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Antioxidant and antiapoptotic effects of *Hydroclathrus clathratus* on hepatic dysfunction in alloxan-induced *Diabetes mellitus* in male albino rats

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ABSTRACT

Background: *Hydroclathrus clathratus* is considered to have protective effects against several diseases. The hepatic dysfunction associated with *Diabetes mellitus* (DM) has been reported and was found to be associated with oxidative damage. This study was conducted to evaluate the role of *H.clathratus* to protect against alloxan -induced liver dysfunction in rats. **Method**: Alloxan was administered i.p. in a single dose (150 mg/kg) to adult male rats. Alloxan-induced diabetic rats were orally administered hot water extract of *H.clathrus* (HWHC) 400 mg/kg body weight of rats daily for 30 days after alloxan injection. **Result**: Alloxan administration to rats resulted in significant elevation of serum transaminases (sALT and sAST), depletion of hepatic reduced glutathione (GSH), catalase (CAT) and glutathione peroxidase (GPx), elevation of lipid peroxides (LPO) expressed as malondialdehyde (MDA). Significant rises in liver tumor necrosis factor-alpha (TNF-α) and caspase-3 levels were noticed in alloxan-induced diabetic. Treatment of the alloxan-induced diabetic rats with HWHC significantly prevented the elevations of sALT and sAST, inhibited depletion of hepatic GSH, GPx, CAT and inhibited MDA accumulation. Furthermore, HWHC had normalized serum total proteins and hepatic CAT, TNF- α and caspase-3 levels of alloxan-induced diabetic rats In addition, HWHC prevented the alloxan -induced apoptosis and liver injury as indicated by the liver histopathological analysis. Results showed significant correlation in either alloxan HWHC group between TNF- α and each of serum ALT, AST and liver GPX, CAT, GSH, MDA and caspase-3 levels. **Conclusion**: our data indicate that HWHC protects against alloxan -induced liver injury in rats through antioxidant, anti-inflammatory and antiapoptotic mechanisms. However, further merit investigations are needed to verify these results and to assess the efficacy of HWHC therapy to counteract the liver dysfunction and oxidative stress status. © 2015 Trade Science Inc. - INDIA

KEYWORDS

Alloxan;
Hydroclathrus clathratus;
Hepaatic dysfunction;
Oxidative stress;
Caspase-3;
Tumor necrosis factor- α.

INTRODUCTION

Although, the spread of folk traditional medicine, recent pharmaceutical research is also focusing on marine organisms that have developed biologically unique molecules as sulphated poly saccharides for their biological activity[1]. As a consequence of the research efforts, it is clear that the marine environment represents an important source of unknown natural compounds whose medicinal potential must be evaluated. Recent studies in the field of diabetic research have revealed promising compounds, isolated from natural sources, with proven antidiabetic activity[13].

Brown marine algae to be rich sources of antioxidant compounds with potential free radical scavenging activity that may be useful in prevention and treatment of various diseases caused by oxidative damage. Fucoidans, polysaccharides containing substantial percentages of L-fucose and sulfate ester groups, are constituents of brown algae that have numerous other biological properties such antioxidant, anti-inflammatory immuno-modulatory and apoptosis-inducing activities[33].

Hydroclathrus clathratus (C. Agardh) Howe is brown marine algae and considered to be a traditional drug and health food in Korea, Japan and China. Hot water extract of H. clathratus (HWHC) is rich in of water-soluble sulfated polysaccharides that exhibit valuable biological effects as anticancer, anti-herpetic and anti-coagulant activities.

Alloxan, a β-cytotoxin, has demonstrated severe physiological and biochemical derangements of the diabetic state. The alloxan rats exhibited severe glucose intolerance and metabolic stress as well as hyperglycemia due to a progressive oxidative insult interrelated with a decrease in endogenous insulin secretion and release[2].

