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Majoon-e-Dabeed-ul-Ward protects lung cells against ethanol-induced cytotoxicity

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Abstract
The purpose of this work was to investigate the effect of Majoon-e-Dabeed-ul-Ward (MD) on ethanol (EtOH) induced cytotoxicity in human liver cells. Cells were treated with either EtOH (30mM) alone or together with MD (25µM) for 12 hours. It was found that exposure with EtOH induced different cytotoxicity processes like formation of oxygen radical formation (ROS), decrease in the activities of SOD and Catalase, lactate dehydrogenase leakage, lipid peroxidation, decrease in glutathione (GSH) and increased oxidized glutathione (GSSG) levels in liver cells as compared to untreated cells. EtOH induced lactate dehydrogenase (LDH) leakage was significantly prevented by MD treatment. Activity of Superoxide dismutase (SOD) and catalase were significantly increased in EtOH challenged cells upon treatment with MD. Other beneficial effects associated with MD treatment in EtOH challenged cells were reduction in generation ROS and lipid peroxidation. Our findings suggest that MD exert cytoprotective action against EtOH induced liver cell damage.

Keywords: Majoon-e-Dabeed-ul-Ward, reactive oxygen species, ethanol, glutathione

Introduction
Ethanol consumption is considered to be the main cause of liver diseases and is responsible for about 3.8% of global mortality [1]. It has been found that chronic ethanol consumption leads to cellular and tissue damages [2]. Studies have shown that ethanol metabolism is associated with the production of reactive oxygen species (ROS), inhibition in antioxidant enzymes, decrease in antioxidants like glutathione (GSH), enhanced lipid peroxidation etc. [3, 4]. These molecular changes associated with the ethanol consumption in liver cells leads to cell death [5, 6].
Majoon-e-Dabeed-ul-ward (MD) is a popular unani herbal formulation with hepatoprotective roles and is prepared from around 21 medicinal plants like Sumbul-ut-teeb (Nardostachys jatamasnsi), mastagi (Pistaca lentiscus), Zafran (Crocus sativa), Tabashee (Bambusa bambos), Darchini (Cinnamoum zeylanicum), Izkhar (Cymbopogon jwarrncusa), Asaroon (Cinnamoum zeylanicum), Qust Shireen (saussurea hypoleuca), Gul-e-Ghafis (Gentiana oliverii), Tukhm-e-kasoos (Cuscuta reflexa), Majeeht (Rubia cordifolia), Luk Maghsool (Coccus lacca), Tukhm-e-karafs (Apium graveoleus), Tukhm-e-kasni (Cichorium intybus), Zarawand Taweel (Aristolochia longa), Habb-e-Balsan (Commiphora opobalsamum), Ood Handi (Aquilaria agollocha), Qaranfal (Syzygium aromaticum), Heel Khurd (Eletharia cardamomum), Waraq-e-Gul Surkh (Rosa domaseena), and Asal or Qaind Safaid [7, 8]. Till date its different hepatoprotective roles anti-oxidant, anti-cancerous and anti- inflammatory roles have been explored [7, 9]. The present study was carried out to investigate its possible role in preventing ethanol associated cytotoxicity in liver cells.

Materials and Methods
1. Cell culture and treatments
Human Chang liver cell line was purchased from NCCS, Pune, India. The cells were maintained in Dulbecco's minimal essential medium (DMEM) containing 10% heat-inactivated foetal calf serum (FBS) and supplemented with antibiotics penicillin-streptomycin (100U/ml) at 37ºC in 5% CO_2. Cells were grown and treated with either EtOH (30mM) alone or together with MD (25µM). *Majoon-e-Dabeed-ul-Ward* was collected from Regional Research Institution of Unani Medicine, Srinagar.

