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Evaluation of Hepatoprotective Activity of *Ocimum sanctum* in HepG2 Cell Line.

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The aqueous extract and steam distilled oil obtained from *Ocimum sanctum* Linn. (OS) were evaluated for hepatoprotective activity against ethanol-induced cell damage in HepG2 cells. The results of this study indicated that morphological changes in HepG2 cells induced by 120*mM* ethanol were restored to normal by the aqueous extract of OS and steam distilled oil at different doses (3-100µg/ml). Cell viability studies further confirmed the hepatoprotective activity of aqueous extract of OS as it significantly increased the percent cell viability ranged between 85 to 133 percent at concentration 3 -100 µg/ml, when compared to ethanol intoxicated cells. However, steam distilled oil has shown hepato-protective action at lower dose as it increased percent cell viability ranged between 89 to 115 per cent at 3 -25 µg/ml concentration.Both aqueous extract and steam distilled oil were found to reduce elevated enzyme levels such as Aspartate amino transferase(AST) and Alanine amino transferase (ALT). The finding suggests that the aqueous extract and steam distilled oil obtained from O*cimum sanctum* Linn. exhibited hepatoprotective effect against ethanol-induced hepatotoxicity in HepG2 cell line.

Keywords: Ethanol; Hepatototoxicity; HepG2 Cell Line; steam distilled oil; *Ocimum sanctum*

INTRODUCTION

Ocimum sanctum Linn. (OS) commonly known as tulsi (Family Labiateae) is the most useful traditional medicinal plant in India. It is an herb that has immense medicinal properties such as hepatoprotectiveanti-stress, anti-oxidant, adaptogenic, anticancer, radioprotective activities. [4, 19] Its modern medicinal applications are receiving widespread attention day by day. The aqueous extract of *Ocimum sanctum* exhibited significant antioxidant activity against several paradigms of oxidative stress induced by a variety of techniques in different rat tissues, which was comparable to that of vitamin E[2]. Preliminary studies have reported that ethanol extract (90%) of the leaves showed hepatoprotective effect against paracetamol-induced liver damage in rats[4]. However, there has been no scientific report on the *in-vitro* hepato-protective activity of OS in the literature. The quest to identify hepato-protective activity from plant sources prompted us to evaluate the activity of OS in both forms (aqueous extract and steam distilled oil) against ethanol induced damage in HepG2 cell line, as these cells retain many of the morphological and biochemical characteristics of normal hepatocytes. The present *in vitro* studies benefit over the *in vivo* studies that had been done in the past in such a way that the *in vitro* screening is less time consuming, specific, accurate and have the potential to provide information about the function of a specific organ and how a toxic agent can adversely affect them. Silymarin was used as standard in this experiment as it has antioxidant property which is responsible for its beneficial effects against liver damage[20].

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MATERIALS AND METHODS

Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), Dulbecco's Phosphate Buffer saline (PBSA), Trypan blue (0.5%) solution, 0.25% Trypsin & 0.02% EDTA,MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide]were purchased from Hi-media, Mumbai. Silymarin was obtained as a gift sample from Ranbaxy Research Laboratory, New Delhi.

Cell line

The human derived HepG2 cell line was obtained from the National Center for Cell Science, Pune, India.

Plant material

The leaves of OS were procured from Delhi, India in the month of October 2004. The plant material was authenticated by comparison with the reference specimen, preserved at the National Herbarium of Cultivated Plants, by Dr. E. R. Nayar at National Bureau of Plant Genetic Resources (Indian Council of Agricultural Research), Pusa campus, New Delhi.

Preparation of aqueous extract

Ocimum sanctum (dry extract) was supplied by Sanat Products Ltd. and the aqueous extract was prepared by dissolving the powder in Millipore water. The solution was then passed through the membrane filter (0.22 microns) and stored at -20°C prior to use.

Extraction of volatile oil

Steam distilled volatile oil was extracted from freshly collected leaves of *Ocimum sanctum* using steam distillation were used for the experimental studies. Fresh leaves of OS were collected locally and steam distilled *oil* was obtained by refluxing with double distilled water at 100^{o}C in Clevenger apparatus. The greenish yellow oil obtained from the fresh leaves of OS was dissolved in dimethyl sulfoxide (DMSO) and then passed through the membrane filter (0.22 microns) and stored at -20°C prior to use.

Preparation of Positive Control

Silymarin powder was dissolved in DMSO and the whole of the solution was then passed through membrane filter (0.22 microns) and stored at -20°C prior to use.

