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RESEARCH ARTICLE

RHEUM EMODI AMELIORATES ETHANOL INDUCED CYTOTOXICITY IN LIVER CELLS

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ABSTRACT

The aim of this work was to investigate the effect of *Rheum emodi* (MER) on ethanol (EtOH) induced cytotoxicity in human liver cells. Cells were treated with either EtOH (30mM) alone or together with MER (150µM) for 12 hours. It was found that exposure with EtOH induced different cytotoxicity processes like formation of oxygen radical formation (ROS), lactate dehydrogenase (LDH) leakage, lipid peroxidation, decrease in glutathione (GSH) as compared to untreated cells. EtOH induced ROS formation was significantly prevented by MER treatment. Other beneficial effects associated with MER treatment in EtOH challenged cells were increase in GSH level and reduction in LDH leakage and lipid peroxidation. Our findings suggest that MER exert cytoprotective action against EtOH induced liver cell damage.

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INTRODUCTION

Ethanol consumption is main cause of liver diseases and causes about 3.8% of global mortality (Li *et al*., 2015). Chronic ethanol consumption is responsible for cellular and tissue damages (Wlodek and Rommelspacher, 1994). Ethanol metabolism mediates production of reactive oxygen species (ROS) like hydroxyl radical, hydrogen peroxide and superoxide (Wu and Cederbaum, 2003) (Kurose *et al*., 1996). Apart from ROS generation, ethanol induces inhibition in antioxidant enzymes, decrease in antioxidants like glutathione (GSH), enhanced lipid peroxidation etc in liver (AI, 2001) (Meagher *et al*., 1999). These molecular changes associated with the ethanol consumption in liver cells leads cell death (Wu and Cederbaum, 1999) (Masalkar and Abhang, 2005). Antioxidants are considered to be the potential pharmacological agents for treating alcoholic liver diseases. Several herbal formulations are presently in use as hepato-protectants. *Rheum emodi* (*Himalayan rhubarb)* is one such herb used as tonic for several years in India. *Rheum emodi* (locally known as *Pam chalan*) is a well known herb with immense medical potential. It is an effective antispasmodic, antiseptic, anticholesterolemic, antitumor, aperients, astringent, cholagogue, diuretic and stomachic. Its chemical constituents are mainly hydroxyanthracene derivatives (emodin, rhein, aloe emodin and physcion, chrysophanol and their glycosides) (Alam *et al*., 2005a) Aqueous extract of *Rheum emodi* has been shown to protect the proximal tubule segments of kidneys against cadmium chloride, potassium dichromate, mercuric chloride and gentamicin-induced nephrotoxicity in rats (Alam *et al*., 2005b). Anthraquinone derivatives isolated from *Rheum emodi* like emodin, aloeemodin and rhein has been shown to found to exhibit antiproliferative activity against different cancer cells. Emodin exhibits antiproliferative activity against colorectal, breast, lung prostate and cervical cancers cells (Chan, 1993) (Zhang *et al*., 1995) (Kuo *et al*., 1997) (Cha *et al*., 2005). Ethanolic extracts of *Rheum emodi* has been found to show hepatoprotective effects against carbon tetrachloride (CCl4)-induced liver damage in Wister rats (Ibrahim *et al*., 2008). Similarly aqueous extract of *Rheum emodi* has been found to function as hepatoprotective entity against paracetamol induced damage in albino rats (Akhtar MS, 2009). So, *Rheum emodi* was chosen for present study as it possesses tremendous therapeutic potential and may pave way for amelioration of ethanol induced cytotoxicity in liver cells.

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MATERIALS AND METHODS

Preparation of the methanolic extract of *Rheum emodi*

The dried rhizome of plant was powdered and extracted with methanol. The alcohol was distilled off at 45°C and aqueous

part was dried to obtain the extract. The extract of *Rheum emodi* was prepared in dimethyl sulfoxide (DMSO) for cell line studies.

(1) Cell culture and treatments

Human Chang liver cell line was 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. Cells were sub-cultured by trypsinization and cultured in plates according to the requirement of the experiment. After 24 hrs of seeding, liver cells were treated with ethanol EtOH (30mM) in presence/absence of Rheum emodi (MER) extract (150µM).

(2) LDH leakage

Cells were cultured in a 24- well plate for 24 hrs and subsequently treated with EtOH (30mM) alone or together with MER (150µM). 100 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

Cells were cultured in a 24-well plate for 24 hrs and were treated with EtOH (30mM) alone or together with MER (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 Thiobarbituric acid reactive substances (TBARS) was used to measure lipid peroxidation (Niehaus and Samuelsson, 1968).

(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 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 MER (150µM) for 12 hrs and later on fluorescence detection was done.

(5) GSH assay

Chang liver cells were cultured in a 6-well plate for overnight. Cells were treated with either EtOH (30mM) alone or together with MER (150µM) for 12 hrs**.** Media was removed and cells were rinsed four times with 1x phosphate buffer saline (PBS). Cells were collected by centrifugation (3000rpm for 4 minutes), supernatant was removed and 200µl 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 15 min at 4°C). Supernatant was extracted five times with diethyl ether to remove TCA, followed by measurements of GSH (F, 1969).

(8) Statistical analysis

In the present study, 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

Effect of MER 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 (Bailey, 1998.) (SHANNON M. BAILEY, 1999). As shown in figure 1, Chang liver cells when treated with EtOH increased the ROS production (Bar 2) as compared to control (Bar 1). However, incubation together with MER decline EtOH associated ROS production (Bar 3) in liver cells.

