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Parotoid gland secretions of the Egyptian toad (*Bufo relgularis*): *In vivo* antitumor effect on Ehrlich ascites carcinoma bearing mice

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Running title: Treatment with PGS ameliorates the pathophysiological changes in the liver of EAC- bearing mice

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Abstract

Background: Parotoid gland secretions (PGS) contain several bioactive compounds with potential applications for drug development. **Objective:** This study was conducted to evaluate the *in vivo* antitumor effect of PGS collected from the Egyptian toad, *Bufo relgularis* using Ehrlich ascites carcinoma-bearing mice (EAC). **Methods:** The median lethal dose (LD_{50}) of PGS was determined, then forty CD-1 female mice were divided into 4 groups (n=10) as follows: Group (Gp1) served as a negative control. Gp2-Gp4 had inoculated intraperitoneally (i.p) with 1×10^6 EAC cells/mouse. Then, Gp2 was left as a positive control (EAC- bearing mice). After 24 hours, Gp3 had injected i.p with Cisplatin (Cis) (2 mg/kg) on day 1 for 7 consecutive days. Gp4 had injected with 1/10 LD_{50} of PGS (7.85 mg/kg, b.wt) i.p for 7 consecutive days. All groups were sacrificed on day 14 to collect blood samples. The percentages of total body weight (% bwt) change, tumor volume, and total tumor cell counts were determined. Alanine and aspartate transaminases (ALT and AST), antioxidant /oxidant biomarkers (SOD, CAT, and MDA), and histological investigations of liver tissues were evaluated. **Results:** The results showed that the % b.wt changes were increased in EAC- bearing mice, while the treatment of EAC- bearing mice with PGS decreased its % b.wt changes. The treatment of EAC- bearing mice with PGS decreased the tumor volume and its counts. PGS treatment led to an improvement in AST, ALT, and antioxidant enzyme activities, and ameliorated the histopathological changes in the liver induced by EAC inoculation. **Conclusion:** We concluded that PGS had a potential anticancer effect against EAC- bearing mice.

Keywords: Parotoid gland, Secretions, EAC- bearing mice, Pathophysiological

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Introduction

Cancer is the second leading cause of mortality after cardiovascular disease worldwide (1). Currently, several settings for cancer treatment including chemotherapy are in use. Even though chemotherapeutic agents are potential agents for cancer treatment, however, they induce a variety of side effects on vital organs (2). Cisplatin (Cis) is a well-known chemotherapeutic drug for the treatment of cancer (3). However, it has numerous undesirable side effects including severe kidney problems and allergic reactions (4). Therefore, selective and more efficient new drugs are urgently needed to replace the current treatment settings for cancer patients (5). Natural products (NPs) represent available sources for developing new anticancer agents (6). In addition, NPs have antioxidant effects that can eliminate free radicals (7). NPs can also reduce or minimize the toxic side effects of chemotherapy (8). Parotoid gland secretions (PGS) released from the skin are involved in defense against germs (9). PGS contains several bioactive components including a large number of biogenic amines, alkaloids, steroids, peptides, and proteins (10). These biomolecules play a potent character in molecular drug development for pharmacological evaluation, drug synthesis, and biomedical applications (11). PGs have been used clinically as an antimicrobial, local anesthetic, and antineoplastic agent (12). Proteins, peptides, poisonous substances, steroids, alkaloids, and biogenic amines depending on the species and other chemical components are among the substances released by these glands (13). Hundreds of peptides have been discovered in amphibian skin (14). The crude extracts of PGS of *Rhaebo guttatus* and *R. marina* showed strong toxic effects on rat breast carcinoma (15). A previous study by Giri et al (16) reported that Indian toad skin extract (*Bufo melanostictus*) inhibited EAC cell growth in EAC-bearing mice. This study aims to evaluate the impact of PGS that were collected from the Egyptian toad (*Bufo regularis*) as an antitumor agent using EAC-bearing mice.

Materials and Methods

Chemicals

Cisplatin (Cis) was purchased from sigma Company, Egypt. Alanine transaminase (ALT), aspartate transaminase (AST), Superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) were determined by using kits purchased from Bio-diagnostic Company, Egypt.

