Silymarin and Potassium Bromate in EAC-bearing Mice: histopathological and Immunohistological study

Mona A. Elhadad  
Kafrelsheikh University

Prof. Dr. Magdy E. Mahfouz  
Kafrelsheikh University

Prof. Dr. Nora F. Ghanem  
Kafrelsheikh University

Prof. Dr. Eman A. Moussa (mailto:aeman123@hotmail.com)  
Kafrelsheikh University

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Abstract

Cancer is one of the leading causes of death worldwide. Reactive oxygen species induce pathology by damaging lipids, proteins, and DNA. Elevated reactive oxygen species levels, accompanied with down-regulation of cellular antioxidant enzyme systems, result in malignant transformation. Potassium bromate is a chemical oxidizing agent that is a famous flour improver. It can attack cellular constituents through reactive oxygen species and destroy the cellular structures so it may lead to cancer as a result of administration of it for a long time. Plant polyphenols such as silymarin reported to be a safe nontoxic agent to treat several diseases such as cancer. The anticancer mechanism of action of silymarin has been attributed to its antioxidant activity, antiproliferation, and cell cycle arrest, as it causes apoptosis in cancer cells. We aimed to investigate the effect of silymarin, and administration of potassium bromate on mice with Ehrlich ascites carcinoma cells. Histopathological, ultrastructural and immunohistochemical studies were applied on the liver, kidney, and Ehrlich ascites carcinoma cells. Silymarin was found to destroy tumors and improve the histological changes induced by Ehrlich ascites carcinoma cells in liver and kidney of Ehrlich ascites carcinoma-bearing mice. Potassium bromate showed an improvement in the histological changes induced by Ehrlich ascites carcinoma cells in liver and kidney of Ehrlich ascites carcinoma-bearing mice and caused mitochondrial degeneration and nuclear distortion in Ehrlich ascites carcinoma cells but showed no effect on non Ehrlich ascites carcinoma-bearing mice. The present study revealed that silymarin and potassium bromate could be very promising anticancer agents.

Introduction

Cancer is considered as a serious human health problem despite much progress in understanding its biology and pharmacology. Cancer results from the fast development of abnormal cells that spread to neighboring parts of the body and spread to other organs, this process is called metastasis. Metastasis is the major reason for cancer deaths [1]. Reactive oxygen species (ROS) can damage DNA leading to mutations [2]. These events, if the cell is unable to repair the damage, are lethal. If not lethal, these changes in genetic material result in tumor growth by losing cell cycle control [3]. Potassium bromate (KBrO$_3$) is one of the materials that cause the creation of ROS. Because of KBrO$_3$ biotransformation, free radicals formation can cause oxidative damage to essential cellular macromolecules, leading to cancer in experimental animals [4]. KBrO$_3$ is a famous flour improver that acts as a maturing agent [5]. It has been in use as a food additive for the past 90 years [6]. It acts mainly in the late dough stage giving strength and elasticity to the dough during the baking process while also stimulating the rise of bread. KBrO$_3$ is also used in beer making, cheese production and is commonly added to fish paste products [7]. Additionally, it is used in pharmaceutical and cosmetic industries [6].

Natural agents are believed to suppress the inflammatory process that lead to neoplastic transformation, hyper-proliferation, progression of carcinogenic process and the formation of blood vessels (angiogenesis). It is estimated that approximately one-third of all cancer deaths in the United States could be prevented via suitable dietary modification. Accumulating research evidence suggests that several
dietary agents/medicinal plants may be used alone or in combination with chemotherapeutic agents to prevent the development of cancer or even to treat cancer such as silymarin (SM) [8, 9].

SM has been used for more than 2000 years as a natural medicine for treating hepatitis and cirrhosis and to protect liver from toxic materials. SM acts by anti-oxidative, anti-fibrotic, anti-inflammatory, immunomodulatory and liver regenerating mechanisms in experimental liver diseases. Furthermore, SM has been studied, both in vivo and in vitro, for its cancer chemo-preventive potential against different types of cancer [10].

The transplant ability of certain tumors in rodents has provided a useful tool for cancer research. Ehrlich ascites carcinoma (EAC) is a tumor which provides a reasonably homogenous sample of malignant tissue; it is available in large quantities and grows at a fairly expected rate [11].

The present work aimed to study the histopathological and immunohistochemical changes after treatment of EAC-bearing Swiss albino mice by using SM and KBrO₃.

Materials And Methods

Experimental animals

Adult female Swiss albino mice (average 25–30 g in weight) were obtained from the National Cancer Institute of Cairo University. All mice reared under strict standard hygienic conditions and were fed a balanced diet. Ad libitum water was provided for mice.

EAC cells

The parent line of EAC cells was kindly supplied by the National Cancer Institute of Cairo University. The tumor line was maintained by serial intraperitoneal transplantation of EAC 1 x 10⁶ EAC tumor cells / 0.2 ml in female Swiss albino mice.

Materials Used

Dimethyl sulfoxide (DMSO), SM, KBrO₃ and trypan blue dye, were purchased from Sigma -Aldrich (St. Louis, Mo., USA).

Experimental Design

A total of 90 female Swiss mice were equally divided into nine groups at random (10 mice per group) as shown in Table (1). Group 1 was kept as the negative control group. Group 2 was injected intraperitoneally (ip) with DMSO daily for 14 days. Group 3 was injected (ip) with SM daily for 14 days (50 mg/kg body weight) (SM was dissolved in DMSO). Group 4 was treated orally with water soluble KBrO₃ daily for 14 days (100 mg/kg body weight) according to Altoom, Ajarem et al [12]. Group 5 was treated orally with water soluble KBrO₃ (100 mg/kg body weight) and also injected (ip) with SM (50 mg/kg body weight) daily for 14 days. Group 6 was treated orally with water soluble KBrO₃ (100 mg/kg body weight) and also injected (ip) with SM (50 mg/kg body weight), then SM was injected (ip) daily for 14 days. Group 7 was treated orally with water soluble KBrO₃ (100 mg/kg body weight) and also injected (ip) with SM (50 mg/kg body weight) and then injected (ip) with SM daily for 14 days. Group 8 was treated orally with water soluble KBrO₃ (100 mg/kg body weight) and Sm was injected (ip) daily for 14 days. Group 9 was treated orally with water soluble KBrO₃ (100 mg/kg body weight) and Sm was injected (ip) daily for 14 days.
mg/kg body weight) daily for 14 days. **Group 6** was injected *(ip)* with $1 \times 10^6$ EAC cells and kept as the positive control group according to El-Far, Salah et al. **Group 7** was injected *(ip)* with $1 \times 10^6$ EAC cells in the first day and then was injected *(ip)* with SM daily for 14 days (50 mg/kg body weight) according to El-Far, Salah et al [13]. **Group 8** was injected *(ip)* with $1 \times 10^6$ EAC cells in the first day and then was treated orally with water soluble KBrO$_3$ daily for 14 days (100 mg/kg body weight) and **Group 9** was injected *(ip)* with $1 \times 10^6$ EAC cells in the first day and then was injected *(ip)* with SM daily (50 mg/kg body weight) and also was treated orally with water soluble KBrO$_3$ (100 mg/kg body weight) daily for 14 days.

**Collection Of Samples**

After 14 days of treatment, each mouse of each group was anesthetized by diethyl ether and sacrificed. Animals were dissected; ascitic fluid was collected from EAC-bearing mice groups and then EAC cells pellet was used in histopathological and ultrastructural studies. Livers and kidneys were removed and washed with 0.9% NaCl then undergo fixation process in 10% formalin for histopathological and immunohistochemical investigations.

