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RESEARCH ARTICLE

Antioxidant role of Holothuria atra extract against nephrotoxicity induced by 7, 12-dimethylbenz (a) anthracene in male albino rats

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Abstract

The kidney is the target of numerous xenobiotic toxicants, including environmental chemicals. Nephrotoxicity is one of the most common kidney problems and occurs when the body is exposed to a drug or toxin. The aim of this study is to assess the antioxidant activity of *Holothuria atra* extract (HaE) against nephrotoxicity induced by 7, 12-dimethylbenz (a) anthracene (DMBA). Fifty male Wistar albino rats were assigned into two main groups, the pre-treated group (25 rats) and post-treated group (25 rats). Each group divided into 5 subgroups; healthy control (received 1ml distilled water daily prior to a single dose of corn oil), DMBA groups (received 1ml distilled water for 7 and 14 days prior to or after a single dosage of 15 mg/ kg body weight DMBA) and HaE treated groups (received 1ml HaE at a dosage of 200 mg/kg body weight, orally for 7 and 14 days prior to or after a single dosage of 15 mg/ kg body weight DMBA). The nephrotoxic effect of DMBA was assessed by the significant increase in malondialdehyde (MDA) concentration and the significant decrease in glutathione reduced (GSH) concentration, Glutathione-S-Transferase (GST), superoxide dismutase (SOD), and Catalase (CAT) activities, as compared with healthy controls. However, an antioxidant effect of HaE was noticed by a significant decrease in MDA concentration and the significant increase in GSH concentration, GST, SOD, and CAT activities in kidney as compared to DMBA groups. In conclusion, HaE has potential protective and ameliorative effects against the nephrotoxicity induced by DMBA.

INTRODUCTION

An exposure to environmental pollutants increased risks for heart, respiratory and kidney disease (Hendryx, 2009). The kidney is the target of numerous xenobiotic toxicants, including environmental chemicals. An environmental chem. icals has the potential to adversely affect human health through the disruption of renal functions (Van Vleet & Schnellmann, 2003). Chronic kidney disease has recently been recognized as a public health problem (Stevens et al., 2006). Nephrotoxicity is one of the most common kidney problems and occurs when the body is exposed to a drug or toxin (Porter & Bennett, 1981). Nephrotoxic effects may develop in glomerular and tubular epithelial cells as a result of mechanisms that disrupt normal cellular functions of mitochondria and/or membrane integrity which induces renal injury (Anzai & Endou, 2008).

Polycyclic aromatic hydrocarbons (PAHs) are a class of organic pollutants that is released into the environment in large quantities, mainly due to human activities (Sharma et al., 2012a). As a derivative of PAHs, 7, 12-dimethylbenz (a) anthracene (DMBA) is hazard chemical in mammalian and other organisms (Li et al., 2002;

Nagar et al., 2004). The DMBA is well known as cytotoxic, carcinogenic, mutagenic and immunosuppressive agent (Manoharan & Vasantha, 2012). It induces potentially malignant tumors in vital organs such as liver, kidney, brain and bladder (Berber et al., 2014). In addition, there are emerging evidences suggest that DMBA induces the production of reactive oxygen species (ROS) that result in lipid peroxidation, DNA damage, and depletion of cell antioxidant defense systems (Bharali et al., 2003). Oxidative stress may alter the structure and function of the glomerulus because of the effect of reactive oxygen species on mesangial and endothelial cells (Kandeel et al., 2011). So, the kidney may be a major target organ of DMBA toxicity, after mammary glands, skin, and liver and that the epithelial cells of proximal convoluted tubules and Bowman's capsule seem to be more sensitive to DMBA induced nephrotoxicity (Sharma & Paliwal, 2012).

Countless marine plants and animals contain biochemical secrets that, if unlocked, can provide new insights and understanding of human diseases and their treatment (Murti & Agrawal, 2010). Chemicals produced by or found in marine organisms have been shown to have a wide variety of applications as pharmaceuticals for humans and other animals (Fahmy & Soliman, 2013). The search for bioactive principles of marine origin has allowed valuing the biological diversity present in aquatic systems (Sottorff et al., 2013). Marine organisms were studied for their bioactive compounds with many applications (Zotchev, 2012). Sea cucumber is among the marine organisms a traditional food item, especially in Asia (Farshadpour et al., 2014). They are cylinder-shaped invertebrates that live in a variety of sea floor habitats from warm tropical waters to cold deep-sea trenches (Wu et al., 2014). Sea cucumber contains various bioactive substances such as saponins, polysaccharides, cerebrosides, gangliosides, and phospholipids (Bordbar et al., 2011). A number of unique biological and pharmacological activities including anti-angiogenic, anticancer, anticoagulant, anti-hypertension, anti-inflammatory, antimicrobial, antioxidant, antithrombotic, antitumor and wound healing have been ascribed to various species of sea cucumbers (Bordbar et al., 2011). Moreover, Xu et al. (2013) found that sea cucumber had a protective effect on renal injury in diabetic rats. *Holothuria atra* is abundant in the Red sea on the Saudi Arabia coast (Hasan, 2009.). The aim of work is to evaluate the antioxidant activity of the sea *Holothuria atra* extract (HaE) against nephrotoxicity induced by DMBA in male albino rats.