Collection of toads and preparation of PGS

Between November and January 2021, two hundred toads (*B. regularis*) were collected from fields in Egypt's Monufia Governorate's Sadat City. The toads were carefully placed into wooden cages before being transported to the Zoology Department, Faculty of Science, Tanta University. Toads washed with distilled water, the laterodorsal region of the parotoid glands was gently squeezed by hand. PGS were collected and put in autoclaved beakers. The toads were released to their nature after sample collection. The collected secretions were lyophilized after being dissolved in distilled water, sonicated, stored at -20 ˚C, and dried under decreased pressure. The PGs were weighed and kept for further processing **(**17**)**.

Determination of median lethal dose

The median lethal dose (LD_{50}) was determined using probit analysis. Briefly, a total number of 24 female Swiss albino mice (20 ± 2 g) were divided into six groups (N=4). These groups were injected with a single dose of different concentrations of PGS (40 to 700 mg/kg). Groups were noticed for 24 h to assess the acute toxicity of PGS. LD_{50} value was detected by plotting the mortality percentage of animals against the doses (18).

EAC-cells expansion and inoculation

The first inoculum of the EAC cell line was purchased from the Department of Tumor Biology, National Cancer Institute, Cairo University. The cell line was

intraperitoneal (i.p.) inoculated 1×10^6 cells/mouse. Cells were grown in the peritoneal cavity of mice and transferred every ten days to new animals. Mice were monitored daily for signs of tumor progression, including the amount of abdominal distension and signs of illness and distress. The volume of ascites fluid was determined by needle (18–22 gauge) aspiration. Withdrawal of ascites fluid was performed under aseptic conditions. To run the experiment on EAC-bearing mice, an individual mouse was inoculated with 1×10^6 EAC-cells (8).

Animals and experimental protocols

Forty healthy female Swiss albino mice (18–20 g) were obtained from the Animal Facility of Cairo University, Egypt. Mice allowed for acclimating for 1 week. Target values for temperature and relative humidity were about 22 ± 1ºC and 55 ± 5% respectively, light-dark (day/night) cycle was achieved. Mice were given drinking tap water and normal experimental pelleted animal food *ad libitium*. The experiments were done in compliance with the guiding principles for the care and use of laboratory animals at the Faculty of Science, Tanta University, under the ethical number (IACUC-SCI-TU-0244).

Mice were randomly divided equally into four groups (n = 10) according to body weights to minimize the standard errors between groups as follows. Gp1 was left as a negative control. Gp2 had inoculated with EAC-cells (1×10^6/ mouse) i.p and was kept as a positive control. Gp3 and Gp4 had inoculated with EAC (1×10^6/ mouse) i.p followed by injection of Cis (2 mg/kg) or with (1/10) of LD_{50} of PGS for 7 consecutive days, respectively. On day 14, the total body weight changes were assessed, and all groups were sacrificed. Blood samples were collected, and the sera were separated and frozen at -20 ˚C until used for the determination of liver transaminases. By using 10 ml syringes, the ascitic fluids were harvested from all groups under the study. The volume of ascitic tumor fluids was measured. To determine the live and dead tumor cells count, the trypan blue exclusion method was used.

Determination of the percentages of body weight change

All groups of mice were weighted at the beginning (initial b.wt) and at the end of the experiment (final b.wt). The percentage of body weight change (% T.B.W) was calculated as follows: (final b.wt – initial b.wt / initial b.wt) × 100.

Biochemical analysis

ALT and AST activities in serum were assayed by using a commercial kit according to the method of Xing-Jiu (19). Hepatic SOD and CAT were assayed according to the method of Christine and Joseph (20). Furthermore, hepatic MDA level was determined according to the method of Prima (21).

Histopathological investigations

The liver specimens (0.5 cm^3) from all groups will be collected and immersed in 10% neutral buffered formalin. The samples will be dehydrated in ascending graded series of ethanol, cleared in xylene, impregnated, and embedded in paraffin wax. Sections of 5-7µm will be cut by using Leica Microtome (RM 20352035; Lecia Microsystems, Wetzlar, Germany) and mounted on glass slides. Paraffin sections will be stained by hematoxylin and eosin (H&E) stain according to Suvarna (22). The stained sections will be examined with a BX50/BXFLA Microscope (Olympus, Tokyo, Japan).

