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Protective effect of polyherbal formula nalpamaram on the ethanol-induced toxicity in Hep G2 cell lines

S. Jyothilekshmi*[1], Jini Joseph[2]

[1]*Department of Biosciences, Union Christian College, Aluva, Kerala, India*

[2]*Department of Biotechnology, St. Peter's College, Kolenchery, Kerala, India*

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ABSTRACT

Plants are rich sources of bioactive secondary metabolites with well-established antioxidant and therapeutic properties. Nalpamaram, a classical Ayurvedic formulation composed of the barks of four *Ficus* species—*Ficus racemosa*, *Ficus microcarpa*, *Ficus benghalensis* and *Ficus religiosa*—is traditionally credited with cooling, antioxidant, anti-inflammatory and wound-healing properties. Despite its long-standing use in Ayurvedic medicine, its hepatoprotective potential has not been scientifically validated. Since hepatic disorders are closely associated with oxidative stress and inflammation, the presence of antioxidant phytochemicals in Nalpamaram suggests possible liver-protective effects. In this study, the hepatoprotective activity of the aqueous extract of Nalpamaram (NMAE) was evaluated using human hepatoma (Hep G2) cells, an established *in-vitro* model for screening hepatoprotective agents. Hep G2 cells retain key biochemical and morphological characteristics of normal hepatocytes and are suitable for assessing cytotoxicity, oxidative stress and protective effects of medicinal plant extracts. The present investigation assessed the ability of NMAE to protect Hep G2 cells from ethanol-induced toxicity by examining changes in cell viability, apoptosis and oxidative stress markers. The findings provide preliminary evidence supporting the hepatoprotective potential of NMAE and highlight the need for further mechanistic and *in-vivo* evaluations to substantiate its therapeutic relevance.

***Corresponding Author:** S. Jyothilekshmi ✉ jyothilekshmis@uccollege.edu.in
* https://orcid.org/0009-0007-5522-780X

Co-authors:
Jini Joseph: https://orcid.org/0009-0008-7409-3023

INTRODUCTION

Plants are powerful biochemical factories that have been used in all the traditional systems of medicine. The medicinal effects of plants result from the combination of secondary metabolites, present in different parts of plants - bark, leaves, flowers, roots, fruits and seeds.

They are known to contain antioxidants such as phenols, flavonoids, flavonols, isoflavones, flavones, anthocyanins, coumarins and lignans. The antioxidant potential of compounds such as vitamins C and E, betacarotene and tocopherol present in natural foods are also well documented (Shwetha *et al.*, 2011).

Nalpamaram is an ayurvedic formulation which has not been scientifically evaluated for its therapeutic effect. It is a classical Ayurvedic formulation which constitutes a group of four plants of *Ficus* sp., Family Moraceae. The four plants are - *Ficus racemosa* (FR), *Ficus microcarpa* (FM), *Ficus benghalensis* (FB) and *Ficus religiosa* (FG). They are called Athi, Ithi, Peral and Arayal respectively in malayalam. The aqueous extracts of the barks of FR, FM, FB and FG form an ingredient in many of the Ayurvedic medicines. Nalpamaram is credited with cooling, soothing, anti-inflammatory and antipruritic properties and are used in the treatment of various diseases to relieve burning sensation, haemoptysis, wounds and ulcers (Chopra *et al.,* 1992, Warrier *et al.*, 1995).

Since the medicines which contain Nalpamaram are used for the treatment of diseases associated inflammation, and hepatic disorders are associated with oxidative stress and inflammation, Nalpamaram may exhibit hepatoprotective action. But no reports are available on the scientific studies exploring its hepatoprotective action. Systematic studies on the toxicological evaluation, therapeutic action, and its mechanism may lead to an increase in the acceptability of Nalpamaram. Hence the present study focuses on the hepatoprotective activity of the aqueous extract of Nalpamaram (NMAE).

Immortalised cell lines are widely used in biological research for toxicity testing as well as screening of plant extracts for biological activities. In Ayurveda numerous herbs, PHF and herbo-mineral formulations are mentioned for the treatment of liver disorders (Subramanian *et al.*, 1978). The scientific evaluation of their hepatoprotective activity using animal models and freshly isolated hepatocytes is often cumbersome. Hence for the screening of plant extracts for hepatoprotective action human hepatoma cell lines (Hep G2) have been proposed as an alternative.

