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Biochemistry

Ameliorative Effect of *Azolla Pinnata Ethanolic* Extract on Ranitidine-Induced Hepatotoxicity in Rats

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ABSTRACT

Intoxication with ranitidine is considered as one of the major causes of hepatotoxicity. The current study aimed to investigate the protective effect of an ethanolic extract from Azolla pinnate (*APE*) against ranitidine -induced hepatotoxicity in rats. The present study was carried out on 40 male Wister albino rats, which were randomly divided into four groups (n=10). The 1st group, *control* group; orally administered saline. The second group, was given ranitidine (150 mg/kg body weight (BW) orally for 30 days); The third group, orally administered *APE* (10 mg/kg BW), daily for 30 days;The fourth group, was orally administrated ranitidine (as group 2), and *APE* (as group 3) together for 30 days. At the end of the experiment samples from blood and liver were obtained. Ranitidine increased serum activities of alanine and aspartate aminotransferases and serum levels of urea, creatinine, tumor necrosis factor alpha and interleukin-1β and hepatic tissue malondialdehyde contents. However, it decreased serum levels of interleukin-10 and GSH contents and catalase and superoxide dismutase activities in hepatic tissue. On the contrast, administration of rats with *APE* ameliorated ranitidine-induced alterations in liver function and structure stating the benefits of Azolla's phytochemical contents. Therefore, A. pinnate extract is a potential protective against ranitidine-induced hepatotoxicity via its antioxidant (presence of tamarixetin, rutin and quercetin) anti-inflammatory and anti-apoptotic activities.

Keywords: *Ranitidine, Hepatotoxicity, Azollapinnata, TNF-α, IL-1β, IL-10*

INTRODUCTION

The main cause of hepatotoxicity in vivo system is exposure to drugs as ranitidine, toxins or compounds such as carbon tetrachloride, sodium oxalate, ethylene glycol, and heavy metals (Pal *et al.*, 2011).

Ranitidine is a H_2 antagonist used in peptic ulcer and gastro-intestinal reflux disorder (GIRD) to neutralize the acid content in the stomach and duodenum (Hemieda *et al.*, 2005). It causes steatosis, cholestasis and induces fibrosis in portal track (Hemieda *et al.*, 2005). It also induces proliferation in the bile duct, and sometimes presence of plasma cell, eosinophils and lymphocytes are seen due to long-term use of ranitidine (Maddox *et al.*, 2006).

Ranitidine is a member of the class of histamine H_2-receptor antagonists with antacid activity. It is a competitive and reversible inhibitor of the action of histamine, released by enterochromaffin-like (ECL) cells, at the histamine H_2-receptors on parietal cells in the

stomach, thereby inhibiting the normal and meal-stimulated secretion of stomach acid and this results in decreased gastric acid secretion and gastric volume, and reduced hydrogen ion concentration (Palmer, 2019).

Ranitidine is a histamine type 2 receptor antagonist (H_2 blocker) which is widely used for treatment of acid-peptic disease and heartburn. Ranitidine has been linked to rare instances of clinically Apparent acute liver injury. Ranitidine HCl is white to pale yellow, crystalline, practically odorless powder, sensitive to light and moisture. Melts at about 140°C with decomposition. The empirical formula is $C_{13}H_{22}N_4O_3S.HCl$ (Grant *et al.*, 1989).

Under the influence of ranitidine, onset of oxidative stress occurs on account of two different pathways operative simultaneously; first comes the generation of ROS, like hydroperoxides (H2O2), singlet oxygen and hydrogen peroxide (H2O2), and second, the depletion of antioxidant reserves (Flora *et al.*, 2007).

These free radicals also damage other tissues, including liver (Oberley, 1988) by decreasing levels of antioxidant enzymes and increasing lipid peroxidation, ranitidine lead to hepatic oxidative stress and consequently the destruction of vital organs of the rat's body (Atawodi, 2011).

AzollaPinnata is the only genus in the family Salviniaceae and has a worldwide distribution from temperate to tropical climates, Azolla pinnata is a species of fern known by several common names, including mosquitofern, feathered mosquitofern and water velvet. It is native to much ofAsia and Africa (Sumit and Nayak, 2014).