When alloxan monohydrate is injected into various laboratory animals, destruction of insulin-secreting β cells in the islets of Langerhans occurs, while other cells (α, g, d) are resistant to alloxan. Disappearance of β cells within a few days is accompanied by typical and permanent hypoinsulinaemia and hyperglycaemia. Alloxan-treated animals were considered as excellent tools to study the pathogenesis of human diabetes, although in alloxan diabetes, in contrast to T1D in humans, there is no autoimmune component and no insulin resistance as in T2D. Thus alloxan diabetes can be regarded as a pure form of hypoinsulinaemia[20].

Alloxan is toxic glucose analogues that preferentially accumulate in pancreatic beta cells *via* the Glucose transporter -2 (GLUT2). In the presence of intracellular thiols, especially glutathione; alloxan generates reactive oxygen species (ROS) in a cyclic redox reaction with its reduction product, dialuric acid[1]. Autoxidation of dialuric acid generates superoxide radicals, hydrogen peroxide and, in a final iron-catalysed reaction step, hydroxyl radicals. These hydroxyl radicals are ultimately responsible for the death of the beta cells, which have a particularly low antioxidative defence capacity[19].

In this study, we aimed to evaluate the role of *H. clathratus* intake to aloxan-intoxicated rats via monitoring the liver histopathological changes and the go insight the changes of different biochemical parameters such as serum alanine transaminase (sALT), aspartate transaminase (sAST) and endogenous hepatic antioxidants e.g. reduced glutathione (GSH), and catalase (CAT) enzyme levels; lipid peroxides expressed as malondialdehyde (MDA). Moreover, the hepatic tissue damage marker; tumor necrosis factor (TNF-α) and an apoptotic marker; caspase-3 were measured.

MATERIALS AND METHODS

Animal license

Maintenance and treatment of all the animals was done in accordance with the principles of Institutional Animal Ethics Committee constituted as per the directions of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Egypt

Animals and experimental design

130–190 g were used as experimental animals in this study. total of 30 adult male Swiss albino rats, weighing The animals were kept in wire-floored cages under standard laboratory conditions of 12 h/12 h light/dark, 25±2 °C with free access to food and water.. The rats were randomly divided into three groups of ten animals, each as follows:

- **Group 1:** NC rats: normal control untreated rats received orally an equivalent volume of

normal saline based on body weight

- **Group 2:** (DC)Alloxan-induced diabetic rats: rats were treated with single dose of alloxan i.p. (150 mg kg-1) dissolved in normal saline
- **Group 3: (**HWHC) H.clathrus rats: rats were orally administered HWHC (400 mg kg-1 day-1) for 30 days.

The selection of HWHC doses used in this study, was based on the work conducted by other investigators[21]. After the last treatment, rats were fasted for 8 h. Animals were subjected to light ether anesthesia and sacrificed by cervical dislocation. The blood sample were collected and centrifuged to obtain serum in order to estimate total proteins, sALT, sAST and sALP. Each right hepatic lobe sample was washed thoroughly in ice-cold saline to remove the blood after thawing, blotted the saline gently using filter paper. A 10% of liver homogenate was prepared in ice-cold 0.1M potassium phosphate buffer, pH 7.5. The obtained rat liver homogenate aliquoted and immediately frozen at -80 °C for biochemical analysis.

Estimation of serum hepatic enzymes (sAST and sALT)

To assess the liver function, the serum activity (U/l) of sAST and sALT were analyzed. The sAST was determined spectrophotometrically at 340 nm in presence of α-ketoglutarate, aspartate,NADHand malate dehydrogenase. The sALT was assayed in presence of α-ketoglutarate, pyruvate, NADH and lactate dehydrogenase at 340 nm[30].

Determination of MDA in liver homogenate

The lipid peroxidation level in rat liver homogenate was measured as MDA which is the end product of lipid peroxidation that reacts with thiobarbituric acid (TBA) as a TBA reactive substance (TBARS) to produce a red colored complex which has peak absorbance at 532 nm[19]. Phosphoric acid 1% (3 ml) and TBA 0.6% (1 ml) was added to 0.5 ml of liver homogenate in a centrifuge tube and the mixture was heated for 45 min in boiling water bath. After cooling, 4ml of n-butanol was added to the mixture and vortexed for 1 min followed by centrifugation at 20,000 rpm for 20 min. Organic layer was transferred to fresh tube and its absorbance was measured at 532 nm[6].

