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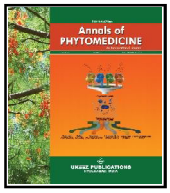

Polyherbal formulation : Open Access

Protective effect of the polyherbal formulation Nalpamaram on the oxidative stress induced by ethanol

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Abstract

Oxidative stress plays a prominent role in the pathogenesis of alcoholic liver diseases. The present study focuses on the protective effect of Nalpamaram on ethanol induced oxidative stress. Male albino rats of wistar strain weighing 200-250 g were divided into different groups. Ethanol, 20% w/v, was administered orally (3 ml/kg b.wt./day), to animals of all the groups for 120 days except normal group of animals. Aqueous extract of Nalpamaram (NMAE) at doses 100, 250 and 500 mg/kgb.wt./day and silymarin at a dose of 100 mg/kg b.wt./day were administered orally for 120 days, to the animals of different groups. After 120 days the rats were fasted overnight, sacrificed and liver was collected and used for various assays. The increased alcohol dehydrogenase levels in ethanol administered rats were found to be decreased in NMAE and silymarin treated groups. The indicators of oxidative stress, thiobarbituric acid reacting substances, conjugated dienes, conjugated trienes and hydroperoxides in the liver of ethanol treated rats were found to be decreased in ethanol + NMAE treated rats. The decreased activities of the antioxidant enzymes superoxide dismutase and catalase, and the levels of reduced glutathione in ethanol administered rats, were increased significantly in ethanol + NMAE treated rats. The activities of glutathione reductase and glutathione peroxidase decreased significantly in ethanol administered rats, when compared with pair fed controls. In ethanol + NMAE treated rats, the activities of these enzymes increased in a dose dependent manner. However, the activity of glutathione S transferase was significantly higher in ethanol-treated rats, which was brought down towards near normal values in NMAE treated rats. The hepatoprotective effect of NMAE can be attributed to the amelioration of oxidative stress induced by ethanol. The effect of NMAE at a dose of 500 mg/kg b.wt./day was comparable to that of the standard drug silymarin at a dose of 100 mg/kg b.wt./day.

1. Introduction

Free radicals have been implicated in the pathogenesis of diseases, such as Alzheimer's, Parkinson's, arthritis, rheumatism, atherosclerosis, AIDS, cataract, cancer, diabetes and degenerative diseases (Hemnani and Parihar, 1998;Venkatesh *et al*., 2001; Tiwari, 2001; Halliwell and Gutteridge, 2015). In biological systems, the highly reactive free radicals also called reactive oxygen species (ROS) are generated from molecules which possess unpaired peripheral electrons (Muriel, 2009). The term ROS refers to chemical species such as superoxide, hydrogen peroxide and hydroxyl radical, which are generated in the human body by the normal metabolic reactions. ROS are generated in the hepatocytes by the action of CYP2E1, mitochondrial respiratory chain alterations and NADPH oxidase (Jezek and Hlavata, 2005).

Oxidative stress plays an important role in the pathogenesis of alcoholic liver diseases by inducing mitochondrial injury, enhancing endoplasmic reticulum stress and lysosomal fragility, and activating the proinflammatory signalling pathways (Ambade and Mandrekar, 2012). The ROS and reactive nitrogen species (RNS) relevant in ethanol induced liver damage include superoxide radical, hydroxyl radical, hydroperoxide radical, lipid radical, peroxy lipid radical, hydrogen peroxide, peroxynitryl radical, nitrogen dioxide and nitric oxide (Galicia-Moreno and Gutiérrez-Reyes, 2014). These ROS and RNS disrupt the cellular integrity by damaging the cellular macromolecules like DNA, proteins and lipids.

Under normal physiological conditions the endogenous antioxidants of the cell balances or neutralises the toxic effect of the ROS and RNS. However, a shift in balance between the prooxidants and antioxidants create oxidative stress (Halliwell and Gutteridge, 2015). Hence, dietary or exogenous, plant derived antioxidants play a significant role in mitigating the cellular oxidative stress.

The antioxidant potential of the plant extracts and polyherbal formulations (PHF) has been reported to have a crucial role in the hepatoprotective action. Their protective effects can be attributed to the secondary metabolites such as phenolic compounds, flavonoids and terpenoids (Mukherjee *et al.*, 2015; Mahmoodzadeh *et al*., 2017; Bakr *et al*., 2018). Many of these compounds have been claimed to possess antioxidant activity due to their free radical scavenging and redox properties (Soni and Sosa, 2013; Kuntal Das *et al*., 2019; Punit *et al*., 2019).

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Nalpamaram, a polyherbal formulation used by Ayurvedic practitioners comprises the four trees: *Ficus racemosa* L. (FR), *Ficus microcarpa* L. (FM), *Ficus benghalensis* L. (FB) and *Ficus religiosa* L. (FG). The aqueous extracts of the barks of FR, FM, FB and FG form an ingredient in many of the Ayurvedic medicines. In a previous study carried out in our laboratory, the aqueous extract of Nalpamaram (NMAE) showed protective action against ethanol induced toxicity in male albino wistar rats as evidenced by the decreased activities of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma glutamyl transferase (GGT), and serum bilirubin in NMAE treated rats compared to the ethanol administered group (Jyothilekshmi *et al.*, 2020). In the present study, the protective effect of NMAE on the ethanol induced oxidative stress has been carried out using male albino wistar rats.

