Research Paper

Majoon-e-Dabeed-ul-Ward protects lung cells against ethanol-induced cell death and activates Nrf2/HO-1 signaling pathway

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Abstract

Majoon-e-Dabeed-ul-ward (MD) is a hepatoprotective unani formulation with strong antioxidative effects. The purpose of the work was to investigate the effect of Majoon-e-Dabeed-ul-Ward (MD) on ethanol (EtOH) induced cell death and its probable role in activating Nrf2/HO-1 pathway. Cytoprotective role of MD in preventing ethanol induced cell death in liver cells was determined by MTT assay. Protein expression levels of Nrf2 and HO-1 was determined by immunoblotting using antibodies against the target protein and their mRNA expression was studied by RT-PCR. Our results showed that MD treatment increases cell viability in EtOH induced liver cells. Nrf2 expression level (both at mRNA and protein) was increased by MD treatment. It was further found that Nrf2 inturn increases expression of Heme oxygenase-1 (HO-1): an antioxidant phase II enzyme. Our findings suggest that MD exert cytoprotective effect in EtOH induced liver cell and causes activation of Nrf2/HO-1 signaling pathway.

Keywords: Majoon-e-Dabeed-ul-Ward, Nrf2, HO-1, Alcoholic liver disease, hepatoprotective

Introduction

Liver diseases are a worldwide heath issue, caused by excessive alcohol consumption, toxic chemicals, autoimmune disorders and infections[1]. Alcohol consumption accounts for about 3.8% of global mortality and Alcoholic liver disease (ALD) are considered to be one of the major causes of liver associated death. Chronic ethanol consumption is associated with cellular and tissue damages[2]. Ethanol metabolism has been linked to reactive oxygen species (ROS) production (like 1-hydroxy ethyl radicals), decrease in antioxidants like glutathione (GSH), inhibition in antioxidant enzymes etc[3]. The molecular changes in the liver cells leads to the degenerative changes like mitochondrial injury and ultimately to apoptotic associated cell death[4,5].

Nuclear factor (erythroid-derived 2)-like 2, also called NFE2L2 or Nrf2 (a transcriptional factor) is a major regulator of cellular defense mechanisms in various organs including the liver, lung, GI tract, bladder, kidney, brain, skin and ovary[6]. Additionally, Nrf2 has been implicated in a number of disease states including a role in the progression of hepatic and gastrointestinal disease[7], drug-induced cellular toxicity[8], pulmonary fibrosis[9] etc. It is considered as the master regulator of the anti-oxidant response, since it modulates the expression of a set of anti-oxidant genes encoding phase II enzymes and anti-oxidant enzymes such as glutathione S-transferase (GST), NAD(P)H:quinone acceptor oxidoreductases (NQOs), Heme oxygenase-1 (HO-1), multidrug resistance-associated proteins (Mrps), the UDP-glucuronosyltransferase (UGT) family, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS)[10,11] (see figure 1).

Within cells Nrf2 level is maintained at low levels in cytoplasm by Kelch-like ECH-associated protein 1 (KEAP1): a cytoskeleton-associated inhibitory protein. KEAP1 is responsible for Nrf2 degradation via KEAP1-dependent ubiquitin conjugation[12]. Oxidation stress causes modification of KEAP1, which results in loss of Nrf2[13]. As a result, Nrf2 gets accumulated, followed by phosphorylation and finally is translocated to the nucleus[14]. Nrf2 within nucleus causes up regulation of cytoprotective genes via binding to a promoter sequence called antioxidant response element (ARE)[15]. Nrf2/ARE pathway works as an important endogenous anti-oxidation mechanism within cells and can be activated by exogenous factors[16]. Downstream genes of Nrf2 support cellular redox homeostasis, cell growth and apoptosis, mitochondrial biogenesis, inflammatory functions and upregulate phase II enzymes. A few phase II enzymes include HO-1, Catalse (CAT), Glutathione peroxidase (GPx), Superoxide dismutase (SOD), thioredoxin, NQO-1, and Glutathione S-transferase (GST)[17]. The coordinated induction of Nrf2-mediated enzymes is crucial for cells to maintain redox homeostasis and avoid the adverse effects of oxidative stress. These cytoprotective proteins serve to directly or indirectly scavenge free radicals and as a result, decrease ROS toxicity.
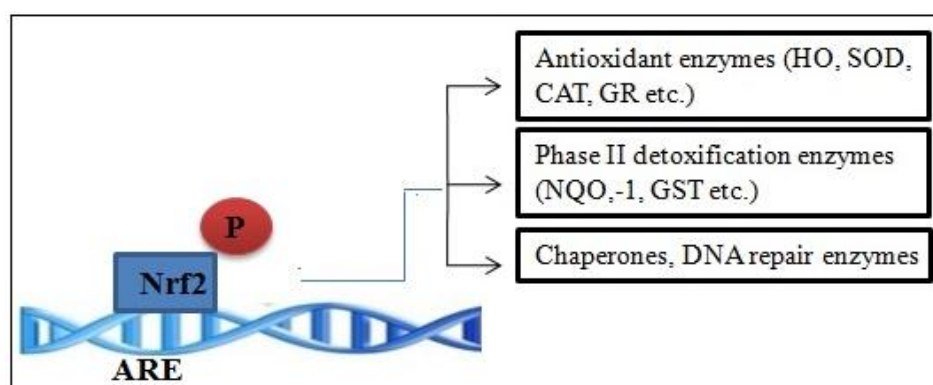
Figure 1: Downstream targets of Nrf2 protein

