ABSTRACT. In this study we have investigated the protective effect of Trigonella seed extract against ethanol induced toxicity in BRL3A rat liver cells. Cells were treated with either 30 mM ethanol alone or together with Trigonella seed extract for 24 h. We have performed various assays in treated cells to evaluate the ability of seeds to prevent the toxic effects of ethanol. Ethanol treatment suppressed the growth of BRL3A rat liver cells and induced cytotoxicity, oxygen radical formation and mitochondrial dysfunction. Incubation of TSE with ethanol increased cell viability in a dose-dependent manner. It caused a decrease in lactate dehydrogenase outflow. Trigonella seed extract reduced the production of TBARS. Apoptosis was observed in ethanol treated cells while TSE reduced apoptosis by decreasing the accumulation of G1 phase cells. The cytoprotective effects of TSE were comparable with those of a positive control. For this we have used silymarin, a known hepatoprotective agent. The data obtained suggest that fenugreek seeds can be considered cytoprotective in ethanol induced liver damage due to their polyphenolic compounds.

INTRODUCTION

A worldwide problem regarding the socio-economic and medical impact is the chronic ethanol consumption. Ethanol, also called ethyl alcohol, pure alcohol, grain alcohol, or drinking alcohol, is a volatile, flammable, colorless liquid. It is a psychoactive drug, best known as the type of alcohol found in alcoholic beverages and in modern thermometers. Ethanol is converted into acetaldehyde by alcohol dehydrogenase and then into acetic acid by acetaldehyde dehydrogenase. The product of the first step of this breakdown, acetaldehyde, is more toxic than ethanol. Acetaldehyde is linked to most of the clinical effects of alcohol. It has been shown to increase the risk of developing cirrhosis of the liver, multiple forms of cancer, and alcoholism (Mos L. et al., 2008). The mechanisms by which ethanol causes cell injury are many and the effects can be on the short or long-term. Some of the major hypotheses suggested to play a significant part in ethanol-induced cell injury are redox state changes, damage to mitochondria, direct effects of ethanol on membrane and excessive generation of free radicals (Cederbaum, 2001).

Ethanol is a central nervous system depressant and has significant psychoactive effects in sublethal doses; for specifics, see effects of alcohol on the body by dose (Table 1). Based on its abilities to change the human consciousness, ethanol is considered a drug. Death from ethyl alcohol consumption is possible when blood alcohol level reaches 0.4%. A blood level of 0.5% or more is commonly fatal. Levels of even less than 0.1% can cause intoxication, with unconsciousness often occurring at 0.3-0.4%.

<table>
<thead>
<tr>
<th>BAC (mg/dL)</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>Euphoria, talkativeness, relaxation</td>
</tr>
<tr>
<td>&gt;100</td>
<td>Central nervous system depression, impaired motor and sensory function, impaired cognition</td>
</tr>
<tr>
<td>&gt;140</td>
<td>Decreased blood flow to brain</td>
</tr>
<tr>
<td>&gt;300</td>
<td>Stupification, possible unconsciousness</td>
</tr>
<tr>
<td>&gt;300</td>
<td>Possible death</td>
</tr>
<tr>
<td>&gt;550</td>
<td>Death</td>
</tr>
</tbody>
</table>

Table 1

Effects of alcohol on the body by dose (after Pohorecky and Brick, 1988)

Trigonella foenum graecum and Trigonella corniculata are annual herbs that belong to the family Fabaceae. It has a long history as both a culinary and medicinal herb. The seeds of Trigonella are commonly used as a spice in food preparations due to the strong flavour and aroma. The seeds are reported to have restorative and nutritive properties (Pribac et al., 2008). Trigonella seeds are used in remedies for diabetes and hypercholesterolaemia in Indian, Arabic and Chinese medicine. Its utility has been proved experimentally in

Possible protective effects on the liver cells may be controlled by supplementation with antioxidant substances of vegetal origin. *Trigonella* seeds are supposed to have antioxidant activity and have been shown to produce beneficial effects. Numerous effects were reported, the most significant are neutralization of free radicals and enhancement of antioxidant apparatus (Anuradha and Ravikumar, 2001). A study of *Thirunavukkarasu*, has shown that the administration of fenugreek seeds protects rat liver from ethanol-induced oxidative stress (Thirunavukkarasu et al., 2003). Furthermore, to explore the protective role of the seeds in detail, the effect of the polyphenolic extract of the seeds was evaluated on EtOH-induced cytotoxicity in Chang liver cells and compared it with a standard hepatoprotective drug silymarin (extracted from *Silybum marianum*) (Kaviarasan S. et al, 2006).