**Cell Viability And Counting Of Eac Cells**

After the dissection of mice, the peritoneal ascitic fluid was obtained from the cavity of each mouse using a needle and the volume of fluid was measured in a measuring tube. The number of cells present in the ascitic fluid was counted using a haemocytometer to calculate the percent in the inhibition of tumor growth by comparing the number of cells in the ascitic fluid of treated EAC-groups mice in comparison with non-treated EAC- group of mice. In addition, tumor cell growth in the positive control group was taken as 100 percent cell growth. Viability test was performed by staining the ascitic fluid using trypan blue dye (0.4% in normal saline). Cells were counted where the cells that cannot take up the dye were viable, with an intact membrane, and those which can take the stain were considered not viable, with a damaged membrane [14].

**Histopathological Investigations**

The collected samples were fixed in 10% formalin for 24 h, and then dehydrated, cleared, and impeded in paraffin wax. Obtained tissue blocks were sectioned by microtome at a 5 $\mu$m thickness, and stained with haematoxylin and eosin (H&E). Finally, sections were examined for investigating histopathological changes. Tissues of the EAC groups were studied in comparison with the normal control and the other treated groups for finding and assess the histopathological changes [15].

**Immunohistochemical Investigations**
For immunohistochemical investigations, immediately after dissection, small pieces of tissues were fixed in 10% neutral formalin for 24 hours. Sections of 5 µm thickness were prepared by using rotary microtome for immunohistochemical staining of alpha-smooth muscle actin (αSMA), transforming growth factor β1 (TGF-β1) and cathepsin D (Cath-D) using suitable antibodies [16, 17].

Ultrastructural Studies

TEM was used for the investigation of samples. EAC cell samples (pellets) were treated with a mixture of glutaraldehyde and paraformaldehyde prepared in 0.1 M cacodylate buffer. Subsequently, samples were fixed in a buffer of pH 7.2 made of 0.1 M cacodylate including 1% osmium tetroxide, 0.8% potassium ferricyanide and 2 mM calcium chloride. Then, samples were dehydrated and established in resin. Solidification was accomplished for three consecutive days at 60°C. Resin molds were then sectioned and gathered on nickel grids, stained with 5% lead citrate and uranyl acetate and imaged with a TEM [13].

Statistical analysis

Values of the obtained results were expressed as means ± standard error (SE) for each animal group. Differences between groups were statistically analyzed using the Statistical Package for Social Science (SPSS), version 22 software. The significant differences were performed by One-way analysis of variance (ANOVA) test followed by Tukey’s post hoc test for comparison of means with control. Data were statistically significant when the P values ≤ 0.05.

Results

Effect of SM, KBrO₃ and a combination of them on body weight, tumor volume and count of EAC Cells

Body weight, tumor volume and count of EAC cells in mice (non EAC-bearing and EAC-bearing mice) treated with SM (50 mg/kg), KBrO₃ (100 mg/kg) and a combination of them (50 mg/kg and 100 mg/kg) are shown in Table 2. SM, KBrO₃ and a combination of them did not show any significant effect on the body weight of non EAC-bearing mice. In contrast, SM, KBrO₃ and a combination of them significantly affected the body weight of EAC-bearing mice when compared with EAC-treated group (positive control group). Treatment with SM, kBrO₃ and a combination of them in EAC-bearing mice were found to significantly reduce the tumor volume by 46.5%, 15% and 75.6% as well as the EAC cell count by 61.5%, 32% and 85%, respectively when compared with EAC-treated group (positive control group).

Histological Examination Of Liver

Control liver sections of mice stained with H&E were normal as showing in Fig. (2). Also, liver sections of mice groups treated with DMSO, SM, KBrO₃ and a combination of SM and KBrO₃ revealed the same appearance of the control group as showing in Figs. (3–6), while liver sections of EAC-bearing mice
groups either untreated or treated showed different abnormalities (Figs. 7–11). The abnormalities were remarkably severe in the untreated groups of EAC-bearing mice compared to the treated groups of EAC-bearing mice. These abnormalities are summarized as massive infiltration of hepatic capsule with EAC cells, neoplastic cells surrounded by necrotic area of hepatocytes and perivascular and portal aggregation of EAC cells admixed with lymphocytes (Figs. 7–8). In EAC-bearing mice group that was treated with SM showed decreased capsular infiltration with EAC cells as well as small clumps of EAC cells perivascular and in portal areas with decreased hepatic damage (necrosis, congestion and hemorrhage) compared to EAC-bearing mice (Fig. 9). Treatment with KBrO$_3$ resulted in less congestion of blood vessels as well as decreased capsular infiltration with EAC cells and small clumps of EAC cells perivascular and in portal areas with decreased hepatic damage (necrosis, congestion and hemorrhage) compared to EAC-bearing mice (Fig. 10). EAC-bearing mice group that was treated with a combination of SM and KBrO$_3$ revealed a decrease in capsular infiltration with EAC cells and small clumps of EAC cells perivascular and in portal areas without hepatic damage (necrosis, congestion and hemorrhage) compared to EAC-bearing mice (Fig. 11).

**Histological Examination Of Kidney**

Control kidney sections of mice stained with H&E were normal as showing in Fig. (12). Moreover, kidney sections of mice groups treated with DMSO, SM, KBrO$_3$ and a combination of SM and KBrO$_3$ revealed similar appearance to the control group as showing in Figs. (13–16), while kidney sections of EAC-bearing mice groups either untreated or treated showed different abnormalities (Figs. 17–21). The abnormalities were severe in the untreated groups of EAC-bearing mice compared to the treated groups. These abnormalities appeared as massive infiltration of renal capsule with EAC cells, tubular hydropic degeneration, necrosis, interstitial aggregation of EAC cells, congestion of blood vessels and perivascular infiltration of EAC cells (Figs. 17–18). The abnormalities of kidney sections in EAC-bearing mice group that was treated with SM revealed decreased capsular infiltration with EAC cells compared to EAC-bearing mice and perivascular aggregation of EAC cells admixed with lymphocytes (Fig. 19). EAC-bearing mice group treated with KBrO$_3$ showed decreased capsular infiltration with EAC cells compared to EAC-bearing mice and perivascular aggregation of EAC cells admixed with lymphocytes (Fig. 20). Treatment with a combination of SM and KBrO$_3$ proved a decrease in capsular infiltration with EAC cells compared to EAC-bearing mice and perivascular aggregation of EAC cells admixed with lymphocytes (Fig. 21).

**Histological Examination Of Ascitic Fluid**

Smears of ascitic fluid stained with H&E in EAC-bearing mice group and in EAC-bearing mice groups that were treated with SM, KBrO$_3$ and a combination of SM and KBrO$_3$ appeared as showing in Figs. (22–25). the ascitic fluid smear from EAC-bearing mice group showed intact live tumor cells with mitotic figures (Fig. 22). The ascitic fluid smear from EAC-group treated with SM showed mild degeneration of the cancer cells (Fig. 23), while those treated with KBrO$_3$ showed moderate degeneration of cancer cells and
mitotic figures (Fig. 24). The ascitic fluid smear from EAC-group treated with a combination of SM and KBrO$_3$ showed marked degeneration and necrosis of cancer cells with complete loss of cellular details “ghosts” (Fig. 25).