I. Materials and methods

1. Chemicals and reagents

DPPH (2, 2-diphenyl-1-picrylhydrazyl) and ketamine were purchased from Sigma–Aldrich (St. Louis, MO, USA). Kits of oxidative stress parameters were purchased from Bio-diagnostic Company (Giza, Egypt).

2. Preparation of *Holothuria atra* extract (HaE)

1.1 Sample collection

Sea cucumbers (*Holothuri atra)*, were collected from Thowal area, Saudi Arabia's Red Sea coast. The taxonomic identity of the samples will be confirmed by Professor of invertebrate. The animals were transported to our laboratory in an ice box containing ice cubes and a few pinches of table salt. They immediately washed under running tap water and cut open, and then all visceral organs were removed. The animals were rinsed thoroughly of any internal organs or body fluids, and then the body walls of the animals were stored at $^{-}20^{0}$C until processing.

1.2 Preparation of *Holothuria atra* extract (HaE):

Preparation of the phosphate buffer done according to the method of (Yasumoto et al., 1967).The body walls of the animals were cut into small parts and blended in phosphate buffer (volume = 4 X tissue weight) and extracted at room temperature (25^{o}C) with pH 7.2 for 5 hours. The filtered was collected immediately, concentrated and lyophilized using a lyophilizer (LABCONCO, shell freeze system, USA).

3. Free radical scavenging activity

The free radical scavenging activities of each HaE and ascorbic acid were analyzed by the DPPH assay (Sanchez-Moreno et al., 1998). A 1.0 ml of the test extract, at gradient final concentrations of 10 - 60 mg/ml, was mixed with 2 ml of 0.3 mM DPPH solution in MeOH in a cuvette. The absorbance was taken at 517 nm after 20 minutes of the incubation in the dark at room temperature. The experiment was done in triplicates. The percentage antioxidant activity was calculated as follows:

%Antioxidant Activity [AA] = 100 - [{(Abs $_{sample}$ – Abs $_{blank)}$ X 100}/Abs $_{control]}$. Where Abs $_{sample}$ was the absorbance of sample solution (2.0 ml) + DPPH solution (1.0 ml, 0.3 mM), Abs $_{blank}$ was the absorbance of Methanol

(1.0 ml) +sample solution (2.0 ml), $Abs_{control}$ was the absorbance of DPPH solution (1.0 ml, 0.3 mM) + methanol (2.0 ml).

4. Toxicity study (OECD 420)

Eighteen Wistar rats weighing (130-150 g) were used for acute toxicity studies. The animals were divided into three groups containing six animals each. Two groups administered orally with HaE at dose levels of 5 g/kg (high dose) and 2 g/kg (low dose). The third group used as a control and received the same amount of distilled water only. Animals were observed carefully for 24 hours after extract administration and then for the next 14 days. At the end of this experimental period, the rats were observed for signs of toxicity, morphological behavior, and mortality. Acute toxicity was evaluated based on the number of deaths (if any). Acute toxicity was calculated as per OECD guidelines 420 (Fixed dose method) (Whitehead & Curnow, 1992). The effective dose of the HaE will be calculated as 10% of the safety tested dose of OECD test.

5. Animals

Male Wistar rats (*Rattus norvegicus*) weighing 130-150 $\pm$ 5 g were reared in a room with a constant temperature of 22 ± 1 ºC, a relative humidity of 55 ± 10%, and under the controlled light/dark cycles (12/12 hour). Animals were maintained with free access to water and a standard laboratory diet. The animals were obtained from the National Research Center (NRC, Dokki, Giza). Rats were acclimatized to laboratory conditions for 7 days before commencement of the experiment.

6. **Ethical Consideration**

Experimental protocols and procedures used in this study were approved by the Cairo University, Faculty of Science Institutional Animal Care and Use Committee (IACUC) (Egypt), (CUFS/F/16/14). All the experimental procedures were carried out in accordance with international guidelines for care and use of laboratory animals.

7. Experimental Design

Fifty male Wistar albino rats were assigned into two main groups, the Pre-treated group (25 rats) and Post-treated group (25 rats).

A-The Pre-treated group (protective group) divided into 5 subgroups:

Subgroup 1: served as control and received 1ml distilled water daily prior to a single dosage of corn oil by oral gavage.

Subgroups 2&3: received 1ml distilled water for 7 and 14 days prior to a single dosage of DMBA (15 mg/ kg body, weight orally).

Subgroups 4&5: received 1ml HaE (200mg/kg body weight, orally) for 7 and 14 days prior to a single dosage of DMBA (15 mg/ kg body weight, orally).

The Post-treated group (curative group) which divided also into 5 subgroups:

Subgroup 1: served as a control, received 1ml distilled water daily than administrated a single dosage of corn oil by oral gavage

Subgroups 2&3: received 1ml distilled water for 7 and 14 days, then administrated a single dosage of DMBA (15 mg/ kg body weight, orally)

Subgroups 4&5: received 1ml of HaE (200mg/kg body weight, orally) for 7 and 14 days, then administrated a single dosage of DMBA (15 mg/ kg body weight, orally)

8. Animal handling

Animals were euthanized under sodium pentobarbital. The kidney was removed and immediately blotted using filter paper to remove traces of blood and then each of them was divided into two parts, the first part was suspended in 10% formal saline for fixation preparatory to histopathological processing, while, the second part was stored at –80^{o}C for biochemical studies.

