Moringa oleifera Leaf Ethanolic Extract Subsidized by Low Doses of Gamma Irradiation Modulates the Thioacetamide Induced Fibrotic Signs in Liver of Albino Rats

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Abstract.-The objective was to evaluate the role of ethanolic extract of Moringa oleifera leaf (MOE) administered orally followed by low doses of gamma-irradiation (LDR) on thioacetamide (TAA) induced liver fibrosis in female albino rats. LDR treatment was performed by whole body gamma-irradiation of animals with 0.25Gy applied on the 1st and 15th day. Biochemical analysis in the serum of TAA-treated rats revealed a significant increase in the levels of tumor necrosis factor (TNF-α) and transforming growth factor (TGF-β), as well as, the activities of aspartate amino transferase (AST), alanine aminotransferase (ALT) and gamma glutamyl transferase (GGT). In addition, a significant elevation of malondialdehyde (MDA), hydroxyproline (HYP) and nitric oxide (NO) levels associated to a significant decrease of reduced glutathione (GSH) content was recorded in the liver of TAA-treated rats. Histopathological examination of liver sections depicted fibroblasts proliferation surrounding cytoplasmic hepatocytes. A significant amelioration of liver damage was recorded in rats receiving MOE and/or LDR treatment. It could be concluded that exposure to LDR intensifies the effectiveness of MOE against liver fibrosis induced by TAA in rats.

Key words: Liver fibrosis, Moringa oleifera, gamma-irradiation, thioacetamide.

INTRODUCTION

Liver is a vital organ that plays a role in controlling critical biochemical and physiological activities including homeostasis, growth, energy and nutrient supply, detoxification of drugs and other xenobiotics, and also combating infections (Olorunnisola et al., 2011). Chronic liver diseases are major global health problems causing approximately 800,000 deaths per year worldwide. The Egyptian population has a heavy burden of liver disease, mostly due to chronic infection with hepatitis C virus (Kurbanov et al., 2010), alcoholism, virus-induced chronic liver disease, and using hepatotoxic drugs (antibiotics, carbon tetrachloride, thioacetamide, and acetaminophen) are the major risk factors for liver diseases (Saleem et al., 2010). Thioacetamide (TAA) is a typical hepatotoxin, causing centrilobular necrosis. It induces apoptosis in the rat liver based on histochemical observations (Ledda-Columbano et al., 1991).

Medicinal plants have been used from ancient times for the treatment of a wide variety of diseases (Khosravi-Boroujeni et al., 2012) as well as for hepatotoxicity (Heidarian and Rafieian-Kopaei, 2013). In fact, owing to its lower costs and greater compatibility, herbal medicine has received a great attention in recent decades (Azadbakht et al., 2003; Dkhil et al., 2015). Herbs are rich in different compounds such as triglycerides, flavonoids, and polyphenols that can protect the liver against damages induced by hepatotoxic drugs (Galisteo et al., 2000).

Moringa oleifera Lam belongs to Moringaceae family which accounts fourteen species. Moringa oleifera has been reported to possess anti-cancer (Tiloke et al., 2013) anti-inflammatory (Lee et al., 2013) antioxidant (Hamza, 2010) and hypoglycemic properties (Edoga et al., 2013). In addition, it was found to be thyroid status regulator (Tahiliani and Kar, 2000).

Pretreatment with nonlethal low-dose irradiation has been shown to have a protective effect against oxidative injury in animal tissues. At
low doses, radiation is generally regarded as safe, and its effect, if any, is considered to be negligible. Induction of hormesis and adaptive response by low dose of radiation (LDR) has been extensively indicated (Takahashi et al., 2000). Adaptive response induced by LDR was not only resistant to damage caused by a subsequently high-dose radiation, but also cross-resistant to other non-radiation challenges, such as chemicals. Mechanisms by which LDR induces the preventative effect on radiation - or chemical-induced tissue damage is by expression of protective proteins, such as heat shock proteins and antioxidants. LDR significantly increases endogenous antioxidants in different tissues including liver, spleen, brain and testes (Yamaoka et al., 1991; Zhang et al., 1998; Kojima et al., 1999).

The objective of this study was to elucidate the role of Moringa oleifera leaf ethanolic extract (MOE) either subsidized or not by exposure to LDR against liver fibrosis induced by TAA in female albino rats.