Statistical analysis

The Group's data expressed as means ± S.D. were analyzed by t-test while percentage data were analyzed by SPSS software. $P < 0.05$ was considered a significant value for all statistical analyses in this study.

Results

LD_{50} of parotoid gland secretions

The median lethal dose of PGS that killed 50% of mice was determined by using probit analysis. Six

groups (4 mice/each) were injected i.p with different doses of PGS from 40 to 700 mg/Kg. The LD_{50} value of PGS was determined after 24 hrs of injection and represented 78.52 mg/kg b.wt **(Figure 1)**.

Treatment with PGS decreased the percentages of body weight change

EAC- bearing mice alone showed a significant increase in the % b.wt change when compared to the control group ($P \leq 0.05$). However, treatment of EAC-bearing mice with Cis led to a significant decrease in the % b.wt change when compared to the EAC-bearing mice alone ($P \leq 0.05$). Treatment of EAC-bearing mice with PGS also led to a significant decrease in the % b.wt change, when compared to the EAC-bearing mice alone ($P \leq 0.05$) **(Table 1 and Figure 2)**.

Treatment with PGS decreased the tumor volume and tumor counts in EAC-bearing mice

Treatment of EAC-bearing mice with Cis led to a significant decrease in tumor volume (0.45 ± 0.05 ml/mouse) with a reduction percentage (–93.28%) ($P \leq 0.05$). Treatment of EAC-bearing mice with PGS also led to a significant decrease in tumor volume (3.8 ± 1.3 ml/mouse) with a reduction percentage (-43.28%) ($P \leq 0.05$) (**Table 2 and Figure 3)**. The total count of EAC cells (TCC) in EAC-bearing mice was $510 \pm 27 \times 10^6$/mouse, however, the treatment of EAC-bearing mice with Cis led to a significant decrease in the number of EAC cells to $36 \pm 3.4 \times 10^6$/mouse with a reduction percentage (-92.9%) when compared to EAC-bearing mice alone ($P \leq 0.05$). Treatment of EAC-bearing mice with PGS led to a significant decrease in the TCC ($284 \pm 8.5 \times 10^6$/mouse) with a reduction percentage (-44.3%). Treatment with Cis or PGS led to a significant decrease in the total EAC-live cells (T.L.C) and an increase in the total EAC-dead cells (T.D.C) when compared to EAC-bearing mice ($P \leq 0.05$) **(Table 2 and Figure 3)**.

Treatment with PGS ameliorated the liver transaminases activity

EAC-bearing mice showed a significant increase in ALT and AST activities when compared to the control group ($P \leq 0.05$). Treatment of EAC-bearing mice with Cis led to a significant decrease in the activities of ALT and AST activities when compared to EAC-bearing mice alone ($P \leq 0.05$). Furthermore, treatment of tumor-bearing mice with PGS led to a significant decrease in ALT and AST activities when compared to tumor-bearing mice alone ($P \leq 0.05$) **(Table 3 and Figure 4)**.

Treatment with PGS enhanced the antioxidant enzymes

The results showed that EAC-bearing mice showed a significant decrease in SOD and CAT activities with a significant increase in the MDA level when compared to the control group ($P \leq 0.05$). Compared to the EAC-bearing mice alone, treatment with Cis showed a significant increase in the hepatic SOD and CAT activities ($P \leq 0.05$), and a significant decrease in the MDA level ($P \leq 0.05$). Similarly, the treatment of tumor-bearing mice with PGS led to a significant increase in the SOD and CAT activities and a significant decrease in the MDA level when compared to EAC-bearing mice alone ($P \leq 0.05$) **(Table 4 and Figures 5, 6)**. [1]