Determination of GPx and CAT activities in liver homogenate

Determination of GPx activity in rat liver homogenate is based on the oxidation of GSH by GPx, using t-butyl hydroperoxide as a substrate, coupled to the disappearance of NADPH by glutathione reductase[7]. The results expressed as mU/mg protein. The CAT activity was measured using H2O2 as substrate that can be decomposed by CAT enzyme. A mixture of 50mMphosphate buffer (pH 7.0), 20mMH2O2 and 0.1 ml liver homogenate in a final volume of 3ml was incubated at room temperature for 2 min. The change in absorbance at 240 nm in 2 min was calculated. One unit of CAT is defined as the amount needed to decompose 1 nmol H2O2 of per minute and the specific activity is expressed as μ moles H2O2 decomposed/min/mg protein[4].

Estimation of GSH in liver homogenate

To estimate the GSH liver homogenate content, liver homogenate (0.5 ml) was mixed with 0.5 ml of 10% trichloroacetic acid. The contents were mixed well for complete precipitation of proteins and centrifuged at 2000 rpm for 5 min. An aliquot of clear supernatant (0.1 ml) was mixed with 1.7 ml of 0.1M potassium phosphate buffer (pH 8). A 0.1 ml of DTNB was added. After 5 min, the absorbance was measured at 412 nm against blank[5]. The GSH value was expressed as mg/gm tissue.

Determination of TNF-α in liver homogenate

The determination of TNF-α in rat liver homogenate involved solid phase sandwich ELIZA using two kinds of high specific antibodies. Tetra methyl benzidine was used as chromogen. The strength of color measured at 450 nm is proportional to the quantities of rat TNF-α that expressed as pg/gm liver.

Estimation of caspase-3 level in liver homogenate

The caspase-3 colorimetric assay in liver rat homogenate (U/mg protein) based on the spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA (acetyl-Asp-Glu-Val-Asp p-nitroanilide). The pNA can be quantified using spectrophotometer at 405 nm.

Liver histological examination

The liver tissues were removed, plotted with normal saline between filter paper and fixed in 10% neutral buffered formalin and subsequently embedded in paraffin and sliced into slices of 5_m thickness followed by staining with hematoxylin and eosin and examined under light microscope (Olympus BX-200, Tokyo, Japan).

Statistical analysis

Statistical analysis was carried out using GraphPad Instat software (version 3, ISS-Rome, Italy). Unless differently specified, groups of data were compared with un-paired t-test and one-way analysis of variance (ANOVA) followed by Tukey-kramer (TK) multiple comparisons post-test. Values of P<0.05 were regarded as significant. The data, as clearly indicated are reported in tables and figures as mean ± standard error (S.E).

TABLE 1 : Effect of treatments on sALT and sAST in alloxan induced diabetic rats.

Group	sALT	sAST
	U/l	U/l
Normal (N)	19.51±0.84	23.17±0.75
Diabetic Control (DC)	62.39±0.87***[a]	84.29±0.94***[a]
Diabetic+ HWHC (DC + HWHC)	30.58±0.81[b]	32.89±0.69[b]

Values significantly different compared to normal P‹0.001; * Values are expressed as means ± SE.Means not sharing common letter are significantly different (p‹0.05) based on one –way ANOVA with Tukey's post –hoc test.**

RESULTS

Alloxan intake to normal rats showed significant elevations of sALT and sAST levels compared to the normal control rats, $p < 0.001$. The oral administration of HWHC to diabetic rats at a dose of 400 mg/kg body weight of rats for 30 days blocked the Alloxan-induced elevations of sALT and sAST as noticed by significant decrease compared to diabetic rats, $p < 0.001$. (TABLE 1).

Alloxan –induced diabetic rats produced significant increment in hepatic MDA levels as compared to normal control rats, $p < 0.001$. HWHC administration reduced significantly the liver MDA level by compared to diabetic rats, $p < 0.001$(TABLE.2).