**Electron Microscopic Studies**

All results of TEM of EAC cells are displayed in Figs. (26–29). EAC cells of EAC-bearing mice group showed rounded shape large nucleus with defined nucleolus, normal mitochondria and normal microvilli like processes over the cell surface (Fig. 26). While that EAC cells of EAC-bearing mice groups that were treated with SM, KBrO$_3$ and a combination of SM and KBrO$_3$ showed abnormalities as showing in (Figs. 27–29). EAC cells of the EAC-group that was treated with SM showed nucleus abnormality with condensation of chromatin, moderate cytoplasmic vacuoles, marked shrinkage of mitochondria and marked loss of microvilli (Fig. 27). EAC cells of the EAC-group that was treated with KBrO$_3$ showed distortion in the nuclear membrane and necrotic chromatin of the nucleus, pronounced cytoplasmic vacuoles and marked loss of microvilli (Fig. 28). EAC cells of the EAC-group that was treated with a combination of SM and KBrO$_3$ showed major loss of cellular framework of cytoplasm, extensive cytoplasmic vacuolation, severe fragmented nuclei, condensation of chromatin material around the nuclear membrane, and completely loss of mitochondria and microvilli (Fig. 29).

**Liver αSMA**

αSMA immunopositive expression was revealed among all groups, whether in the control, untreated EAC-bearing mice or treated EAC-bearing mice with SM, KBrO$_3$ and a combination of SM and KBrO$_3$ (Figs. 30–34). The control group of mice showed very mild positive brown expression on vascular wall (Fig. 30). Meanwhile the EAC-group of mice showed increased positive brown expression on vascular walls (Fig. 31). EAC-group treated with SM showed slightly decreased positive brown expression on vascular walls (Fig. 32) while that treated with KBrO$_3$ showed moderately decreased positive brown expression on vascular walls (Fig. 33). In EAC-group of mice treated with a combination of SM and KBrO$_3$ a marked decrease in positive brown expression on vascular walls was pronounced (Fig. 34).

**Kidney αSMA**

αSMA immunopositive expression was proved among all groups, whether in the control, untreated EAC-bearing mice or treated EAC-bearing mice with SM, KBrO$_3$ and a combination of SM and KBrO$_3$ (Figs. 35–39). Control group of mice showed very mild positive brown expression in interstitial tissue (Fig. 35). While that of EAC-group showed increased positive brown expression in interstitial tissue (Fig. 36). EAC-group of mice treated with SM showed slightly decreased positive brown expression in interstitial tissue (Fig. 37). EAC-group of mice treated with KBrO$_3$ showed moderately decreased positive brown expression in interstitial tissue (Fig. 38). EAC-group of mice treated with a combination of SM and KBrO$_3$ showed markedly decreased positive brown expression in interstitial tissue (Fig. 39).
Liver TGF-β1

TGF-β1 immunopositive expression was shown among all groups, whether in the control, untreated EAC-bearing mice or treated EAC-bearing mice with SM, KBrO₃ and a combination of SM and KBrO₃ (Figs. 40–44). Control group of mice showed mild to weak positive expression (+1) of TGF-β1 protein in most hepatocytes cytoplasm with diffuse pale brown color, while a blue negative color appeared in the vesiculated nuclei and lymphocytes infiltrating between hepatocytes and in the sinusoid, yellow color of RBCs was also seen (Fig/40). EAC-group of mice showed intense positive expression (+4) of TGF-β1 protein in most hepatocytes’ cytoplasm and cell membrane with diffuse brown color, mostly in proliferating hepatocytes and bile duct epithelial cells at the portal tract. A blue negative color appeared in the nuclei and lymphocytes infiltrating at portal tract (Fig. 41). EAC-group of mice treated with SM showed moderate positive expression (+2) of TGF-β1 protein in most hepatocytes’ cytoplasm and cell membrane with diffuse pale brown color. A blue negative color appeared in the nuclei and lymphocytes infiltrating at central vein and yellow RBCs in the sinusoid was seen (Fig. 42). EAC-group of mice treated with KBrO₃ showed moderate to strong positive expression (+2) of TGF-β1 protein in most hepatocytes’ cytoplasm and cell membrane with diffuse pale brown color. A blue negative color appeared in the nuclei and lymphocytes infiltrating at portal tract and bile duct epithelial cells and yellow color of RBCs was seen (Fig. 43). EAC group of mice treated with a combination of SM and KBrO₃ showed moderate positive expression (+2) of TGF-β1 protein in most hepatocytes’ cytoplasm and cell membrane with diffuse brown color, mostly in hyperchromatic hepatocytes and a foci area of proliferating hepatocytes. A blue negative color appeared in the nuclei and lymphocytes cells (Fig. 44).

Kidney TGF-β1

TGF-β1 immunopositive expression was noticed among all groups, whether in the control, untreated EAC-bearing mice or treated EAC-bearing mice with SM, KBrO₃ and a combination of both (Figs. 45–49). Control group of mice showed moderate positive expression (+2) of TGF-β1 protein in most renal tubules with diffuse brown color in the cytoplasm of epithelial tubular cells and Bowman's capsule cells of glomeruli. Negative blue color of the nuclei of tubules cells and mesangial cells nuclei and yellow color of the RBCs was seen (Fig. 45). EAC group of mice showed intense positive expression (+4) of TGF-β1 protein in the dilated renal tubules. Other tubules have moderate (+2) diffused brown color in the cytoplasm and cell membrane of most epithelial tubular cells and Bowman's capsule cells of glomeruli. Negative blue color appeared in the nuclei of tubules and mesangial cells (Fig. 46). EAC-group of mice treated with SM showed strong positive expression (+3) of TGF-β1 protein in the marked dilated renal tubules (nephrosclerosis). Other tubules have moderate (+2) diffuse brown color in the cytoplasm and cell membrane of most epithelial tubular cells and Bowman's capsule cells of glomeruli. Negative blue color appeared in the nuclei of tubules and mesangial cells (Fig. 47). EAC-group of mice treated with KBrO₃ showed intense positive expression (+4) of TGF-β1 protein in most renal tubules with diffuse brown color in the cytoplasm and cell membrane of the most epithelial tubular cells and Bowman's capsule cells of glomeruli. Negative blue color appeared in the nuclei of tubular cells and mesangial cells.
EAC-group of mice treated with a combination of SM and KBrO$_3$ showed intense positive expression (+ 3 or + 4) of TGF-β1 protein in most renal tubules with brown color in the cytoplasm and cell membrane of the epithelial tubular cells and Bowman's cells of glomeruli. Negative blue color appeared in the nuclei of tubules and mesangial cells (Fig. 49).

