group in comparison with rosemary group.. Moreover, MDA showed significant decrease in Rosemary + Ehrlich group in comparison with Ehrlich+ Rosemary group.

Finally, Serum total antioxidant capacity (TAC) showed significant decrease in Ehrlich group, Ehrlich + Rosemary group and in Rosemary + Ehrlich group in comparison with rosemary group. However, there were no statistical significance difference among Ehrlich, Rosemary + Ehrlich and Ehrlich+ Rosemary groups in TAC.

Liver

In control mice as well as methanolic rosemary –extract-treated groups; the liver displays the normal histological structure feature. It is composed of anastomosing plates, usually one layer thick. The hepatocytes are arranged in trabeculae running radially from the central vein. Hepatic sinusoids are localized in between the cords and contained fine arrangement of Kupffer cells (Fig.2A-B).

Experimental Erlich ascites carcinoma revealed massive pathological alterations distributed throughout the hepatic tissue. The liver showed abnormal hyperplasia of their capsule peripheral layer associated with numerous focal lesions of leukocyte infiltration. Focal collection of leukocytic infiltration was also detected throughout hepatic lobules as well as around the blood vessels. The hepatocytes appeared either necrotic with characteristic eosinophilic-staining or showed vacuolar degeneration (Fig. 3 A-C).

In experimental Erlich ascites carcinoma treated with alcoholic rosemary, there was a marked amelioration of the hepatic picture except slight foci of periportal leukocyte infiltration and small numbers of vacuolar degenerated hepatocytes (Fig.4 A-B).
However, in experimental rosemary protected Ehrlich ascites carcinoma, amelioration was less than alcoholic-rosemary-treatment. The liver capsule retained to their normal collagenous appearance. There was a detected reduced size of grouping leukocytes within the hepatic tissues as well as around the central vein. Small numbers of hepatocytes were still possessed either vacuolar degeneration or pyknotic nuclei (Fig. 5 A- B).

Kidney

In control mice as well as alcoholic rosemary – treated group, the kidney is composed of two main regions; the renal cortex and medulla which possess normal histological features. The renal cortex enclosed by numerous renal corpuscles, each made up of a glomeruli and the Bowman's capsule. There is a characteristic normal space between the glomeruli and Bowman's capsule to allow renal filtration. The glomeruli are composed of capillaries with characteristic flattened nuclei projecting into the capillary lumen. The parietal layer of its renal capsule is composed of simple squamous epithelium. The renal corpuscles are surrounded by proximal and distal convoluted tubules. Bundles of parallel tubules can be identified running into the cortex. The tubules have inner wide luminal space lined externally with cuboidal epithelium. These are the cortical medullary rays, made up of collecting tubules, and loops of Henle from the corpuscles located in the outer part of the cortex and run internally in the renal medulla (Fig. 6 A-C).

Experimental Ehrlich ascites carcinoma revealed marked damage of renal tissues characterized by mild peritubular inflammatory cellular infiltration associated with degeneration of tubular lining epithelial cells and widening of the tubular lumina. Some renal tubules surrounding the glomeruli exhibited hyaline degeneration. There was a detected inflammatory cell

infiltration within the glomeruli. The size of the glomeruli become reduced and filling almost of the Bowman's capsule space. There is a marked degeneration of endothelium cells within the glomeruli (Fig. 7 A-C).

In experimental Ehrlich ascites carcinoma treated with alcoholic rosemary, there was a marked amelioration of the renal tissues except slight foci of internal haemorrhage and few hyalinized renal tubules (Fig.8 A-B).

However, in experimental rosemary protected Ehrlich ascites carcinoma, amelioration was less than alcoholic-rosemary-treatment. The renal tissues showed a slight degeneration of epithelium lining the tubules, besides focal spots of hyalinization of tubules especially in the surrounding of the glomeruli (Fig.9 A& B).

Rosemary leaf extracts are proposed as important human dietary factors and have been investigated as potential therapeutic agents including antioxidants against several diseases (33). Hepatic injury is a fundamental pathological process in most chronic hepatic diseases and long-standing hepatic injury can lead to hepatic fibrosis, liver cirrhosis, and even hepatocellular carcinoma. Various herbal extracts, and their chemical constituents, have been used in the treatment of liver disease and have been shown to inhibit pathologic processes and protect hepatocytes against injury (34). Rosemarinus officinalis, has been considered as a hepatoprotective and antimutagenic anti-hyperglycemic and anti-ulcerogenic actions (10, 35, 36).

The biolobical activities for rosemary extract are attributable to its content of phenolic diterpenes such as carnosol, carnosic acid, rosmanol, epirsomanol, isorosmanol, methyl carnosate and other phenolic acids, such as rosmarinic acid. These polyphenols have shown biological activities in vitro as anti-tumor, chemopreventive and anti-inflammatory agents and may play a role by regulating the activity and/or expression of certain enzymatic systems implicated in relevant physiological processes like apoptosis, tumor promotion, intracellular signal transduction or xenobiotic-metabolizing enzymes in the liver (37).

Characterization of the Rosemary extract

In the present study gas, chromatography (GC) revealed that the methanolic extract of Rosemary leaves consisted of 10 compounds. The α-pinene represented the highest concentration, while, the predominant alcohol was 1,8-Cinole which was also the major component of volatile fractions mainly responsible for their aromatic profiles. On the other hand, Thujene and B-Caryophyllene were detected as trace compounds. In agree with (**2**) who reported that the major component of dried leaves of Rosmarinus officinalis (L.) was monoterpene oxide 1,8 cineole (36.1%), and identified α - pinene, B-pinene, B – caryophylene, camphene,limonene, myrcene and p-cymene. They also detected the monoterpene ketone camphor (12.8%) and monoterpene alcohols (9.6%), such as borneol. Others reported that rosemary leaves contained about twenty six compounds and the major compounds were verbenone, camphor, 1,8-cineole, borneol, linalool, α-pinene and caryophyllene after extraction using supercritical fluid extraction technique (3, 38).The present study demonstrated that volatile oil was found to be the most important component of rosemary leaves and its content was about 1.7432 with a specific gravity of 0.9088 ± 0.205 gm/cm^3.