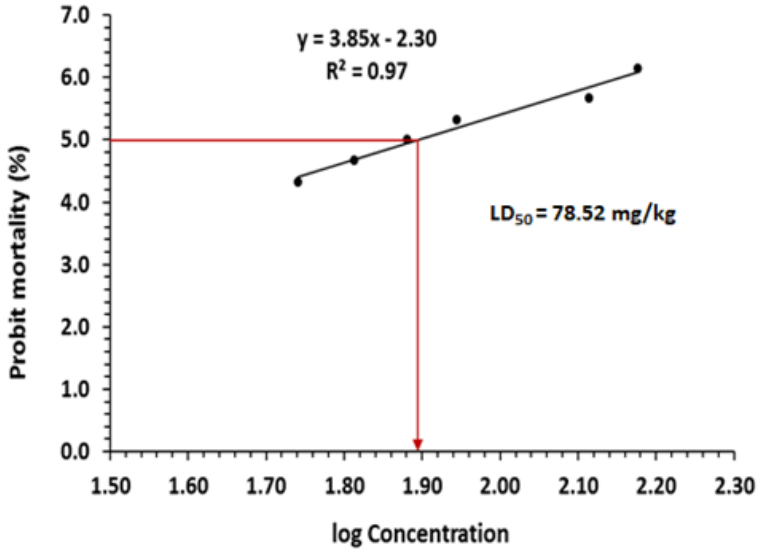

Figure 1. LD_{50} of parotoid gland secretions

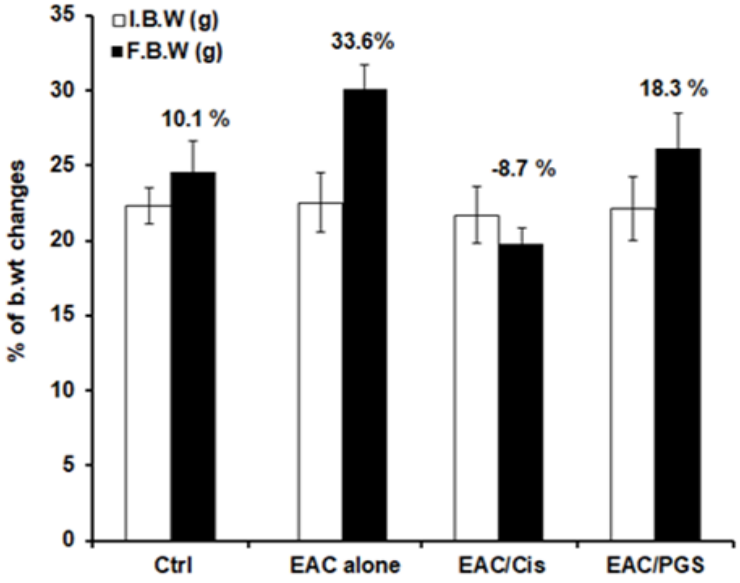

Figure 2. The initial, and final body weight and their percentages of change in the different groups.

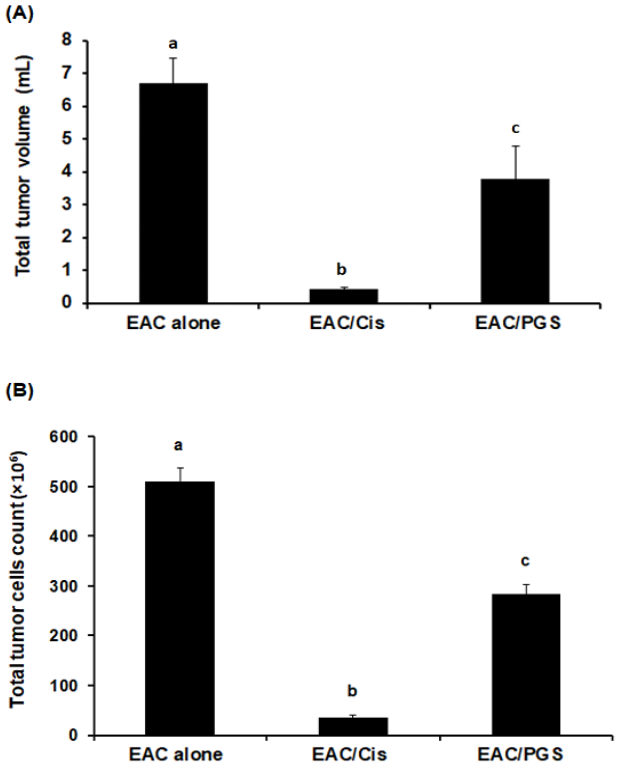

Figure 3. The T.T.V (A) and T.C.C (B) in the EAC-bearing groups

Table 1. The initial, final body weight, and % b. wt change in different groups.