**Liver Cath-D**

Cath-D immunopositive expression was evident among all groups, whether in the control, untreated EAC-bearing mice or treated EAC-bearing mice with SM, KBrO$_3$ and a combination of SM and KBrO$_3$ (Figs. 50–54). Control group of mice showed moderate positive expression (+ 2) of Cath-D protein in some hepatocytes cytoplasm with small brown granules at foci area at the central vein and infiltrating lymphocytes. A blue negative color appeared in the liver nuclei and lymphocytes (Fig. 50). EAC-group of mice showed intense positive expression (+ 4) of Cath-D protein in most proliferating hepatocytes cytoplasm with diffuse brown color at foci area of the portal tract surrounded by necrotic hepatocytes, also a blue negative color appeared in the liver nuclei (Fig. 51). EAC-group of mice treated with SM showed strong positive expression (+ 3) of Cath-D protein in most hepatocytes cytoplasm with small brown granules surrounding the nuclei of vacuolated hepatocytes at the central vein area. In addition, a blue negative color appeared in liver nuclei (Fig. 52). EAC-group of mice treated with KBrO$_3$ showed intense positive expression (+ 4) of Cath-D protein in most hepatocytes cytoplasm with aggregated brown granules at foci area at the central vein, and moderate expression in the vacuolated hepatocytes cytoplasm surrounding the nuclei. A blue negative color appeared in liver nuclei and lymphocytes (Fig. 53). EAC group of mice treated with a combination of SM and KBrO$_3$ showed strong positive expression (+ 3) of Cath-D protein in most hepatocytes cytoplasm with aggregated brown granules at the central vein while a blue negative color appeared in liver nuclei and lymphocytes (Fig. 54).

**Kidney Cath-D**

Cath-D immunopositive expression was noticed among all groups, whether in the control, untreated EAC-bearing mice or treated EAC-bearing mice with SM, KBrO$_3$ and a combination of both (Figs. 55–59). Control group of mice showed moderate positive expression (+ 2) of Cath-D protein in the cytoplasm of most renal tubular epithelial cells and most mesangial cells of glomeruli. Negative blue color of tubular epithelial cell's nuclei was seen (Fig. 55). EAC-group of mice showed intense positive expression (+ 4) of Cath-D protein in the cytoplasm of renal tubular epithelial cells distributed with diffuse brown color and differentiated through the both renal tubules and most mesangial cells of glomeruli. Negative blue color of the nuclei of the tubular epithelial cells was seen (Fig. 56). EAC-group of mice treated with SM showed strong positive expression (+ 3) of Cath-D protein in the cytoplasm of renal tubular epithelial cells distributed with diffuse brown color and intense immunostaining in destructive renal tubules. The negative blue color of the nuclei of the tubular epithelial cells was seen (Fig. 57). EAC-group of mice treated with KBrO$_3$ showed intense positive expression (+ 4) of Cath-D protein in the cytoplasm of some renal tubular epithelial cells distributed with diffuse brown color and moderate in most mesangial cells of glomeruli. The nuclei have negative blue color (Fig. 58). EAC-group of mice treated with a combination of
SM and KBrO\textsubscript{3} showed strong positive expression (+ 3) of Cath-D protein in the cytoplasm of few renal tubular epithelial cells distributed with diffuse brown color and in most mesangial cells of glomeruli. The nuclei were negative with blue color (Fig. 59).

**Discussion**

Cancer is a complicated disease where cell growth is being abnormal, aggressive, invasive, and may metastasizes many times leading finally to death [18]. EAC is one of the experimental breast tumors derived from spontaneous mouse adenocarcinoma. Similar to other tumors developing in body cavities, EAC cells fill the peritoneal cavity by rapid division of cells, accumulation of a fluid named ascitic fluid and usually animal dies 17–18 days following EAC transplantation [19]. EAC likens human tumors in their sensitivity to chemotherapy [20]. Free radicals can cause damage to various important biomolecules, including lipids, proteins, and nucleic acids, leading to oxidative stress and damaging different human tissues. Elevated ROS levels, accompanied with down-regulation of cellular antioxidant enzyme systems, result in malignant transformation via different molecular targets [21&22]. There are many oxidizing substances that lead to the formation of free radicals such as KBrO\textsubscript{3}. KBrO\textsubscript{3} is one of the materials that cause the formation of ROS. As a result of KBrO\textsubscript{3} biotransformation, free radicals generation can cause oxidative damage to cellular macromolecules, leading to cancer [23]. Efforts are still being in growth to search for an impressive anticancer therapy, from natural sources, that would lessen or even impede the cancer progress such as SM [24]. SM has been extensively studied, both in vivo and in vitro, for its cancer chemopreventive potential against various cancers [25].

The present study was performed to evaluate the potential use of a non-toxic and stable SM as a promising anticancer agent. Furthermore, the study also aimed at providing the first assessment of KBrO\textsubscript{3} as an agent against EAC-bearing mice as tumor models. It has been shown [26] that this investigated tumor model is perfect for such in vivo or in vitro studies where the investigated SM compound, when injected intraperitoneally (ip), would be in direct contact with target tumor cells in peritoneal cavity where tumor is growing, and would also show immediate effect that allows perfect evaluation of tumor inhibitory effect. Previous studies on this experimental model [27&28] showed that the antitumor effect was mainly assessed by monitoring and measuring changes in ascetis tumor volume expressed as milliliter as well as total number of its viable tumor cell count. This was found to be in agreement with our present results either in the treatment of EAC-bearing mice with SM, KBrO\textsubscript{3} or a combination of them. Herein, SM had an antitumor effect against EAC tumors cells due to its antioxidant activity, while KBrO\textsubscript{3} had a possible antitumor effect against EAC tumors cells due to its toxicity. The SM's anticancer activity was previously reported both in vitro and in vivo [29], and SM was also used clinically against certain types of cancers [30]. The present results (Figs. 1A, 1B & 1C) showed that SM at a dose of 50 mg/kg has an effective antitumor effect. It exhibited a destruction and disappearance of tumor cells, thus represents a potentially high tumor growth inhibitory effect and anticancer activity when compared with that obtained from treatments with KBrO\textsubscript{3} at dose of 100 mg/kg. Meanwhile a combination of SM and KBrO\textsubscript{3} represents a highest tumor growth inhibitory effect when compared with that obtained from treatments
with SM or KBrO₃ at the examined doses. Therefore, this combination is of superior activity over SM as an antitumor agent upon investigation under the tested experimental conditions.

The histopathological examination in the liver of untreated EAC-bearing mice group in the present study showed that, EAC induced liver injury and many histological changes including massive infiltration of hepatic capsule with EAC cells, presence of clumps of EAC cells perivascular and in portal areas admixed with lymphocytes, necrosis of hepatic tissue and congestion of blood vessels. The present findings are in accordance to that of previous studies [31&32], which described EAC induced liver injury as remarkable degenerative changes characterized by marked diffuse necrosis of hepatic tissue, marked inflammatory cells, congested blood sinusoids. Infiltration of EAC cells was attributed to cancer cells proliferation and transport to the internal organs [33], whereas the aggregations of inflammatory cells may happen because of degeneration of mitochondria or disorganization of the cytoplasm [34].

Herein, the histopathological examination in the liver of SM-treated and KBrO₃-treated EAC-bearing mice groups demonstrated decreased capsular infiltration with EAC cells, small clumps of EAC cells perivascular and in portal areas with decreased hepatic damage (necrosis, congestion and hemorrhage). SM can improve liver structure in EAC-bearing mice because it can efficiently reduce intracellular ROS levels of hepatocytes, thus preventing oxidative stress-induced hepatic cellular damage [35]. In the EAC-bearing mice group treated with a combination of SM and KBrO₃, liver examination revealed similar appearance as those in groups treated with SM or KBrO₃ but with no pronounced hepatic damages (necrosis, congestion and hemorrhage).

The histopathological examination in the kidney of untreated EAC-bearing mice group revealed that, EAC induced kidney impairment and histological changes due to massive infiltration of renal capsule with EAC cells, tubular hydropic degeneration, necrosis and congestion of blood vessels. These findings agree with previous studies [33, 36&37], which described EAC induced cellular infiltration, necrosis and degeneration in the glomeruli and renal tubules cells.