Experimental design

HepG2 cells were grown and subcultured as monolayer in DMEM supplemented with 10 percent FBS and antibiotic antimycotic solution. A total of 8 groups were used for the experimental studies which are as follows:

Group1 (Control): No treatment was given to these cells.

Group2 (Ethanol treated): The cells were treated with ethanol (120 m*M*) for 24h.

Group3 (Aqueous extract control): These cells were treated with different concentrations (3, 6, 12.5, 25, 50 and 100 µg/ml) of aqueous extract of OS alone.

Group4 (Aqueous extract Treated): These cells were treated with different concentrations (3, 6, 12.5, 25, 50 and 100 µg/ml) of aqueous extract of OS along with (120 m*M*) ethanol.

Group5 (Silymarin Control): In this group, cells were treated with different concentrations (3, 6, 12.5, 25, 50 and 100 µg/ml) of silymarin alone and used as positive control, as it has antioxidant property, which was responsible for its beneficial effects against liver damage.

Group 6 (Silymarin Treated): In this group, cells were treated with different concentrations (3, 6, 12.5, 25, 50 and 100 µg/ml) of silymarin along with ethanol (120 m*M*).

Group7 (steam distilled oil Control): These cells were treated with different concentration (3, 6, 12.5, 25, 50 and 100 µg/ml) of steam distilled oil alone.

Group 8 (steam distilled oil Treated): These cells were incubated with different concentrations (3, 6, 12.5, 25, 50 and 100 µg/ml) of steam distilled oil along with ethanol (120 mM).

Time period kept constant i.e., 24 h.

The hepato-toxic dose of ethanol was optimized as we did not observe significant toxicity, even after culturing cells with 80 *mM* concentration. Thus the hepatotoxic dose of ethanol was selected as 120 *mM* as dose-dependent cytotoxicity was found at higher concentrations (100-120 *mM*).

Cell Culture and Cell Viability Estimation

Cellular viability was determined using standard MTT assay[11] and Trypan blue dye exclusion method. Mitochondrial functional assay includes MTT Assay which detects the mitochondrial function of dying cells during the early stages of cell death while Membrane Integrity Assay includes Trypan blue dye uptake by hemocytometer counting which detects loss of membrane integrity during the later stages of cell death.

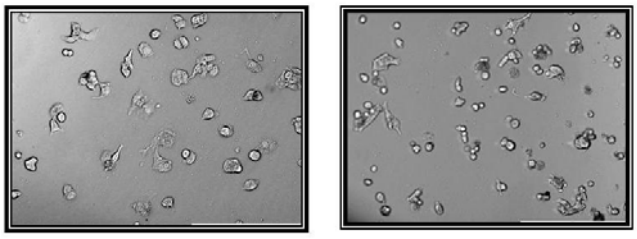


Fig1. Phase contrast microscopy of HepG2 cells. A-Control Cells, B-Ethanol treated cells

Cell viability estimation by MTT assay

HepG2 cells were grown and subcultured as monolayer in DMEM supplemented with 10%FBS and antibiotic antimycotic solution.Cells were seeded in a 96 well microplate (Nunclon D_3 technologies) at a concenteration of 5 x 10^3 cells/well , in a final volume of 100µl culture medium per well and left for 24h at 37^{o}C in a humidified atmosphere of 5 percent CO_2/95% air. The cells were then pretreated with different doses of aqueous extract of OS and steam distilled oil as a test,silymarin as a positive control for 2h.After 2h, cells were exposed to medium containing toxicant(ethanol) or medium alone, in treated and control wells respectively for 24h. MTT(25 µl) reagent was added to each well including control and incubated for a further 2h at 37^{o}C to allow for intracellular reduction of the soluble yellow MTT to the insoluble purple formazan crystals.100 µl of the lyses buffer was then added into each well in order to solubilize the formazan crystals. The plate was incubated overnight at 37^{o}C in order to solubilize the formazan crystals. Quantification of cell viability was done on a microplate reader at a wavelength of 600nm and the mean absorbance for cells grown in the presence(treated) or absence(control) of ethanol was taken. The number of viable cells was proportional to the extent of formazan crystals produced. The percentage cell viability was calculated using the following formula:

$$= \frac{\text{Absorbance of treated group x 100}}{\text{Absorbance of treated group}}$$