Alloxan significantly decreased the GSH level compared to control rats, $p < 0.001$ (TABLE. 3C). The HWHC intake to diabetic rats produced significant elicited an increase in hepatic GSH level compared to diabetic, $p < 0.001$ (TABLE. 2).

HWHC exhibited significant increase in liver CAT level compared to the control rats, $p < 0.001$ (TABLE 3). Alloxan significantly decreased hepatic diabeeetic rats produced significant increase in liver CAT level compared to diuabetic rats, $p < 0.001$ (TABLE. 3).

On the other hand, alloxan significantly decreased

TABLE 2 : Effect of treatments on level of hepatic MDA and hepatic GSH in alloxan induced diabetic rats.

Group	Hepatic TBARS	Hepatic GSH
	nM TBARS/mg protein	mg/gm tissue
Normal (N)	46.39±5.2	16.23±1.23
Diabetic Control (DC)	1248±133***[a]	10.23±0.69***[a]
Diabetic+ HWHC (DC + HWHC)	542.36±29.8 [b]	24.89±1.88 [b]

Values significantly different compared to normal P‹0.001; * Values are expressed as means ± SE.Means not sharing common letter are significantly different (p‹0.05) based on one –way ANOVA with Tukey's post –hoc test.**

TABLE 3 : Effect of treatments on activity of hepatic CAT, hepatic GSH-Px and hepatic SOD in alloxan induced diabetic rats.

Group	Hepatic CAT	Hepatic GSH-Px	Hepatic SOD
	µ moles H2O2 decomposed/min/mg protein,	µg GSH consumed/min/mg protein	Units/min/mg protein
Normal (N)	234.25±9.58	681.23±26.36	308.23±30.25
Diabetic Control (DC)	174.36±11.89***[a]	453.69±33.26***[a]	235.16±23.06***[a]
Diabetic+ HWHC (DC + HWHC)	248.69±17.06[b]	648.32±30.41 [b]	299.32±23.28 [b]

Values significantly different compared to normal P‹0.001; * Values are expressed as means ± SE.Means not sharing common letter are significantly different (p‹0.05) based on one –way ANOVA with Tukey's post –hoc test.**

TABLE 4 : Effect of treatments on activity of hepatic caspase-3 and level of hepatic TNF-α in alloxan induced diabetic rats.

Group	Hepatic caspase-3 (U/mg protein)	Hepatic TNF-α (pg/gm liver)
Normal (N)	0.6±0.06	86±7.5
Diabetic control (DC)	1.8±0.05***[a]	498±40.25***[a]
Diabetic+ HWHC (DC + HWHC)	0.7±0.06 [b]	189±9.84 [b]

Values significantly different compared to normal P‹0.001; * Values are expressed as means ± SE.Means not sharing common letter are significantly different (p‹0.05) based on one –way ANOVA with Tukey's post –hoc test.**

the liver GPx level compared to control rats, $p < 0.001$ (TABLE. 3A). HWHC intake to alloxan –induced diabetic rats produced significant increase in GPx level compared to alloxan-intoxicated rats, $p < 0.001$ (TABLE. 3).

Caspase-3-activity was significantly increased upon administration of alloxan to normal rats as compared to diabetic rats (Figure 4B). This increase was significantly declined upon HWHC intake to diabetic compared to alloxan –induced diabetic rats, $p < 0.001$ (TABLE. 4).

Additionally, alloxan significantly increased the hepatic level of TNF-α compared to control rats (TABLE. 4A). Administration HWHC to alloxan -treated rats decreased the hepatic TNF-α level significantly compared to the diabetic rats, $p < 0.001$ (TABLE. 4).

To confirm the protective effect of HWHC on alloxan-induced liver tissue damage, we performed histological examinations. The normal control rat liver showed normal architecture of hepatic lobules and hepatocytes. The hepatocytes form columns of cells adherent to each other by one or more surfaces. The sinusoids were variable in diameter and lined with discontinuous sheet of endothelial cells with flat nuclei (Figure 1).

