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Evaluation of Hepatoprotective Potential of Leaf Extracts of Some Medicinal Plants Using HepG2 Cell line

Khadijeh Alsadat Tahamtan[1] and M. S. Sharada[2*]

*[1]Research Scholar, Department of Studies in Botany, Manasagangotri, University of Mysore, Mysore - 570006, Karnataka, India.

[2*]Professor, Department of Studies in Botany, Manasagangotri, University of Mysore, Mysore-570006, Karnataka, India

ABSTRACT

The present study was carried out to evaluate the hepatoprotective activity of *Adina cordifolia, Careya arborea, Cassia angustifolia, Hiptage benghalensis* and *Lannea coromandelica* against alcohol induced toxicity using HepG2 cell lines. Prior to the determination of hepatoprotective property, leaf extracts were subjected to the toxic dose study. The degree of hepatoprotection of extracts was determined by measuring cell viability percentage by MTT assay. The preliminary phytochemical analysis of leaf extracts was carried out by qualitative analysis. HepG2 cells were pretreated with the different concentrations (below toxic dose) of leaf extracts for 72 hours followed by alcohol intoxication. Results revealed that *Cassia angustifolia* ethanolic leaf extract pretreated HepG2 cells show 92% cell viability compared to the standard silymarin pretreated HepG2 cells which showed 80% cell viability. Out of the other plants tested, *Adina cordifolia* exhibited the significant hepatoprotection. The bio-efficacy study confirms the presence of promising secondary metabolites of hepatoprotective nature in leaf extracts of *Cassia angustifoliaand Adina cordifolia*.

KEY WORDS: ALCOHOL TOXICITY, HEPATOPROTECTION, CASSIA ANGUSTIFOLIA, MTT ASSAY, HEPG2 CELLS.

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INTRODUCTION

Plants as a source of medicine can be traced back over five millennia to written documents of the early civilisation of India and China. Plants have been frequently used to treat various ailments and some of the traditional medicines are still part of habitual treatment of various maladies. Popular observations on the usage and efficacy of medicinal plants contribute to the disclosure of their therapeutic properties so that they are frequently prescribed even if their chemical composition is not fully known. At global level, use of medicinal plants has significantly supported primary health care (Maciel et al., 2002)The herbal products today symbolise safety in contrast to the synthetic pharmaceutical products which are regarded unsafe for humans and environment. Alcohol abuse and alcoholism are the very serious current health and socioeconomic problems throughout the world. Alcohol induces a number of adverse metabolic changes in liver. Excessive consumption of alcohol for a long time leads to the steatosis, alcoholic hepatitis and cirrhosis resulting in weight and volume change of liver, (Kumar and Cotran, 2003 Londonkar et al., 2015 Sharma et al., 2016 Saha et al., 2019).

Alcohol Liver Disease (ALD) is one of the most serious consequences of the chronic alcohol abuse and is the second leading cause of death among all liver diseases (Rehm et al., 2003) .Liver cirrhosis, the culmination of the illness, is one of the main causes of the mortality in western countries (Fernández-Checa et al., 1993) .Researchers have found in the studies on animal models that liver injury in chronic alcoholics is due to oxidative stress which leads to fibrosis, impaired function of liver and increases apoptosis (Schuppan et al., 1995). Excessive alcohol intake elevates reactive oxygen species (ROS) production and this enhanced ROS production results in cell damage and death causing serious health concerns. One of the important characteristics of alcohol induced liver injury is an impaired vitamin A nutritional status (Ronis et al., 2004).

Studies in human HepG2 cell lines have shown that ethanol is cytotoxic and is apoptotic in nature predominantly in liver (Ibrahim et al., 2008). There are no satisfactory remedies available for liver diseases; hence search for effective hepatoprotective drugs from natural products is continued, (Wu and Cederbaum, 1999 Alla et al., 2014 Saha et al., 2019). Natural products play an important role in health care rehabilitation programmes worldwide. Medicinal plants are the significant source of hepatoprotective drugs. It has been reported that about 170 phytoconstituents isolated from 110 plants belonging to 55 families do possess hepatoprotective activity (Panda et al.,2006).Only a small number of traditionally used medicinal plants have been scientifically evaluated for the hepatoprotective property, (Alla et al., 2014). In our study, an attempt has been made to screen *Adina cordifolia, Careya arborea, Cassia angustifolia, Hiptage benghalensis* and *Lannea coromandelica* for hepatoprotective activity against ethanol induced toxicity using HepG2 cell lines.