2. LDH leakage
Cells were cultured in a 24- well plate containing 500 µl medium/well at a seeding density of 2 x 10^5 for 24 hrs., and subsequently treated with EtOH (30mM) alone or together with MD (25µM). 200 µl of medium was taken out for the extracellular LDH activity analysis. Total LDH activity was determined by using the LDH assay kit after cells were disrupted by sonication. The percentage of LDH released was calculated using the formula

$$\text{LDH release} = \frac{\text{Absorbance of the supernatant}}{\text{Absorbance of the supernatant and cell lysate}} \times 100$$

3. Lipid peroxidation
Thiobarbituric acid reactive substances (TBARS) production was used to measure lipid peroxidation [10]. Cells were cultured in a 24-well plate containing 500µl medium/well at a

seeding density of 2 x 10^5 for 24 hrs., and were treated with EtOH (30mM) alone or together with MD (25µM) for 12 hrs. Cells were incubated with 1ml (0.5 M KCl in 10 mM Tris-HCl), mixed properly and then treated with 0.5 ml (30% trichloroacetic acid (TCA)) and 0.5 ml (52 mM thiobarbituric acid) and finally heated in water bath (90°C for 30 min). The mixture was cooled and later on centrifuged (3000 rpm for 10 min). Supernatant was collected and its absorbance was measured at 532 nm and the amount of TBARS was expressed as nmol/mg protein.

4. Measurement of ROS

ROS level was measured by DCFDA - Cellular Reactive Oxygen Species Detection Assay Kit (Abcam, USA). The kit contains 2', 7'–dichlorofluorescin diacetate (DCFDA) a fluorogenic dye that measures peroxyl, hydroxyl and other reactive oxygen species (ROS) activity. Human Chang liver cells were cultured in a 96-well plate containing 200µl medium/well at a seeding density of 25 x 10^3 for overnight. Media was removed, followed by addition of 100 µl/well of 1X buffer. Buffer was removed and cells were stained with diluted DCFDA solution (100µl/well) for 45 minutes at 37°C. DCFDA solution was removed followed by treatment with either EtOH (30mM) alone or together with MD (25µM) for 12 hrs and later on fluorescence detection was done.

5. GSH/GSSG assay

Chang liver cells were cultured in a 6-well plate containing 3ml medium/well at a seeding density of 1 x 10^6 cells/ well for overnight. Cells were treated with either EtOH (30mM) alone or together with MD (25µM) for 12 hrs**.** Media was removed and cells were rinsed three times with 1x phosphate buffer saline (PBS). Cells were collected by centrifugation (3000rpm for 3 minutes), supernatant was removed and 300µl of ice-cold [(10% trichloroacetic acid (TCA) and 0.01 N HCl)] was added to the cell pellets. Tubes were vortexed, kept on ice for 10 minutes and centrifuged (12,000 rpm for 20 min @ 4°C). Supernatant was extracted 4 times with diethyl ether to remove TCA, followed by measurements of GSH and GSSG [11].

6. SOD activity assay

SOD was quantified by xanthine-oxidase (XOD) method. This assay is based on the fact that conversion of xanthine to H_2O_2 and uric acid by xanthine-oxidase (XOD) results in the formation of $O2^{-}$. The O_2 free radical inturn converts nitroblue tetrazolium (NBT) to NBT diformazan dye that enables the system to absorb light at 560 nm. Chang liver cells were cultured in a 96-well plate overnight followed by treatment with either EtOH (30mM) alone or together with MD (25µM) for 12 hrs**.** Media was removed followed by addition of 160µl of 0.1 M glycine-NaOH buffer (pH 9), 6.75µl each of xanthine (3 mM), ethylenediaminetetraacetic acid (EDTA) (3 mM), bovine serum albumin (BSA) (0.15%), NBT (0.75 mM), and 20µl of sample to each well. After equilibration at 20˚C for 10 min, the reaction was initiated by adding 6mU of XOD and incubated further at 20˚C for 20 min. The reaction was terminated by addition of 6.75 ml of 6 mM $CuCl_2$ and absorbance at 560 nm was determined using a microplate reader.