Hep G2 cells are immortalized and can be cryopreserved without losing their drug metabolizing enzyme activities (Duthie *et al.*, 1994). But being transformed cells, the mechanisms underlying drug metabolism and toxicity may be abnormal. However, Hep G2 cells possess many of the biochemical and morphological characteristics of normal hepatocytes and therefore they are widely used in the studies on the evaluation of liver function and drug toxicity (Bouma *et al.*, 1989; Sies, 1997; González *et al.,* 2017). Since they retain characteristics of normal liver cells, they are used to study the protective effects of medicinal plants also (Krithika *et al.*, 2009; Pareek *et al.*, 2013).

In the present study, the protective effect of NMAE on ethanol induced toxicity was evaluated using Hep G2 cells. The protective effect of NMAE on cell viability, apoptosis and ethanol induced oxidative stress have been evaluated.

MATERIALS AND METHODS

Culture and maintenance of cell lines

Hep G2 cell line was purchased from National Centre for Cell Sciences (NCCS), Pune, India and maintained in DMEM. The cells were cultured in 25 cm^2 tissue culture flasks with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphoteracin B (2.5µg/ml). The cultured cell lines were stored at 37°C in a humidified 5% CO_2 incubator.

After attaining 70% confluency, the cells were exposed to ethanol for one hour to induce toxicity. Ethanol exposed cells were treated with silymarin and NMAE for 24 hours. Untreated well was also maintained.

Determination of apoptosis by AO-EtBr double staining

Hep G2 cells were cultured as described above. Ethanol, NMAE and silymarin treated cells and untreated control cells were maintained. The effect of NMAE on alcohol induced apoptosis was determined by AO-EtBr double staining.

Assay of toxicity marker enzymes and lipid peroxidation

The cell lysate was prepared after treatment of Hep G 2 cells with ethanol, NMAE and silymarin. The cell lysate was used for the assay of ALT, AST, ALP and lipid peroxides as per standard protocols.

Assay of ALP

The cells (3×10^5 cells) were seeded in 6 well plates and groups were maintained as mentioned previously. After 24 hours of incubation cell supernatant and lysates were prepared as per standard procedures and used for further studies.

Alkaline phosphatase estimation was done using ALP assay kit as per manufactuer's instructions. 0.02ml of cell culture supernatant was added with 1ml of working reagent provided by manufacturer, mixed well. The initial absorbance and absorbance after 5minutes were read at 405 in a UV VIS spectrophotometer. Alkaline phosphatase activity was quantified by the spectrophotometric evaluation at 405nm (Kind and King, 1954).

ALP Activity (U/L)= (▲OD/minute) × 2754

Assay of AST

AST catalyzes the transfer of amino group between L-Aspartate and α- ketoglutarate to form oxaloacetate and glutamate. The oxaloacetate formed reacts with the NADH in the presence of Malate dehydrogenase to from NAD^+. The rate of oxidation of NADH to NAD^+ is measured as a decrease in absorbance which is proportional to the AST activity in the sample.

AST activity was determined using AST assay kit. 0.2ml of culture supernatant was taken in a test tube. 0.8mL of enzyme reagent was added and incubated for one minute at 30°C. After incubation, 0.2ml of starter reagent was added and the contents were mixed well and read on spectrophotometer at 340nm. Repeated the absorbance for 3 minutes and the mean absorbance change per minute was calculated.

AST Activity (U/L) = Mean absorbance change per minute × 952 (Reitman and Frankel, 1957)

Assay of ALT

ALT catalyses the transfer of amino group between L-Alanine and α- ketoglutarate to form pyruvate and glutamate. The pyruvate formed reacts with the NADH in the presence of LDH to from NAD. The rate of oxidation of NADH to NAD^+ is measured as a decrease in absorbance which is proportional to the ALT activity in the sample.

ALT activity was calculated using ALT assay kit. 0.2ml of culture supernatant was taken in a test tube. 0.8mL of enzyme reagent was added and incubated for one minute at 30°C. After incubation, 0.2ml of starter reagent was added and the contents were mixed well and read on spectrophotometer at 340nm. Repeated the absorbance after 3 minutes and the mean absorbance change per minute was calculated.