**MATERIALS AND METHODS**

*Plant material collection and preparation of extract*

The leaves of *M. oleifera* were harvested from different trees cultivated in Egypt. The leaves were first rinsed with distilled water, dried in shade and were completely extracted with ethanol (70%) using Soxhlet apparatus for 3 days. The percolated extract was then dried in vacuum using Rotary Evaporator apparatus (Model RE52A, China), weighed and dissolved in double-distilled water to give the final concentration of 250 mg extract /kg body weight with the help of a cyclomixer just before oral administration (Sinha et al., 2012).

*Chemicals and reagents*

TAA and all chemicals were purchased from Sigma-Aldrich Corporation (St Louis, MO, USA). *Moringa oleifera* leaves was obtained from the Egyptian Society of *Moringa*, National Research Center, Giza, Egypt.

*Experimental design*

The study was performed on 6 weeks old female albino rats weighing 120-150g, obtained from the Egyptian Holding Company for Biological Products and Vaccines Cairo, Egypt. The animals were kept in an environment with controlled temperature (25°C), humidity (45–75%), and photoperiod (12-h/12-h light/ dark cycle). All animals had free access to chow and water. All animal care protocols were in accordance with and approved by the Institutional Animal Ethics Committee. Rats were divided into 8 equal groups (6 rats/group) (a) Control group: animals received via oral tube 0.5 ml normal saline for four weeks, (b) MOE group: rats received via oral tube 0.5 ml of MOE (250 mg/kg) for four weeks (Gunjal et al., 2010), (c) LDR group: rats whole body exposed to 0.25Gy gamma radiation on the 1st and 15th day of the experiment to reach 0.5Gy and received 0.5 ml normal saline via oral tube during the four weeks, (d) MOE + LDR group: rats received via oral tube 0.5 ml of MOE for four weeks and exposed to 0.25Gy on the 1st and 15th day, (e) TAA: rats received TAA (200 mg/kg, i.p.) (Amin et al., 2012) dissolved in normal saline three times/week for four weeks. (f) TAA + MOE group: rats received TAA and via oral tube 0.5 ml of MOE (f) TAA + LDR group: rats received TAA and exposed to 0.25Gy on the 1st and 15th day (g) TAA +MOE + LDR group: rats received TAA, 0.5 ml of MOE and exposed to 0.25Gy on the 1st and 15th day. At the end of MOE treatment, the animals were sacrificed, blood samples and organ tissues were collected for biochemical and histopathological examinations.

*Radiation facility*

Whole body gamma-irradiation of rats was performed with a Canadian gamma cell-40, (137Cs) at the National Center for Radiation Research and Technology (NCRRT), Cairo, Egypt at a dose rate of 0.45 Gy /min.

*Biochemical assay*

Lipid peroxidation in liver homogenate was measured by thiobarbituric acid assay, which is based on malondialdehyde (MDA) reaction with thiobarbituric acid forming thiobarbituric acid reactive substances (TBARS), a pink colored complex exhibiting a maximum absorption at 532 nm according to (Yoshioka et al., 1979). The reduced glutathione (GSH) content in liver
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homogenate was determined photometrically at 412 nm using 5, 5-dithiobis-2-nitrobenzoic acid according to (Ellman, 1959). Serum transforming growth factor-beta (TGF-β) and tumor necrosis factor alpha (TNF-α) were detected by using ELISA procedure as described by Kim et al. (1994) and Corti et al. (1992), respectively by Avi-Bion ELISA Kit (Orgenium Laboratories, Finland), using ELISA microplate reader (DV 990 BV 4/6; Gio. De Vita & Co., Rome, Italy). Serum activities Aspartate (AST) and alanine amino-transferase (ALT) were determined according to Reitman and Frankel (1957) and gamma glutamyl transferase (GGT) according to Szasz (1974). Serum Nitric oxide (NO) was measured according to the method of Miranda et al. (2001). Hydroxyproline (HYP) in liver homogenate was hydrolyzed with 12N HCl at 110°C for 18 hours then oxidized into pyrrole followed by coupling with p-dimethyl-amino-benzaldehyde and the developed red color was measured spectrophotometrically at 456nm according to (Bergman and Loxley, 1963). All photometric determinations were done using (Thermo Electron UV-Visible spectrophotometers U.S.A.

Histopathological study

Part of the liver was fixed in 10% formalin and embedded in paraffin. Sections of tissues were stained with Haematoxylin and eosin according to the method adopted by Stevens et al. (1982) and examined by light microscope for histopathological investigation.