Cell viability estimation by Trypan blue exclusion method

HepG2 cells were grown until they are confluent, then harvested and the amount of viability was counted using the haemocytometer.Cells were seeded in a microplate (tissue culture grade,6 wells, flat bottom) at a concentration of 0.02x 10^6 cells/well, in a final volume of 400µl culture medium per well and incubated overnight at 37^{o}C in a humidified atmosphere of 5 percent CO_2/95% air prior to testing. Cells were then exposed to toxicant (ethanol) along with /without various concentration of the aqueous extract of OS or steam distilled oil as a test and silymarin as a positive control or the medium alone for the time period of 24h.At the end of the period, cytotoxicity was assessed by estimating the cell viability of HepG2 cells by haemocytometer counting and levels of AST and ALT were measured in cells treated with ethanol, aqueous extract of OS and silymarin.

$$\% \text{ cell viability} = \frac{\text{Total viable cells (unstained cells) x 100}}{\text{Total cells}}$$

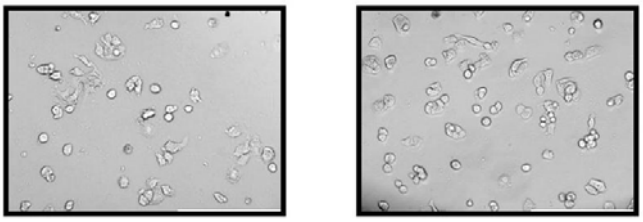


Fig.2. Phase contrast microscopy of protective effects of aqueous extract of OS against damage induced by ethanol (120m*M*) in HepG2 cells. A-OS extract 3µg/ml, B- OS extract 100µg/ml

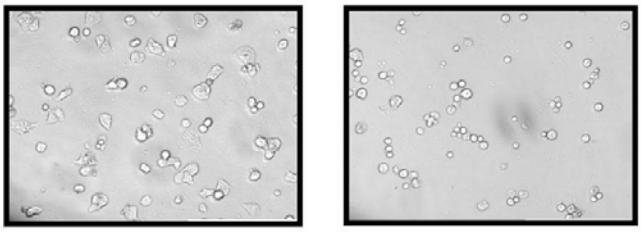
Statistical analysis

All the values are expressed as mean ± standard deviation. The statistical difference was analyzed by student's t-test and significance was calculated as P value and P value of less than 0.05 was regarded as significant.

RESULTS

Hepatoprotective effect of OS in HepG2 cell line

The hepato-protective activity of aqueous extract and steam distilled oil extracted from *Ocimum sanctum* against ethanol induced cell damage in HepG2 cell line was assessed by observing the morphology of the cells, estimating the percent cell viability by MTT assay and Trypan blue dye uptake and measuring the decrease in the levels of AST and ALT into the medium.



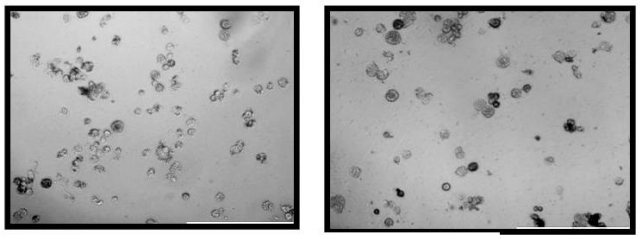
A B

Fig.3. Phase contrast microscopy of protective effects of Silymarin against damage induced by ethanol (120m*M*) in HepG2 cells. A- Silymarin 3µg/ml, B- Silymarin 100µg/ml

Morphology of the Cells

HepG2 cells were spindle shaped; they usually grow in clumps they were adherent to the walls, clear cell borders and nuclei with darker cytoplasm (Fig.1a.).When cells were exposed to ethanol (120mM) for 24 h, the morphology of HepG2 cells were changed from spindle shaped to round (cells were swollen), irregular membrane shape with plasma membrane blebbing (Fig.1b.), the ability of HepG2 cells adhering to walls were decreased, the ability of HepG2 cells in suspension were increased and debris emitted around the cells which may be due to the hepatotoxic effect of ethanol on HepG2 cells.

The changes in the cells, which were caused by ethanol, were largely prevented with the increasing concentration of aqueous extract of OS (Figs.2a, 2b). Thus aqueous extract of OS exhibited dose dependent effect which implies that the effect increases with the increasing concentration, where as Silymarin (Figs.3a, 3b,) prevent changes in the cells which were caused by ethanol at a concentration less than 50µg/ml which may be due to the protection of Silymarin against ethanol-induced toxicity. However, cells treated with steam distilled oil (Figs.4a,4b) prevent changes in the cells, which were caused by ethanol. But with the increase in concentration, the anti-oxidative activities of Silymarin and steam distilled oil could contribute to its cytotoxic properties as the morphology of HepG2 cells were changed from spindle shaped to round (cells were swollen) and ability to adhere to the walls was decreased which suggests that Silymarin had antiproliferative activity at higher concentration whereas exhibit potent hepatoprotective activity at a concentration less than 50µg/ml against ethanol-induced cell damage in HepG2 cells.