*Silybum marianum*, commonly known as ‘milk thistle’ (Family: Asteraceae/Compositae) is one of the oldest and thoroughly researched plants in the treatment of liver diseases. The extracts of milk thistle is being used as a general medicinal herb from as early as 4th century B.C. The active constituents of the plant are obtained from the dried seeds and consist of four flavonolignans which are collectively known as silymarin. Wagner et al characterized these active compounds and Flora et al reviewed its history, properties and the clinical effects (Wagner et al. 1974, Flora et al., 1998). The safety and efficacy of this herbal drug has been analyzed by a systematic approach in a review by Saller et al., 2001.

In the present study we evaluated the protective role of the seeds by measuring cell viability, MTT metabolism, LDH leakage, intracellular ROS production and apoptosis in BRL3A rat liver cells exposed to ethanol in the presence and absence of *Trigonella* seeds and or silymarin.

**MATERIALS AND METHODS**

**Preparation of *Trigonella* seed extracts.** *Trigonella f.g.* and *Trigonella c.* seeds (100 g each) were finely powdered mixed with 75% methanol and kept at room temperature. After 5 days it was filtered and the solvent was evaporated. The residue was dissolved in water and the aqueous layer was washed with petroleum ether several times until a clear upper layer of petroleum ether was obtained. The lower layer was then treated with ethyl acetate containing 100º acetic acid (10 ml/l). Extraction of polyphenols was carried out for 36 h at room temperature and the combined ethyl acetate layer was concentrated. The residue was lyophilized and stored at -70ºC. The result was about 5-7 g per 100 g of seed powder. An aqueous extract was prepared and used for the following studies.

**Cell culture.** Monolayer cultures of BRL3A rat liver cells were grown in DMEM growth medium containing 10% heat inactivated fetal calf serum, 100 mg/ml penicillin and 100 mg/ml streptomycin. Cells were incubated in 25 cm² tissue culture flasks at 37ºC in a humidified atmosphere (5% CO₂).

Cells were plated at different seeding densities in complete medium and allowed to settle for at least 4 h. The cultures were washed twice in warm phosphate-buffered saline (PBS) and incubated at 37ºC in a humidified atmosphere: 5% CO₂.

At 75-80% confluence the cells were treated with either ethanol (30 mM) or ethanol + seed extract (20, 40, 60 mg/ml) or ethanol + silymarin (30 mg/ml) for 24 h. Preliminary studies were done to fix the time of exposure and dosage of ethanol, seed extract and silymarin using cell viability and MTT assay.

**Cell viability.** This was measured by the ability of living cells to eliminate trypan blue vital dye. Cells were seeded in 96-well microplates at a density of 10⁵ cells/well and were treated with ethanol in the presence and absence of seed extract/silymarin for 24 h. After this, the cells were trypsinized and pelleted by centrifugation at 1000 g for 10 min at 4ºC. Cells were then washed twice with PBS, pH 7.2–7.4, and trypan blue was added at a final concentration of 0.2%. Living cells were counted in a haemacytometer and expressed as the percentage of the total count in untreated control. Moreover, a dose dependent study was carried out for the seed extract to find out the maximum inhibition.

**MTT metabolism.** Mitochondrial function was evaluated by following the conversion of MTT to a purple formazan product. Briefly, cells were seeded in a 96-well microtiter plate (2 x 10⁴ cells/well in 100 ml of complete medium) and then incubated. After 24 h, 50 ml of MTT (5 mg/5 ml) was added to each well, and the cells were incubated in the dark at 37ºC for an additional 4 h. Afterwards, the medium was removed, the formazan crystals were dissolved in 200 ml of dimethyl sulfoxide, and the absorbance was measured at 570 nm.

**LDH outflow.** The release of LDH from cells was used as a measure of membrane damage. Cells were seeded in 24-well plates at a density of 2 x 10⁵ cells/well in 500 ml of complete medium and incubated with additives. At the end of the treatment, one aliquot of medium (0.2 ml) was taken out for extracellular LDH activity analysis. The total LDH activity was determined after the cells were disrupted thoroughly by sonication. In order to reflect the cytotoxicity, the percentage of LDH released into the medium against the total activity present in the hepatocytes was calculated.

**Flow cytometry.** In order to detect the presence of apoptotic cells and the cell-cycle distribution we used flow cytometry techniques. After cultivation in the medium alone or in a medium containing 30 mM of ethanol in the presence and absence of seed extracts or
silymarin, the cells were harvested and washed with PBS. Then, they were fixed and stained. The cell-cycle distribution was quantified using FACScan Flow Cytometry (Becton–Dickinson) and analysed by Cell Quest software.