The histopathological examination in the kidney of EAC-bearing mice groups treated with SM, KBrO₃ and a combination of them in the present study showed decreased capsular infiltration with EAC cells and perivascular aggregation of EAC cells admixed with lymphocytes. SM improved kidney structure in EAC-bearing mice because it has shown anticancer activities against renal cell carcinoma [38&39]. So that, it can be demonstrated that, treatment with SM, KBrO₃ and a combination of them resulted in improvement in the histological changes induced by EAC cells in liver and kidney of EAC-bearing mice.

The cytological examinations of EAC cells in untreated EAC-bearing mice group in the present study demonstrated intact live tumor cells with mitotic figures. This result agrees with a previous study [40], which described the ascites fluids cells in EAC group as elevation in the volume of the ascites fluid and increasing in the number of mitotic cells and tumor cells with many of pleomorphism. On the other hand, the cytological examinations of EAC cells in EAC-bearing mice groups treated with SM, KBrO₃ or a combination of them showed degeneration in the cancer cells, in EAC-bearing mice group treated with a
combination of SM and KBrO$_3$ marked degeneration in cancer cells with necrosis and complete loss of cellular details were revealed. SM has a negative effect on EAC cells in this study and this agree with the previous study [13], which described EAC cells in SM-treated group (50 mg/kg) of EAC-bearing mice with obvious destruction.

Ultrastructurally, EAC cells in the untreated EAC-bearing mice group showed rounded shape with large nucleus and defined nucleolus, normal mitochondria and normal microvilli like processes over the cell surface. These findings are compatible to that of a previous study [13], which described EAC cells with prominent nuclei with obvious nucleoli. In addition, there were small mitochondria and the cell surface showed numerous thread-like projections over it. On the other hand, EAC cells in EAC-bearing mice groups treated with SM, KBrO$_3$ or a combination of them in the present study showed abnormalities as indicated by nucleus abnormality with condensation of chromatin, appearance of moderate cytoplasmic vacuoles, marked shrinkage of mitochondria, and loss of microvilli, completely loss of mitochondria and microvilli in EAC-bearing mice treated with a combination of SM and KBrO$_3$ were noticed. These results are parallel to that of a previous study [13], which described EAC cells in mice treated with SM with cytoplasmic and nuclear degeneration characterized by the segregation of condensed chromatin and remarkable presence of vacuoles and condensed mitochondria.

The expression of TGF-β1 was noticed among all groups, whether in control group of mice, untreated EAC-bearing mice or treated EAC-bearing mice with SM, KBrO$_3$ and a combination of them. Control group of mice showed mild to weak positive expression of TGF-β1 protein in most hepatocytes’ cytoplasm with diffuse pale brown color, while that in EAC-group demonstrated intense positive expression in most hepatocytes’ cytoplasm. EAC groups of mice treated with SM showed moderate positive expression of TGF-β1 protein in most hepatocytes’ cytoplasm. EAC-group of mice treated with KBrO$_3$ showed moderate to strong positive expression of TGF-β1 protein in most hepatocytes’ cytoplasm while those treated with a combination of SM and KBrO$_3$ showed moderate positive expression of TGF-β1 protein in most hepatocytes’ cytoplasm. In kidney TGF-β1 immunopositive expression was among all groups whether in control, untreated EAC-bearing mice group or in treated EAC-bearing mice with SM, KBrO$_3$ and a combination of them. In control group of mice, moderate positive expression of TGF-β1 protein was revealed in most epithelial tubular cells cytoplasm, while that of EAC-group of mice showed intense positive expression of TGF-β1 protein represented as a diffuse brown color in the cytoplasm of most epithelial tubular cells and Bowman's capsule cells of the glomeruli. EAC-groups of mice treated with SM, KBrO$_3$ and a combination of them showed intense positive expression of TGF-β1 protein in most renal tubules that appeared as brown color in the cytoplasm and cell membrane of most epithelial tubular cells. TGF-β1 is a cytokine involved in the regulation of cell proliferation, differentiation and survival or apoptosis of numerous cells and may add to tumor pathogenesis by backing tumor growth as well as influencing local microenvironment, causing immunosuppression, induction of angiogenesis, and modification of extracellular matrix [41]. Reduction of TGF-β1 activity and production may be a promising target of therapeutic strategies to control tumor growth because obstructing TGF-β1 activity inhibits tumor viability, migration and metastasis in mammary cancer, melanoma and prostate cancer model [42].
The immunohistochemistry detection of TGF-β1 protein in the mice kidney tissue showed a diffuse brown color of the (TGF-β1) protein immunoeexpression in the cytoplasm and cell membrane [17]. A comparable results were obtained in the present study.

In the herein study, TGF-β1 protein elevated significantly in EAC group in comparison to control group and this agrees with a previous study [43], which reported that, there is a positive correlation between an elevated plasma TGF-β1 level and malignant progression in colorectal, prostate, bladder and liver cancers. The present results revealed that TGF-β1 protein level in treated EAC-bearing groups of mice was decreased in comparison to EAC group.

In liver, Cath-D immunopositive expression was remarked among all tested groups, whether in the control, untreated EAC-bearing or in treated EAC-bearing mice groups with SM, KBrO₃ and a combination of them. Control group of mice showed moderate positive expression of Cath-D protein in some hepatocytes cytoplasm, while EAC group of mice showed intense reaction of Cath-D protein in most proliferating hepatocytes cytoplasm. EAC-group of mice treated with SM showed strong positive expression while EAC-group of mice treated with KBrO₃ showed intense positive expression of Cath-D protein in most hepatocytes cytoplasm. EAC-group of mice treated with a combination of SM and KBrO₃ showed strong positive expression of Cath-D protein in most hepatocytes cytoplasm.

In kidney, Cath-D immunopositive expression was realized among all examined groups; control, untreated EAC-bearing mice, or treated EAC-bearing groups with SM, KBrO₃ and a combination of SM and KBrO₃. Control group of mice showed moderate positive expression of Cath-D protein in the cytoplasm of most renal tubular epithelial cells and most mesangial cells of glomeruli, while in EAC-group of mice there were intense positive expression of Cath-D protein in the cytoplasm of renal tubular epithelial cells. EAC-group of mice treated with SM showed strong positive expression of Cath-D in the cytoplasm of renal tubular epithelial cells. EAC-group of mice treated with KBrO₃ showed intense positive expression of Cath-D protein in the cytoplasm of renal tubular epithelial cells. EAC-group of mice treated with a combination of SM and KBrO₃ demonstrated strong positive expression of Cath-D protein in the cytoplasm of renal tubular epithelial cells and most mesangial cells of glomeruli. The enzyme Cath-D in various rat tissues and blood cells was determined by immunohistochemistry. The enzyme behaves as a soluble cytosolic enzyme localized in tissue used the specific discriminative antibodies. The immunohistochemical localization of Cath-D was clearly different in the stomach, large intestine, kidney and urinary bladder. Increased expression of Cath-D in benign tumor tissue samples might indicate a role for the development and progression of renal cell cancer [44].

