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Majoon-e-Dabeed-ul-Ward protects neuronal cells against glutamate toxicity

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Abstract
The aim of this work was to investigate the effect of Majoon-e-Dabeed-ul-Ward (MD) on glutamate induced cytotoxicity in neuronal cells (IMR-32). Cells were treated with either glutamate (30mM) alone or together with MD (25µM) for 12 hours. It was found that exposure with glutamate induced formation of oxygen radical formation (ROS) and Nitric oxide (NO) as compared to untreated cells. Glutamate induced ROS and NO formation was significantly prevented by MD. Our findings suggest that MD exert cytoprotective action against glutamate induced cytotoxicity in neuronal cells.

Keywords: majoon-e-dabeed-ul-ward, nitric oxide, reactive oxygen species, glutamaate

Introduction
Glutamate is a well-known neurotransmitter responsible for communication between nerve cells under normal conditions. It is maintained at an intracellular concentration of 10^{-3} M in the brain, whereas extracellularly it is present at a concentration of 10^{-5} to 10^{-6} M (Coyle and Puttfarcken, 1993; Meldrum, 2002) [3, 10]**.** But under certain pathological conditions such as ischemia, hypoxia, and brain injury, there is huge elevation in its concentration in the brain (Collard *et al*., 2002) [2], and at these excitotoxic concentrations, it can elicit damage and death of neurons (Dore *et al*., 2000; Dore *et al*., 1999) [5, 6]. Glutamate toxicity has been implicated in stroke, alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), parkinson's disease (PD) and other neurodegenerative conditions. However, the molecular mechanisms responsible for glutamate-induced toxicity have not been fully elucidated (Wang and Qin, 2010) [13]. Though, over-activation of glutamate receptors during glutamate toxicity has been hypothesized to impair calcium homeostasis by increasing the influx of calcium especially in mitochondria which results in energy failure, enhanced ROS) and NO production and ultimately cell damage and death (Duchen, 2000; Henshall and Simon, 2005; Wong *et al*., 2002) [7, 8, 14]. The increased levels of ROS and NO initiate certain neurotoxic cascades that are responsible for mediating neuronal cell death in many neurodegenerative disorders (Dawson and Dawson, 1998; Meng *et al*., 2000; Zhang *et al*., 2000) [4, 11, 15]. Therefore, the anti-oxidant substances that can scavenge ROS and NO could prove to be effective in either preventing the onset or delaying the progression of diverse neurodegenerative diseases.
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 (Shukla, 2011) [12] (Kaviarasan *et al*., 2007) [9]. Till date its different hepatoprotective roles anti-oxidant, anti-cancerous and anti- inflammatory roles have been explored (Shukla, 2011) [12] (Bilal A Bhat, 2015) [1]. The present study was carried out to investigate its possible role in preventing glutamate associated cytotoxicity in neuronal cells

Materials and methods
Cell culture and treatments
IMR 32 cells were purchased from National Centre for Cell Science (NCCS, Pune, India). The cells were grown in dulbecco's modified eagle's medium (DMEM), supplemented with 10% foetal bovine serum (FBS) and 1% pencillin-streptomycin at 37 °C in a humidified incubator containing 5% CO_2. After 24 hrs of seeding, IMR 32 cells were treated with MD for 12 hrs in presence/absence of glutamate (30mM).

NO assay
NO estimation was performed as per standard Griess assay. The quantity of nitrite in the culture medium was measured as an indicator of NO production. Amount of nitrite, a stable metabolite of NO, was measured using Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride (NED) in 2.5% phosphoric acid). Briefly, IMR-32 cells were cultured in a 24-well plate for overnight. Media was removed, followed by treatment with either glutamate (30mM) alone or together with MD (25µM) for 12 hrs. 100µl of cell culture medium was taken from control and

treated cells and firstly mixed with 100µl of Griess reagent 1 (Sulfanilamide) and incubated at room temperature for 10 min. Subsequently, 100µl of Griess reagent 2 (NED) was added to the mixture and the absorbance was measured at 540 nm in a microplate reader.

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. IMR-32 cells were cultured in a 96-well plate 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 glutamate (30mM) alone or together with MD (25µM) for 12 hrs and later on fluorescence detection was done.

Statistical analysis

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

MD impairs NO production in glutamate-activated IMR 32 cells

NO has been observed to contribute to neurotoxicity in various neurodegenerative diseases like Alzheimer's disease and has been observed to rise to a considerable level during stress conditions. Thus its regulation can have therapeutic role in various neurodegenerative diseases. We accordingly investigated the effect of MD on elevated NO levels during glutamate-induced toxicity in IMR 32 cells. As NO is secreted out by cells, we estimated its level after various treatments in culture supernatant (media) by standard Griess assay (described in materials and methods). As shown in figure 1, the exposure of neuronal cells to the glutamate stress resulted in the appreciable rise in NO levels after 24 hrs (Bar 2), compared to control cells (Bar 1). Moreover, treatment with MD resulted in significant decrease in glutamate associated NO levels in IMR 32 cells (Bar 3).

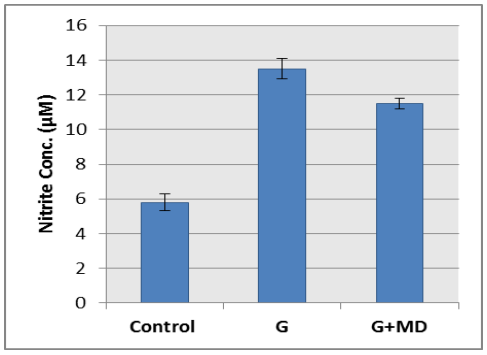

Fig 1: Estimation of NO levels in IMR 32 cells: NO levels were determined in culture supernatants of IMR 32 cells by Griess assay after treatments for 12 hrs. The data is expressed as mean ± SEM of three experiments in triplicates.

MD impairs ROS production in glutamate-activated IMR 32 cells:

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 neuronal cells and therefore leads to cell injury. As shown in figure 2, IMR-32 cells when treated with glutamate increased the ROS production (Bar 2) as compared to control (Bar 1). However, incubation together with MD decline glutamate associated ROS production (Bar 3) in IMR-32 cells.

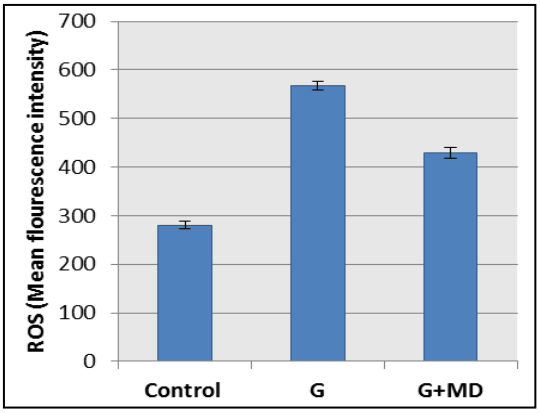

Fig 2: Estimation of ROS levels in IMR 32 cells: ROS level was determined by measuring DCFH fluorescent dye. Bar 1 (control) represents ROS level of untreated cells. Bar 2 (G) represents ROS level of glutamate-treated IMR-32cells. Bar 3 (G+MD) represents ROS level from IMR-32 cells treated with MD in presence of glutamate stress.

Conflict of interest statement

We declare that we have no conflict of interest.

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