Majoon-e-Dabeed-ul-ward (MD): a unani herbal formulation prepared by mixing parts of around 21 medicinal plants[18] (see table 1). It is a hepatoprotective mixture with strong anti- inflammatory, anti-oxidant and anti cancerous effects[18,19]. In the present study, we investigated the relation between MD and Nrf2/HO-1 pathway liver cells and role in preventing EtOH induced cell death.

Table 1: Ingredients of Majoon-e-Dabeed-ul-ward

Unani name	Botanical name
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
Asal or Qaind Safaid	

Materials and Methods

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 and supplemented with antibiotics penicillin-streptomycin (100U/ml) at 37°C in 5% CO_2. Cells were grown and treated with EtOH (30 mM) alone or together with MD (25 µM).

MTT viability assay

The effect of different treatments on cell proliferation was determined by MTT (3-(4,5 Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) colorimetric assay. Liver cell line was cultured in a 24- well plate containing 1 ml medium/well at a seeding density of 5×10^5 for 24 hrs and subsequently treated with EtOH (30 mM) alone or together with MD (25 µM). The controls consisted of cells without any treatment. After treatment for 24 hrs, media was aspirated from each well and 200ul of 5mg/ml MTT (pH 4.7) was added to each well and incubated for 4 hrs. The supernatant was removed and 200 ul MTT solvent (acidified isopropanol) was added to each well. The absorbance was then measured at 570 nm in microplate reader (Epoc biotech), using wells without cells as blanks. All experiments were performed in duplicates and repeated at least three times. The effect of different treatments on proliferation of cells was expressed as percentage proliferation of treated cells with respect to controls taken as cent percent.

Reverse transcription and real-time PCR

Total RNA from liver cells was isolated using TRIzol® reagent (Invitrogen, USA) in accordance to the manufacturer's instructions. The mRNA expression level of Nrf2 and HO-1 was detected by qRT-PCR assay based upon the SYBR® Green gene expression analysis detection system (Thermo scientific). β-actin was used as the loading control. The sequences for gene-specific forward and reverse primers were designed using NCBI-Primer Blast software. After DNase treatment, RNA was subjected to reverse transcription using standard protocol. The 3.5 µl of synthesized cDNA was amplified in a total volume of 25µl in Real Time PCR 7500 (Applied Systems, USA) using maxima SYBR® green mix and in accordance to manufacturer's protocol. To determine the expression level of each gene qRT-PCR was done with specific primer pairs for each gene and β-actin as a loading control, as shown in table 2.

Table 2: Primer sequence of Nrf2, HO-1 and β-actin

Gene name	Forward primer	Reverse primer
Nrf2	CCTCAACTATAGCGATGCTGAATCT	AGGAGTTGGGCATGAGTGAGTAG
HO-1	GGGCCAGCAACAAAGTG	AGTGTAAGGACCCATCGGAGAA
β-actin	AGGCATCCTCACCCTGAAGTA	CACACGCAGCTCATTGTAGA

Protein extraction

Cell lysate was prepared using 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 5000 rpm for 5min. Protein concentration in the samples was determined using Bradfords assay. For immunoblotting, each protein fraction was separated on 12% SDS PAGE and processed as described previously[20]. Immuno-detection was performed using primary antibodies against the desired protein; Nrf2, HO-1 and anti-β actin.

Preparation of nuclear and cytoplasmic fractions

Cytoplasmic and nuclear extracts were prepared from liver cells using a nuclear extraction kit (Abcam, USA) and in accordance to the protocol supplied by the manufacturer. The protein concentration of each extract was measured using Bradford assay. The purity of the nuclear protein extract was checked by immunoblotting using anti-Histone 4 (H4) antibody (nuclear marker) and anti-GAPDH antibody (cytosolic marker).