AzollaPinnata is an aquatic fern consisting of a short, branched, floating stem, bearing roots which hang down in the water. The leaves are alternately arranged and each consists of a thick aerial dorsal lobe containing green chlorophyll and a thin floating ventral lobe of slightly larger size that is colorless (Debashis *et al.*, 2016).

Medicinal plant possesses an important role in human health care system. Herbal medicine has a tremendous demand in primary health sector because of their safety, efficacy, and less side effect (Baghbanan *et al.*, 2014).

In traditional health system, herbal treatment for the liver disorder is claimed to be safest and effective, hence development and validation of newer herbal drug is of prime concern. In the development of newer therapeutic agent, animal model plays vital role (Boonstra *et al.*, 2009). For the past 30-40 years it is the animal model only that helped in better understanding various liver disorders (Arteel *et al.*, 2010).

The chronic effect of Profenofos (broad spectrum insecticide) on Swiss albino mice can be eliminated by giving the azolla filiculoides which was a suitable antioxidant. (Arun *et al.*, 2014).

The APEshows the presence of many bioactive compounds where considered a good source of high-quality protein (Kumar and Chander, 2017), It also contains almost all essential amino acids, vitamins, Beta-carotene, minerals, sapeonin and flavonoid (Muraleed *et al.*, 2011).

The present study aimed to investigate the protective potential of ethanolic extract of APE against ranitidine acetate induced-hepatotoxicity in rats, and to elucidate the underlying molecular mechanism of its protective potentials as part of our ongoing project of phytochemical analysis of Egyptian plants showed antioxidant, anti-inflammatory and ant apoptotic activities (Mousa,*et al.*, 2019).

MATERIALS AND METHODS

Experimental animals

A total of 40 male Wistar albino rats, weighing 95–117 g, were purchased from Vac Sera lab., Helwan City, Cairo Governorate, Egypt. The animals were housed in polypropylene cages and kept under standard laboratory conditions of temperature 22–25 °C and 12 h light/12 h dark cycle. Rats were provided with clean water and diet *ad libitum*. A balanced diet of commercial pellets, (table 1), (Atmida Company for international commerce and development, Egypt). The animals were kept for 10 days before the beginningof the experiments for acclimatization. Animal rearing and handling and the experimental design and procedures were approved by the Research Ethics Committee of the Faculty of Veterinary Medicine, University of Sadat City, Egypt (VUSC-006-2-20).

Chemicals

A ranitidine was purchased from the Egyptian international Pharmaceuticals industries company (EIPICO), Golf city, Cairo, Egypt.

Diagnostic kits for assaying serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and diagnostic kits for assaying serum levels of total urea, and creatinine were purchased from Diamond Company, Egypt, while kits for assaying reduced glutathione (GSH),malondialdehyde (MDA), and superoxide dismutase (SOD) and Catalase activity (CAT) content in hepatic tissue were purchased from Bio-Diagnostics Ltd., Egypt. Other chemicals used in this study were of analytic grades.

Preparation of plant extract

For preparation of *A. pinnata*ethanolic extract *(APE),* fresh green leaves of *A. pinnata* were obtained from the botanical gardens of National Research Centre, Giza governorate, Egypt. The extract was prepared according to **Selvaraj and Ranjana, (2015).** Briefly, fresh leaves of *A. pinnata* were collected and dried under shade at room temperature (22°C and 65% relative humidity) for 7 days. The dried leaves were ground into powderand soaked in ethanol 70% (500 g/ L) for 48 hrs later with gentle shaking in incubator shaker at 37ºC. The content wasfiltered through Whatman No. 1 filter paper, and the filtratewasevaporated till dryness. This extract was kept in airtight bottle in a refrigerator at 4º C till usage.

Experimental design

A total of 40 male Wistar albino rats were equally assigned into 4 groups, (n=10).

Control group: Rats were given normal physiological saline (0.9% sodium chloride) orally.

A ranitidine group: Rats were orally administered ranitidine at a dose of 150 mg/kg BW for 30 daysand prepared at dose 150mg/kg BW according to **Hemieda *et al.* (2005)**.