ALT Activity (U/L) = Mean absorbance change per minute × 952 (Reitman and Frankel, 1957)

Estimation of hydroperoxides by Fox assay (Gay and Gebicki, 2003)

Reagents

20 mM ammonium ferrous sulphate

2.5 mM xylenol orange

Phosphate buffer 0.1 M pH 7.5

Procedure

The lipid hydroperoxide formed in reaction system was quantitatively measured by FOX assay. Oxidation of xylenol orange red salt by ferric ion formed during the reduction of ferrous ion by peroxides is measured. The general reaction is as follows:

$H_2O_2 + Fe^{2+} \rightarrow OH^- + OH^- + Fe^{3+}$

Fe^{3+} + xylenol orange→ Coloured complex.

The coloured complex absorbs maximally at 560 nm. Approximately 60 µl of tissue homogenate in

phosphate buffer, 0.1 M, pH 7.5, was diluted to 300 µl and was allowed to react with 100 µl of ammonium ferrous sulphate (20 mM) and 100 µl of xylenol orange (2.5 mM), in dark for 15 minutes. After incubation 1.5 ml of phosphate buffer was added in all test tubes.

The absorbance of resulting orange yellow solution was measured at 560 nm. Lipid hydroperoxides content was determined using standard graph prepared with various concentrations of H_2O_2. The values are expressed as mmol /100 g tissue.

RESULTS

AO-EtBr bromide double staining

The inhibition of apoptosis by NMAE was determined by AO-EtBr double staining. From the images it can be observed that untreated control cells show green nucleated live cells whereas ethanol administration show both apoptotic (orange) and necrotic cells. NMAE treatment decreased the orange nucleated and red nucleated cells suggesting anti-apoptotic effects of NMAE which is comparable with silymarin groups (Fig. 1).

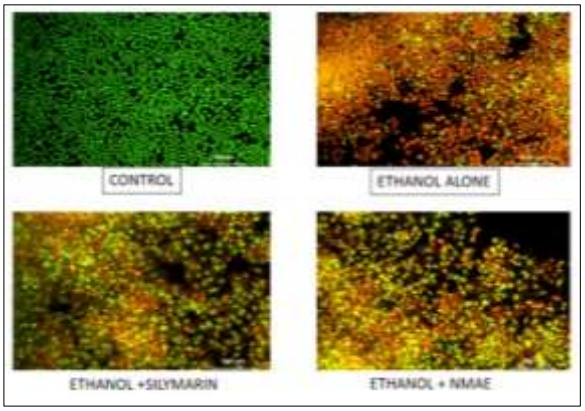

Fig. 1. Inhibition of apoptosis by NMAE and silymarin illustrated by AO and EtBr double staining.

Assay of toxicity marker enzymes and lipid peroxidation

Effect of NMAE on ALP, AST and ALT

The culture supernatant prepared after treatment with ethanol, NMAE, and silymarin was used for the assay of ALP, AST and ALT. The increased activities of all the three enzymes in ethanol treated groups was decreased on treatment with NMAE and silymarin indicating reduction in ethanol induced toxicity (Fig 2, 3, and 4).

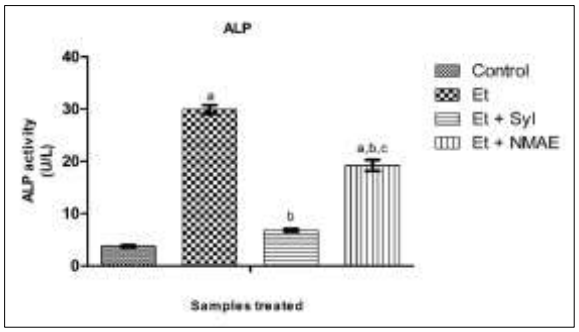

Fig. 2. Effect of NMAE on the activity of ALP

Values are expressed as mean± SD for three experiments. a = $p< 0.05$ when compared to control. b= $p< 0.05$ when compared to ethanol administered group. c = $p< 0.05$ when compared to silymarin administered group