Total phenolic and flavonoids content in Rosemary extract

1,1-diphenyl-2-picrylhydrazyl (DPPH), is a stable free radical, which has a spare electron that makes delocalization over the whole molecule. The reaction takes place by the delocalization which causes a deep violet color with absorption maxima (λmax) around 520 nm. When a solution of DPPH is mixed with a substrate acting as a hydrogen atom donor, a stable non radical form of DPPH is obtained with simultaneous change of the violet color to pale yellow (39).It was reported that, the rosemary alcoholic extract had higher antioxidant activity than the oregano alcoholic extract, despite the lower total phenolic content (40).They found that the percent inhibition of DPPH was 90.14% for rosemary alcoholic extract and 41.16% for oregano alcoholic extract. This suggests that the physicochemical nature of the individual phenolics in the extracts may be more important in contributing to the antioxidant activity than the total phenolic content (41). This observation indicates that a critical concentration of phenolic compounds is enough to obtain a desired antioxidant activity after which there is a saturation effect and the presence of additional phenolics does not increase the antioxidant activity.

The total phenolic content of rosemary methanolic extract in the present study was 20.16 mg/g dry matter as gallic acid equivalent while, the total dried methanolic extract was 13.52 ± 0.421%. Extraction of phenolic compounds by HLPC demonstrated that, the cinnamic acid was the most abundant phenolic compound in rosemary leaves (192.929 mg/100g) followed by Vanaillic acid (152.607 mg/100g). Moreover, Ferulic acid, Caffeic, Catechol, Protocatchuic acid and Pyrogallol were also detected in medium amounts. On the other hand, the Coumarine, Synergic acid and P.(OH)-Benzoic acid were also detected in small amounts. Also, nine flavonoids compounds were separated in the present study. Rosmarinic was the most abundant flavonoids compound in rosemary leaves (77.83 mg /100 g), followed by quercitrin, rutin and hesperetin (35.29, 33.44 and 21.38 mg/100 g, respectively). Moreover, hesperidin, apegnin, narengenin, kampferol and quercetin were also detected in small amounts, these results was in agreement with (42).

Debersac *et al.* (2) found that, only 28.1% of the compounds present were quantified in the dichlormethane extract (DCME) from rosemary leaves. The main compounds characterized in DCME were flavonoids and phenolic diterpenes. Two flavones were identified as cirsmaritin and genkwantin. Six structures of phenolic diterpenes were detected in that extract, namely carnosic acid (14.7 %) and its methylated form (7.4%), carnosol (3.8 %), rosmanol (1.4%), epirosmanol methyl ether (0.8 %) and epirosmanol (traces). Dichlormethane extracted also other lipophilic chemicals, probably corresponding to pigments, sterols and hydroxylated fatty acids.

Rosemary essential oil and Erlich ascitic carcinoma (EAC)

Preparations from the Rosemary have been recently investigated for their ability to exert antiproliferative and antioxidant properties and protect against skin tumorigenesis and DNA damage (43).Therefore, in the current work we investigated the effect of rosemary extract on EAC mice model. Where Ehrlich group showed significant increase in body weight and this increase significantly reduced in animals treated with rosemary especially before induction of tumor more than those given rosemary after induction of

tumor. Reliable criteria for judging the value of any anticancer agents is the prolongation of life span of animals (44).The mean survival time (MST) of mice per days in the present study showed significant increase in both animal groups treated with the Rosemary extract. Moreover, the percentage of increasing life span was significantly high in both animal groups treated with Rosemary. In line with these findings (45). using the same animal model of EAC , reported that methanol extract (50, 100, and 200 mg/kg) of *Bauhinia racemosa* stem bark increased the animal survival time and enhanced the animal life span of EAC bearing mice.

Regarding its effect on tumor size and volume and tumor cell count, the present study showed that, the tumor volume and tumor cell count significantly decreased in both animal groups treated with rosemary and the effect was more profound in animal group treated with rosemary before tumor induction. In agreement with these findings, Gupta et al. (45), reported that methanol extract of *Bauhinia racemosa* stem bark decreased the tumor volume and viable tumor cell count and finally reduced the tumor burden of EAC bearing mice. Several studies tried to explain the anticancer effect of rosemary extract. One mechanism through which Rosemary components may exert anticancer effects is by reducing the expression of the proinflammatory gene cyclooxygenase-2 (46), which has been regarded as a risk factor in tumor development. Furthermore, the addition of Rosemary extracts is an important factor in decreasing carcinogenic compounds such as heterocyclic amines those are mutagenic compounds formed during cooking muscle foods at high temperature (47).The competent inhibiting effect of Rosemary extracts on heterocyclic amines formation has been reported. In animal models, rosemary components were found to inhibit the initiation and tumor promotion phases of carcinogenesis. In addition, oral administration of Rosemary extract was found to be effective in decreasing the tumor incidence (48). Amagase (49) also reported that rosemary can prevent the binding of cancer causing chemicals (carcinogens) to cellular DNA. Binding of a carcinogen to DNA, leads to mutations in the DNA, and is an early step in the development of cancer. They found that whole rosemary extract given in the diet prevented the binding of the known carcinogen, 7, 12-dimethylbenz(a)anthracene, (otherwise known as DMBA) to DNA in breast cells. These are in line with the findings in the present study which found the use of rosemary before induction of tumor is more powerful than after induction of tumor.

It is worthy to mention that, some recent studies detected a contradictory effect of Rosmarinic acid, a component of Rosemary, which showed proliferative effects rather than cytotoxic activity in almost all tested cell lines (50). In addition, cotreatment with Rosemary extract slightly induced, TPA-dependent AP-1 activation in transfected Jurkat T cells (51).

Rosemary essential oil and hematological parameters

The anemia encountered in tumor bearing mice is mainly due to reduction in RBC or hemoglobin percentage and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions (**52**).In the current study, hemoglobin (Hb) content, RBCs count and hematocrite (Hct) value showed significant decrease in EAC bearing mice. Treatment with rosemary extract caused significant elevation of Hb, RBCs count and Hct in both rosemary-treated groups compared control untreated group.

Moreover, the increase of Hb, RBCs and Hct was higher in EAC bearing mice treated before induction of tumor than treated after induction of tumor.

Regarding white blood cell (WBC) count, there were significant increase in WBC in EAC bearing mice and this elevation was significantly attenuated by rosemary treatment before and after induction of tumor. Moreover, the improvement of WBCs by rosemary was significantly high in EAC bearing mice treated before induction of tumor than treated after induction of tumor. So, treatment with rosemary extract brought back the hemoglobin content, RBC and WBC cell count near to normal values. These findings suggest that rosemary extract posses protective action on the heamoto-poietic system. The same effect was observed by (45) for the extract of Bauhinia racemosa stem bark on EAC bearing mice.