MATERIAL AND METHODS

Fresh and healthy leaves of *Adina cordifolia, Careya arborea, Cassia angustifolia, Hiptage benghalensis* and *Lannea coromandelica* were collected from in and around the University of Mysore campus. The collected leaves were thoroughly washed under running tap water and shade dried in the laboratory. Dry leaves were ground to coarse powder by laboratory grinder and stored in air tight containers for further use.25 grams of dry leaf coarse powder was filled in a thimble separately and extracted sequentially with 200 ml of petroleum ether, chloroform, ethyl acetate, ethanol and methanol in Soxhlet extractor for 48 hours. The solvent extracts were concentrated under reduced pressure and were stored at 50C in vials for further use. Leaf extracts were dissolved in 1% DMSO for bio-efficacy evaluation. HepG2 (Normal Human Liver Cell Line) cell culture was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells of HepG2 cell line were sub cultured as monolayers in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS (Fetal Bovine Serum) and penicillin (100 μg/ml) (Trease and Evans, 1983).

Prior to the screening of hepato protective activity, toxic dose studies of leaf extracts and silymarin were carried out by MTT assay (3-(4,5-dimethylthiazole-2 yl)-2,5 diphenyl tetrazolium bromide). The IC50 value of ethanol was also calculated by MTT assay (Santhosh et al., 2007). HepG2 cells were subcultured, after 72 hours medium supernatant was flicked off, cell monolayer was trypsinized and the cell count was adjusted to the 1.0 x 105 cells/ml. To each well of the 96 well plate 200 μl of diluted cell suspension

(1x105 cells approximately) was added and the plates were incubated for 72 hours at 370C and 5% CO2 for 24 hours. After 72 hours when monolayer of cells was formed, medium supernatant was flicked off and the 200 µl of DMEM containing leaf extracts in concentrations below toxic dose was added to each well containing cell monolayer. The plates were then incubated for 72 hours at 370C and 5% CO2. This boosting of cells with the different extracts is also known as pre-treatment protocol. After 72 hours, the medium containing extracts was flicked off and DMEM containing ethanol at 150 mM concentration was added to the each well containing extract and silymarin pretreated cell monolayers. The plates were again incubated for 72 hours at 370C and 5% CO2.

After 72 hours of incubation, medium containing ethanol was flicked off and 100 µl of 5% MTT reagent in DMEM was added to each well and the plates were again incubated at 370C for 3 hours. After 3 hours, the supernatant was discarded and 100 µl of solubilisation solution (1% DMSO) was added to each well and the plates were gently shaken in gyratory shaker to solubilise the formed formazan (Kamel et al., 2010).The absorbance was then measured by using microplate reader at 630 nm and the percentage of growth inhibition was calculated using formula given as under:

Percentage growth inhibition =(Mean OD of the individual test group)/(Mean OD of control group)x100

All the assays were carried out in triplicates and the data analysis was carried out by using SPSS, DMRT. Preliminary phytochemical analysis of leaf and leaf callus extracts was carried out by the method described by (Sharma et al., 2016).

RESULTS AND DISCUSSION

Cytotoxic study of leaf extracts of all selected plants was carried out to standardize extract concentrations and evaluate their hepatoprotective activity. Results revealed that *Cassia angustifolialeaf* extracts above 225 µg/ml concentration is toxic to the HepG2 cells where the cell viability percentage was reduced to 40%. MTT assay for cytotoxicity of standard drug silymarin revealed that concentration above 75 µg/ml is toxic to the cells and hence 75 µg/ml was used as the test concentration for the subsequent study. IC50 value of ethanol was reported to be

Table 1. The cell viability percentage of the HepG2 cells pre-treated with leaf extracts of selected plants

Plant name	The cell viability percentage of the HepG2 cells pre-treated with leaf extracts of selected plants				
	Conc. (µg/ml)	Chloroform	Petroleum ether	Ethyl acetate	Ethanol
Adina cordifolia	100	58	57	60	68
	200	64	62	64	72
Careya arborea	100	56	58	52	56
	200	59	60	58	68
Cassia angustifolia	100	65	68	72	89
	200	71	70	74	92
Hiptage	100	54	62	65	70
benghalensis	200	56	66	68	72
Lannea	100	55	60	60	65
coromandelica	200	58	63	65	68
Silymarin	75	81			
Ethanol (Toxicant)	150 mM	50			
Control	Only cells	100			

*All the MTT assays were repeated thrice. Each value represents Mean±S.D. Statistical analysis done by DMRT (P≤0.5)

150 mM (0.69% ethanol. Out of the different extracts, polar solvent extracts like aqueous, ethanol and methanol pretreated cells showed significant viability percentage after intoxication with ethanol. HepG2 cells pretreated with leaf extracts showed a dose dependent increase in percentage viability. The cell viability ranged from 65% to 92% in leaf extracts. The maximum cell viability percentage (92%) was reported in C. angustifolia ethanolic leaf extract pretreated HepG2 cells at 200 µg/ml. The cell viability percentage of HepG2 cells pretreated with leaf extracts is presented in Table 1. Preliminary phytochemical analysis of leaf extracts revealed the presence of Alkaloids, flavonoids, saponins, phytosterols, phenols, terpenoids, triterpenoids and sterols. The study was aimed to evaluate the hepatoprotective property of leaf extracts of some ethnomedicinal plants. Drug, whether synthetic or natural, if consumed in excess dose damages the liver cells.