**Statistical data.** The data were analysed for statistical significance using Student’s t-test. Triplicate experiments were performed and the results are presented as means ± SD. A value of $P < 0.05$ was considered significant.

**RESULTS AND DISCUSSIONS**

The viability of cells as a percentage of the total count in control cells is presented in figure 1. Using 30 mM ethanol, only 40% of cells were viable. Simultaneous incubation of the same dose of ethanol with 20, 40, and 60 mg/ml TSE, increased the cell viability to 50, 55, and 80%, respectively. Silymarin treatment increased the cell viability to 85%.

**Fig. 1** The chart represents the percent viability of BRL3A rat liver cells. TSE increased cell viability in a dose-dependent manner in ethanol treated BRL3A rat liver cells. Data are means ± SD (n = 3). *Significantly different from control ($P < 0.05$). **Significantly different from ethanol treated cells ($P < 0.05$)

Ethanol is shown to cause cytotoxicity in BRL3A rat liver cells, as proved by the release of LDH. Cultured cells with TSE significantly reduced ethanol induced LDH outflow in a dose-dependent manner ($P < 0.05$) (Fig. 2). Silymarin, on the other hand, significantly inhibited ethanol induced membrane leakage.

Mitochondrial function was assessed by its ability to metabolize MTT. In figure 3 we have shown that cells exposed to ethanol had a decreased MTT metabolism (40% of control, $P < 0.05$), whereas cells exposed to TSE or silymarin using also ethanol showed a significant increase in MTT metabolism.
Fig. 2 Dose-dependent effect of TSE on ethanol induced LDH outflow in BRL3A rat liver cells. Data are the means ± SD (n = 3). *Significantly different from control (P < 0.05). **Significantly different from ethanol treated cells (P < 0.05)

Fig. 3 Mitochondrial assessed by MTT metabolism in BRL3A rat liver cells. Cells were incubated for 24 h with 30 mM ethanol alone or in combination with either *Trigonella* seed extracts (TSE) or silymarin. Data are the means ± SD (n = 3). *Significantly different from control (P < 0.05). **Significantly different from ethanol treated cells (P < 0.05)
Trigonella foenum-graecum and Trigonella corniculata seed extracts exert a protective action on alcohol toxicity in BRL3A rat liver cells

Fig. 4 Dose-dependent inhibition of TSE on ethanol induced TBARS production in BRL3A rat liver cells. Rat liver cells were treated with ethanol with and without concentrations of TSE or silymarin. Data are means ± SD (n = 3). *Significantly different from control (P < 0.05). ** Significantly different from ethanol treated cells (P < 0.05)

From Figure 4 it can be seen that treatment of cells with ethanol led to significantly higher release of Thiobarbituric Acid Reactive Substances (TBARS). Trigonella seed extract caused a dose-dependent reduction of TBARS production. Among the three concentrations tested, the reduction was the highest at the concentration of 60 mg/ml. Addition of silymarin also reduced TBARS production in rat liver cells treated with ethanol.

In table 2 are indicated the alterations in the percentage of cells in the cell cycle stages: G1, S and G2/M phase. In cells exposed only to ethanol, the percentage of cells at S phase and G2/M phase were decreased (5.17 and 2.35%), corresponding to an increase in the percentage of cells at G1 phase (92.5%). An increase in the percentage of cells in the G2/M and S phase with a decrease in G1 phase was observed in control cells, TSE or silymarin treated cells.

As previous studies show, Trigonella seeds seem to have multiple protection effects and other therapeuitic results such as: lower blood sugar levels, lower seric cholesterol and lipids etc. (Pribac et al. 2003, 2006). Other published studies have shown that Trigonella seeds have a various benefits under various experimental conditions. The most significant effects of these seeds are antidiabetic (Sharma et al., 1996) antiiinflammatory (Thakur et al., 1994) and antineoplastic effects (Sur et al., 2001). Nevertheless, there is limited data concerning its protection against ethanol toxicity. Moreover, there few reports concerning the effects of fenugreek seeds on the cytotoxicity induced by ethanol (Kaviarasan S. et al., 2006).

For this study we used BRL3A rat liver cells to assess ethanol toxicity. In a 1998 study, Cameron et al. reported that hepatoblastoma cells (HepG2) exposed to 60-80 mM ethanol for 24 h have exerted cells toxicity, as dose-dependent. Cytotoxicity in normal human liver cells after exposure to 69 -174 mM ethanol was obtained in a previous study by Walker et al., 1974. In a previous study, we reported that Trigonella seed administration may induce apoptosis in culture cells and that we observed the increase of oxidative stress biomarkers (Pribac et al., 2008).