Rosemary and liver function tests

Liver damage induced by tumor cells generally reflects disturbances in liver cell metabolism, which lead to characteristic changes in serum enzyme activities. The increased levels of AST, ALT, ALK and albumin in serum may be interpreted as a result of liver damage or as changes in membrane permeability indicating the severity of hepatocellular damage by EAC. Serum liver enzymes (ALT, AST and ALK) showed significant increase in EAC bearing mice. The increment in serum enzymatic activities is related to hepatic parenchymal damage since ALT and AST are released from mitochondrial and cytosolic localization and γ-GTP from membranal sites, and cellular rupture allows the enzyme to escape into the blood (53). Also, in the present study treatment with rosemary caused significant attenuation of this elevation in liver enzymes and the decrease in liver enzymes was more significant in Rosemary + Erlich group than Erlich+ Rosemary group. Also, serum albumin showed significant decrease in Ehrlich group and this decrease was improved by Rosemary treatment. In line with these findings, (54) and (55) showed that the methanol extract of *Careya arborea* bark (MECA) has hepatoprotective activity in Ehrlich ascites carcinoma (EAC) tumor-bearing mice.

Findings of the present study suggest hepatoprotective role for rosemary extract in case of tumor. Furthermore, the hepatoprotective effects of rosemary extract have been shown in studies on experimental acute liver damage (10) and on liver experimental cirrhosis (56) and (57).Carnosic acid also provides protection from the liver carcinogen aflatoxin A (58). Enzymes found in the liver, known as P450, glutathione S-transferases (GSH), and quinone reductases (QR) can affect the toxicity of some chemicals. Although the main role of the liver P450 enzymes is to detoxify compounds, the aromatic hydrocarbons such as DMBA are actually activated into much more potent carcinogens. Thus, DMBA, benzo(a)pyrene and aflatoxin are considered pro-carcinogens rather than direct acting carcinogens. The second group of enzymes, GSH and QR, act by detoxifying these active carcinogenic metabolites and thus protect against cancer. Singletary and Rokusek (59) found that, when rats fed diets containing whole rosemary extract, the enzymes GST and QR were increased significantly compared to controls. Also, they found that, animals fed carnosol in their diet did not exhibit an increase in these liver enzymes. These experiments show that rosemary has a protective effect by increasing the amount of enzymes that the liver uses for detoxification of cancer causing chemicals, and that the

effect of whole rosemary is greater than that of its component, carnosol.

Rosemary and oxidative stress state

Oxygen free radicals are known to stimulate cancer development at all three stages: initiation, promotion and progression. A high level (up to 5 nM/h x 10^6 cells) of hydrogen peroxide is constitutively released from a wide variety of human tumors (60) SOD and CAT are involved in the clearance of superoxide and H_2O_2 (45, 54) Ehrlich tumor growth induces inhibition of SOD and CAT. In EAC-bearing mice, the antioxidant acts by a mechanism that involves modulating lipid peroxidation and augmenting the antioxidant defense system (45).

Serum malondialdehyde MDA, the end product of lipid peroxidation which causes degeneration of tissues (61) was reported to be higher in carcinomatous tissue than in non diseased organs (62). In the current study, serum malondialdehyde (MDA) showed significant increase in EAC bearing mice. This rise in MDA was significantly reduced by rosemary extract treatment before and after induction of tumor and the degree of lowering was more in Rosemary + Ehrlich group than Erlich+ Rosemary group. In agreement with these findings, Gupta et al. (45) reported that the extract of Bauhinia racemosa stem bark reduced the elevated levels of lipid peroxidation.

On the other hand, the free radical scavenging system, GSH, SOD and catalase are present in all oxygen-metabolizing cells and they provide a defense against the potentially damaging reactivities of superoxide and hydrogen peroxide. Glutathione, a potent inhibitor of neoplastic process plays an important role as an endogenous antioxidant system that is found particularly in high concentration in liver and is known to have key function in the protective process (61). It was reported a decrease in SOD activity in EAC bearing mice which might be due to loss of Mn SOD activity in EAC cells and the loss of mitochondria, leading to a decrease in total SOD activity in the liver (63). The inhibition of SOD and CAT activities as a result of tumor growth was also reported (64). Similar findings were observed in the present investigation with EAC bearing mice in which the serum markers of antioxidants including total antioxidant capacity (TAC), GSH, SOD and catalase enzymes showed significant decrease in EAC bearing mice. The administration of rosemary either before or after induction of tumor increased the GSH, SOD, catalase and total antioxidant levels, which may indicate the antioxidant and free radical scavenging property of rosemary extract.

Rosemary and Histopathological examination for liver and kidney

The liver specimens from control mice shows normal histological structure feature, in comparison with the liver specimens from EAC bearing mice revealed massive pathological alterations distributed throughout the hepatic tissue. The liver showed abnormal hyperplasia of their capsule peripheral layer associated with numerous focal lesions of leukocyte infiltration. Focal collection of leukocytic infiltration was also detected throughout hepatic lobules as well as around the blood vessels. The hepatocytes appeared either necrotic with characteristic eosinophilic-staining or showed vacuolar degeneration. Treatment with alcoholic rosemary caused marked amelioration of the hepatic picture except slight foci of periportal leukocyte infiltration and small numbers of vacuolar degenerated hepatocytes.

Table.1 Volatile oil content of Rosemary leaves

Volatile oil content %	1.7432
Specific gravity gm/cm3	0.9088

Table.2 Rosemary volatile oil constituents

No	Identified Constituents	% area	KI
1	Tricyclene	22.42	926
2	Thujene	0.73	931
3	α- pinene	49.77	1010
4	myrcene	1.51	1141
5	α- phelandrene	1.63	1155
6	limonene	1.43	1188
7	1,8-Cinole	16.97	1200
8	Bornyl acetate	3.99	1293
9	neo-Dohyro carveoil acetate	1.05	1317
10	β -Caryophyllene	0.51	1432

KI: Kovat Indices calculated by comparison with standard hydrocarbons from C6-C22

Table.3 Antioxidant measurements of methanolic extract of Rosemary leaves

Total dried methanolic extract	13.52 ± 0.42%
Total phenolic content as gallic acid equivalent	25.56 (mg/g gallic acid).
Radical Scavenging activity	94.30 %

Table.4 Fractions of phenolic compounds of rosemary leaves

Identified constituents	Phenolic compound (mg/100g) dry matter
Catechol	53.9923
Caffeic acid	68.7443
Synergic acid	10.9817
Cinnamic acid	192.9290
Ferulic acid	76.8758
Coumarin	29.9932
P.OH-Benzoic acid	3.6586
Vanaillic acid	152.6070
Pyrogallol	43.3969
Protocatchuic acid	46.3041

Table.5 Fractions of Flavonoids compounds of Rosemary Leaves

Flavonoids compounds	Flavonoids (mg/100g dry matter)
Hesperidin	8.81
Rosmarinic	77.83
Quercitrin	35.29
Narengenin	6.65
Quercetin	2.78
Hesperetin	21.38
Kampferal	4.42
Apegnin	7.44
Rutin	33.44

.