Groups	I.B.W (g)	F.B.W (g)	% b. wt
Ctrl	22.3 ± 1.22	24.53 ± 2.1	10.12 ± 1.18 [a]
EAC alone	22.5 ± 1.98	30.06 ± 2.9	33.6 ± 2.87 [b]
EAC/Cis	21.7 ± 0.98	19.8 ± 1.02	-8.7 ± 0.98 [c]
EAC/PGS	22.1 ± 1.87	26.1 ± 2.4	18.3 ± 1.95 [d]

The values represented means ± S.D; **Ctrl:** Control; **PGS:** Parotoid gland secretions; **EAC:** EAC-bearing mice; **Cis:** Cisplatin; **I.B.W:** Initial body weight; **F.B.W:** Final body weight; **% b. wt:** Percentage of body weight changes. P value ≤ 0.05 was considered to be statistically significant. This means that do not share a significantly different letter

Table 2. The total tumor volume, tumor cells count, and live and dead tumor cells in different groups.

Groups	T.T.V (mL)/m	% r	T.C.C ($\times 10^6$)/m	% r	T.L.C ($\times 10^6$)/m	T.D.C ($\times 10^6$)/m
EAC alone	6.7 ± 0.95 [a]	-	510 ± 27 [a]	-	494 ±7.5 [a]	16 ±1.52 [a]
EAC/Cis	0.45 ± 0.05 [b]	-93.28%	36 ± 3.4 [b]	-92.9%	9 ± 0.8 [b]	27 ± 3.7 [b]
EAC/PGS	3.8 ± 1.3 [c]	-43.28%	284 ± 18.5 [c]	-44.3%	170 ±225 [c]	116 ± 9.5 [c]

The values represented means ± S.D.; **EAC:** EAC-bearing mice; **Cis:** Cisplatin; **PGS:** Parotoid gland secretions; **T.V.:** Total tumor volume per mouse; **T.C.C.:** Total tumor cell count; **T.L.C.:** Total live cells; **T.D.C.:** Total dead cells. P value ≤ 0.05 was considered to be statistically significant. This means that do not share a letter is significantly different.

Table 3. ALT and AST activities in the different groups.

Groups	Liver enzymes parameters	
	ALT (U/L)	**AST (U/L)**
Ctrl	39.5 ± 4.6 [a]	151.5 ± 13.5 [a]
EAC alone	118.7 ± 8.5 [b]	381 ± 18.8 [b]
EAC/Cis	70 ± 6.6 [c]	263 ± 17.8 [c]
EAC/PGS	85 ± 5.5 [c, d]	286 ± 20.1 [c, d]

The values represented means ± S.D.; **Ctrl:** Control; **PGS:** Parotoid gland secretions; **EAC:** EAC-bearing mice; **Cis:** Cisplatin; **ALT:** Alanine transaminase; **AST:** Aspartate transaminase. P value ≤ 0.05 was considered to be statistically significant. This means that do not share a letter is significantly different.

Table 4. Hepatic SOD, and CAT activities, MDA level in the different groups.

Groups	SOD (U/mg tissue)	CAT (U/mg tissue)	MDA (nmol/g tissue)
Ctrl	4.98 ± 0.13 [a]	83.5 ± 4.7 [a]	38.2 ± 2.2 [a]
EAC alone	1.5 ± 0.17 [b]	37.2 ± 2.3 [b]	89.8 ± 6.67 [b]
EAC/Cis	2.8 ± 0.07 [c]	61.8 ± 3.1 [c]	42.3 ± 3.92 [a, c]
EAC/PGS	1.97 ± 0.16 [b, c]	47.1 ± 4.5 [d]	64.1 ± 3.9 [d]

The values represented means ± S.D.; **Ctrl:** Control; **PGS:** Parotoid gland secretions; **EAC:** EAC-bearing mice; **Cis:** Cisplatin; **SOD:** Superoxide dismutase, **CAT:** Catalase, **MDA:** Malondialdehyde. P value ≤ 0.05 was considered to be statistically significant. This means that do not share a letter is significantly different.