***A. pinnata*ethanolic extract (*APE*) group:** Rats were orally administered 10 mg/kg BW of *APE.* extract daily for 30 days prepared at dose 150 mg/kg BW according to **Arun *et al.* (2014**), which illustrated in the previous section.

A ranitidine and *A. pinnata*ethanolic extract group: Rats were orally administrated ~~with~~ranitidine as the 2nd group and *APE* as the 3rd group simultaneously for 30 days.

Sampling

At the end of the experiment, animals were anaesthetized, then blood samples were collected from the medial canthus of the eye with heparinized capillary tube. Sera samples were separated and stored at -20^{0}C until being used for measuring the biochemical parameters. Animals were sacrificed, then liver was soon removed and kept at -80^{0}C for further investigation of lipid peroxidation and antioxidant activities biomarkers.

Methods:

Preparation of tissue homogenate:

Hepatic tissue homogenate was prepared according to **Combs *et al.* (2011)**

Hematological analysis: At the end of the experiment, animals were anaesthetized, then blood samples were collected from the medial canthus of the eye with heparinized capillary tube. Blood samples were left at room temperature for clotting. Sera samples were separated and stored at -20^{0}C until being used for measuring the biochemical parameters. Liver was removed and kept at -80^{0}C for further investigation of lipid peroxidation and antioxidant activities biomarkers.

Complete blood count (CBC) was estimated according to the methods of Tefferi *et al.* (2005)

Biochemical assays

Liver and kidney functions biomarkers were determined by using specific commercial diagnostic kits. Serum ALT and AST activities were analyzed according to the methods of Reitman and Frankel, (1957).

Serum levels of urea were measured according to the methods of Fawcett and Scott (1960). Serum creatinine concentration was measured according to the methods of Bartels *et al.* (1972).

Liver homogenate was analyzed for determination of malondialdehyde (MDA) according to the procedure described by Satoh, (1978), reduced glutathione (GSH) concentration according to the procedure described by Beutler *et al.* (1963), superoxide dismutase (SOD) activity according to the procedure described by Nishikimi *et al.* (1972) and catalase activity according to the procedure described by Fossati *et al.* (1980).

Determination of serum level of interleukin 1 beta (IL-1β) using kit from (Bio-Diagnostics Ltd, Egypt) (Catalog No. IL 1234) according to Vidal *et al.* (2000), tumor necrosis factor alpha (TNF-α) according to Brynskov *et al.* (2002) using kit from (Bio-Diagnostics Ltd, Egypt) (Catalog No: TF 1691), and Serum concentration of interleukin-10 (IL-10)by using kit from (Bio-Diagnostics Ltd, Egypt)

(Catalog No: IL 2341) according to methods described by Odewumi *et al.* (2015).

Statistical analysis:

Analysis of results was performed by using SPSS program software version 16 (IBM®, USA). Data were subjected to analysis of variance (ANOVA) and Duncan's post-hoc tests to determine significant differences among the data. The differences between means were analyzed at the 5% probability level ($P \leq 0.05$), which was statistically significant.

RESULTS

A. pinnata extract modulated the toxic effects of ranitidine and normalized hematological parameters in different albino rat groups after 30 days (one month):

Intoxication of the rats with ranitidine elevated significantly ($P < 0.05$) RBCs countandWBCs count (Lymphocytes and Granulocytes) ascompared with the control group. However, supplementation of rats with *APE*during their intoxication with ranitidine reducedsignificantly WBCs countespecially (Lymphocytes andGranulocytes) as compared with the ranitidine group. Treating of rats with *APE* had no significant effect on hematological parametersascompared with the control rats ($P < 0.05$) (Table 2).

pinnata extract modulated the toxic effects of ranitidine on liver and kidney functions biomarkers: Intoxication of the rats with ranitidine elevated significantly ($P < 0.05$) activities of serum ALT and AST and serum levels of urea and creatinine compared with the control group. However, supplementation of rats with *APE* during their intoxication with ranitidine 4thgroup reduced significantly activities of serum ALT and AST and serum levels of urea and creatinine as compared with rats intoxicated with ranitidine only ($P < 0.05$).Treating of rats with *APE* had no significant effect on liver and kidney functions biomarkersas compared with the control rats ($P < 0.05$) (Table 3).