Table.6 Effect of rosemary extract on hematological parameters of Ehrlich ascites carcinoma (EAC)- bearing mice

Parameters / Group	Group (1) Rosemary gp Mean ± SD (n = 10)	Group (2) Ehrlich ascites carcinoma gp Mean ± SD (n = 10)	Group (3) Rosemary + Ehrlich gp Mean ± SD (n = 10)	Group (4) Ehrlich+ Rosemary gp Mean ± SD (n = 10)
Hemoglobin (g/dl)	$12.60^{a} \pm 0.64$	$9.12^{d} \pm 0.83$	$11.62^{b} \pm 0.42$	$10.48^{c} \pm 0.8$
Red Blood Cells ($x\ 10^{6} mm^{3}$)	$5.88^{a} \pm 0.52$	$3.98^{c} \pm 0.30$	$5.16^{b} \pm 0.29$	$4.44^{c} \pm 0.28$
Hematocrit (Hct) (%)	$55.00^{a} \pm 5.24$	$37.40^{c} \pm 2.1$	$47.80^{b} \pm 2.95$	$4.44^{c} \pm 0.28$
White Blood Cells ($x\ 10^{3} mm^{3}$)	$5.78^{d} \pm 1.18$	$19.84^{a} \pm 1.92$	$10.84^{c} \pm 1.27$	$13.40^{b} \pm 1.99$

All data are expressed as mean ± SD. Values in the same raw with different superscript differ significantly at $P \leq 0.05$

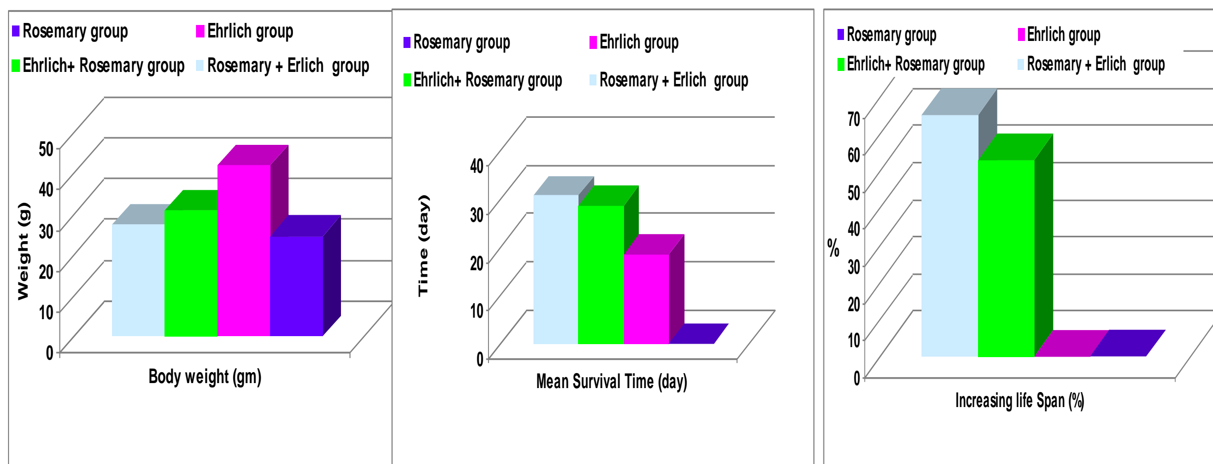

Figure.1 A-C: Effect of rosemary extract on body weight, mean survival time and increasing life span of Ehrlich ascites carcinoma (EAC) bearing mice

Table.7 Effect of rosemary extract on tumor size parameters (volume and cell count) of Ehrlich ascites carcinoma (EAC)- bearing mice

Parameters / Group	Tumor volume (ml)	Tumor cell count (10^6/ml)
Group (1) Rosemary group Mean ±SD (n = 10)	0.00 ± 0.00	0.00 ± 0.00
Group (2) Ehrlich ascites carcinoma group Mean ±SD (n = 10)	$9.26^a \pm 1.03$	$49.18^a \pm 3.31$
Group (3) Rosemary + Ehrlich group Mean ±SD (n = 10)	$2.86^c \pm 0.34$	$16.44^c \pm 3.21$
Group (4) Ehrlich+ Rosemary group Mean ±SD (n = 10)	$5.74^b \pm 0.43$	$29.90^b \pm 4.29$

All data are expressed as mean ± SD. Values in the same raw with different superscript differ significantly at $P \leq 0.05$.

Table8 Effect of rosemary extract on liver function test parameters of Ehrlich ascites carcinoma (EAC) - bearing mice

Group / Parameters	Group (1) Rosemary (n = 10)	Group (2) Ehrlich ascites carcinoma (n = 10)	Group (3) Rosemary + Ehrlich (n = 10)	Group (4) Ehrlich+ Rosemary (n = 10)
Alkaline Phosphatase (IU/L) Mean ±SD	23.42^{b} ±13.87	104.08^{a} ± 26.99	68.42^{c} ±18.29	82.42^{a} ± 19.06
Asparate Transaminase (U/L) Mean ± SD	302.8^{c} ±26.69	1036.2^{a} ± 47.94	603.2^{d} ±71.32	791.0^{b} ± 96.88
Alanine Transaminase (U/L) Mean ± SD	41.20^{c} ±2.39	90.40^{a} ± 8.77	52.60^{d} ± 7.77	77.20^{b} ± 9.23
Total bilirubin (mg/dl) Mean ± SD	0.168^{c} ±0.03	0.266^{a}±0.02	0.206^{a} ±0.02	0.260^{b} ± 0.02
Albumin (g/dL) Mean ± SD	3.196^{a} ±0.36	2.090^{c} ± 0.12	2.570^{b} ±0.16	2.322^{bc} ± 0.33

All data are expressed as mean ± SD. Values in the same raw with different superscript differ significantly at P ≤ 0.05.