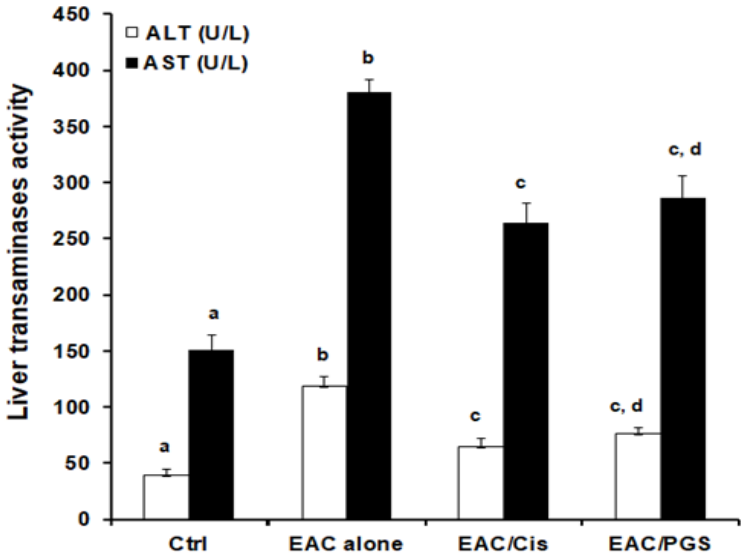

Figure 4. ALT and AST activities in the different groups.

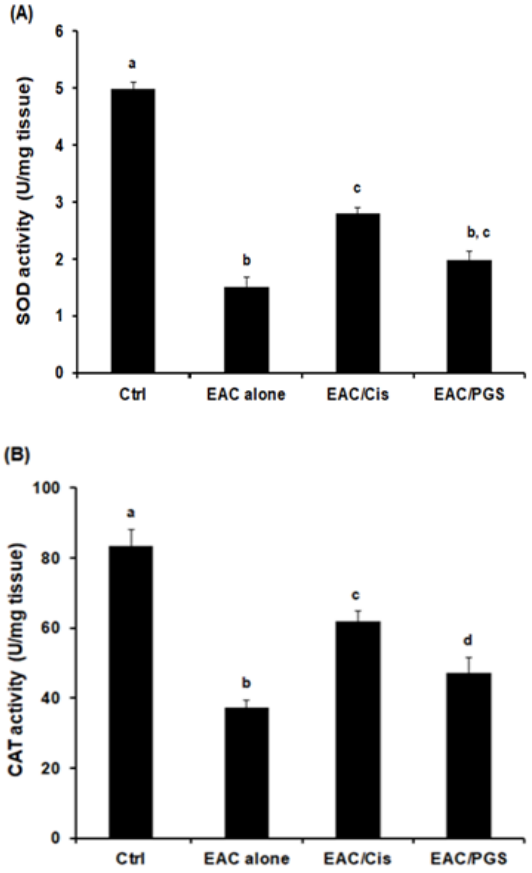

Figure 5. SOD (A) and CAT (B) activities in the different groups.

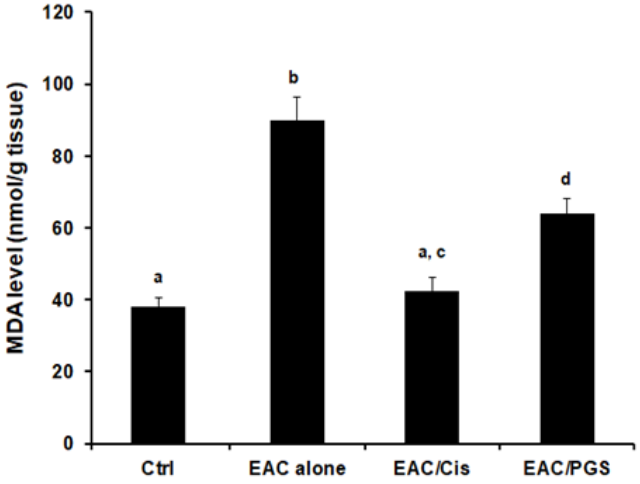

Figure 6. MDA levels in the different groups

Treatment with PGS decreased the histopathological changes in the liver tissues of EAC- bearing mice