A. pinnata extract revered the effects of ranitidine on serum levels of inflammatory and anti-inflammatory cytokines

The administration of rats of the 2nd group with ranitidine elevated significantly ($P < 0.05$) serum levels of TNF-α and IL-1β while it reduced serum level of IL-10 as compared with the control group. However, administration of APE with ranitidine reduced significantly ($P < 0.05$) the elevated serum levels of TNF- α and IL-1β, while it increased serum level of IL-10 as compared with the ranitidine intoxicated group. Supplementation of rats with *APE* elevated significantly ($P < 0.05$) serum levels of IL-10 while it had no significant effects of serum levels of TNF-α and IL-1β as compared with the control rats.

A. pinnata extract ameliorated the deleterious effects of ranitidine on oxidative/antioxidant statues in hepatic tissues of rats:

The effects of ranitidine and/or *APE* on hepatic tissue lipid peroxidation and antioxidant defense system biomarkers of rats were shown in table 5. Oral administrated of the rats with ranitidine increased significantly hepatic tissue level of MDA ($P < 0.05$) as compared with the normal control rats. However, administration of APE with ranitidine reduced significantly MDA contents in the hepatic tissue ($P < 0.05$) as compared with ranitidine intoxicated group (2nd group). On the opposite, ranitidine reduced significantly GSH contents and SOD and CAT activities in hepatic tissues of the 2nd group as compared with the control group. However, supplementation of ranitidine intoxicated rats with *APE* elevated significantly GSH contents and SOD and CAT activities in hepatic tissues of 4th group as compared with the rats intoxicated with ranitidine alone (2nd group). *APE* itself had no significant effect on MDA and GSH contents and SOD and CAT activities in hepatic tissues of rats of the 2nd group as compared with normal control rats (table 5).

Table (1): The ration ingredient and chemical composition

Ingredient	Composition (g %)
Protein	17%
Fat	4.9%
Vitamin mixture	1%
Salt mixture	3.5%
Carbohydrates	68.16%
Fiber	3.44%
Choline chloride	٪٢

Table (2): Hematological changes in different albino rat groups after 30 days.

parameters	ControlI	RanitidineII	*APE*III	Ranitidine and *APE*IV
RBCs	6.33±0.13^{b}	7.63±0.29^{a}	6.44±0.28^{b}	6.36±0.92^{b}
Hb	11.72±0.58^{a}	8.17±0.33^{b}	10.96±0.39^{a}	8.2±0.73^{b}
WBCs	12.15±0.55^{c}	20.42±2.87^{a}	11.23±0.26^{c}	14.2±0.55^{b}
Granulocytes	7.28±0.28^{c}	8.19±0.47^{a}	7.3±0.24^{c}	8.68±0.75^{b}
Lymphocytes	3.35±0.37^{b}	11.45±0.74^{a}	3.15±0.63^{b}	5.14±0.75^{a}

- The values are expressed as the means ±SE.- Number of rats=10 - Values carrying different letters in the same row are significantly different, at P≥ 0.05- APE = Azollapinnataethanolic extract; WBCs = White blood cells; RBCs = Red blood cells, Hb = Haemoglobin.

Table (3): Changes in some liver and kidney functions in serum theef different albino rat groups after 30 days

parameters	ControlI	Ranitidine II	*APE*III	Ranitidine and *APE*IV
ALT (U/L)	19±2.82^{c}	31±0.25^{a}	18±2.79^{c}	26±2.61^{b}
AST (U/L)	112±2.35^{c}	161±3.50^{a}	113±2.48^{c}	132±5.69^{b}
Creatinine (mg /dl)	0.68±0.02^{b}	1.02±0.04^{a}	0.61±0.02^{b}	0.72±0.03^{b}
Urea (mg /dl)	18.41±1.03^{c}	33.03±0.75^{a}	20.60±0.46^{bc}	21.85±1.49^{b}

- The values are expressed as the means ±SE.- APE = Azollapinnataethanolic extract; ALT = Alanine aminotransferase; AST = Aspartate aminotransferase. - Number of rats=10- Values carrying different letters in the same row are significantly different, at P≥ 0.05

Table (4): Changes of TNF-α, IL-1β and IL-10 in serum of different albino rat groups after 30 days. The values are expressed as the means ±SE.