Table.9 Effect of rosemary extract on redox state parameters (MDA, CAT, SOD, reduced glutathione and total antioxidant capacity) of Ehrlich ascites carcinoma (EAC) - bearing mice

Group / Parameters	**Group (1) Rosemary (n = 10)**	**Group (2) Ehrlich ascites carcinoma (n = 10)**	**Group (3) Rosemary + Ehrlich (n = 10)**	**Group (4) Ehrlich+ Rosemary (n = 10)**
SOD (U/g)* Mean ± SD	38.90^{a} ± 3.17	18.00^{c} ± 2.95	26.06^{b} ± 3.27	23.84^{b} ± 4.84
CAT (U/g)* Mean ± SD	42.56^{a} ± 8.15	22.08^{c} ± 2.51	35.02^{b} ± 4.88	25.38^{c} ± 4.44
GSH (mmol/g)* Mean ± SD	0.1366^{a} ± 0.01	0.0636^{d} ± 0.01	0.1036^{b} ± 0.01	0.0872^{c} ±0.01
MDA (nmol/g) * Mean ± SD	4.24^{d} ± 0.62	11.1^{a} ± 0.76	8.24^{c} ± 0.73	9.94^{b} ± 0.56
TAC (mM/L)* Mean ± SD	2.406^{a} ± 0.20	1.568^{b}± 0.338	1.946^{b} ± 0.264	1.704^{b} ± 0.33

All data are expressed as mean ± SD. Values in the same raw with different superscript differ significantly at P ≤ 0.05.

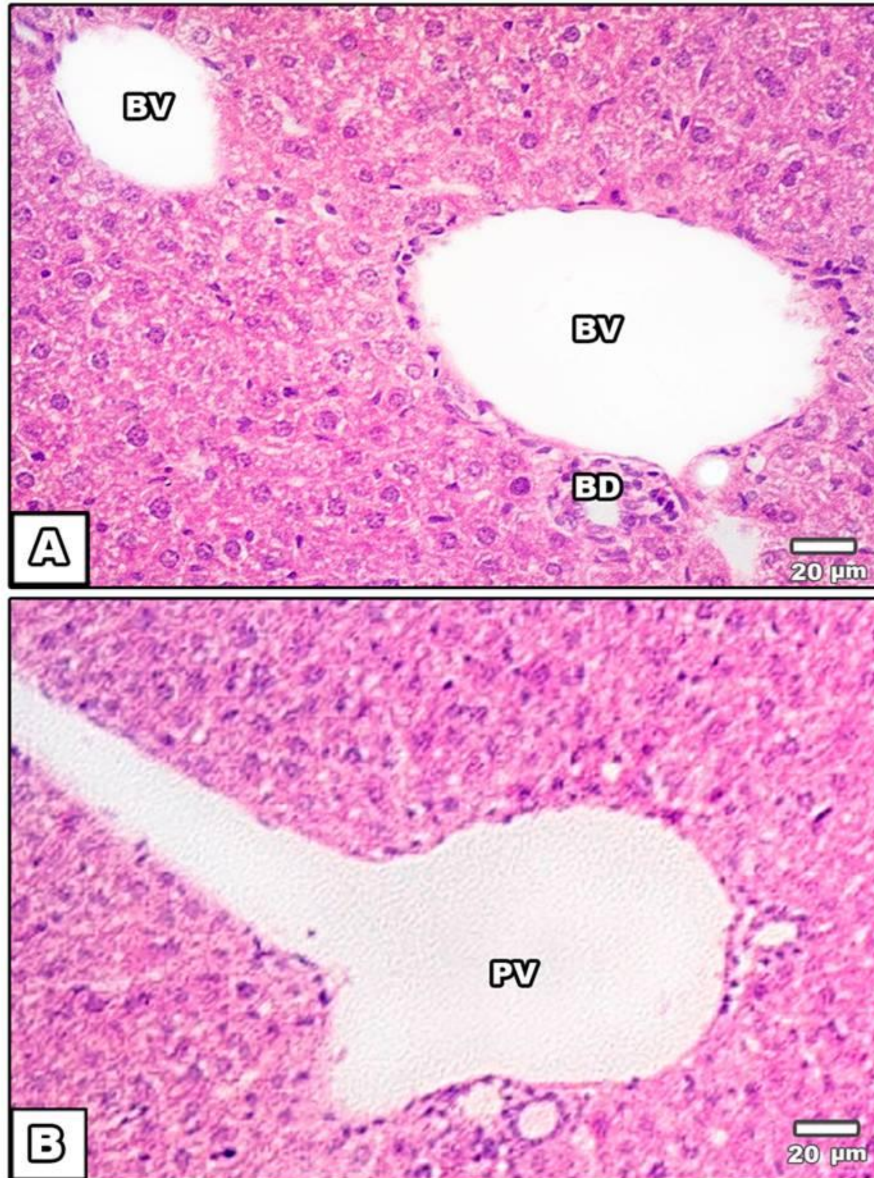

Fig.2 (A-B) Photomicrographs of histological section of methanolic rosemary-extract-treated liver showing normal hepatocytes and regular pattern of hepatic cord around central vein. Abbreviation; BD, Bile ductule; BV, Blood vessel; PV, Portal vein. Hx-E.