In the present study, the cytotoxic effect in BRL3A rat liver cells was obtained after 24 h exposure to 30 mM ethanol. Ethanol inhibited the viability of rat liver cells as a consequence of apoptosis induction asserted by flow cytometry. Ethanol also caused accumulation of cells in the G1 peak, as the hallmark of apoptosis (Wu and Cederbaum, 1999). Cells treated with ethanol displayed enhanced density and more apoptotic nuclei than the control cells.

As shown in previous studies, acute intoxication with ethanol induces oxidative stress and apoptosis in primary cultured hepatocytes (Higuchi et al., 2001).
Oxidative stress and cytokines have been suggested to play an important role in liver cell apoptosis in alcoholic liver disease (Adachi et al., 2004). The cells exposed to ethanol showed important increase in the production of TBARS, and significant alterations in redox status.

Other studies have shown that ethanol metabolism in the liver cell causes depolarization of the mitochondrial membrane, resulting in a mitochondrial permeability transition followed by cytochrome c release, caspase activation, and apoptosis (Nakayama et al., 2001). In our study, mitochondrial dysfunction and membrane damage are evident in cells with ethanol.

Treatment with TSE resulted in an increase of cell viability as compared with ethanol treated cells. The data show that TSE caused a dose-dependent reduction in TBARS production and LDH outflow. TSE show also an inhibition of the formation of apoptotic nuclei.

**CONCLUSIONS**

As shown also in Kaviarasan studies, these data may suggest that the polyphenols, present in the extract, might have a cytoprotective effect (Kaviarasan et al., 2008). For example the antioxidant, probucol protects against hypochlorite-induced endothelial dysfunction (Witting et al., 2005). Quercetin, one of the constituents of the extract, has been reported to have protective effects on cells suffering from overload of intracellular Ca2+ (Sakanashi Y. et al., 2008). There are few studies that can explain the mechanism involved in the protection effects of phenols. Some studies suggest that the scavenging activities of phenols are the consequence of the active hydrogen ability resulting from the hydroxyl substitutions (Bors et al., 1996).

Additionally, the flavonoids separate into the hydrophobic core of the membrane similar to cholesterol and may cause a modification in lipid fluidity (Arti et al., 2000). As a consequence, these structures, may react with the membrane domains and intracellular structures, and protect the cells from oxidative alteration.

<table>
<thead>
<tr>
<th>Groups / Cell cycle phases</th>
<th>Percentage of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1</td>
</tr>
<tr>
<td>Control</td>
<td>5.5±0.25</td>
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<tr>
<td>Ethanol</td>
<td>92.5±0.20</td>
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<tr>
<td>Ethanol + TSE</td>
<td>31.28±1.75</td>
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<tr>
<td>Ethanol + silymarin</td>
<td>23.8±1.56</td>
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<table>
<thead>
<tr>
<th></th>
<th>S</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>65.1±2.23</td>
</tr>
<tr>
<td>Ethanol</td>
<td>5.17±0.66</td>
</tr>
<tr>
<td>Ethanol + TSE</td>
<td>58.11±2.12</td>
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<tr>
<td>Ethanol + silymarin</td>
<td>63.12±1.96</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22.20±0.92</td>
</tr>
<tr>
<td>Ethanol</td>
<td>2.35±0.33</td>
</tr>
<tr>
<td>Ethanol + TSE</td>
<td>6.52±0.22</td>
</tr>
<tr>
<td>Ethanol + silymarin</td>
<td>7.50±0.85</td>
</tr>
</tbody>
</table>

**Values are means ± SD of three independent experiments**

Concluding, we have shown that Trigonella seeds may protect cell structure and function from the toxic effects of ethanol. TSE has a similar action as the hepatoprotective agent, silymarin. These information offer prove for the use of Trigonella seeds in the treatment of chronic alcoholic liver disease. There is also a need for further studies on the molecular mechanisms of TSE protective effects.

**REFERENCES**


Trigonella foenum-graecum and Trigonella corniculata seed extracts exert a protective action on alcohol toxicity in BRL3A rat liver cells


Wagner H, Diesel P, Seitz M. The chemistry and analysis of silymarin from Silybum marianum Gaertn. Arzneimittelforschung 1974; 24, pp. 466-71

Flora K, Hahn M, Rosen H, Benner K. Milk thistle (Silybum marianum) for the therapy of liver disease. J Gastroenterol 1998; 93, pp. 139-43


Pribac G.C., Ardelean A., (2008) Diosgenin, the active principle of Trigonella sp. extracts may induce apoptosis on MCF7 cancer cells through caspase activation, Studii de Stiinta si Cultura, pp. 7-11