parameters	Control I	Ranitidine II	*APE* III	Ranitidine and *APE* IV
TNF-α (pg/ml)	81.20±2.19^{c}	98.79±2.23^{a}	80.40±2.53^{c}	87.50±2.66^{b}
IL-1β (pg/ml)	132.20±3.22^{c}	174.60±2.33^{a}	139.20±1.93^{c}	162.75±3.07^{b}
IL-10 (pg/ml)	5.74±0.74^{b}	3.79±0.26^{c}	7.34±0.46^{a}	5.63±0.37^{b}

- Number of rats=10- Values carrying different letters in the same row are significantly different, at P≥ 0.05- APE = Azollapinnataethanolic extract; TNF-α = tumor necrosis factor alpha; IL-1β = Interleukin 1 beta; IL-10 = Interleukin 10,

Table (5): Changes in lipid peroxidation and antioxidant biomarkers in hepatic tissue of different albino rat groups after 30 days

parameters	ControlI	Ranitidine II	APEIII	Ranitidine and *APE* IV
MDA (nmol/g tissue)	7.75±0.28^{c}	13.44±0.27^{a}	8.12±0.44^{c}	10.42±0.33^{b}
GSH (mmol/g tissue)	2.64±0.18^{a}	0.69±0.06^{c}	2.57±0.14^{a}	1.82±0.13^{b}
CAT (U/ g tissue)	0.64±0.01^{a}	0.29±0.03^{c}	0.60±0.01^{a}	0.38±0.02^{b}
SOD (U/g tissue)	3.23±0.07^{a}	1.87±0.05^{c}	3.69±0.07^{a}	2.93±0.09^{b}

- The values are expressed as the means ±SE.- Number of rats=10 - Values carrying different letters in the same row are significantly different, at P > 0.05- APE = Azollapinnataethanolic extract ; MDA = Malondialdehyde,; GSH = Reduced glutathione,; - CAT = Catalase, ; SOD = Super Oxide Dismutase,.

DISCUSSION

Ranitidine toxicity has been shown to disturb hepatic function (Parente *et al.*, 2003). The results of the current study showed that intoxication of rats with ranitidine induced hepatotoxicity represented by elevated activities of serum ALT and AST. This finding was in line with those of Gisbert *et al.* (2001) who indicated that ranitidine increases the activities of serum ALT and AST. This finding may due to ranitidine induced oxidative stress in hepatic tissues as it increased hepatic tissue contents of MDA while it deceased hepatic tissues contents of GSH and activities of SOD and CAT activity (table 4). These findings agreed with that of Luyendyk,*et al.* (2004) who indicated that ranitidine induces oxidative stress in hepatic tissues as a result of increasing of lipid peroxidation and disturbance of the antioxidant defense system in hepatocytes. Thus, ranitidine and its metabolites induce redox cycle with the generation of superoxide radicals and hydrogen peroxide, which subsequently increase lipid peroxidation and decrease antioxidant enzyme activities

resulting in hepatocytes destruction, activation of innate immunity by producing pro-inflammatory cytokines such as TNF-α and IL-1β in hepatic tissues (Francis *et al.,* 2007). Ranitidine damages many tissues through induction of oxidative stress (Bandyopadhyay *et al.,* 2001) also, ranitidine leading to lipid peroxidation, which induces inflammatory processes (Hitesh *et al.,* 2012). In addition, occupational exposure of human to ranitidine increases serum levels of some pro-inflammatory cytokines such as IL-1, IL-6, and TNF-a (Francis *et al.,* 2007). TNF-α is produced at the site of inflammation by activated macrophages and lymphocytes and participates with IL-1β and IL-6 to induce systemic inflammatory reactions (Turner *et al.,* 2014). Furthermore, intoxication of rats with ranitidine in the current study decreased serum level of the anti-inflammatory cytokine IL-10 (table 4). This finding was parallel with that of Mobarakeh *et al.* (2000) who indicated that exposure to ranitidine decreased IL-10 in the area of cerebral cortex of rats, that finding confirmed the role of ranitidine in the development of inflammatory response in rat brain tissue (Brijesh *et al.,* 2004). Such decrease in IL-10 due to ranitidine exposure may be implicated in the increased serum levels of IL-1β and promotion of inflammatory condition in ranitidine intoxicated rats as it has been indicated that IL-10 can block IL-1β gene expression (Wong *et al.,* 1997). These inflammatory cytokines may injury the hepatic tissues and TNF-α has been indicated to induce apoptosis of hepatocytes,while inhibition of TNF-α production or signaling pathways reduces hepatic injury induced by TNF-α (Turner *et al.,* 2014).In addition, ranitidine causes activation of innate immunity system by producing pro-inflammatory markers such as TNF-α and IL-1. Further, our study was in accordance with that of Francis *et al.* (2007) who reported that the ranitidine intoxication of rats induces hepatic tissues necrosis and increases serum transaminase activity and hepatic lipid peroxidation. Collectively ranitidine induced oxidative stress and increased pro-inflammatorymarkers.Finally, hepatic tissue injury damages hepatocytes and discharge of liver enzyme and consequently raised their activities (Selvaraj and Ranjana, 2015).