7. Catalase assay

Catalase activity was determined by spectrophotometric method and involves the removal of peroxide by the Catalase. For Catalase assay, Chang liver cells were cultured in a 6-well plate overnight and were treated with either EtOH (30mM) alone or together with MD (25µM) for 12 hrs**.** Cell lysate was prepared using Nonidet P-40 (NP-40) lysis buffer (150mM NaCl, 1% NP-40, 50 mM Tris-Cl pH 8.0, 1mM PMSF). Protease inhibitor cocktail (Sigma) was freshly added to the cell lysate and insoluble material from the cell lysate was removed by centrifugation at 10000 rpm for 10 min, supernatant was collected for the assay. First of all, spectrophotometer was blanked with 3 ml of Phosphate buffer. 2 ml of phosphate buffer was taken in another cuvette and 80µg protein was added to it. Then the cuvette was placed in spectrophotometer and immediately 1 ml of H_2O_2 working solution was added to it. Its absorbance change versus time was recorded for two minutes.

Catalase activity was calculated as;

$k = 1/60 \ln Ao/A60$

$K_{total/ml} = k \times 6/ml$ sample

$k/mg = k_{total}$ ml-1/ mg ml-1

8. Statistical analysis

In the present study, representative experiments from three independent experiments are shown. Results for each experiment are given as mean of triplicates ± SE. Statistically significant differences between sample groups were determined using Student's t-test. A p value of <0.05 was considered significant.

Results and Discussion

Protective role of MD on LDH release

LDH is an enzyme responsible for cellular respiration and is found within the cells. Disruption of cell membrane by any stress results in release of LDH to the external medium. Presence of this enzyme in the culture medium is considered to be a death call. Treatment of Chang liver cells with 30mM EtOH resulted in membrane damage as shown by LDH release. Prevention of LDH leakage by MD treatment reflects its role in protecting cells against EtOH-induced toxicity.

As shown in figure 1, EtOH-challenged liver cells showed significant increase in the LDH leakage (Bar 2) as compared to control untreated cells (Bar 1)**.** This enhanced LDH leakage may partly explain the basis of stress. However, treatment with MD significantly decreased EtOH associated LDH leakage (Bar 3) in liver cells.

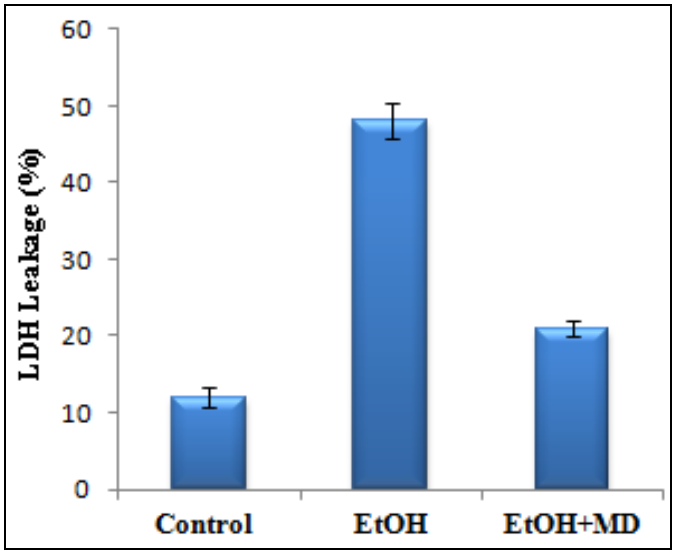

Fig 1: Showing effect of MD on LDH leakage

LDH leakage was determined using LDH assay kit. Bar 1 (control) represents LDH leakage from untreated liver cells. Bar 2 (EtOH) represents LDH leakage level from EtOH-treated liver cells. Bar 3 (EtOH+MD) represents LDH leakage level from liver cells treated with MD in presence of EtOH stress.