Herein, a relation between cancer and releasing of Cath-D was revealed as Cath-D immunoexpression was intense in EAC group, while treated groups of EAC-bearing mice showed less expression. This agrees with a previous studies [45] that reported that many breast and colorectal cancer cells secrete increased amounts of pro-Cath-D into the extracellular space to contribute to the invasive and metastatic potential of cancer cells.
Bile duct proliferation was detected in the liver of mice treated with EAC. This is in agreement with a previous study [46] that observed bile duct proliferation in rats treated with Nolvadex. The proliferating ductules in liver may originate from actually activated hepatic oval cells during hepatocyte regeneration [47]. The endogenous stem cells, located in the junction between hepatocytes and the terminal bile ductules, may also play an important role in bile duct proliferation [48]. Proliferation of bile duct is a hepatic cellular reaction observed in most forms of human liver disease and in a variety of experimental conditions associated with liver injury [49].

In the present study, the immunohistochemical observations showed strong expression of αSMA in the hepatic and renal tissues after EAC development. In liver tissues, many studies reported that there was a significant correlation between αSMA expressed by activated hepatic stellate cells (HSCs) and the degree of fibrosis [50]. The development of hepatic fibrosis is related to increase in the expression of αSMA. In the present study, Lipid peroxidation (increased MDA) caused by EAC may activate tumor necrosis factor TNF and consensus interferon, which directly stimulate the production of matrix by activated HSCs [51]. The activation of HSCs, involve increase in the cellular proliferation, synthesis of extracellular matrix proteins and activate the expression of αSMA [52]. αSMA expression was elevated in EAC group and this is parallel to [53], who described fibrotic changes occurred in tumor bearing mice hearts and attributed that the generation of myofibroblasts as indicated by the expression of myofibroblast markers such as α-SMA, which was significantly upregulated in tumor bearing mice hearts.

After EAC development, an increase in the expression of αSMA was detected in the damaged renal tissues. This increase may be due to the renal tissue damage that caused by the elevated creatinine and urea after EAC growth. The increase of αSMA was also observed by many authors. During renal fibrosis process, the renal interstitial fibroblasts are activated and display proliferative properties, which express αSMA in the renal tissues [54]. It was reported that the higher immunexpression of αSMA was related to renal function assessed by creatinine level at biopsy moment. The authors suggested that expression of αSMA proved to be a good marker in decreasing the early stages of kidney dysfunction [55].

**Conclusion**

This study aimed to evaluate the potential use of a safe and stable SM as antitumor agent as well as, for the first time, the use of KBrO₃ and the combination of SM and KBrO₃ as agents against EAC-bearing mice as tumor models. SM, KBrO₃ and a combination of them were found to destroy tumors and improve the histological changes induced by EAC cells in liver and kidney in EAC-bearing mice, a combination of them was found to destroy tumors completely by significantly decreasing tumor volume and EAC cell count as well as significantly improve the histological alterations by decreasing capsular infiltration with EAC cells and elimination the damages as congestion. Electron microscopic and histological investigations of EAC cells demonstrated the superiority of a combination of SM and KBrO₃ treatment over SM or KBrO₃ treatment in causing necrosis of the cancer cells with complete loss of cellular details, completely loss of mitochondria and microvilli, severe fragmentation of nuclei and extensive cytoplasmic vacuolation. According to immunohistochemical investigations, it was noticed that TGF-β1 and Cath-D
protein levels were decreased in treated EAC-bearing groups of mice in comparison to EAC group. This study reveal that SM can be used to kill cancer cells due to its antioxidant and anti-inflammatory properties, KBrO\(_3\) can be used to kill cancer cells due to its toxicity on EAC cells and a combination of SM and KBrO\(_3\) can kill cancer cells due the antioxidant property of SM and toxicity of KBrO\(_3\) so they have a synergistic effect. The present study revealed that a combination of SM and KBrO\(_3\) is of superior activity over SM or KBrO\(_3\) as an anticancer agent upon investigation under this experimental condition.

**Declarations**

**Author contributions**

Mona A. Elhadad performed the research., Magdy E. Mahfouz¹, Nora F. Ghanem¹ & Eman A. Moussa¹ analyzed the data. Elhadad wrote the manuscript and All authors wrote and edited the manuscript, and provided funding. All authors accepted the final version of the manuscript.

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**Availability of data and materials**

The data used to support the findings of this study are included within the article.

**Ethics approval and consent to participate**

This study was approved by the Ethical Committee of Kafrelsheikh University (Egypt).

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**Authors details**

**Affiliation of the all authors:** Faculty of Science, Zoology Department, Kafrelsheikh University, Egypt

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Tables

Table 1 and 2 are available in the Supplementary Files section.

Figures
Figure 1

(A): Body weight in non-bearing EAC mice treated with DMSO, SM (50 mg/kg), KBrO₃ (100 mg/kg) and a combination of SM and KBrO₃ (50 mg/kg & 100 mg/kg) and body weight in EAC-bearing mice treated with SM (50 mg/kg), KBrO₃ (100 mg/kg) and a combination of SM and KBrO₃ (50 mg/kg & 100 mg/kg) compared with normal mice negative control and the EAC-bearing mice positive control group (mean ± standard error). (B): Tumor volume in EAC-bearing mice treated with SM (50 mg/kg), KBrO₃ (100 mg/kg) and a combination of SM and KBrO₃ (50 mg/kg & 100 mg/kg) compared with the EAC-bearing mice positive control group (mean ± standard error). (C): EAC cell count in EAC-bearing mice treated with SM (50 mg/kg), KBrO₃ (100 mg/kg) and a combination of SM and KBrO₃ (50 mg/kg & 100 mg/kg) compared with the EAC-bearing mice positive control group (mean ± standard error).
**Figure 2**

**Fig 2:** H&E stained liver section of the control group of albino mice showing normal hepatocytes (H) with nuclei, central vein (CV), sinusoids (S) and Kupffer cells (K), X:400 bar 50.

**Fig 3:** H&E stained liver section of a group of mice treated with DMSO showing no structural alterations, high magnification X:400 bar 50.

**Fig 4:** H&E stained liver section of a group of mice treated with SM showing no structural alterations, X:400 bar 50.

**Fig 5:** H&E stained liver section of a group of mice treated with KBrO$_3$ showing no structural alterations, X:400 bar 50.

**Fig 6:** H&E stained liver section of a group of mice treated with a combination of SM and KBrO$_3$ showing no structural alterations, X:400 bar 50.
**Fig 7:** H&E stained liver section of a group of mice treated with EAC showing neoplastic cells (thick black arrows) surrounded by necrotic area of hepatocytes (thin black arrows), X:400 bar 50.

**Fig 8:** H&E stained liver section of a group of mice treated with EAC revealing perivascular and portal aggregation of EAC cells admixed with lymphocytes (double yellow arrows), X:400 bar 50.

**Fig 9:** H&E stained liver section from EAC-group treated with SM showing small clumps of EAC cells perivascular and in portal areas (single yellow arrows) with decreased hepatic damage (necrosis, congestion and hemorrhage), X:400 bar 50.

**Fig 10:** H&E stained liver section from EAC-group treated with KBrO$_3$ showing congested blood vessels (red arrows) and small clumps of EAC cells perivascular and in portal areas (single yellow arrows) with decreased hepatic damage (necrosis, congestion and hemorrhage), X:400 bar 50.

**Fig 11:** H&E stained liver section from EAC-group treated with a combination of SM and KBrO$_3$ showing small clumps of EAC cells perivascular and in portal areas (single yellow arrows) without hepatic damage (necrosis, congestion and hemorrhage), X:400 bar 50.
**Figure 3**

**Fig 12:** H&E stained kidney section of a control group of albino mice showing normal structure of the cortex region, normal glomeruli (G) with normal Bowman's space (Bs) and normal renal tubules (R), X:400 bar 50.