Regarding the ameliorative effects the *azollapinnata* alcoholic extract against ranitidine induced hepatotoxicity, our results showed that oral administration of *APEE* with ranitidine prevented increase inactivities of serum ALT and AST and kept serum levels of urea and creatinine (table 3) and hematological parameters (table 2) within normal range. These findings were in line with those of Debashis *et al.* (2016), who reported that *A. pinnata* reduces serum activities of ALT and AST. The ameliorative effects of *APE*against ranitidine induced hepatotoxicity may be attributed to the antioxidant and anti-inflammatory activities of its constituents.*A. pinnata* ethanolic extract was found to be rich in flavonoids, which well-known cause their antioxidant and anti-inflammatory activities (Chen *et al.,* 2019). In addition, quercetin and its glucoside moieties that produced by loss of glucoside annotated as quercetin-O-glucoside (hyperoside) or by loss of two glucoside unites that annotated as rutinwhich considered antioxidant (Dohaei *et al.,* 2020). Vitexin, which is flavone C-glycoside has been demonstrated possessing anti-inflammatory activity inhibiting IL-1β, (Borghi *et al.,* 2013) and TNF-α (Park *et al.,* 2016). Tamarixetin was investigated to exhibit superior anti-inflammatory activity by reducing the secretion of several inflammatory cytokine; besides, it showed higher anti-inflammatory activity (Park *et al.,* 2016). These compounds may reduce lipid peroxidation and inflammatory cytokines while increased antioxidant and anti-inflammatory activities as shown in tables (3 and 4) which consequently, modulatedranitidine inducedapoptosis and injury of hepatic tissues. These beneficial effects of *APE* against ranitidine induced hepatotoxicity confirmed the findings of Debashis *et al.* (2016) who indicated that *APE* suppresses lipid peroxidation and scavenges free radicals preventing the pathological changes in hepatic tissues architecture (Selvaraj and Ranjana, 2015). Quercetin normalizes thioacetamide increased liver function biomarkers, ALT and AST, through inhibition of change of p-ERK1/2 and the increase in Bax/Bcl-2 ratio preventing cell apoptosis(Russo *et al.,* 2014). In addition, quercetin suppresses prenatal stressincreased serum IL-1β levels through increasing serum IL-10 levels in rats (Wang,*et al.,* 2012). Thus, the hepatoprotective effect of *APE* might attribute to free radical scavenging activity

(De David *et al.,* 2011). Rutin suppresses the activity of pro-inflammatory cytokines through diminishing TNF-α and IL-1β levels (Mkhize *et al.,* 2017)

CONCLUSION:

Ranitidine induced hepatotoxicity in rats through oxidative stress increased pro-inflammatory cytokines and pro-apoptotic protein in hepatic tissue. However, *A. pinnata* alcoholic extract ameliorated ranitidine induced hepatotoxicity through reducing oxidative stress and pro-Apoptotic protein expression in hepatic tissue and pro-inflammatory cytokines production and increasing ani-inflammatory cytokine production and antioxidant activities in hepatic tissue. Thus, *A. pinnata* alcoholic extract is a considered hepatoprotective agent against ranitidine induced hepatotoxicity.

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