Effect of MD on EtOH-induced lipid peroxidation

Lipid peroxidation is associated with oxidation of unsaturated fatty acids (FA) within the cell membrane and therefore leading to cell damage. TBARS are the byproducts of the lipid peroxidation and their detection is used to measure the cell damage. As shown in figure 2, treatment with EtOH resulted in almost four times increase in TBARS levels (Bar 2) as compared to control (Bar 1) in liver cells. However incubation together with MD decline EtOH associated lipid peroxidation (Bar 3).

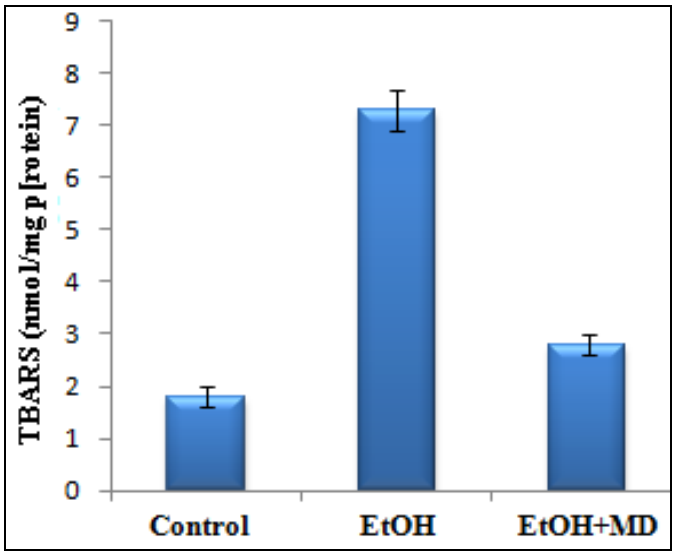

Fig 2: Showing effect of MD on lipid peroxidation

Lipid peroxidation was determined by measuring TBARS release. Bar 1 (control) represents lipid peroxidation from untreated liver cells. Bar 2 (EtOH) represents lipid peroxidation level from EtOH-treated liver cells. Bar 3 (EtOH+MD) represents lipid peroxidation level from liver cells treated with MD in presence of EtOH stress.

Effect of MD on ROS levels

ROS production is a major factor in oxidative damage of cells and effect main biological molecules like nucleic acids, proteins and lipids. It has been earlier found that ethanol stimulates ROS production in hepatocytes and therefore leads to cell injury [12, 13]. As shown in figure 3, Chang liver cells when treated with EtOH increased the ROS production (Bar 2) as compared to control (Bar 1). However, incubation together with MD decline EtOH associated ROS production (Bar 3) in liver cells.

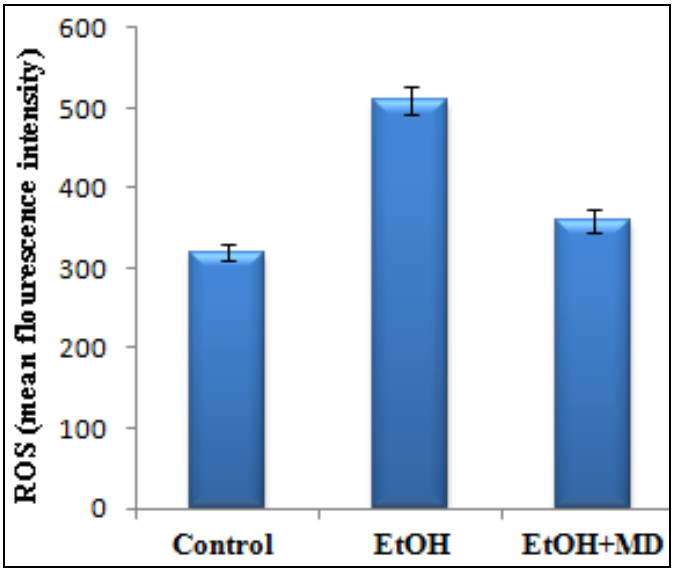

Fig 3: Showing effect of MD on ROS level

ROS level was determined by measuring DCFH fluorescent dye. Bar 1 (control) represents ROS level of untreated liver cells. Bar 2 (EtOH) represents ROS level of EtOH-treated liver cells. Bar 3 (EtOH+MD) represents ROS level from liver cells treated with MD in presence of EtOH stress.