Statistical analysis

Statistical analyses of all data were presented as the mean ± standard derivation (SD). Statistical analyses were performed by one-way ANOVA test. Differences were considered statistically significant for values of P < 0.05. All data were analyzed by SPSS PC-software version 15.0 for Microsoft Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

The administration of TAA to female albino rats induced a significant increase in serum TNF-α and TGF-β compared to control (Table I).
Administration of the ethanolic extract of *Moringa oleifera* leaves (MOE) and/or LDR has significantly reduced this increase (*P*<0.05). However, the amelioration was more pronounced (*P*<0.05) when MOE treatment was subsidized by LDR (Table I).

The administration of TAA to female albino rats provoked a significant elevation (*P*<0.05) of MDA, NO and HYP contents comonitmit with a significant decrease (*P*<0.05) of GSH content compared to control (Table I). The toxic effect of TAA was significantly improved by the oral administration of MOE and/or LDR. The exposure of TAA+MOE treated rats to LDR has significantly reduced the MDA content associated with a significant increase of GSH when compared to TAA+MOE treated rats, while had no significant effect on NO and HYP contents (Table I).

In TAA-treated rats the activity of aspartate (AST) and alanine aminotransferase (ALT) and gamma glutamyl transferase (GGT) showed a significant increase (p<0.05) compared to control (Table I). Administration of MOE and/or exposure to LDR has significantly decreased the activity of liver enzymes compared to TAA-treated group. Nevertheless, exposure to LDR had no significant effect on the amelioration recorded by MOE treatment (Table I).

The histological examination of the liver obtained from control, MOE, LDR and MOE+ LDR groups showed the normal histological structure of hepatic lobule which consists of central vein and concentrically arranged hepatocytes (Fig. 1). However, the liver of TAA-treated rats revealed pseudolobulation of hepatocytes surrounded by fibroblasts proliferation (arrow) (Fig. 1E). While the liver of TAA+MOE rats showed oval cells proliferation, newly formed bile ductules and deposition of haemosidrin pigments (arrow) (Fig.1F). The liver of TAA+LDR showed strands of fibroblasts proliferation surrounding cytomegalic hepatocytes (arrow) (Fig.1G). The liver of TAA + MOE+LDR showed preserved normal architecture but with scanty fibroblasts between vacuolated hepatocytes (arrow) Figure 1H.