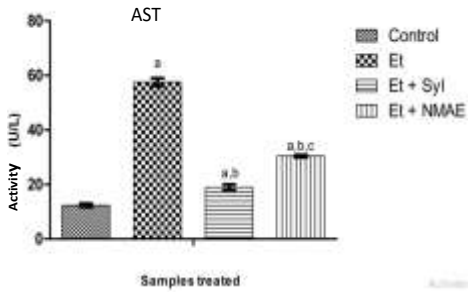

Fig. 3. Effect of NMAE on the activity of AST

Values are expressed as mean± SD for three experiments. a = $p< 0.05$ when compared to control. b= $p< 0.05$ when compared to ethanol administered group. c = $p< 0.05$ when compared to silymarin administered group

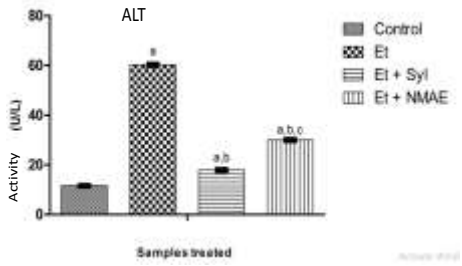

Fig. 4. Effect of NMAE on the activity of ALT

Values are expressed as mean± SD for three experiments. a = $p< 0.05$ when compared to control. b= $p< 0.05$ when compared to ethanol administered group. c = $p< 0.05$ when compared to silymarin administered group

Lipid peroxidation

The cell lysate prepared after treatment with ethanol, NMAE, and silymarin was used for the assay of lipid peroxidation. The higher levels of lipid peroxides was observed in the cells administered ethanol when compared to control group (Fig 5).

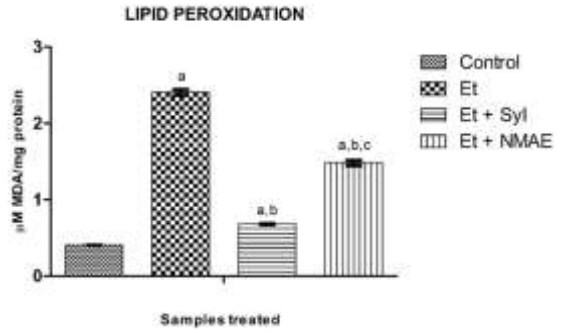

Fig. 5. Effect of NMAE on the level of lipid peroxides Values are expressed as mean± SD for three experiments. a = p< 0.05 when compared to control. b= p< 0.05 when compared to ethanol administered group. c = p< 0.05 when compared to silymarin administered group

DISCUSSION

Hep G2 cells have been widely used for the studies on the screening and evaluation of plant extracts for hepatoprotective activity (Krithika *et al.*, 2009; Pareek *et al.*, 2013). In the present study the protective effect of NMAE on ethanol induced toxicity has been evaluated using Hep G2 cell lines.

Induction of hepatocellular apoptosis and necrosis is commonly associated with ethanol administration and we screened the anti-apoptotic effects of NMAE on Hep G2 cells by EtBr – AO staining. The anti-apoptotic effect of NMAE was shown by the decrease in the orange nucleated and red nucleated cells suggesting its protective effect which is comparable with silymarin treated group.

The increased activity of the toxicity marker enzymes in the culture supernatant (ALP, AST and ALT) on ethanol administration and the significant reduction of their activity on treatment with NMAE also demonstrates the protective effect of NMAE. Praneetha *et al.*, 2017 have demonstrated the protective effect of the methanolic extract of *Echinochloa colona* against ethanol induced cytotoxicity in Hep G2 cell lines. Studies on the protective effect of PHF on Hep G2 cells have been carried out by other workers also. Bhatt *et al.*, 2017 showed that the commercially available PHF - LIVT mitigates the D galactosamine induced toxicity in Hep G2 cell line. Another classical Ayurvedic polyherbal formulation Punarnavasak Kwath is reported to increase in the viability of Hep G2 cells treated with CCl_4 (Shah *et al.*, 2011).

In the present study, the cell lysate prepared after treatment of the cells with NMAE and silymarin was analysed for lipid peroxidation and antioxidants. Lipid peroxidation was found to be increased by ethanol administration as evidenced by the increase in the level of MDA The reduction in the levels of lipid peroxides and significant increase in the antioxidant levels on treatment with NMAE clearly indicates amelioration of the oxidative stress induced by ethanol.

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