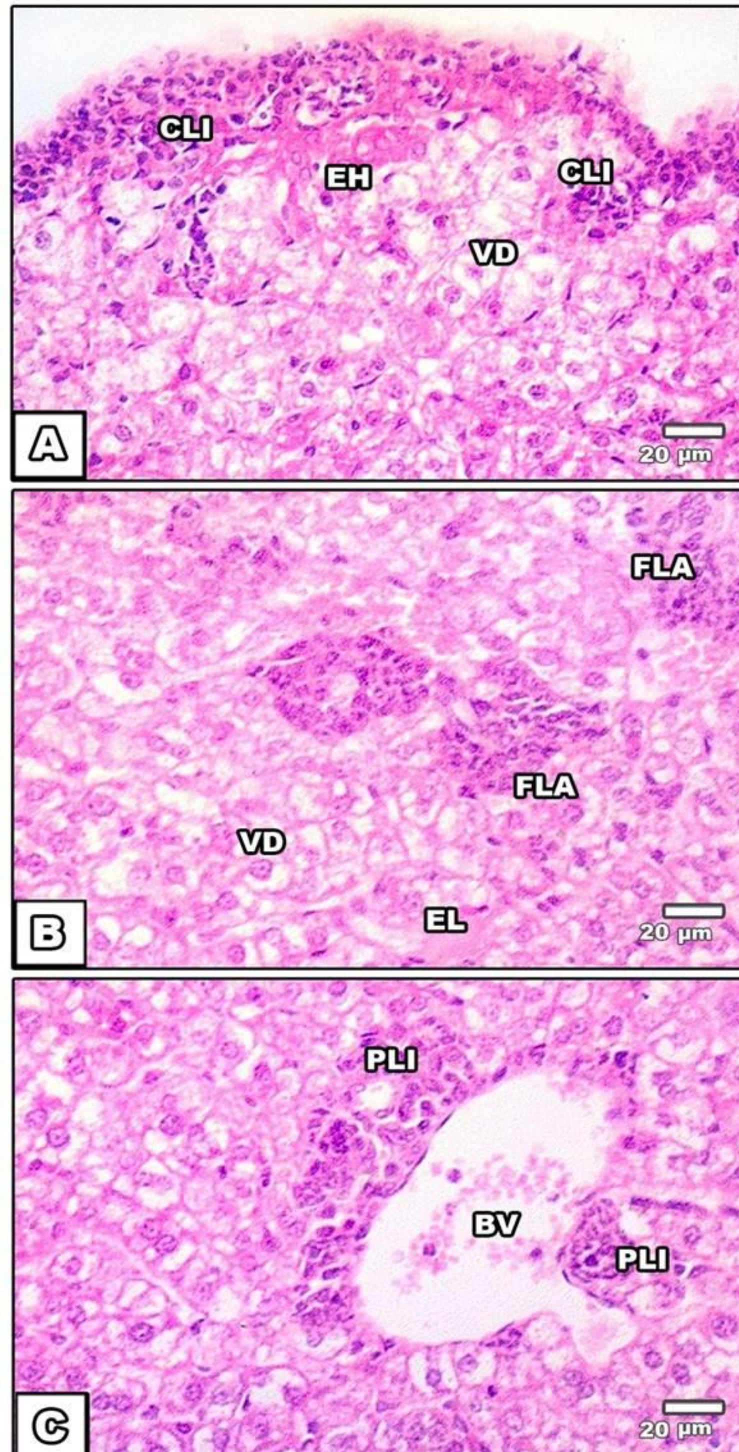

Fig.3 (A-C). Photomicrographs of histological section of liver of Erlich ascites carcinoma. (A) Showing hyperplasia of liver capsule associated with leukocyte infiltration. (B) Showing focal collection of leukocytes. (C) Showing periportal leukocyte infiltration. Abbreviations; BV, Blood vessel; CLI, Capsule leukocyte infiltration; EH, Eosinophilic hepatocyte; FLA, Focal leukocyte aggregation; PLI, Periportal leukocyte infiltration; VD, Vacuolar degeneration. Hx-E.

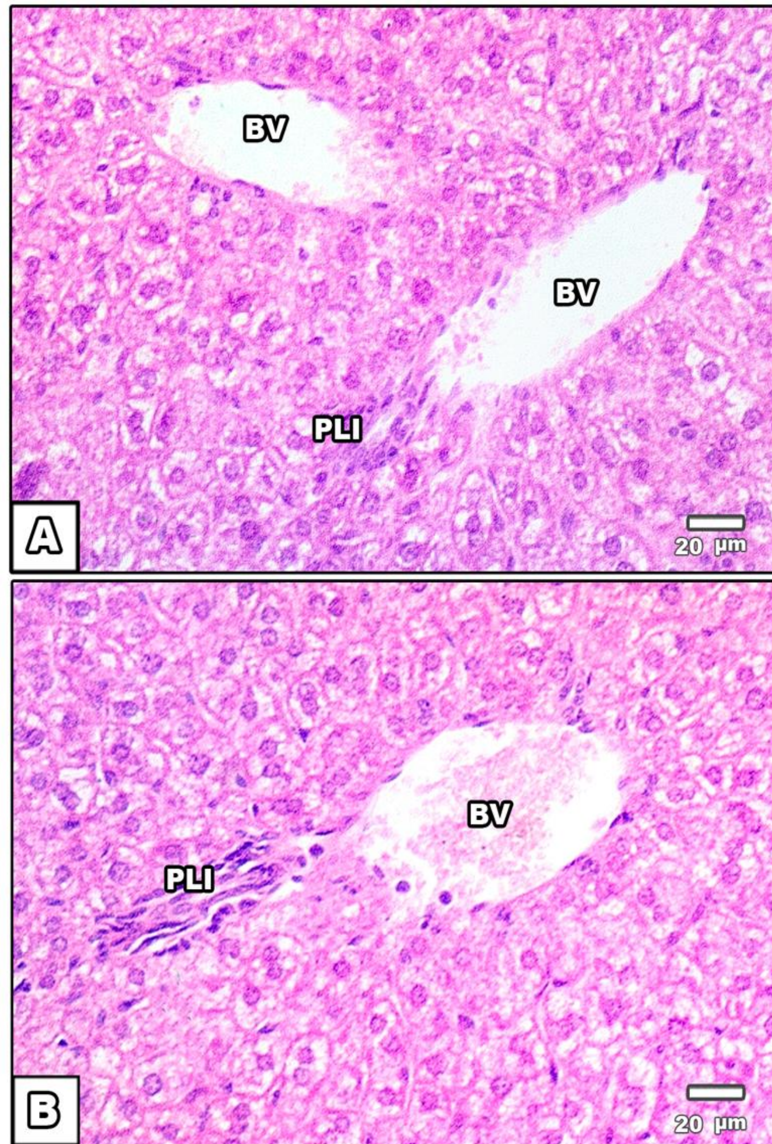

Fig.4 (A-B) Photomicrographs of histological section of alcoholic rosemary-treated Erlich ascites carcinoma liver showing amelioration of hepatic picture
Abbreviation; BV, Blood vessel; PLI, Periportal leukocyte infiltration. Hx-E