This is the reason; toxic dose study of drug is carried out. In the present study, cytotoxic study of leaf extracts was carried out by MTT assay before the extracts were subjected to the hepatoprotective activity. The principle involved is the cleavage of tetrazolium salt into its blue coloured derivative formazan; the process takes place in the inner mitochondrial membrane where an enzyme succinate dehydrogenasec leaves the tetrazolium salt. Only the living cell mitochondria reduce the MTT to coloured formazan, therefore the concentration of the dye is directly proportional to the number of metabolically active cells. The MTT assay revealed that concentration of leaf extracts above 225 µg/ml kill 60% of the cells. Toxic dose studies of the plant extract lays the foundation for the formulation of any type of herbal remedy (Babbar et al., 2011).

In the present investigation, ethanol was used as hepatotoxicant. Ethanol induced live injury is encompassed by wide spectrum of lesions, the most characteristic being alcoholic steatosis (fatty liver), alcoholic hepatitis, alcoholic fibrosis and cirrhosis (Kamali et al., 2016). Results revealed that ethanol treated groups of HepG2 cells showed a drastic decrease in the cell viability when compared to the HepG2 cell pretreated with the leaf extracts. Increase in the percentage of cell viability in pretreated HepG2 cells indicates that the cells get boosted up upon treatment with extract and does not allow oxidation to take place upon intoxication with ethanol. In our study, HepG2 cells pretreated with leaf extracts showed percentage viability more than that of positive control silymarin. The cell viability relies on the structure of membrane and any damage to cell membrane causes leakage of the cellular enzymes and consequently a cell death. Results revealed that HepG2 cells pretreated with leaf extracts show a dose dependent increase in the cell viability. The dose dependent cytoprotection has been reported in other plants like Cassia roxburghii (Kanchana and Jayapriya, 2013), *Polygonum multiflorum* (Londonkar et al., 2015), *Andrographis paniculata* (Sivaraj et al., 2011) and *Rumexvesicarius*, Kanchana and Jayapriya, (2013) (Arulkumaran et al., 2009).

Described in their study that the increase in the cell viability percentage maybe a consequence of membrane stabilisation boosted by phytochemicals and they further demonstrated that the plant extracts elevate the tissue antioxidant defence enzymes and thus tackles oxidative stress. Several researchers have concluded that the antioxidant activity of plant extract is generally attributed to the presence of phenolic compounds (Ibrahim et al., 2008) .Preliminary qualitative phytochemical studies revealed the rich presence of phenolic compounds in leaf and leaf callus extracts which could possibly be attributed to hepatoprotective nature of the extracts. The chemical constituents of leaf extracts may have interrupted the reaction of ROS with cell proteins and nucleic acids and thus prevented the formation of adducts by acting as scavengers and thereby stabilising the cell membrane resulting in increased cell viability. Our inference is in concurrence with the earlier studies carried out by (Santhosh et al., 2007), (Sharma et al., 2016) (Kamel et al., 2010) (Patil et al., 2011; Thabrew et al., 1997).

CONCLUSION

Ethanol induces production of reactive oxygen species (ROS), leading to huge oxidative stress which damages the liver cells. Leaf extracts of selected medicinal plants have shown the dose dependent hepatoprotection against ethanol. The results suggest the presence of active phytoconstituents in leaf extracts which strengths antioxidant defense in cells and thus minimizing the chances of production of free radicals. Furthermore, phytochemical analysis needs to be carried out to isolate and characterize

the bioactive compounds from leaf extracts with hepatoprotective activity and for further authentication pharmacological studies also need to be carried out.

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Authors' Contribution Kh: Tahamtan and Dr. M.S Sharada developed ideas and drafted the manuscript. Dr. M.S Sharada provided the required facilities. Kh. Tahamtan conducted the experiments. Kh .Tahamtan and Dr. M.S Sharad had contributed to the design and analysis of data. Authors contributed to revise the manuscript and approved the final version for publication.

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