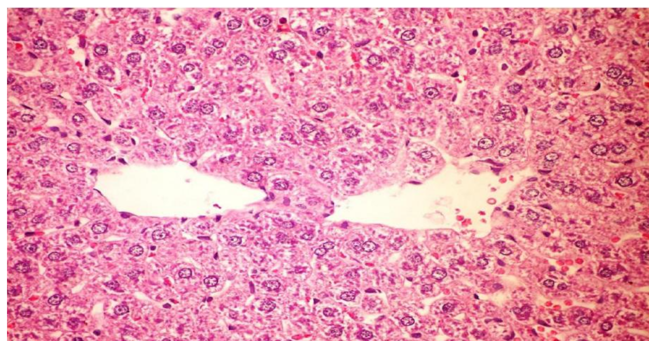

Figure 1

However, Alloxan-treated rat liver elicited decrease in the number of hepatocytes and widely dilated central veins. The cytoplasm showed area of hemorrhage and

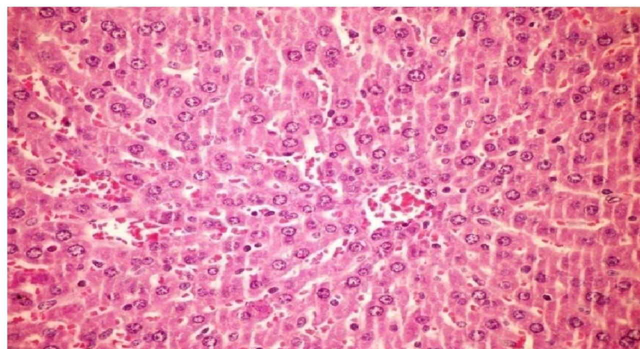

Figure 2

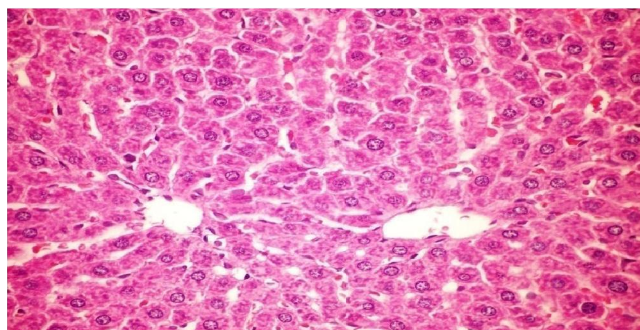

Figure 3

inflammatory cell infiltration around the blood sinusoids which appeared widely dilated (Figure 2).

Alloxan –treated rats administered HWHC revealed degeneration of some hepatocytes and normal architecture of the others. The blood sinusoids are still widely dilated and appeared congested and showed inflammatory cell infiltration. The diabetic rats administered HWHC showed marked regeneration of the hepatocytes with preservation of the normal hepatic architecture (Figure 3).

DISCUSSION

DM causes a disturbance in the uptake of glucose, as well as glucose metabolism. The liver plays an important role in the maintenance of blood glucose levels

by regulating its metabolism[23].

The present investigation indicated that, a single dose of alloxan (150 mg/kg) intraperitoneally to adult male albino rats (210-220g) was suitable to induce histological changes in the liver of alloxan induced diabetic rats with characterized appearance, enlarged and swollen hepatocytes.

DM induces the growth of HSCs via MAP kinase pathways, which are activated by ROS produced by the NADPH oxidase system under the regulation of protein kinase C. On other hand, hepatic oxidative stress induces proinflammatory cytokines, such as TNF-α, transforming growth factor-β (TGF-β), interleukin-1β, and interleukin-6, which are critical for HSC activation and perpetuation[29].

The enlarged hepatocytes showed intense cytoplasmic staining with Periodic Acid-Schiff stain, and negative staining with Periodic Acid-Schiff Diastase. This is suggestive of glycogen accumulation and consistent with glycogenic hepatopathy. The present dose as well as the observed histopathological and biochemical manifestations agree with the literature of[30].