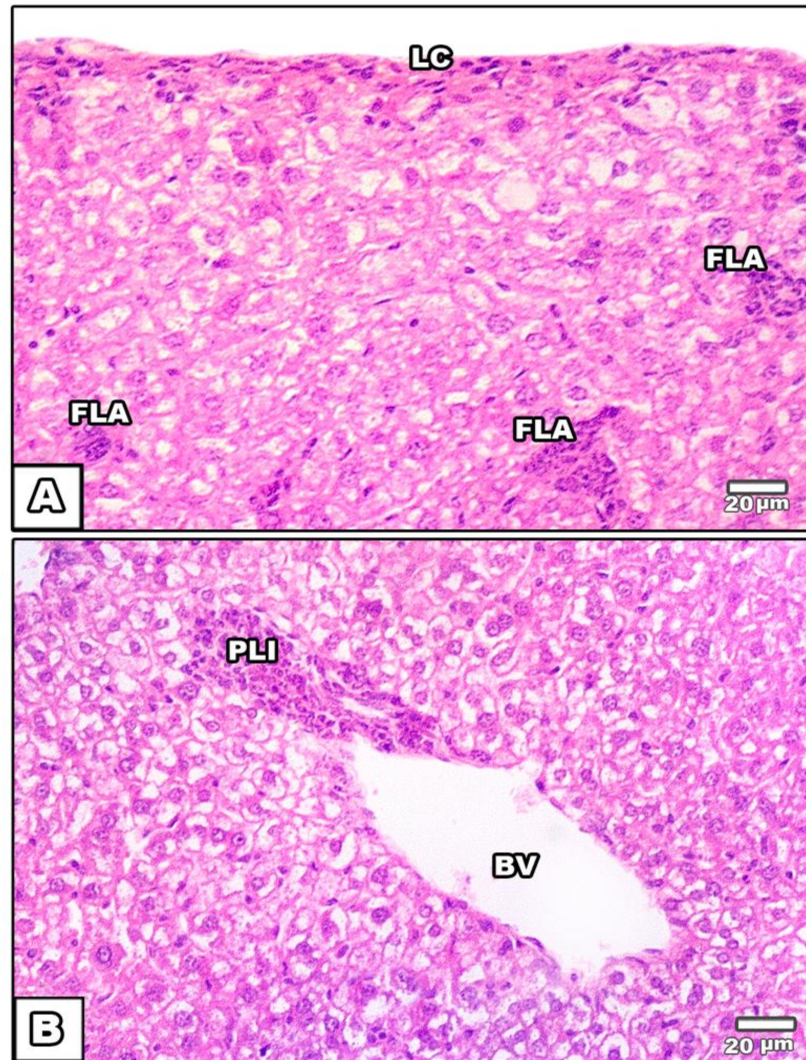

Fig.5 (A-B). Photomicrographs of histological section of alcoholic rosemary-protected Erlich ascites carcinoma liver showing amelioration of hepatic picture.
Abbreviation; BV, Blood vessel; FLA, Focal leukocyte aggregation; LC, Liver capsule; PLI, Periportal leukocyte infiltration. Hx-E.

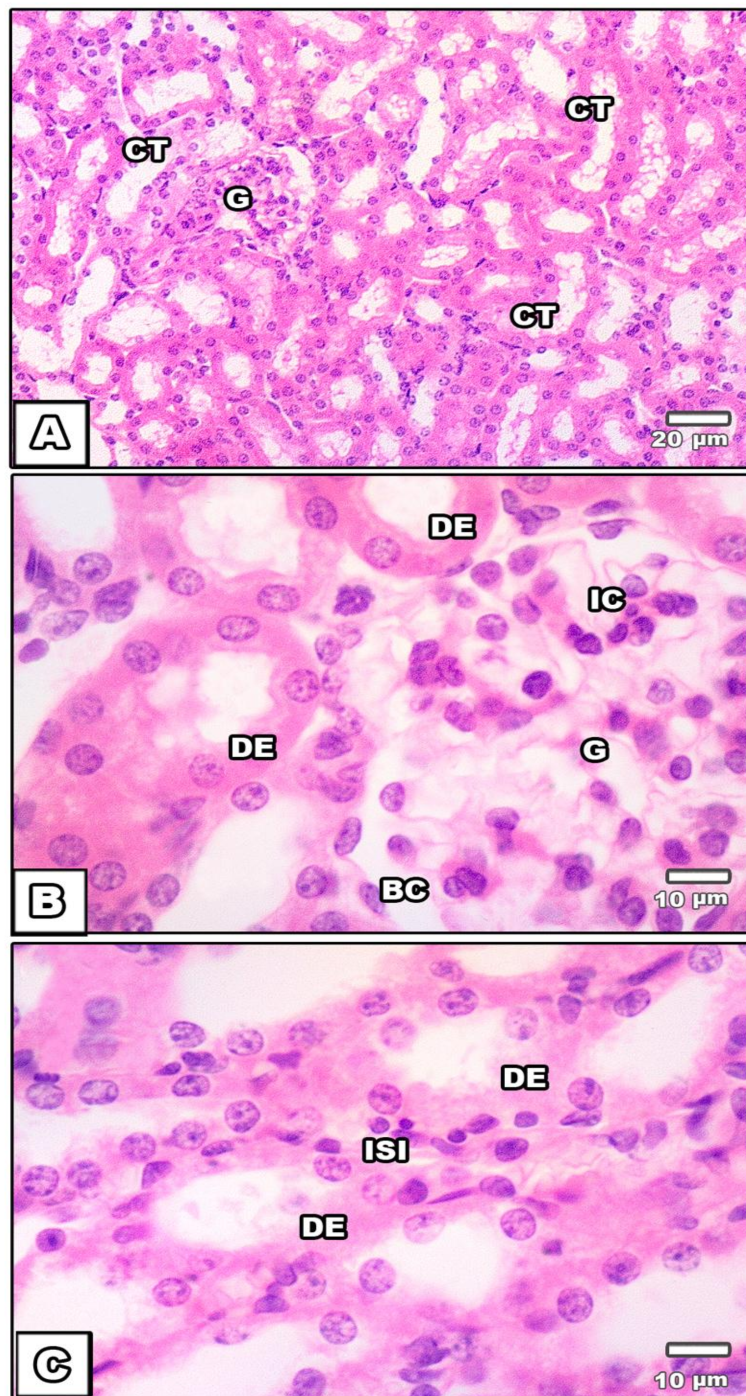

Fig.6 (A-C) Photomicrographs of histological section of alcoholic rosemary-treated kidney. A. Showing normal renal structure including renal corpuscles and tubule. B. Showing normal glomeruli with characteristic endothelial cells and capsules having lining squamous endothelium. C. Showing proximal and distal tubules lined with cuboidal epithelium and enclosed by tubular lumina. Abbreviation; BC, Bowmans capscule; CT, Collecting tubule;G, Glomeruli; . Hx-E.