9. Preparation of Kidney Homogenate

Kidney homogenate was prepared in cold 50 mM potassium phosphate buffer (pH 7.4), using Remi homogenizer. The unbroken cells and debris were removed by centrifugation at 10,000 rpm for 15 min at -4 ^{o}C using a Remi cooling centrifuge and the supernatant was used for the estimation of the antioxidant properties (Paliwal et al., 2011).

10. Histopathological examination

Histological sections (4 µm) thick were prepared from paraffin blocks of kidney tissues fixed in 10% formal saline. Sections were stained with hematoxylin and eosin (H & E) (Mayer, 1998).

11. Determination of Oxidative Stress parameters

Oxidative stress markers were detected in the resultant supernatant of kidney homogenate. The appropriate kits (Biodiagnostic kits, Biodiagnostic Dokki, Giza, Egypt) were used for the determination of malondialdehyde

(MDA) (Okhawa et al., 1979), glutathione reduced (GSH) (Beutler et al., 1963), Catalase (CAT) (Aebi, 1984), Glutathione-S-Transferase (GST) (Habig et al., 1974)and superoxide dismutase (SOD) (Nishikimi et al., 1972)

12. Statistical Analysis

The experimental results obtained were expressed as mean ± SE. The data were subjected to one way analysis of variance (ANOVA) and the differences between samples were determined by Duncan post hoc comparison test using SPSS 20.0 (Statistical program for Social Sciences) program. The level of significance was set at $P<0.05$.

II. Results

1. Acute toxicity

The present results revealed that the *Holothuria atra* extract (HaE), has been found to be toxic at the higher dosage (5000 mg/kg body weight) to the experimental animals as assessed by the occurrence of morbidity in the first 6 hours. According to this result, we used the lower dosage of 2000 mg/kg. With the lower dose there was no mortality and the rats did not show any sign of toxicity in the first 24hour or during the experiment. The single lethal dosage of (IE) that kills half of the animals (LD_{50}) was therefore taken as above 2000 mg/kg. The median effective dose (ED_{50}) was selected based on the proposed LD_{50} obtained from the acute toxicity study. This dose was considered one tenth of the proposed LD_{50} (200 mg/kg body weight).

2. Free radical scavenging activity

The radical scavenging activities were estimated by comparing the percentage of inhibition of DPPH radicals by HaE and the ascorbic acid. Effect of HaE on DPPH free radical scavenging activity has been done at various concentrations (10, 20, 30, 40, 50, 60, 70 and 80 mg/ml) each in three replications (Fig. 1). The data were displayed with mean ± SE of three replications. The present results showed that HaE has *in vitro* antioxidant activity as indicated by dose dependent inhibition of DPPH radicals ranging from (81 to 94%) as compared to ascorbic acid.

3. Oxidative stress parameters

The toxic effect of DMBA was assessed by the significant ($P<0.05$) increase in MDA level (Fig.2) and the significant ($P<0.05$) decrease in; GSH concentration (Fig.3), GST (Fig.4), SOD (Fig.5), and CAT (Fig.6) activities in rat kidneys as compared with those of healthy controls. The antioxidant effect of HaE (200 mg/kg body weight orally) was noticed by a significant ($P<0.05$) decrease in MDA concentration (Fig.2) and the significant increase in GSH concentration (Fig.3), GST (Fig.4), SOD (Fig.5), and CAT (Fig.6) activities in rat kidneys of both protective and curative groups, as compared to the corresponding DMBA groups. In general, HaE administration after 14 days showed amelioration in antioxidant system more than after 7 days.

4. Histopathological analysis of kidney

Histopathology of control rat showed cortical region of normal kidney with healthy glomeruli and renal tubules (Fig. 7.A). While a section of pre-treated rat with DMBA showed severe hemorrhages (H) after 7 days (Fig. 7.B), vasculitis (V) and degenerated renal tubules with renal cast formation (r) after 14 days (Fig. 7.C). However, HaE pre-treated rats kidney sections showed congested peritubular blood capillaries (C) after 7 days (Fig, 7.D), dilated blood vessel with thick wall (D) and perivascular edema (E) after 14 days (Fig. 7.E).

On the other hand, a section of post-treated rat kidney with DMBA showed degenerated glomerular tuft (G), renal tubules (r) after 7 days (Fig. 8.B), and perivascular edema (E) after 14 days (Fig. 8.C); while, section of post-treated rat with HaE showed perivascular edema (E) after 7 days (Fig. 8.D), and degenerated renal tubules with the formation of renal casts (r) after 14 days (Fig. 8. E).

III. Discussion

Nephrotoxicity may be consequent to direct cytotoxic damage to kidney structures by toxicants, to immunologic processes, to indirect toxicity due to alterations in renal hemodynamics, or to the production of endogenous nephrotoxic substances (Blowey, 2005). Renal cortical mitochondria are the source of reactive oxygen metabolites (Padmini & Kumar, 2012). Nephrotoxic effects may develop in glomerular and tubular epithelial cells as a result of mechanisms that disrupt normal cellular functions of mitochondria and/or membrane integrity (Anzai & Endou, 2008), which induces renal injury (Du & Yang, 1994).

Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic compounds with two or more fused aromatic rings that are formed during the incomplete combustion of organic materials such as wood, coal and mineral oil and products derived from them (Koul et al., 2010). DMBA induces substantive nephrotoxicity that is characterized by renal tubular necrosis, proteinuria with upregulation of specific signals like tumor necrosis factor-α, chemokines and cytokines (Sharma et al., 2012b). It also produced marked histological alterations in the kidney include dilation of tubules; sloughing of epithelium indicates advanced disintegration of tubules (Sharma & Paliwal, 2012).

A larger number of chemopreventive agents have been elucidated in epidemiological and experimental studies, preclinical and clinical observations (Radhakrishnan et al., 2007). However, the toxic side effects produced by some of these agents have limited their extensive use (Sharma et al., 2012a). Therefore, there is a need to identify natural compounds that have significant chemopreventive potential without undesirable toxic side effects.

The search for bioactive principles of marine origin has allowed valuing the biological diversity present in aquatic systems (Sottorff et al., 2013). Sea cucumbers, also known as holothurians, are marine invertebrates living in shallow seawater, on reef flats and slopes (Shahrulazua et al. 2013). Various studies have shown that sea cucumber extract possesses several therapeutic properties such as a promoter of soft tissue healing (Nihayah et al., 2000) and as an antibacterial (Haug et al., 2002), antifungal (Shaharuddin et al., 2006), antitumour (Lu & Wang, 2009), antianaphylactic (Kim et al., 2001), antiinflammatory (Hassan et al., 1997), antinociceptive (Ridzwan et al., 2003) and antioxidant agent (Althunibat et al., 2009).

Oxidative damage occurs when the concentration of reactive oxygen species (O_2, H_2O_2 and $^{\cdot}OH$) generated exceeds the antioxidant capability of the cells (Kesba & El-Belagi, 2012). Oxidative stress the major factor lead to hepatotoxicity, neurotoxicity and nephrotoxicity (Kasprzak, 2002). There are many evidences suggest that DMBA induces the production of reactive oxygen species (ROS) that result in lipid peroxidation, DNA damage, and depletion of cell antioxidant defense systems (Bharali et al., 2003). The ROS formed during DMBA metabolism can diffuse from the site of generation to other sites within the cells or even propagate the injury outside the cells (Koul et al., 2010). An endogenous antioxidant system may counteract the ROS and reduce the oxidative stress with the enzymatic antioxidants SOD, CAT and GST. SOD accelerates the conversion of superoxide radical (O^{2-}) to hydrogen peroxide while CAT converts H_2O_2 to H_2O (Sharma & Paliwal, 2013). Change in lipid peroxidation production reactions and antioxidant defense systems were associated with changes in a variety of biochemical pathways (Paliwal et al., 2011).

Lipid peroxides, derived from polyunsaturated fatty acids are unstable and can be decomposed to form a complex series of compounds. These include reactive carbonyl compound, which is the most abundant malondialdehyde (MDA) (Akande & Akinyinka, 2005). The present study revealed that, MDA concentration increase significantly in both pre-treated and post-treated groups with DMBA (15 mg/kg body weight orally) as compared with control groups. These results are in agreement with (Sharma & Paliwal, 2013). This increase in MDA concentration related to the damage occurs in kidney cells due to production of reactive oxygen species by DMBA (Bharali et al., 2003).

On the other hand, MDA concentration decreases significantly in both pre-treated and post-treated groups with HaE (200 mg/kg body weight, orally) as compared with DMBA groups. This decrease revealed the ability of the extract to resistant the oxidative damage occurred by DMBA.

One of the most important antioxidant systems is the glutathione redox cycle (Sharma & Paliwal, 2013). Glutathione is highly abundant in cytosol, nuclei and mitochondria, and is the major soluble antioxidant in these cell compartments (Sharma et al., 2010). Reduced glutathione (GSH), the main component of the endogenous non protein sulfhydryl pool is known to be a major low molecular weight scavenger of free radicals in the cytoplasm (Toklu et al., 2013). The depletion of GSH promotes generation of reactive oxygen species and oxidative stress with a cascade of effects, thereby affecting functional as well as the structural integrity of cell and organelle membranes (Singh et al., 2000). The elevated level of GSH protects cellular proteins against oxidation through the glutathione redox cycle and also directly detoxifies reactive oxygen species and/or neutralizes reactive intermediate species generated from exposure to xenobiotics including chemical carcinogens (Kettrer, 1998).

The current study disclosed that, kidney GSH concentration of pre-treated and post-treated groups decrease significantly after DMBA administration (15 mg/kg body weight orally) as compared with control groups. These results are in agreement with (Bedi & Priyanka, 2012). The depletion in the renal GSH level has been observed in mice in response to oxidative stress caused by DMBA treatment (Sharma & Paliwal, 2013).