Immunoblotting

For immunoblotting, each protein fraction was separated on 12% SDS PAGE and processed as described previously[20]. Immuno-detection was performed using primary antibodies against the desired protein; Rabbit anti-Nrf2 (1:1000) (Cell signaling technology), rabbit Anti HO-1 (:500) (Abcam), mouse

anti-β actin (1:7000) (CST) rabbit anti-GAPDH (1:1000) (Sigma) and mouse anti-Histone 4 (H4) (1:5000) (Sigma). The secondary detection was performed using fluorescent anti-mouse IRDye 680 (1:20,000) and anti-rabbit IRDye 800 (1:10,000) secondary antibodies (LI-COR, Biosciences, St. Lincoln, NE, USA). The fluorescence was detected using the Odyssey infrared detection system (LI-COR).

Statistical analysis
Representative experiments from three independent experiments are shown in the present study. Results 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
### MD increases cell viability in EtOH treated cells
MTT assay was used to determine the effect of MD treatment on viability of liver cell. For this purpose, liver cells were plated in 24- well plates for 24 hrs and subsequently treated with EtOH alone or together with MD for 12 hours, followed by MTT assay. As shown in figure 2, MTT results revealed that EtOH treatment decreases cell viability. However, EtOH/MD treatment together showed significant increase in cell viability.
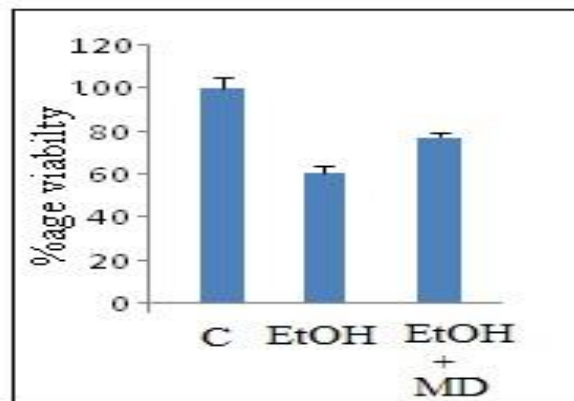
Figure 2: Shows effect of MD treatment on viability of EtOH induced liver cells and was determined by MTT assay

MD increases NrF2 expression level
Nrf2 is known to be regulated at various levels but it is widely accepted that most of the inducers regulate its expression at post-transcriptional level. However, a few drugs have been observed to alter Nrf2 at mRNA levels. We accordingly set out to investigate the effect of MD on Nrf2 mRNA and protein expression in EtOH treated cells. As shown in figure 3, MD treatment increases expression levels of Nrf2 both at mRNA and protein level.
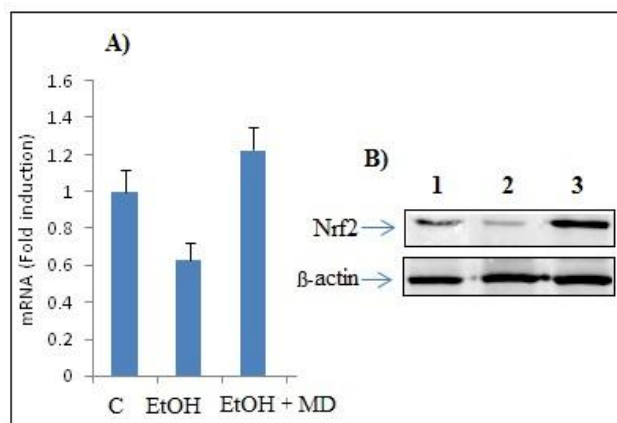
Figure 3: Shows Nrf2 mRNA and protein levels: A) Nrf2 mRNA expression levels was determined by qRT-PCR. B) Nrf2 protein expression level was determined by immunoblotting Nrf2 gets translocated to nucleus with MD treatment

Since MD treatment increases expression of Nrf2 (on mRNA and protein) significantly, we therefore start looking its effect on the translocation of Nrf2 from cytosol to nucleus. For that purpose, firstly cytosolic and nuclear protein fractions were separated and their purity was checked by the immune-detection. Purity of cytosolic and nuclear fractions was confirmed by using anti-GAPDH and anti-Histone 4 (H4) antibodies respectively. As shown in Figure 4. (A), a prominent GAPDH protein band was detected in cytosolic fraction (C, top panel) while nuclear fraction detects no such band (N, top panel). Similarly Histone 4 (H4) protein band was detected in nuclear fraction (lane N, middle panel) and no band was detected in the cytosolic fraction (lane C, bottom panel).

