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Protective effect of *Annona reticulata* Linn againest simvastatin induced toxicity in chang liver cells

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Abstract

The aim of the study was to evaluate the hepatoprotective effect of ethanolic extract of *Annona reticulata* Linn (EEAR) leaves using chang liver cell line. Simvastatin was used to induce the hepatotoxicity in the chang liver cell lines. The extract was evaluated for hepatoprotective effect in simvastatin induced hepatotoxicity in chang liver cells. The percentage viability of in the cell line was evaluated by the MTT assay [(3-(4, 5 dimethylthiazole –2 yl)-2, 5 diphenyl tetrazolium bromide) assay. The EEAR treatment significantly improved viability of chang liver cell line which was evident in morphology. The hepatoprotective activity was confirmed by estimating level of Super Oxide mutase enzyme and lipid peroxidation assay. The EEAR significantly increased super oxide mutase enzyme level and decreased lipid peroxidation. Thus the present study ascertains that the leaf extract of *Annona reticulata* Linn possesses significant hepatoprotective activity.

Keywords: Chang liver cells, Sylimarin, Simvastatin, Super oxide dismutase, MTT.

Introduction

Hepatotoxicity implies chemical-driven liver damage. The various causes of hepatic trouble include parasitic and viral infections, autoimmune diseases and intoxication with various xenobiotics such as chlorinated solvents, alcohol, drugs, herbal medicines, peroxidized fatty acids, fungal toxins, industrial pollutants and radioactive isotopes.[1] Certain medicinal agents, when taken in overdoses and sometimes even when introduced within therapeutic ranges, may injure the organ. Chemicals that cause liver injury are called hepatotoxins.

It is estimated that two billion people around the world are infected with hepatitis B. About 350 million of these have chronic form of the disease. This alarming statistics warrants the immediate necessity for studies to ensure the available formulations or exploration of new herbal therapies to reduce the morbidity and mortality rate due to hepatic complications.

Conventional or synthetic drugs used in treatment of liver disease are inadequate and have serious side effects. Liver diseases remain as one of the serious health problems since we do not have satisfactory liver protective drugs in modern medicine for serious liver disorders [2, 3]

In view of several side effects of these synthetic drugs, there is a growing focus to follow systematic research methodology to evaluate scientific basis of traditional herbal medicine that are claimed to possess hepatoprotective activity. Herbal drugs play a role in the management of various liver disorders most of which enhance the natural healing processes of the liver. A number of plants have been shown to possess hepatoprotective properties by improving the antioxidant status. Therefore, many folk remedies from plant origin are tested for its potential antioxidant and hepatoprotective liver damage in experimental animal model.[4]

The leaves and fruits of *Annona reticulata* Linn were used as a hepatoprotective drug in traditional medicine. It was proved that *Annona squamosa* posses the hepatoprotecive activity.

So there is a possibility of hepatoprotective potential in other species of annonaceae.[5, 6] The present study is intended to explore whether the ethanolic extracts of leaves of this plant could have protective effect on hepatocytes which may contribute to the development of a new formulation for treatment of liver diseases.

Material and Methods

Plant material

The leaves of *Annona reticulata* were collected from local area of village chepparamba in kannur district of kerala. The plant specimen was authenticated by Dr. Jomy Augustine, St.Thomas College, Kottayam and voucher specimen was preserved at St.Thomas College.

Extraction

About 500 gm of dried powder of *Annona reticulata* Linn leaves was extracted using ethanol. It was refluxed for 72 hours and filtered through muslin cloth while hot. The ethanolic extract was dried under vacuum.

Phytochemical Investigation

The preliminary qualitative phytochemical studies were performed for testing the different chemical groups such as alkaloids, tannins, glycosides and saponins etc. present in ethanol extracts [7].

Cell lines and growth media

Chang liver cell lines (Human normal liver cells) were cultured in DMEM (Dulbecco"s modified eagles medium).

Culturing and maintenance of Chang liver cells

Chang liver cells were purchased from National Centre for Cell Science (NCCS), Pune and maintained in Dulbecco's Modified Eagles Medium (DMEM) containing L-glutamine with high glucose at 37°C and 5% CO_2 in a humidified atmosphere in a CO_2 incubator.

Cell culture and treatment

Chang liver cells were cultured in DMEM medium supplemented with 20% heat inactivated Foetal Bovine Serum (FBS). Antibiotics (Streptomycin and penicillin) were added to prevent bacterial contamination. The culture was then filtered and sterilized using 0.2 µm pore size cellulose acetate filter (Sartorious).

Subculturing of Chang liver cells

The subculturing involves transferring a small number of cells in to a new vessel. FBS is used to provide sufficient amount of nutrients for the proper growth of Chang cell line.

Trypsinization

It is the process of using trypsin, a proteolytic enzyme which breaks down proteins, to dissociate adherent cells from the vessels in which they are being cultured. The cell lines were washed with phosphate buffer saline and the fully confluent cells were trypsinized using 500 µl of trypsin (0.025% Trypsin in PBS/ EDTA solution) for 2 minutes at 37^0C and passed to T flasks in complete aseptic conditions. After disaggregation the cells are transferred to other flask and supplemented with media.