However, HaE administration (200 mg/kg body weight orally) causes significant increase in kidney GSH concentration of pre-treated and post-treated groups as compared with DMBA groups. The depletion in GSH concentration represents the antioxidant activity of HaE.

Enzymes have been proposed as biomarkers of oxidative stress include catalase (CAT), glutathione-S-transferase (GST), and superoxide dismutase (SOD) (Radovanović et al., 2010). These enzymes prevent the generation of hydroxyl radicals and protect the cellular constituents from oxidative damage (Halliwell & Gutteridge, 1984). The present study showed that, the kidney GST, SOD and CAT activities of the pre-treated and post-treated rats with DMBA (15 mg/kg body weight orally) decreased significantly as compared with control groups. Our results are in agreement with (Sharma & Paliwal, 2013). Decreased activity of the antioxidant enzyme (SOD), reflecting an oxidative stress state (Bedi & Priyanka, 2012; Vijayabaskaran et al., 2010). In this present observation an increase in MDA was presumably associated with increased free radicals, confirming the fact that these free radicals inhibited the activities of SOD, CAT and GST.

On the other hand, the treatment with HaE (200 mg/kg body weight orally) leaded to significant increase in kidney GST, SOD and CAT activities as compared by DMBA groups. This indicates the efficacy of HaE as antioxidant and so preventing the inactivity of these enzymes from ROS.

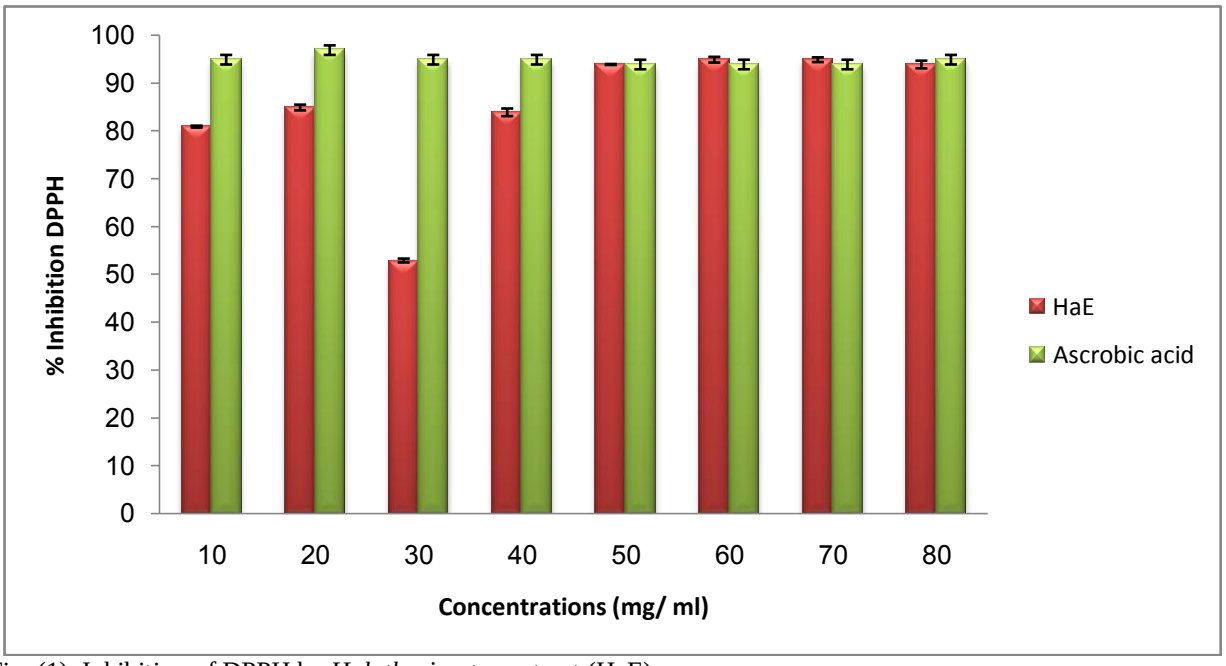

Fig. (1): Inhibition of DPPH by *Holothuria atra* extract (HaE).