A B

Fig.4.Phase contrast microscopy of protective effects of *steam distilled oil* against damage induced by ethanol (120m*M*) in HepG2 cells. A-*Steam distilled oil*3µg/ml, B-*Steam distilled oil* 100µg/ml

Percent cell viability

Ethanol at the concentration of 120mM results in maximum decrease in percent cell viability to 46.01 ± 4.5 and 58.81 ± 2.3 % as assessed by Trypan blue dye exclusion method and MTT assay respectively and this dose was therefore used for the induction of hepatotoxicity. Larger difference in percentage cell viability may be obtained which indicates that cells are at an intermediate stage of programmed cell death. (Fig .5) shows a dose dependent increase in percent cell viability ranged between 85 to 133% at 3-100 µg/ml concentration with aqueous extract of OS, However steam distilled oil showed increase in percent cell viability ranged between 89 to 115% at 3-25 µg concentration, when assessed by MTT assay which may be due to the protection of steam distilled oil against ethanol-induced toxicity. But with the increase in concentration, there was decrease in percent cell viability ranged between 89 to 69% at 50-100 µg concentration of *steam distilled* oil. The result was found to be statistically significant ($P<0.05$), when compared to ethanol-intoxicated cells.

The finding of the MTT assay signifies that insignificant difference($P>0.001$) in percentage cell viability between aqueous extract and *steam distilled* oil, was observed at 25µg/ml ,which implies that *steam distilled* oil was found to be as effective as aqueous extract against ethanol induced toxicity in HepG2 cell line. Results obtained in this investigation indicated that the hepatoprotective activity of *Ocimum sanctum* was comparable to that of Silymarin, which was used as positive control in this study. When aqueous extract of OS at 3-50µg/ml was

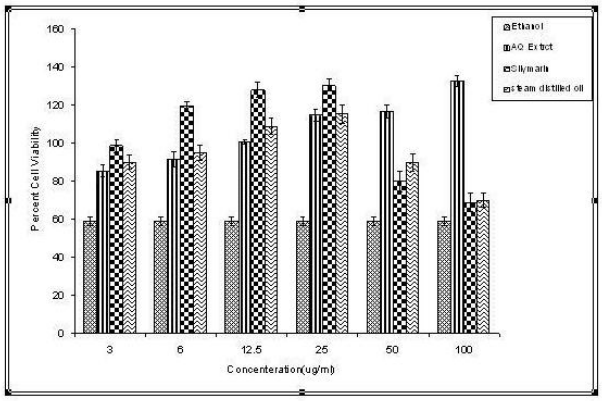


Fig.5. The percent (%) cell viability of different concentrations of OS (aqueous extract and steam distilled oil) and silymarin (positive control) against ethanol 120m*M* (EtOH) induced damage in HepG2 cells as assessed by MTT assay.

compared with silymarin at the same concentration, the result was found to be statistically significant ($P < 0.05$) but found to be insignificant ($P > 0.05$) at 100µg/ml, which implies that OS was found to be as effective as silymarin.

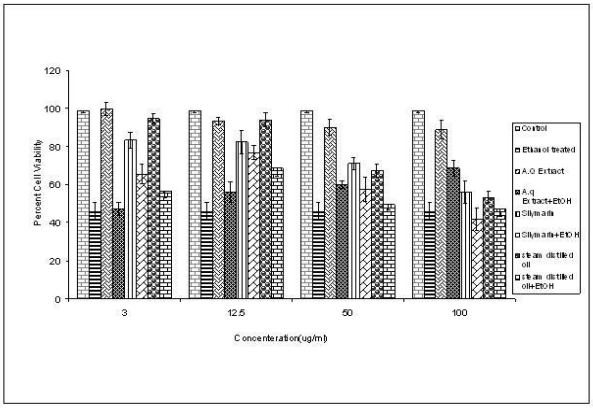


Fig.6. The percent (%) cell viability of different concentrations of OS (aqueous extract and steam distilled oil) and silymarin (positive control) against ethanol 120m*M* (EtOH) induced damage in HepG2 cells as assessed by Trypan blue dye Uptake assay.

Biochemical parameters

Further to signify that ethanol 120m*M* (EtOH) was hepatotoxic, the AST and ALT enzyme levels, which were released from the cells in the medium, were measured. ALT and AST are two of the most reliable markers of hepatocellular injury or necrosis[5]. ALT and AST levels can be elevated in a variety of hepatic disorders. Fig.7a&b depicts significant decrease in liver enzyme levels with increasing concentration of OS (aqueous extract of OS and *steam distilled* oil) when compared to ethanol intoxicated cells.