The control liver sections showed normal structure with several hepatic lobules; each lobule consisted of radially arranged hepatic cords which are separated by a hepatic sinusoid, central vein, and portal area. Normal hepatic cells appeared polyhedral to oval-shaped, and contain a well-defined granular cytoplasm and a large rounded central nucleus with a prominent nucleolus. The portal area includes branches of the bile duct, hepatic artery, and portal vein **(Figure 7A).** The hepatic tissue of tumor-bearing mice showed marked histological alterations including pyknotic darkly stained shrunken nuclei and dilated and congested central vein. Moreover, obvious cytoplasmic vacuolations and severe inflammatory cell infiltration were present **(Figure 7B).** The sections of the liver of EAC- bearing mice treated with Cis, showed marked improvement in the hepatic tissues. In particular, the hepatic cells restore their normal shape with basophilic cytoplasm and large centric rounded nuclei except for a few cells that were shrunken and their nuclei were pyknotic. Meanwhile, few cytoplasmic vacuolations, inflammatory cell infiltration, and dispersed congested dilated blood vessels were observed (**Figure 7C**). The hepatic tissue of EAC- bearing mice that had been treated with PGS, showed normal hepatic structure. Hepatocytes were relatively normal and large with prominent rounded nuclei. Few cells had cytoplasm with deeply stained pyknotic nuclei. The central vein showed an oval-like shape that represents more or less similar to the control. There were no cytoplasmic vacuolations, inflammatory cell infiltration, or congested blood vessels observed **(Figure 7D**).

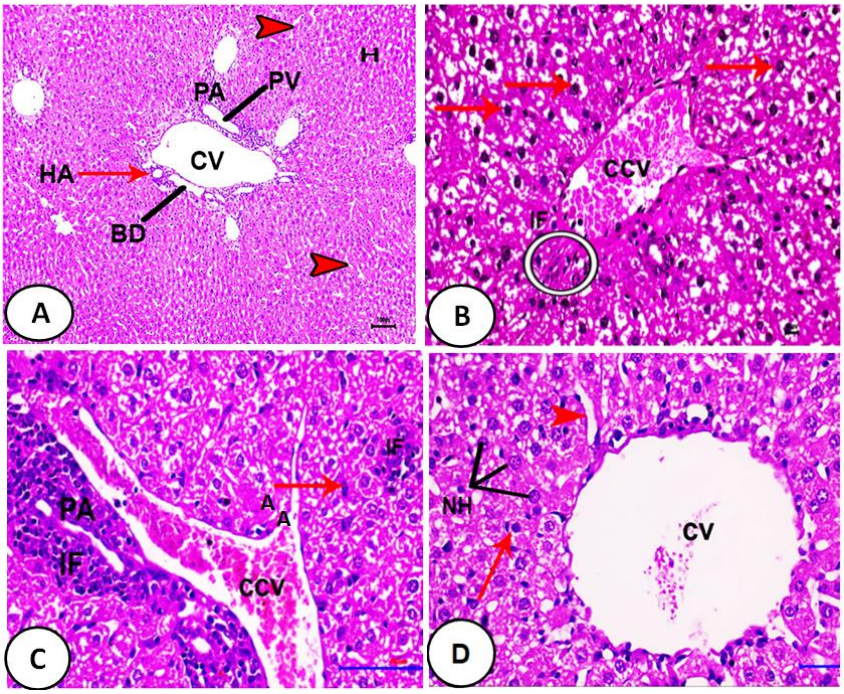

Figure 7 A. Photomicrograph of control liver section showing the normal structure of the liver, hepatic lobules, central vein (CV), portal area (PA), normal hepatic cells (H), sinusoid (red arrowheads), pancreatic duct (BD), portal vein (PV), and hepatic artery (HA). (H&E stain) (X100).

Figure 7 B. Photomicrograph of a liver section of EAC-bearing mice showing, hepatic lobules, irregularly shaped and congested central vein (CCV), pyknotic hepatocytes (red arrows), and inflammatory cells (IF). (H&E stain) (X 400).

Figure 8 C. Photomicrograph of a liver section of EAC-bearing mice treated with Cis showing Few pyknotic hepatocytes (red arrows), portal area (PA), congested central veins (CCV), and inflammatory cells (IF). (H&E stain) (X 400).