Alloxan causes significant increase in activity of sAST, sALT, hepatic glycogen phosphorylase, hepatic G6Pase, hepatic caspase-3 and levels of hepatic MDA and hepatic TNF-α. On other hand, Alloxan causes significant decrease in hepatic GSH content and activity of hepatic SOD, CAT, and GPx in accordance with[9].

In alloxan inducd diabetic rats, the changes in the levels of sAST, sALT, are directly related to changes in metabolism in which the enzymes are involved. The increased activities of transaminases, which are active in the absence of insulin due to the availability of amino acids in the blood of DM and are also responsible for the increased gluconeogenesis and ketogenesis[3].

On other hand[26], revealed that glucose-6-phosphatase is one of the important regulatory enzymes of the gluconeogenic pathway. The activity of glucose-6-phosphatase and glycogen phosphorylase are increased in the liver of alloxan-induced diabetic rats. This results in a decrease in the glycolytic flux. Under normal condition, insulin functions as a suppressor of gluconeogenic enzymes.

Also, Francés et al.[8] revealed that DM promoted a significant increase in hydroxyl radical production which correlated with lipid peroxidation (LPO) levels. Besides, hyperglycemia significantly increased mitochondrial

BAX protein expression, cytosolic cytochrome c levels, and caspase-3 activity leading to an increase in apoptotic index

Hamden et al.[10] revealed that liver is bombarded by ROS that can directly cause inflammation within the liver cells, which then release further pro-inflammatory cytokines, leading to more hepatocyte injury and accelerated apoptosis that affect the integrity and architecture of liver cells[17].

As consequence of DM, the hepatocellular accumulation of triglycerides, initially leads to an altered metabolism of glucose and free fatty acids in the liver. Increased expression of death receptors in response to this altered hepatic metabolism enhances the hepatocytes' susceptibility for pro-apoptotic stimuli, thus eliciting excessive hepatocyte apoptosis and inflammation[31,16].

Ingaramo et al.[16] explained that DM enhances TNF-α in the liver, which may be a fundamental key leading to apoptotic cell death, through activation of caspase-3, NFκB led to an induction of iNOS and consequent increase in NO production.

Oral administration of HWHC causes significant decrease in activity of sAST, sALT, hepatic Glycogen phpsphorylase, hepatic G6P in accordance with. Furthermore, oral administration of HCE causes significant decrease in hepatic MDA and significant enhancement in level of hepatic GSH and the activity of hepatic CAT, hepatic GSH-Px and hepatic SOD in accordance with[4] who suggests that the antioxidant activities of sulphated polysaccharides has been attributed to various mechanisms including the prevention of chain initiation, the binding of transition metal ion catalysts, the decomposition of peroxides, and the prevention of continued hydrogen abstraction and radical scavenging[18].

Oral administrationof HWHC causes significant decrease in level of hepatic TNF-α and activity of hepatic caspase-3 in accordance with[33] who revealed that anti apoptotic activity of HWHC may be mediated via both the death receptor-mediated and mitochondria-mediated apoptotic pathways.

Damage to hepatocytes and activation of hepatic stellate cells are key events in liver fibrosis, and, interestingly, treatment of hepatocytes with HWHC pre-

vented cell death and inhibited the proliferation hepatic stellate cells. So, HWHC might be a promising antifibrotic agent possessing dual functions, namely, protection of hepatocytes and inhibition of hepatic stellate cell proliferation[12].

Hepatic protective effect of HWHC was further evidenced by histological observations made on the hepatic tissue of HCE treated rats that showing no histological changes, moderate PAs particles distributed throughout the hepatocytic cytoplasm

In conclusion, although additional studies are needed, it could be suggested that HWHC could partly protect hepatocytes through antioxidative, anti-inflammatory and antiapoptotic mechanisms against liver injury induced by alloxan. The signaling mechanisms associated with protection against the liver damage and oxidative stress status induced by alloxan via intake of HWHC still need merit further investigations.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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