Cytotoxicity screening of *Annona reticulata* Linn. in Chang liver cells[8]

The cytotoxicity screening of ethanolic extract of *Annona reticulata* Linn. is carried out using the principle of MTT assay in Chang liver cells.

Upon confluency the Chang liver cells were treated with different concentration of EEAR such as 100, 200 mcg/ml. The standard drug used is Silymarin (200 µg/ml), untreated flask was maintained as control and incubated for 24 hours. After 24 hours, cell culture suspension was washed with PBS, then added with 200 µl MTT (3-(4, 5 dimethylthiazole –2 yl)-2,5 diphenyl tetrazolium bromide) solution to the culture and incubated at 37^0C for 3 hours. Then washed each culture with PBS to remove all MTT, and then added 300 µl DMSO. The mixture was incubated at room temperature for 30 min until the cell get lysed and colour is obtained. The solution was transferred to centrifuge tubes and centrifuged at top speed for 2 minutes to precipitate cell debris. Absorbance was read at 540 nm using DMSO as blank. Percentage viability was defined as the ratio (expressed as a percentage) of the absorbance of treated cells to that of untreated cells. The CTC-50 values are determined by plotting the graph of percentage viability vs concentration.

Screening of protective effect of ethanolic extract of *Annona reticulata* in simvastatin induced toxicity in Chang liver cells[8]

Upon confluency the cells were treated with simvastatin (40 µM) in complete aseptic conditions to induce hepatotoxicity, followed by different concentration of EEAR such as 100 and 200 µg/ml, standard drug used is Silymarin in concentration of 200 µg/ml, untreated flask was maintained as control and incubated for 24 hours. All experiments were carried out triplicate. The hepatoprotective effect was determined by morphological analysis using phase contrast microscopy followed by MTT cell viability assay. The percentage viability was calculated. The graph was plotted with percentage cell viability vs concentration.

Enzyme assays in Chang liver cells

Estimation of Lipid Peroxidation activity[9]

Simvastatin was added to each well containing chang liver cells except normal control. Then added different concentration of EEAR and standard sylimarin (200µg/ml) to the respective

wells. Then wells incubated for 24 hours. After 24 hours, supernatant was collected after centrifuging at 2000rpm for 5min. 1ml supernatent was taken in a test tube containing 500µl 70% ethanol, 2ml 1% TBA and then heated in a boiling water bath for 20 minutes. After cooling to room temperature added 50µl acetone. Then centrifuge at 7000 rpm for 10 minutes and read the absorbance at 535 nm in a spectrophotometer. The MDA levels are determined by plotting the optical density in the standard MDA curve.

Estimation of Super Oxide Dismutase Activity (SOD)[10]

Simvastatin was added to each well containing chang liver cells except normal control. Then added different concentration of EEAR and standard sylimarin(200 µg/ml) to the respective wells. Then wells incubated for 24 hours. After 24 hours, supernatant was collected after centrifuging at 2000rpm for 5min. 50µl of supernatant was pipette into the test tube containing 50mM of 300µl potassium phosphate buffer (pH 7.8), 45 µM of 1.35 µl methionine, 5.3 mM of 0.159 µl riboflavin and 84 µM of 2.52 µl NBT and 20µM of 0.6µl potassium ferricyanide. It was incubated for 30 minutes. OD was read at 600 nm.The percentage inhibition and enzyme levels are calculated.

Statistical analysis

The statistical analysis was performed using Graph Pad Prism software version 6.0. All the results were expressed as Mean±SEM. The datas were analysed using One way Analysis Of Variance (ANOVA) followed by Dunnet's multiple comparison test. The P values<0.05 were considered as statistically significant

Results

Cytotoxicity screening in Chang liver cells

The median cytotoxic concentration of *Annona reticulata* Linn.(CTC-50) was found to be 880 µg/ml**.** From this result, the lower concentrations like 100 µg/ml and 200 µg/ml were fixed for the screening of hepatoprotective activity.

Table 1: Cytotoxicity screening of EEAR using MTT assay

S. N.	Sample	Concentration (µg/ml)	Viability (%)
1.	Control (C)	-	100±0.011
2.	Eear	100	96.59±0.025
		500	72.25±0.032
		1000	41.89±0.041

Hepatoprotective in Chang liver cells

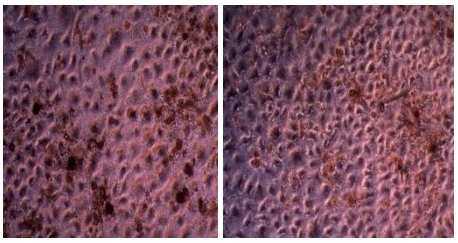
The chang liver cells treated with simvastatin shown the reduction of cell viability. The EEAR treated chang liver cells shows the restoration of cell viability. The extract at concentration of 200µg/ml shows significant increase in cell viability (81.16%). The morphological analysis of chang liver cells confirmed the hepatoprotective activity of EEAR which is evident by the restoration of cellular architecture.