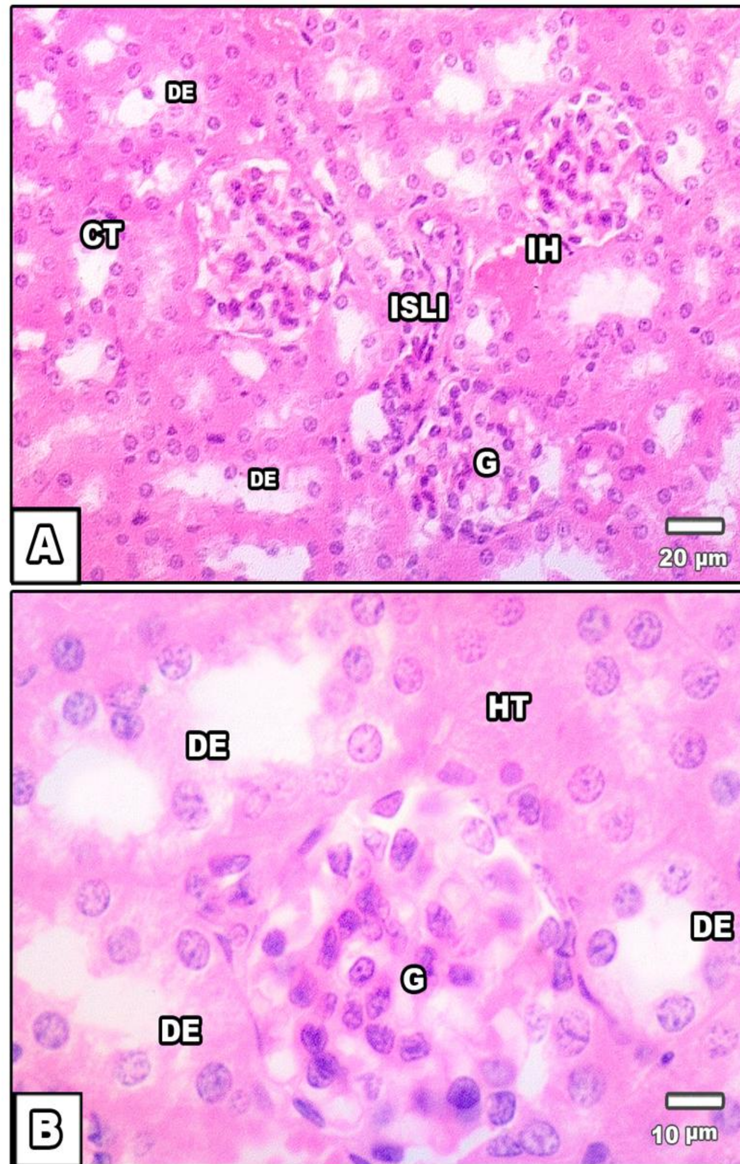

Fig.7 (A-B). Photomicrographs of histological section of kidney of Ehrlich ascites carcinoma. A. Showing atrophied glomeruli and renal tubules with abnormal swelling lumina and damaged lining epithelium. B. Showing abnormal glomeruli with missing Bowman's space and leukocyte infiltration. Abbreviations; BC, Bowman's capsule; CT, Collecting tubules; DE, Degenerated endothelium; IC, Inflammatory cells; ISI, Interstitial inflammatory cell infiltration G, Glomeruli Hx-E.

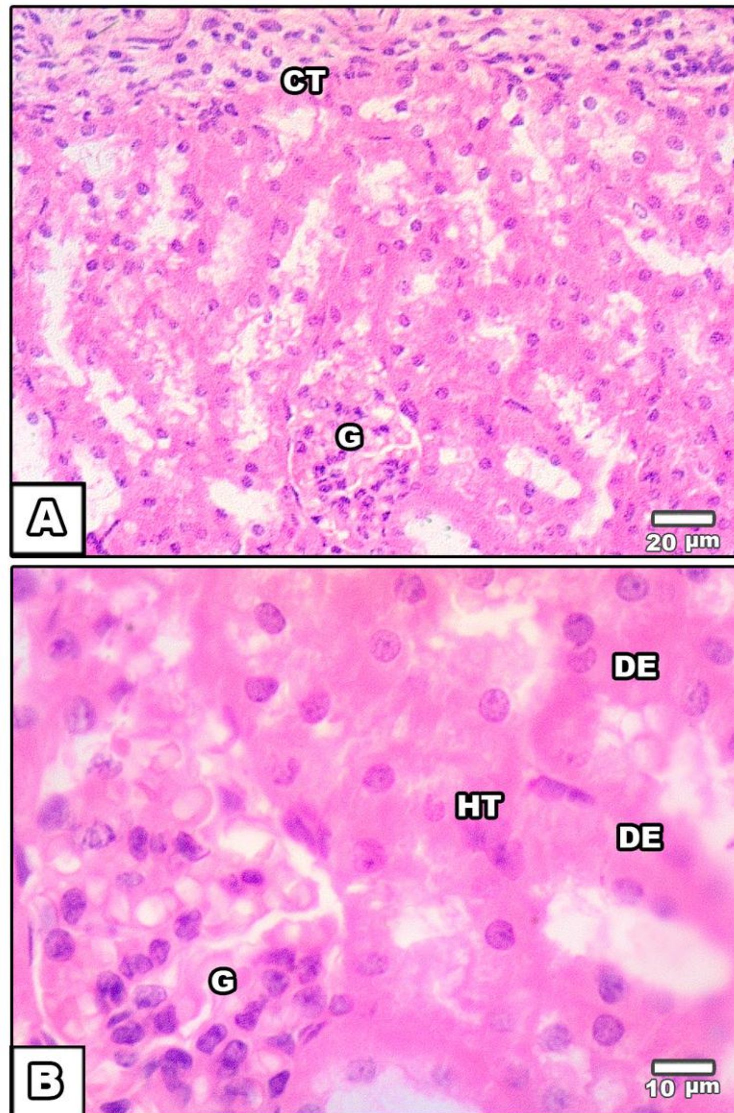

Fig.8 (A-B). Photomicrographs of histological section of alcoholic rosemary-treated Erlich ascites carcinoma renal tissue showing amelioration renal corpuscle and slight damage of epithelium lining the tubules. A Abbreviations; CT, Collecting tubules; DE, Degenerated endothelium; ISLI, Interstitial leukocyte Infiltration , IH internal haemorrhage, HT hyalinized tubule, Hx-E .

However, in experimental rosemary protected Ehrlich ascites carcinoma, amelioration was less than alcoholic-rosemary-treatment.

Regarding the histopathological examination of the kidney from the control rats showed normal renal cortex and medulla with normal histological features i.e. normal structure for glomeruli and the Bowman's capsule with normal space between the glomeruli and Bowman's capsule. The parietal layer of its renal capsule is composed of simple squamous epithelium. The renal corpuscles of glomeruli are surrounded by proximal and distal convoluted tubules. Bundles of parallel tubules can be identified running into the cortex. On the hand, kidney specimens from EAC bearing mice revealed marked damage

of renal tissues characterized by mild peritubular inflammatory cellular infiltration associated with degeneration of tubular lining epithelial cells and widening of the tubular lumina. The size of the glomeruli become reduced and filling almost of the Bowman's capsule space. Treatment of mice with alcoholic rosemary caused marked amelioration of the renal tissues except slight foci of internal haemorrhage and few hyalinized renal tubules. All of these pathological findings indicate liver and kidney damage in EAC as well as protection of liver and kidney structure by treating mice with rosemary extract.

The significant restoration of all of the above biochemical and histopathological parameters towards normal values upon rosemary extract treatment tested in the present study indicates the protection of vital organs such as liver and kidney from damage induced by EAC. Hence, the present study confirms the potent hepatoprotective and antioxidant nature of active phenolic compounds in rosemary extract; the strong antitumor activity observed in this model may be due to the antioxidant nature of the extract. Hence, it will be of great interest to isolate the active constituents of rosemary leave extracts.

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