Immuno-blotting was performed to look for the effect of MD treatment on Nrf2 translocation. Nrf2 antibody detected a prominent protein band in the nuclear fraction obtained from MD treated liver cells (Figure 4. B, lane 2, upper panel), whereas a weak protein band was observed in nuclear fraction of the control sample (Figure 2.B, lane 1, upper panel).
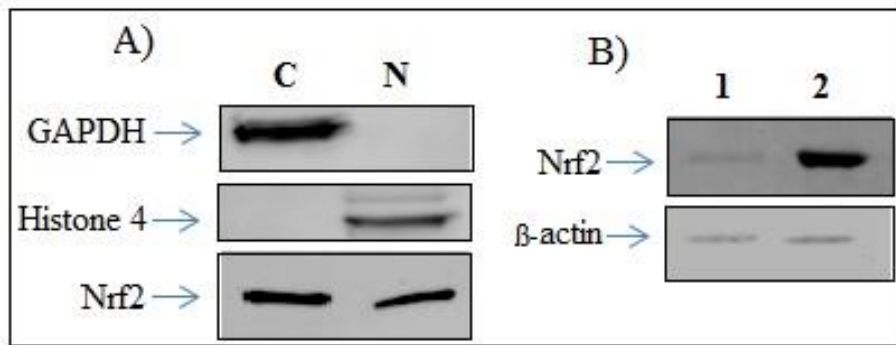
Figure 4: Shows translocation of Nrf2 to nucleus: (A) Immunoblot showing purity of nuclear (N) and cytosolic (C) fractions. (B) Shows increased translocation of Nrf2 to nucleus in response to MD

Effect on HO-1 expression
Nrf2 activates an array of downstream targets and main among them is HO-1. So, we analysed the effect of MD treatment on HO-1 expression in EtOH treated liver cells, as shown in figure 5.
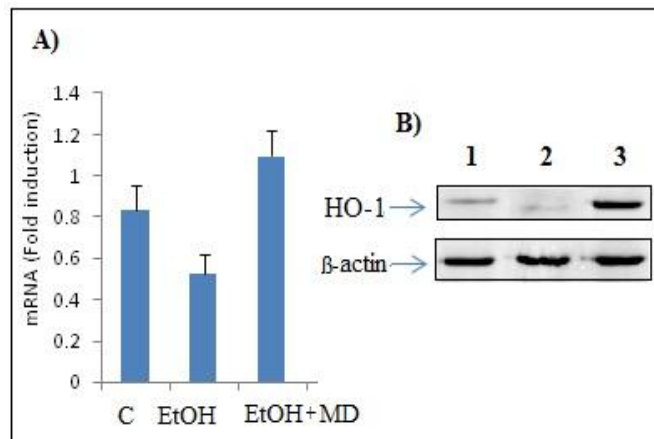
Figure 5: Shows HO-1 mRNA and protein levels: A) mRNA expression level was evaluated by qRT-PCR. B) HO-1 protein expression level was determined by immunoblotting

Discussion
In the present study, we demonstrated that Majoon-e-Dabeed-ul-ward (a unani formulation) prevents EtOH induced cell death in liver cells and activate Nrf2/HO-1 pathway. As earlier said, chronic ethanol consumption is associated with cellular and tissue damages. Ethanol metabolism has been linked to ROS production (like 1-hydroxy ethyl radicals), decrease in antioxidants like glutathione (GSH), inhibition in antioxidant enzymes etc and there is a direct relation between

EtOH consumption and decrease in cell viability [21]. Treatment with EtOH resulted in loss of cell viability which was significantly decreased by MD treatment.

As earlier mentioned, Nrf2 (a transcription factors) is considered as the master regulator of the anti-oxidant response. Nrf2 modulates the expression of a set of anti-oxidant genes encoding phase II enzymes and anti-oxidant enzymes such as glutathione S-transferase (GST), NQOs, Heme oxygenase- 1 (HO-1), multidrug resistance-associated proteins (Mrps), the UDP-glucuronosyltransferase (UGT) family, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS). We therefore start looking effect of MD treatment on Nrf2 levels. As described above, MD increases expression levels of Nrf2 (both mRNA and protein). It is generally believed that Nrf2 confers cytoprotection by translocating from cytoplasm to nucleus, wherein it activates downstream anti-oxidant genes via ARE-binding. Since MD treatment increases expression of Nrf2 (on mRNA and protein) significantly, we therefore start looking its effect on the translocation of Nrf2 from cytosol to nucleus. It was found that MD treatment cause translocation of Nrf2 to nucleus.

Nrf2 activates an array of downstream targets like HO-1, which inturn confers cytoprotection against oxidative and inflammatory injuries caused by a wide array of noxious or pathogenic insults. The cytoprotective response of HO-1 is dependent on the activation of an ARE in the HO-1 promoter by Nrf2. So, next we were prompted to look whether HO-1 is induced by MD treatment, being a downstream target of Nrf2. It was found that MD treatment increases expression levels of HO-1 (both at mRNA and protein level). On the basis of various biochemical assays, we conclude that MD acts as a hepatoprotective unani drug against EtOH induced cell death. Further, MD activates Nrf2/HO-1 pathway and causes accumulation of Nrf2 in nucleus of liver cells.

Conflict of interest statement
We declare that we have no conflict of interest.

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