Table 2: Effect of EEAR on percentage cell viability in Chang liver cells

S. N.	Sample	Sample Concentration (µg/ml)	% Viability
1.	Control (c)	-	100±0.0031
2.	Simvastatin	20	46.11±0.356
3.	Silymarin (s)	200	90.74±0.0251***
4.	Eear	100	62.88±0.0726**
		200	81.16±0.0218***

Values are mean ±SEM, n= 6. (One way ANOVA Followed by Dunnette multiple Comparisons test) Statistically significance of ** P<0.01, *** P<0.001, when compared with Simvastatin control

Control **Simvastatin** **standard**



EEAR-100 **EEAR-200**

Figure 1: Morphologicsal analysis in chang liver cells

Enzyme level assays in Chang liver cells

Lipid Peroxidation assay

The lipid peroxidation is a mechanism of free radical type of cell injury. It is expressed in MDA levels (nM/ mg Protein). The treatment of simvastatin causes lipid peroxidation (MDA level: 8.1nM/ mg Protein). The EEAR treated groups at concentration 200µg/ml shows significant reduction of lipid peroxidation (4.6 nM/ mg Protein) which indicate that *Annona reticulata* possess protective role in lipid peroxidation type of injury.

Table 4: Super Oxide Dismutase assay in Chang liver cells

S. N.	Groups	SOD Level (U/L)
1.	Simvastatin	1.23±0.034
2.	EEAR-100	1.65±0.018 **
3.	EEAR-200	1.87±0.034 ***
4.	Standard	2.21±0.065***
5.	Control	2.00±0.032

Values are mean ±SEM, n= 6. (One way ANOVA Followed by Dunnette multiple Comparisons test)
Statistically significance of ** P<0.01, *** P<0.001, when compared with simvastatin group.

Super Oxide Dismutase assay

The super oxide dismutase is an antioxidant enzyme fights with oxidative free radical injury. The simvastatin treated group shows reduction in SOD level (1.23 U/L). The treatment of EEAR at concentration 200µg/ml shows significant increase in SOD level (1.87 U/L).

Discussion

Liver is an important organ actively involved in metabolic functions and is prone to xenobiotic injury due to its central role in the xenobiotic metabolism. Drug induced liver injury (DILI) is responsible for about 5% of all hospital admissions and 50% of all acute liver failures. DILI represents a clinical challenge due to the large number of reported hepatotoxic drugs in current use, the broad spectrum of hepatic injuries by which it may manifest and the frequent absence of clinical findings that permit its diagnosis with certainty.

Popularity of herbal is increasing globally and at least one quarter of patients with liver diseases use ethnobotanicals. There is a growing demand of methodological scientific evaluation to unravel the mysteries hidden in the plants. This approach will help exploring the real therapeutic value of these natural pharmacotherapeutic agents and standardized the dosage regimen on evidence based findings to become more than a fashionable trend.

In the previous study, it was reported that simvastatin caused oxidative stress mediated hepatotoxicity in the *chang liver* cells[11,12].

The *in vitro* hepatoprotective activity was studied in the chang liver cells by MTT assay, where the toxicity in the cell line was induced by using Simvastatin (40µM). The reduction in the cell viability indicates toxicity in cell line. The cell viability was increased by the plant extract, EEAR. Simvastatin causes the oxidative free radical mediated injury which results increased permeability leading to the leakage of cellular content and disruption of normal cellular architecture with necrosis[13]. The simvastatin exposed Chang liver cells, when treated with different concentrations of ethanolic extracts shown a significant dose dependant increase in percentage viability. Natural antioxidants like flavanoids, phenolic compounds and triterpenoids could prevent the deleterious effect of toxic agents by scavenging free radicals and other reactive oxygen species or by modulation of the inflammatory response. The various phytochemical studies showed that the ethanolic leaf extracts contains these phytoconstituents which supports the *in vitro* hepatoprotective activity. Morphological examination of cell lines showed abnormal cellular architecture and necrosis in simvastatin intoxicated cells. The liver cells treated with EEAR followed by simvastatin intoxication showed a sign of protection that reduces the necrosis and disarrangement of normal cellular architecture, as evident from the morphology of liver cell line.

The *in vitro* hepatoprotective activity was confirmed by enzyme level assays like lipid peroxidation assay and super oxide dismutase assay in chang liver cell lines.

Super oxide dismutase is an antioxidant enzyme. The reduced levels of SOD were seen in the simvastatin treated group, whereas the standard Sylimarin and the extracts ie. EEAR treated group showed significant ($p<0.001$) rise in super oxide dismutase enzyme.

MDA level is a main marker of endogenous lipid peroxidation. In simvastatin treated chang liver cell lines, the MDA level increased significantly indicating the lipid peroxidation. The MDA level in the EEAR treated groups decreased significantly ($p<0.001$) compared with Simvastatin treated group.

Conclusion

It is concluded that the ethanolic extract of *Annona reticulata* Linn. Possesses *in vitro* hepatoprotective potential especially at higher dose and the protection was found to be as comparable that of sylimarin. Further evaluations of the *in vivo* aspects are to be done to confirm the hepatoprotective potential of *Annona reticulata* Linn.

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