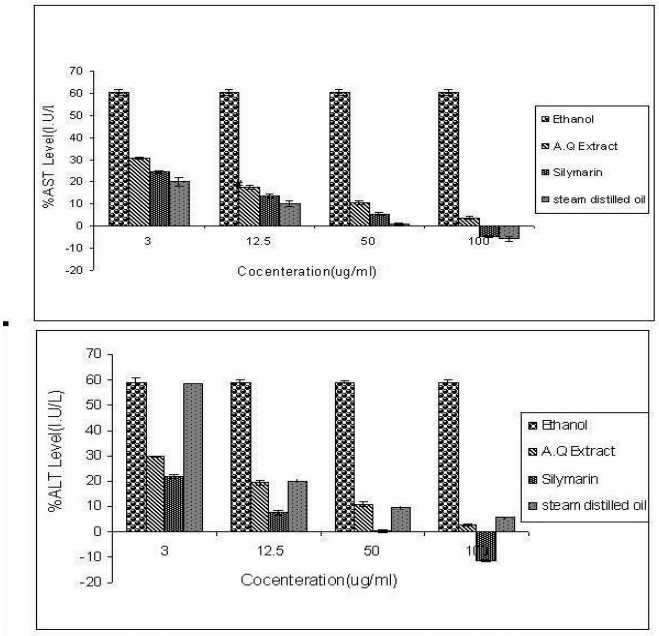


Fig.7. Comparison between ASAT and ALAT (I.U /L) levels of different concentrations of OS in both forms i.e. (aqueous extract and steam distilled oil) and silymarin (control) with Ethanol treated groups in HepG2 cells.

DISCUSSION AND CONCLUSION

Alcohol induced liver diseases is still the most common cause of chronic liver diseases in India and causes serious medical, financial and social problems. HepG2 cells have served as a good model to study the hepatotoxicity of different chemicals or drugs as these cells retain many of the morphological and biochemical characteristics of normal hepatocytes [9]. Although these cells have lost most of their ability to express some enzymes related to ethanol metabolism, such as alcohol dehydrogenase (ADH) and cytochrome P4502E1, which are needed to metabolize ethanol to acetaldehyde, they still retain aldehyde dehydrogenase (ALDH) activity thus results in the production of acetaldehyde which mediate many of the biological effects of ethanol and thus plays an important role in the pathogenesis of alcoholic liver injury.

The hepatoprotective activity of OS was assessed by observing the morphology of the cell, estimating the percent cell viability by MTT assay and Trypan blue dye and measuring the decrease in the level of AST and ALT into the medium.The morphological changes showed that OS and Silymarin largely prevented the damage

that was induced by ethanol in the HepG2 cells, but the morphological approach was difficult to be quantified.. The percent cell viability determined using standard MTT assay[11] and Trypan blue dye exclusion method is useful for predicting whether the cell is damaged or not i.e. basically for detecting the injury that affects cell attachment or may progress to cell death. The finding of our study indicates the significant reduction in percent cell viability when the cells were exposed to ethanol (120m*M*) for 24h. The result of the investigation showed that OS was improving the cell viability, which was decreased by ethanol, and it was observed that a dose dependent increase in percent cell viability was observed with aqueous extract of *Ocimum sanctum* hence favored the ability OS to protect HepG2 cells against ethanol-mediated cytotoxicity. While steam distilled oil at lower concentration was improving the cell viability, which was decreased by ethanol but with the increase in concentration there was decrease in percent cell viability, which may be due to its strong antioxidant action .Further to signify that ethanol (120m*M)* was hepatotoxic, the AST and ALT enzyme levels, which were released from the cells in the medium, were measured. ALT and AST are two of the most reliable markers of hepatocellular injury or necrosis [5]. Their levels can be elevated in a variety of hepatic disorders. With in the two, ALT is thought to be more specific for hepatic injury because it is present mainly in the cytosol of the liver and in low concentrations elsewhere. An increase in the AST level and ALT level in the medium indicated the liver damage by ethanol and there was dose dependent decrease in the AST and ALT levels with aqueous extract of OS and *steam distilled* oil. The hepatoprotective activity of *Ocimum sanctum* was comparable to that of Silymarin, which was used as positive control in this study. The result of the present study suggests aqueous extract and steam distilled oil obtained from O*cimum sanctum* Linn. exhibit hepatoprotective effect against ethanol-induced hepatotoxicity in HepG2 cell line.

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