**DISCUSSION**

Liver fibrosis, a multifactor syndrome could contribute to the presence of serious disorders. It could be a consequence of chemical inducers, chronic alcoholic abuse, viral hepatitis, or inherited metabolic disease (Schuppan and Afdhal, 2008). The administration of chemical inducers (as, TAA) exerts case mimics to human liver cirrhosis (Aydin et al., 2010). The data obtained shows an increase in serum fibrotic signs cytokines, (TNF-α and TGF-β) in TAA rats compared to control rats (Table I). These results are in agreement with those obtained by Darwish and Abdelaziz (2006), Salama et al. (2013) who reported that TAA administration provoked a significant elevation in serum TNF-α and TGF-β. This could be interpreted in the view of hepatotoxic metabolites formation during TAA metabolism. TAA is converted to thioacetamide-S-oxide (TASO) by the action of liver mixed function oxygenases system, this metabolite is further oxidized to other cytoxic species that injured another organs (Spira and Raw, 2000). The hepatotoxins formed during TAA metabolism attack membranes protein and lipids causes alteration in cell permeability (Minnady et al., 2010) and pave to inhibition of mitochondrial activity followed by hepatocyte necrosis (Alshawsh et al., 2011). TAA could increase the level of serum TGF-β, a prominent profibrogenic cytokine with antiproliferative effects (Gressner et al., 2002) through different mechanisms. The free radicals produced during TAA metabolism may activate myofibroblasts that secrete fibrinogen and the growth factors (like TGF-β) (Bassiouny et al., 2011). Further, TAA might activate hepatic stellate cells (HSCs) as reported by Wang et al. (2012). The activated HSCs produce a number of profibrotic mediators (TGF-β) that promote their constrictive, proliferative, and transformative properties in an autocrine manner, promoting the development of liver fibrosis (Borkham-Kamphorst et al., 2007). TNF-α is a multifunctional cytokine that in the liver acts as a mediator of the acute phase response and is a cytotoxic agent in many types of hepatic injury (Liedtke et al., 2013). Liver macrophages (Kupffer cells), could be activated in response to free radicals during TAA metabolism producing mitogens as (TNF-α) (Harstad and Klaassen, 2002; Lochner et al., 2009) which drive apoptosis and steatosis of hepatocytes (Liedtke et al., 2013). The increase of
Fig. 1. Histological structure of rat liver. A, control rat showing the normal architecture of hepatic lobule; B, MOE treated rat showing no histopathological changes; C, exposed to LDR showing normal architecture; D, MOE + LDR treated rat showing Kupffer cells activation; E, TAA treated rat showing pseudo-lobulation of hepatocytes surrounded by fibroblasts proliferation; F, TAA+ MOE treated rats showing oval cells proliferation, newly formed bile ductuoles and deposition of haemosidrin pigments; G, TAA + LDR treated rat showing strands of fibroblasts proliferation surrounding cytomegalic hepatocytes; H, TAA + MOE + LDR treated rat showing scanty fibroblasts between vacuolated hepatocytes (H&E X400).
fibrogenic cytokines observed in TAA rats is accompanied by increase in HYP (Table I). The high content of HYP, a sensitive marker of liver fibrinogenesis (Abd-Allah and Sharaf El-Din, 2008) contributes to the fibrinogenic activities of TAA. Liver fibrosis involves abnormal synthesis and the accumulation of extracellular matrix proteins, in particular collagen, in the liver parenchyma by activated HSCs. HYP is an amino acid unique to all collagens and its level indicates the amount of collagen present and therefore can be used to determine the extent of fibrosis. TAA caused a dramatic increase in hepatic HYP content compared to normal controls, which was supported by the presence of fibrosis and numerous connective tissue strands invaded by inflammatory cells (Esmat et al., 2013). Occasionally, the increased process of lipid peroxidation observed after TAA administration (Table I) could participate greatly in increment of collagen biosynthesis and its deposition in liver cells. Muriel and Moreno (2004) reported that the increase in lipid peroxidation associated with liver damages due to prolonged biliary obstruction induces collagen synthesis. In the present study, the free radicals and ROS generated in abundant during TAA metabolism might responsible for the increased oxidative stress inside the liver cells demonstrated by increased in the liver MDA an index of lipid peroxidation, serum NO and decrease in liver GSH contents (Tables II). This is associated with significant elevations in serum ALT, AST and GGT activities (Tables III). The hydrolysis of TAA to hydrogen sulfide (H$_2$S) as one of the reaction products (Basnar, 2011) could contribute to the alteration of liver MDA, NO and GSH. H$_2$S induced liver cytotoxicity and subsequently increase ROS production (Truong et al., 2006). In the same context, Abdel Salam et al. (2013) reported that TAA provoked oxidative stress leads to the denaturation of cellular biomolecules such as lipids, resulting in lipid peroxidation which was indicated by increased MDA concentration (Sirag, 2007). Whereas, the increased NO production can occur in response to inflammatory cytokines due to the action of unregulated inducible nitric oxide synthase (iNOS). The excessive production of NO can be deleterious to tissue function because of the ability of NO to react with biomolecules or with other free radicals e.g., superoxide anion, yielding the highly reactive peroxynitrite radical capable of evoking the oxidation of important cellular biomolecules. Another consequence of the elevated content of NO is cellular toxicity which can cause lipid peroxidation (Abdel Salam et al., 2013). Additionally, the higher consumption of GSH to scavenge the toxic intermediates formed by TAA led to significantly lower levels of GSH in liver tissue of the TAA rats. The non-enzymatic antioxidant GSH is thought to be a key player in the process of detoxification, which actively participates in reactions which lead to the destruction of H$_2$O$_2$, free radicals, and certain foreign compounds (Fazal et al., 2014). In addition, the changes induced by TAA in liver enzymes could be attributed to the oxidative injuries of hepatocytes. The necrosis of hepatocytes elevates serum ALT, AST and GGT activities as reported by Ajith et al. (2007). TAA interferes with the movement of RNA from the nucleus to the cytoplasm which may cause membrane injury resulting in a rise in serum liver markers. TAA toxic metabolite free radicals induce many changes occur for hepatocytes such as an increase in nuclear volume and enlargement of nucleoli, cell permeability changes, rise in intracellular concentration of Ca$^{2+}$, and effects on mitochondrial activity, which leads to cell death (Alshawsh et al., 2011). The histological structure of the TAA rat’s liver indicated several alterations supports the fibrogenic transformation of hepatocytes expressed biochemically by increase in TNF-α, TGF-β and HYP (Fig. 1). The histopathological examination of TAA rat’s liver demonstrates tissue insults in the form of pseudolobulation of hepatocytes surrounded by fibroblasts proliferation. TAA induced hepatocytes damage was supported by Al-Attar (2012) and Salama et al. (2013). TAA induced oxidative stress and depletion of endogenous antioxidants especially GSH might contribute to hepatocyte damage (Saad et al., 2013). Experimental data obtained in the present study demonstrated that rats receiving oral administration of MOE and/or exposure to LDR results in no mortality over the experimental period. Furthermore, showed no significant changes in fibrotic signs cytokines TNF-α and TGF-β, HYP liver content, oxidative indices (MDA, NO and
GSH) and liver enzymes (ALT, AST and GGT). The histological examination of liver revealed no change in the tissue architecture of rats treated with MOE and/or LDR compared to control group (Table I, Fig. 1). Despite previously mentioned alteration even biochemically or histopathologically, our data reveals that the administration of MOE and/or LDR significantly ameliorates these changes when compared with TAA solely rats (Table I, Fig. 1). The improvement in serum TGF-β after MOE administration could be attributed to the antifibrotic activity of MOE mediated via inhibited phosphorylation of TGF-β which regulates the expression of fibronectin, type I collagen, and plasminogen activator inhibitor-1 (Su-Hyun and Young-Chae, 2012). The amelioration in the serum TNF-α might be due to down regulation of TNF-α expression post MOE administration via inhibition of nuclear factor kappa Beta (Wihastuti et al., 2007; Kooltheat et al., 2014). Also, the LDR induced decreases in the level of TGF-β and TNF-α in rats treated with TAA could be attributed to the fact that LDR produce a group of phenomena generally called radiation hormesis (Kojima et al., 2004). This phenomena include the induction of radio-adaptive response (Ikushima et al., 1996), stimulation of immune function (Nogami et al., 1993), growth rate (Luckey, 1982), and enhance resistance to high doses of radiation (Yonezawa et al., 1996). Worthwhile, Yu et al. (2013) has attributed the efficiency of LDR against bleomycin induced-lung injury through reduction of TNF-α and TGF-β levels. The administrations of MOE and/or exposure to LDR induced significant improvement in oxidative indices and alleviated significantly the liver enzymes (Table I). This could be due to the capability of MOE on to adjust the cellular redox tone. Singh et al. (2014) stated that the antioxidant and hepatoprotective activities of M. oleifera leaves are possibly related to the free radical scavenging activity which might be due to the presence of total phenolics and flavonoids in the extract and/or the purified compounds β-sitosterol, quercetin and kaempferol, which were isolated from the ethanol extract of M. oleifera leaves. Also, Das et al. (2012) reported that MOE prevent liver injury in mice by improving the antioxidant status. Nevertheless, LDR could exert the protection against free radicals by stimulating the radical detoxification system (Feinendegen, 2005). One can attributed the significant reduction in liver HYP of TAA rats treated by MOE and/or LDR to the improvement of the oxidative indices and the fibrotic signs. The antioxidant and anti-inflammatory capacities of MOE contribute to their antifibrotic actions which are reflected on decreasing of HYP content and collagen deposition (Hamza, 2010; Su-Hyun and Young-Chae, 2012). However, the effect of LDR on HYP content might be through mitigating TGF-β and TNF-α expression, a key profibrotic cytokines that stimulate fibrogenesis and collagen synthesis (Lasky and Brody, 2000, Yu et al., 2013), as well as by enhancing the activity of antioxidant enzymes and increasing GSH content (Nomura et al., 2011).

The histopathological structure of TAA treated rat liver treated with MOE showed a marked attenuation of liver damage as represented by oval cells proliferation, newly formed bile ductules and deposition of haemosidrin pigments (Fig. 1). These results are in agreement with the findings of Nanjappaiah and Hugar (2012) and Singh et al. (2014) who reported that MOE exhibited hepatoprotective effect against CCl4 induced liver damage. This hepatoprotective effect of MOE might be attributed to the ability of its constituents to inhibit aromatase activity of cytochrome p-450, thereby favoring liver (Kowalska et al., 1990). Concerning effect of LDR on liver injury induced by TAA, examination of liver sections showed reduced degree of fibrotic signs as depicted by lesser strands of fibroblasts surrounding cytomegalic hepatocytes (Fig. 1). It was shown that low dose of radiation may enhance protection through the stimulation of endogenous antioxidant activities with special emphasis on GSH, which counteract excessive formation of ROS (Kawakita et al., 2003; Fahmy and Gharib, 2014).

CONCLUSIONS

MOE and LDR possess hepatoprotective activity and reduced fibrotic patterns induced by TAA and their combination markedly ameliorated damage and restored tissue vitality. One might deduce that LDR could improve the role of MOE in liver fibrosis.
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