Effect of MD on GSH redox state

GSH plays an important role in neutralizing oxidative stress and mitochondrial injury caused due to various toxins [14]. It has been found that EtOH induces depletion of GSH level in Chang liver cells and causes cytotoxicity [15]. As shown in figure 4, EtOH treatment declines redox status of chang liver cells by decreasing the GSH level (4A) and by increasing GSSG level (4B). Upon EtOH treatment, oxidative stress is increased as shown by low ratio of GSH/GSSG (4C). However, MD treatment of EtOH challenged liver cells, intriguingly recovered the GSH and GSH/GSSG levels (Bar 3rd of the figure 4A and 4C).

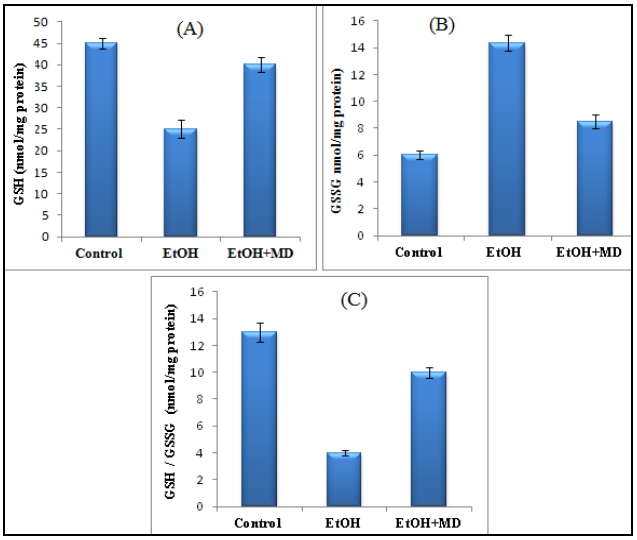

Fig 4: Showing effect of MD on GSH and GSSG levels

GSH levels were determined by DTNB (5, 5'-dithiobis-(2-nitrobenzoic acid) method. Figure (4A), 4(B) and 4(C) represents graphs with GSH, GSSG and GSH/GSSG levels respectively.

Effect of MD on SOD activity

Enhancement of SOD activity or its up-regulation can augment protection against cellular stress. This prompted us to investigate the effect of MD treatment on activity of SOD enzyme. For this purpose, Chang liver cells were treated with either EtOH (30mM) alone or together with MD (25µM) for 12 hrs**.,** followed by spectrophotometric activity of SOD.

As shown in figure 5, EtOH-challenged liver cells showed significant decrease in the activity of SOD (Bar 2) as compared to control untreated cells (Bar 1)**.** This lowered activity of SOD may partly explain the basis of stress. However, treatment of EtOH exposed cells with MD showed much higher SOD activity (Bar 3). This recovery or enhancement of SOD activity upon MD treatment can augment protection against stress.