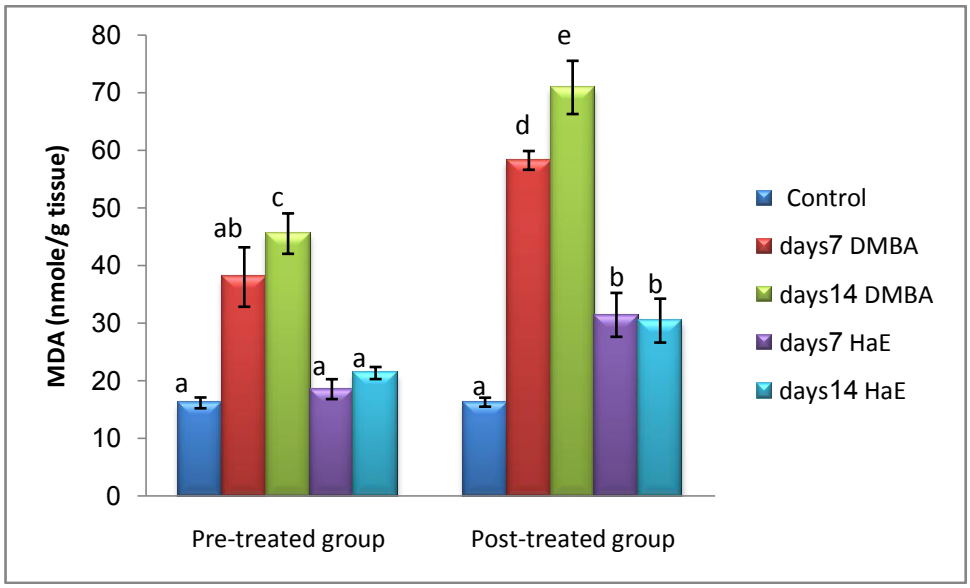

Fig. (2): Renal protective and curative role of *Holothuria atra* extract (HaE) on oxidative Kidney MDA concentration (nmole/g tissue) of DMBA intoxicated rat.

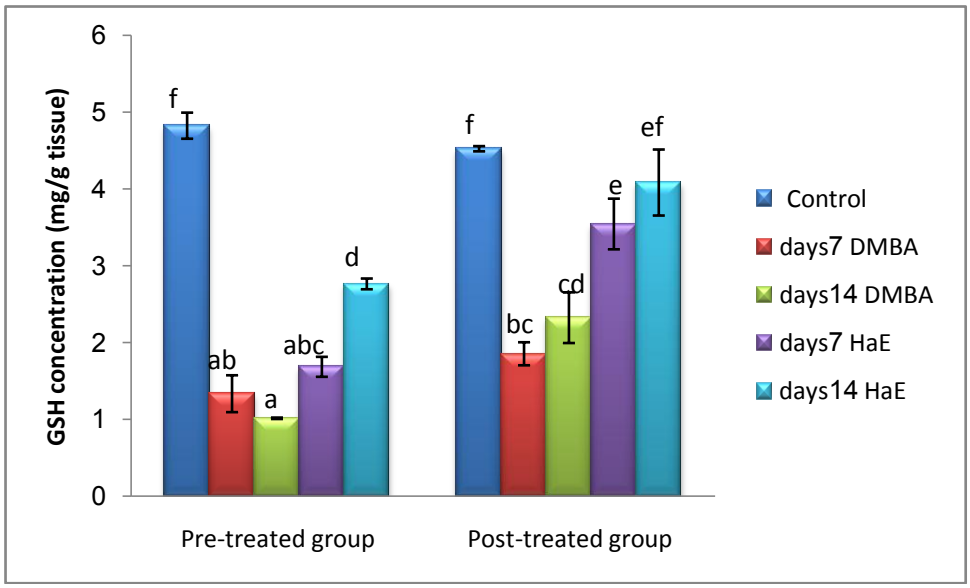

Fig. (3): Renal protective and curative role of *Holothuria atra* extract (HaE) on kidney GSH concentration (mg/g tissue) of DMBA intoxicated rat.

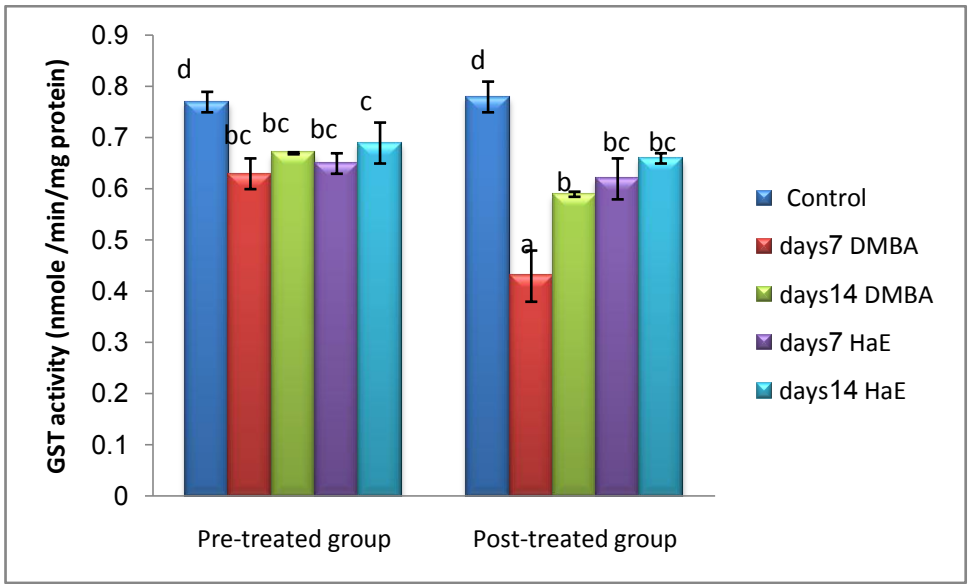

Fig (4): Renal protective and curative role of *Holothuria atra* extract (HaE) on kidney GST activity (nmole /min/mg protein) of DMBA intoxicated rat.