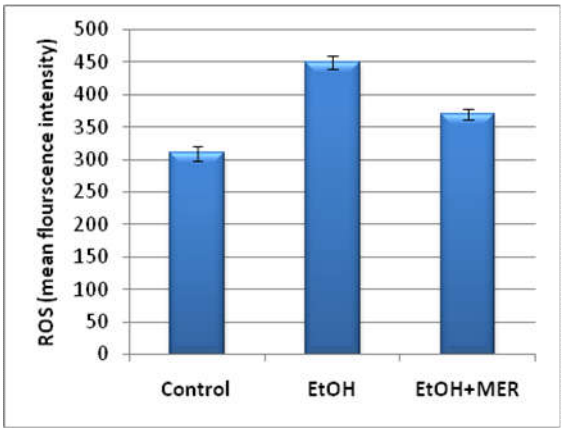

Figure 1. Effect of MER on ROS level: ROS level was determined by measuring DCFH fluorescent dye. Bar 1 (control) represents ROS level of untreated liver cells. Bar 2 (EtOH) represents ROS level of EtOH-treated liver cells. Bar 3 (EtOH+MER) represents ROS level from liver cells treated with MER in presence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

Protective role of MER 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 MER treatment reflects its role in protecting cells against EtOH-induced toxicity. As shown in figure 2, 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 MER significantly decreased EtOH associated LDH leakage (Bar 3) in liver cells.

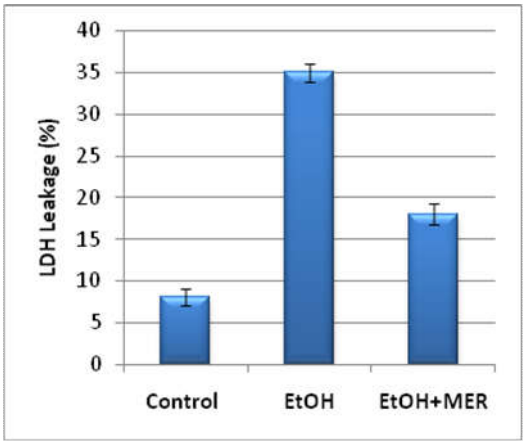

Figure 2. Effect of MER on LDH leakage: LDH leakage was determined using LDH assay kit. Bar 1 (control) represents LDH leakage from untreated liver cells. Bar 2 (EtOH) represents LDH leakage level from EtOH-treated liver cells. Bar 3 (EtOH+MER) represents LDH leakage level from liver cells treated with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

Effect of MER 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 3, 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 MER decline EtOH associated lipid peroxidation (Bar 3).

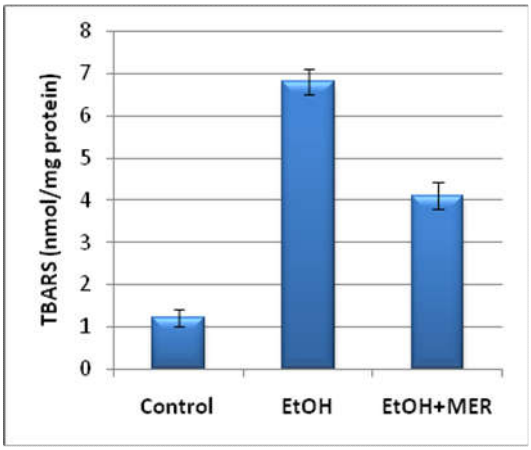

Figure 3. Effect of MER on lipid peroxidation: Lipid peroxidation was determined by measuring TBARS release. Bar 1 (control) represents lipid peroxidation from untreated liver cells. Bar 2 (EtOH) represents lipid peroxidation level from EtOH-treated liver cells. Bar 3 (EtOH+MER) represents lipid peroxidation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

Effect of MER on GSH redox state

GSH plays an important role in neutralizing oxidative stress and mitochondrial injury caused due to various toxins (Dickinson DA, 2002). It has been found that EtOH induces depletion of GSH level in Chang liver cells and causes cytotoxicity (CS., 2000). As shown in figure 4, EtOH treatment declines redox status of chang liver cells by decreasing the GSH level (4). However, MER treatment of EtOH challenged liver cells, intriguingly recovered the GSH (Bar 3rd of the Figure 4).

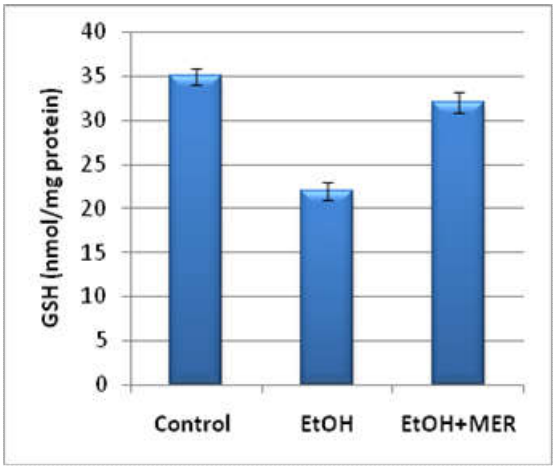

Figure 4. Effect of MER on GSH level: GSH levels were determined by DTNB (5,5'-dithiobis-(2-nitrobenzoic acid) method. Bar 1 (control) represents GSH level from untreated liver cells. Bar 2 (EtOH) represents GSH level from EtOH-treated liver cells. Bar 3 (EtOH+MER) represents GSH level from liver cells treated with MER in presence of EtOH stress

Conflict of interest statement

We declare that we have no conflict of interest.

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