Figure 8 D. Photomicrograph of a liver section of EAC-bearing mice treated with PGS showing normal hepatocytes (NH), Few pyknotic hepatocytes (red arrows), sinusoid (red arrowheads), and normal shaped central vein (CV). (H&E stain) (X 400).

Discussion

Chemotherapy is used for the treatment of different malignancies; however, it has adverse effects on vital organs (23). The present study was conducted to address the antitumor effect of PGS on EAC- bearing mice. The current study showed that LD_{50} of PGS was 78.52 mg/kg b.wt. A previous study by Giri et al (16) reported that LD_{50} of Indian toad (*Bufo melanostictus*) skin extract was 750 mg kg. A significant increase in the % b. wt change in EAC- bearing mice when compared to the control group, this could be due to the proliferation of tumor cells and accumulation of ascetic fluid in the peritoneal cavity (24).

Treatment of EAC- bearing mice with Cis led to a decrease in % b.wt change when compared to EAC-bearing mice alone. This decrease could be due to the antitumor effect of Cis. This finding is consistent with previous studies by Dasari and Tchounwou, (3) and El-Naggar et al (25), who indicated that the decrease in % b. wt due to the antitumor effect of Cis. A similar finding was found after the treatment of EAC- bearing mice with PGS. This finding could be due to the antitumor effect of PGS. This finding also is consistent with a previous study that reported that Indian toad skin extract (*Bufo melanostictus*) was able to inhibit EAC cells growth in EAC-bearing mice (16). In EAC- bearing mice, T.T.V, and T.C.C increased considerably because of the accumulation of ascetic fluid in the peritoneal cavity due to the proliferation of tumor cells. These findings are consistent with Funasaka et al (26) who reported that the cell count of the tumor increased considerably because of the accumulation of ascetic fluid in the peritoneal cavity of EAC- bearing mice. A significant decrease in the T.T.V, T.C.C, and T.L.C after the treatment of EAC-bearing mice with Cis was found when compared to the EAC-bearing mice group. This finding was in agreement with the results obtained by Saad et al (24) and El-Naggar et al (27). Treatment of EAC-bearing mice with PGS led to a decrease in T.V, T.C.C, and T.L.C and an increase in the T.D.C. when compared to EAC-bearing mice alone.

A significant increase in ALT and AST activities in the EAC-bearing mice could be due to hepatic damage such as liver necrosis and inflammation (28). Treatment of EAC-bearing mice with Cis led to a significant decrease in ALT and AST activities, this finding is consistent with the previous study by Salama et al (29), which found that liver transaminases activities in EAC- bearing mice decreased when treated with Cis. The current study showed that the SOD and CAT activities were decreased with a significant increase in MDA levels in EAC-bearing mice. These findings are consistent with a previous study obtained by Ali et al (30), who reported that EAC- bearing mice showed elevation in MDA level accompanied by a decline in SOD and CAT activities in blood and liver. This finding could be due to the tumor growth, the liver exhaustion of CAT and MDA enzymes, and the acceleration of lipid peroxidation (31). Treatment of EAC-bearing mice with Cis or PGS led to an increase in SOD and CA activities, with a decrease in MDA level. This finding could be due to the potent chemotherapeutic effect of Cis or due to the potent antioxidant activity of PGS.

The histopathological examination of the control liver section showed normal structures. In EAC-bearing mice, the liver showed several histological alterations including an obvious pyknotic darkly stained shrunken nuclei as well as, other binucleated hepatocytes.

These findings are consistent with Mutar et al (32) who showed that tumors lead to a defect in liver tissues. EAC-bearing mice that had been treated with Cis, revealed marked improvement in liver tissues, except a very few shrunken cells with pyknotic nuclei, cytoplasmic vacuolations, inflammatory cell infiltration, and dispersed congested dilated blood vessels were observed. Similar results were reported by El-Naggar et al (33) and Fakher El Deen et al (34). While, the treatment of EAC-bearing mice with PGS

led to an improvement in liver tissues, as hepatocytes were relatively normal. This finding could be due to the effect of PGS as a hepatoprotective agent.

Conclusion

This study showed that PGS could have a potential antitumor effect with hepatoprotective properties

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