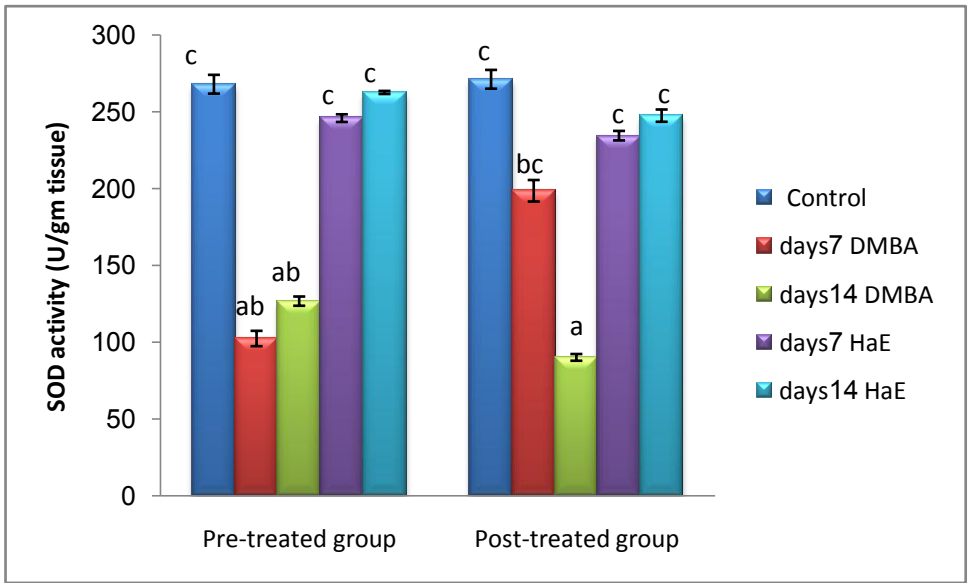

Fig. (5): Renal protective and curative role of *Holothuria atra* extract (HaE) on kidney SOD activity (U/gm tissue) of DMBA intoxicated rat.

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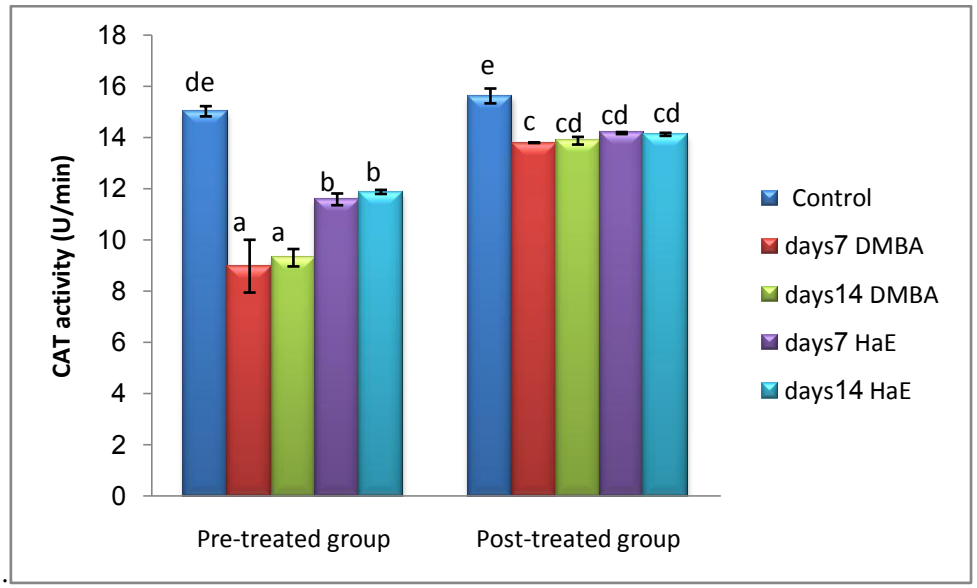

Fig. (6): Renal protective and curative role of *Holothuria atra* extract (HaE) on kidney CAT (U/min) of DMBA intoxicated rat.