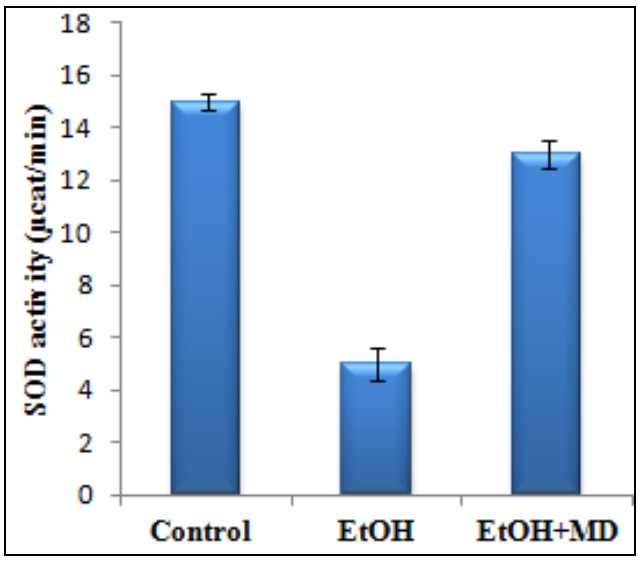

Fig 5: Showing effect of MD on SOD activity

SOD activity was measured by xanthine-oxidase (XOD) method. Bar 1 (control) represents SOD activity of untreated liver cells. Bar 2 (EtOH) represents SOD activity of EtOH-treated liver cells. Bar 3 (EtOH+MD) represents SOD activity of liver cells treated with MD in presence of EtOH stress.

Effect of MD on Catalase activity

Catalase, an H_2O_2-degrading enzyme, is a very important enzyme in protecting the cell from oxidative damage by ROS (especially by H_2O_2). H_2O_2 formation due to mitochondrial superoxide leakage perpetuates oxidative stress in cellular injury. Thus Catalase remains an important target in antioxidant therapy. It is thus argued that enhancement of Catalase activity can protect cells against stress caused by ROS (especially by H_2O_2). This prompted us to investigate the effect of MD treatment on activity of SOD enzyme in EtOH challenged liver cells. Chang liver cells were treated with either EtOH (30mM) alone or together with MD (25µM) for 12 hrs**.,** followed by spectrophotometric activity of catalse.

As shown in figure 6, EtOH-challenged liver cells showed significant decrease in activity of catalse (Bar 2) as compared to control untreated cells (Bar 1)**.** This lowered activity of catalse may partly explain the basis of stress. However, treatment of EtOH exposed cells with MD showed much higher SOD activity (Bar 3).

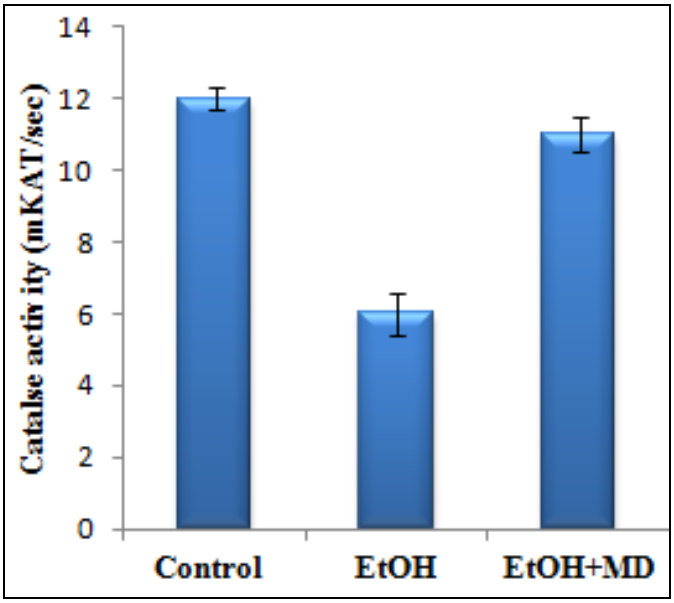

Fig 6: Showing effect of MD on catalse activity

Catalse activity was measured by spectrophotometric method. Bar 1 (control) represents catalse activity of untreated liver cells. Bar 2 (EtOH) represents catalse activity of EtOH-treated liver cells. Bar 3 (EtOH+MD) represents catalse activity of liver cells treated with MD in presence of EtOH stress.

Conflict of interest statement

We declare that we have no conflict of interest.

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