**Fig 13:** H&E stained kidney section of a group of mice treated with DMSO showing no structural alterations, X:400 bar 50.

**Fig 14:** H&E stained kidney section of a group of mice treated with SM showing no structural alterations, X:400 bar 50.

**Fig 15:** H&E stained kidney section of a group of mice treated with KBrO₃ showing no structural alterations, X:400 bar 50.
Fig 16: H&E stained kidney section of a group of mice treated with a combination of SM and KBrO$_3$ showing no structural alterations, X:400 bar 50.

Fig 17: H&E stained kidney section of a group of mice treated with EAC revealing tubular hydropic degeneration (white arrows), necrosis (thin black arrows) and interstitial aggregation of EAC cells (single yellow arrow), X:400 bar 50.

Fig 18: H&E stained kidney section of a group of mice treated with EAC revealing congested blood vessels (red arrows) and perivascular infiltration of EAC cells (double yellow arrows), X:400 bar 50.

Fig 19: H&E stained kidney section from EAC-group treated with SM showing perivascular aggregation of EAC cells admixed with lymphocytes (double yellow arrows), X:400 bar 50.

Fig 20: H&E stained kidney section from EAC-group treated with KBrO$_3$ showing perivascular aggregation of EAC cells admixed with lymphocytes (double yellow arrows), X:400 bar 50.

Fig 21: H&E stained kidney section from EAC-group treated with a combination of SM and KBrO$_3$ revealing perivascular aggregation of EAC cells admixed with lymphocytes (double yellow arrows), X:400 bar 50.
Figure 4

**Fig 22:** H&E stained ascitic fluid smear in the EAC treated group showing intact live tumor cells with mitotic figures (yellow arrows), X:1000 bar 20.

**Fig 23:** H&E stained ascitic fluid smear from EAC- group treated with SM showing mild degeneration of the cancer cells (black arrows), X:1000 bar 20.

**Fig 24:** H&E stained ascitic fluid smear from EAC- group treated with KBrO$_3$ showing moderate degeneration of the cancer cells (black arrows), the smear also showing mitotic figures (yellow arrows), X:1000 bar 20.

**Fig 25:** H&E stained ascitic fluid smear from EAC- group treated with a combination of SM and KBrO$_3$ showing marked degeneration of the cancer cells (black arrows), the smear also showing necrosis of the cancer cells with complete loss of cellular details “ghosts” (blue arrows), X:1000 bar 20.
**Fig 26:** Electron micrograph of Ehrlich ascites carcinoma cell (EAC) ultrastructural features showing it with rounded shape large nucleus (N) with defined nucleolus (NO), normal mitochondria (black asterisk) and normal microvilli (black arrow) like processes over the cell surface (scale bar 5μm).

**Fig 27:** Electron micrograph of SM treated Ehrlich ascites carcinoma cell (EAC) ultrastructural features showing appearance of nucleus abnormality with condensation of chromatin (yellow arrow), appearance of moderate cytoplasmic vacuoles (red arrow), marked shrinkage of mitochondria (black asterisk) and marked loss of microvilli (black arrow). (Scale bar 5μm).

**Fig 28:** Electron micrograph of KBrO$_3$ treated Ehrlich ascites carcinoma cell (EAC) ultrastructural features showing distortion in the nuclear membrane (white arrow) and necrotic chromatin of nucleus (yellow arrow), marked cytoplasmic vacuoles (red arrow) and also a marked loss of microvilli (black arrow). (scale bar 5μm).

**Fig 29:** Electron micrograph of a combination of SM and KBrO$_3$ treated Ehrlich ascites carcinoma cell (EAC) ultrastructural features showing major loss of cellular framework of cytoplasm, extensive cytoplasmic vacuolation (red arrow), severe fragmented nuclei (N), condensation of chromatin material (yellow arrow) around the nuclear membrane, and completely loss of mitochondria (black asterisk) and microvilli (black arrow). (Scale bar 5μm).
Figure 5

**Fig 30:** Paraffin section photomicrograph of immunostained liver section against αSMA showing normal very mild positive brown expression on vascular wall in control group of mice. Black arrows point to positive expression. IHC counterstained with Mayer's hematoxylin, X:400.

**Fig 31:** Paraffin section photomicrograph of immunostained liver section against αASM showing increased positive brown expression on vascular walls in EAC group of mice. Black arrows point to positive expression. IHC counterstained with Mayer's hematoxylin, X:400.

**Fig 32:** Paraffin section photomicrograph of immunostained liver section against αSMA showing slightly decreased positive brown expression on vascular walls in EAC group treated with SM. Black arrows point to positive expression. IHC counterstained with Mayer's hematoxylin, X:400.
Fig 33: Paraffin section photomicrograph of immunostained liver section against αSMA showing moderately decreased positive brown expression on vascular walls in EAC group of mice treated with KBrO₃. Black arrows point to positive expression. IHC counterstained with Mayer’s hematoxylin, X:400.

Fig 34: Paraffin section photomicrograph of immunostained liver section against αSMA showing markedly decreased positive brown expression on vascular walls in EAC group of mice treated with a combination of SM and KBrO₃. Black arrows point to positive expression. IHC counterstained with Mayer’s hematoxylin, X:400.

Fig 35: Paraffin section photomicrograph of immunostained renal section against αSMA showing normal very mild positive brown expression in interstitial tissue in control group of mice. Black arrows point to positive expression. IHC counterstained with Mayer’s hematoxylin, X:400.

Fig 36: Paraffin section photomicrograph of immunostained renal section against αSMA showing increased positive brown expression in interstitial tissue in EAC group of mice. Black arrows point to positive expression. IHC counterstained with Mayer’s hematoxylin, X:400.

Fig 37: Paraffin section photomicrograph of immunostained renal section against αSMA showing slightly decreased positive brown expression in interstitial tissue in EAC group of mice treated with SM. Black arrows point to positive expression. IHC counterstained with Mayer’s hematoxylin, X:400.

Fig 38: Paraffin section photomicrograph of immunostained renal section against αSMA showing moderately decreased positive brown expression in interstitial tissue in EAC group of mice treated with KBrO₃. Black arrows point to positive expression. IHC counterstained with Mayer’s hematoxylin, X:400.

Fig 39: Paraffin section photomicrograph of immunostained renal section against αSMA showing markedly decreased positive brown expression in interstitial tissue in EAC group of mice treated with a combination of SM and KBrO₃. Black arrows point to positive expression. IHC counterstained with Mayer’s hematoxylin, X:400.
**Figure 6**

**Fig.40:** Paraffin section photomicrograph of mouse liver of a control group, showing mild to weak positive expression (+1) of TGF-β1 protein in most hepatocytes cytoplasm with diffuse pale brown color, a blue negative color show in the vesiculated nuclei and lymphocytes infiltrating between hepatocytes and in the sinusoid, the yellow color of RBCs was seen. (Counter stain with hematoxylin) (DAB stain, X:400).

**Fig.41:** Paraffin section photomicrograph of mouse liver of EAC group, showing intense positive expression (+4) of TGF-β1 protein in most hepatocytes cytoplasm and cell membrane with diffuse brown color, mostly proliferating hepatocytes and bile duct epithelial cells at the portal tract, a blue negative color show in the nuclei and lymphocytes infiltrating at portal tract. (Counter stain with hematoxylin) (DAB stain, X:400).
**Fig. 42:** Paraffin section photomicrograph of mouse liver of EAC group treated with SM, showing moderate positive expression (+2) of TGF-β1 protein in most hepatocytes cytoplasm and cell membrane with diffuse pale brown color, a blue negative color show in the nuclei and lymphocytes infiltrating at central vein and yellow RBCs in the sinusoid. (Counter stain with hematoxylin) (DAB stain, X:400).