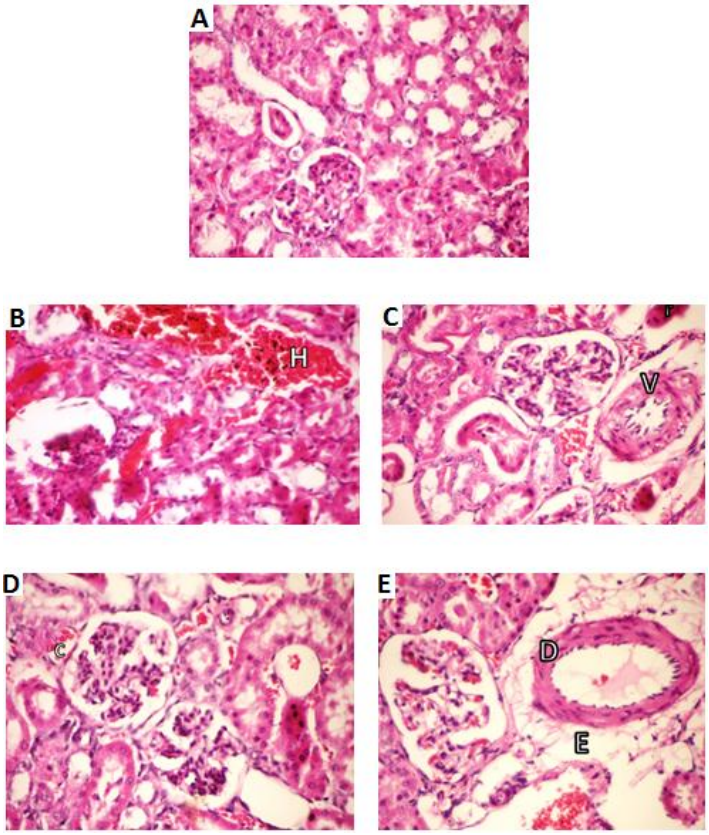

Fig. (7):Photomicrographs of kidney sections for pre-treated group showing control group (A), DMBA groups after 7 days (B) and 14 days (C), HaE treated groups after 7 days (D) and 14 days (E). (H&E X 400).

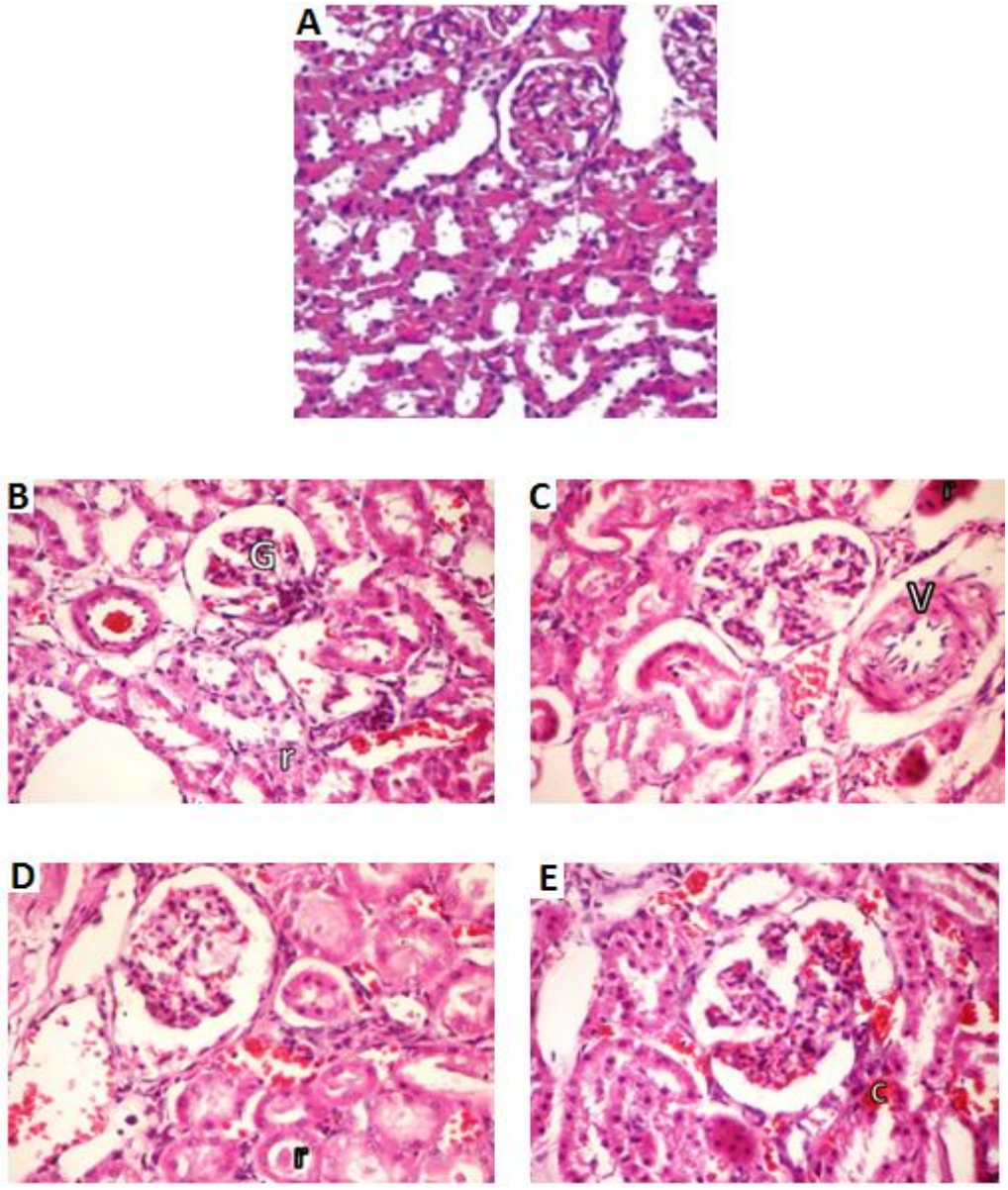

Fig. (8):Photomicrographs of kidney sections for post-treated group showing control group (A), DMBA groups after 7 days (B) and 14 days (C), HaE treated groups after 7 days (D) and 14 days (E). (H&E X 400).

IV. Conclusion

The present study demonstrated that *Holothuria atra extract* has chemopreventive properties which enhancing antioxidant status and quenching of reactive oxygen species. Our results may provide a new insight to explore novel drugs or functional foods for protection against nephrotoxicity.

V. References

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