**Fig. 43:** Paraffin section photomicrograph of mouse liver of EAC group treated with KBrO₃, showing moderate to strong positive expression (+2) of TGF-β1 protein in most hepatocytes cytoplasm and cell membrane with diffuse pale brown color, a blue negative color show in the nuclei and lymphocytes infiltrating at portal tract and bile duct epithelial cells and yellow of RBCs. (Counter stain with hematoxylin) (DAB stain, X:400).

**Fig. 44:** Paraffin section photomicrograph of mouse liver of EAC group treated with a combination of SM and KBrO₃, showing moderate positive expression (+2) of TGF-β1 protein in most hepatocytes cytoplasm and cell membrane with diffuse brown color, mostly in hyperchromatic hepatocytes and a foci area of proliferating hepatocytes, a blue negative color show in the nuclei and lymphocytes cells. (Counter stain with hematoxylin) (DAB stain, X:400).

**Fig. 45:** Paraffin section photomicrograph of a control mouse kidney, showing moderate positive expression (+2) of TGF-β1 protein in most renal tubules with diffuse brown color in the cytoplasm of the most epithelial tubular cells and Bowman's capsule cells of glomeruli. The negative blue color of the nuclei of tubules cells and mesangial cells nuclei, the yellow color of the RBCs. (Counter stain with hematoxylin) (DAB stain, X400).

**Fig. 46:** Paraffin section photomicrograph of mouse kidney treated with EAC, showing intense positive expression (+4) of TGF-β1 protein in the marked dilated renal tubules. Other tubules have moderate (+2) diffuse brown color in the cytoplasm and cell membrane of the most epithelial tubular cells and Bowman's capsule cells of glomeruli. The negative blue color of the nuclei of tubules and mesangial cells. (Counter stain with hematoxylin) (DAB stain, X:400).

**Fig. 47:** Paraffin section photomicrograph mouse kidney of EAC group treated with SM, showing strong positive expression (+3) of TGF-β1 protein in the marked dilated renal tubules (nephrosclerosis). Other tubules have moderate (+2) diffuse brown color in the cytoplasm and cell membrane of the most epithelial tubular cells and Bowman's capsule cells of glomeruli. The negative blue color of the nuclei of tubules and mesangial cells. (Counter stain with hematoxylin) (DAB stain, X400.)

**Fig. 48:** Paraffin section photomicrograph mouse kidney of EAC group treated with KBrO₃, showing intense positive expression (+4) of TGF-β1 protein in most renal tubules with diffuse brown color in the cytoplasm and cell membrane of the most epithelial tubular cells and Bowman's capsule cells of glomeruli. The negative blue color of the nuclei of tubular cells and mesangial cells. (Counter stain with hematoxylin) (DAB stain, X400).
Fig.49: Paraffin section photomicrograph of mouse kidney of EAC group treated with a combination of SM and KBrO₃, showing intense positive expression (+3 or +4) of TGF-β₁ protein in most renal tubule with brown color in the cytoplasm and cell membrane of the most epithelial tubular cells and Bowman's cells of glomeruli. The negative blue color of the nuclei of tubules and mesangial cells. (Counter stain with haematoxylin) (DAB stain, X400).

Figure 7

Fig.50: Paraffin section photomicrograph of mouse liver of a control group, showing moderate positive expression (+2) of Cath-D protein in some hepatocytes cytoplasm with small brown granules at foci area at the central vein and infiltrating lymphocytes, a blue negative color appears in the liver nuclei and lymphocytes (Counterstain with haematoxylin) (DAB stain, X:400).

Fig.51: Paraffin section photomicrograph of mouse liver of EAC group, showing intense positive expression (+4) of Cath-D protein in most proliferating hepatocytes cytoplasm with diffuse brown color at
foci area of the portal tract surrounded by necrotic hepatocytes, a blue negative color appears in the liver nuclei (Counterstain with haematoxylin) (DAB stain, X:400).

**Fig.52:** Paraffin section photomicrograph of mouse liver of EAC group treated with SM, showing strong positive expression (+3) of Cath-D protein in most hepatocytes cytoplasm with small brown granules surround the nuclei of vacuolated hepatocytes at the central vein area, a blue negative color appears in the liver nuclei (Counterstain with haematoxylin) (DAB stain, X:400).

**Fig.53:** Paraffin section photomicrograph of mouse liver of EAC group treated with KBrO₃, showing intense positive expression (+4) of Cath-D protein in most hepatocytes cytoplasm with aggregated brown granules at foci area at the central vein, and moderate in the vacuolated hepatocytes cytoplasm surround the nuclei, a blue negative color appears in the liver nuclei and lymphocytes (Counterstain with haematoxylin) (DAB stain, X:400).

**Fig.54:** Paraffin section photomicrograph of mouse liver of EAC group treated with a combination of SM and KBrO₃, showing strong positive expression (+3) of Cath-D protein in most hepatocytes cytoplasm with aggregated brown granules at the central vein, a blue negative color appears in the liver nuclei and lymphocytes (Counterstain with haematoxylin) (DAB stain, X:400).

**Fig.55:** Paraffin section photomicrograph of mouse kidney of a control group of mice, showing moderate positive expression (+2) of Cath-D protein in the cytoplasm of most renal tubular epithelial cells and most mesangial cells of glomeruli. The negative blue color of the tubular epithelial cell’s nuclei was seen. (Counter stain with hematoxylin) (DAB stain, X:400).

**Fig.56:** Paraffin section photomicrograph of mouse kidney of EAC group, showing intense positive expression (+4) of Cath-D protein in the cytoplasm of renal tubular epithelial cells distributes with diffuse brown color and differentiated through the both renal tubules and most mesangial cells of glomeruli. The negative blue color of the nuclei of the tubular epithelial cells was seen. (Counter stain with hematoxylin) (DAB stain, X:400).

**Fig.57:** Paraffin section photomicrograph of mouse kidney of EAC group treated with SM, showing strong positive expression (+3) of Cath-D protein in the cytoplasm of renal tubular epithelial cells distribute with diffuse brown color and intense immunostaining in destructive renal tubules. The negative blue color of the nuclei of the tubular epithelial cells was seen (Counterstain with hematoxylin) (DAB stain, X:400).

**Fig.58:** Paraffin section of photomicrograph mouse kidney of EAC group treated with KBrO₃, showing intense positive expression (+4) of Cath-D protein in the cytoplasm of some renal tubular epithelial cells distribute with diffuse brown color and moderate in most mesangial cells of glomeruli. The negative blue color of the nuclei of the tubular epithelial cells was seen. (Counterstain with hematoxylin) (DAB stain, X:400).
**Fig.59:** Paraffin section photomicrograph of mouse kidney of EAC group treated with a combination of SM and KBrO₃ showing strong positive expression (+3) of Cath-D protein in the cytoplasm of few renal tubular epithelial cells distribute with diffuse brown color and in most mesangial cells of glomeruli. The negative blue color of the nuclei of the tubular epithelial cells was seen. (Counterstain with hematoxylin) (DAB stain, X:400).

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- table1.jpg
- table2.jpg