2. Materials and Methods

2.1 Preparation of the extract

The preparation of the extract was carried out as per the guidelines given in traditional Ayurvedic text books. Authentic samples of the barks of FR, FM, FB and FG were collected from Arya Vaidyasala, Kottakkal, Kerala. Bark powders (100 g each) of FR, FM, FB and FG were mixed and extracted by boiling with water (6400 ml) and concentrated to one fourth of the volume, filtered and lyophilized. The dry residue (NMAE), thus obtained was used for the present study. NMAE was dissolved in distilled water and used for this study.

2.2 Chemicals and reagents

Silymarin was purchased from Sigma Aldrich, USA. All other chemicals and solvents used in this study were of analytical grade.

2.3 Experimental animals

Animal experiments were conducted at Amala Cancer Research Centre, Amala Nagar, Thrissur, Kerala. The study was approved by the Institutional Animal Ethics Committee (Approval No.ACRC/IAEC/2018[1]P5) and was carried out in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), constituted by the Animal Welfare Division, Government of India. Male albino rats of wistar strain weighing 200-250 g used for the study, were housed in polypropylene cages, maintained at 25 ± 2°C, 50-60 % humidity and 12 h light and 12 h dark cycle and were given standard pellet diet and water *ad libitum*.

2.4 Experimental design

Male albino rats weighing 200-250 g were divided into different groups as follows:

Group I-Normal group

Group II-20% w/v ethanol, 3 ml/kg b.wt./day

Group III-20% w/v ethanol, 3 ml/ kg b.wt./day + Silymarin 100 mg/kg b.wt./day

Group IV-20% w/v ethanol, 3 ml/kg b.wt./day + NMAE 100 mg/kg b.wt./day

Group V-20% w/v ethanol, 3 ml/kg b.wt./day + 250 mg/kg b.wt./day

Group VI-20% w/v ethanol, 3 ml/kg b.wt./day + 500 mg/kg b.wt./day

Administration of ethanol, silymarin and NMAE were done orally for 120 days. The dose of ethanol and silymarin were standardised previously (Sindhu *et al.*, 2010; Kanaujia *et al.*, 2011). Acute toxicity studies showed that LD_{50} of NMAE is greater than 5000 mg/kg b.wt. Subacute toxicity studies carried out by administering NMAE at different doses, less than 1000 mg/kg b.wt/day for 14 days also indicated that NMAE is nontoxic (unpublished data). Hence, three doses of less than 1000 mg/kg b.wt./day were administered to rats. After 120 days the rats were fasted overnight and sacrificed. Liver tissue was collected and used for the following assays.

2.5 Biochemical assays

The assays carried out were thiobarbituric acid reactive substances (TBARS) (Nichans and Samuelson, 1968), conjugated dienes (CD) and conjugated trienes (CT) (Recknagel and Ghosal,1966), hydroperoxides (Fox Assay, Gay and Gebicki, 2003), reduced glutathione (Moron *et al.*, 1979), superoxide dismutase (SOD) (Mc Cord and Fridovich, 1969), catalase (Aebi Method, 1974), glutathione reductase (GR) (David and Richard, 1983), glutathione S transferase (GST) (Habig *et al.*, 1974), glutathione peroxidase (GPx) ((Lawerance and Burk, 1976) and alcohol dehydrogenase (ADH) (Vallee and Hoch, 1955).

2.6 Statistical analysis

Statistical analysis was carried out using one-way analysis of variance followed by Tukey's post hoc multiple-comparison test in GraphPad Prism 5.0. The differences between groups were considered significant at $p<0.05$.

3. Results

3.1 Effect of NMAE on ADH

A significant increase was observed in ADH levels upon ethanol administration compared to the control group. The ADH levels decreased in the silymarin and NMAE treated groups as compared to the ethanol administered group (Figure 1).

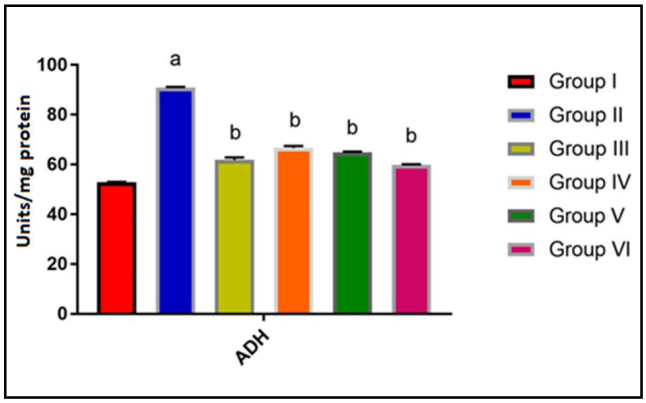

Figure 1: Effect of NMAE on the activity of ADH.

Values are expressed as mean ± SD for six animals in each group. a = $p<0.05$ when compared to normal group.

b = $p<0.05$ when compared to ethanol administered group (Group II).

One unit reduces one micromole of NAD^+ per minute at 25°C under the specified conditions.

3.2 Effect of NMAE on TBARS, CD, CT and hydroperoxides

TBARS, CD, CT and hydroperoxides concentration significantly increased in the liver of ethanol treated rats as compared to normal rats. Their levels were decreased in a dose dependent manner and attained a near normal level in the liver of ethanol + NMAE (500 mg/kg) treated rats (Figures 2 and 3).

3.3 Effect of NMAE on SOD, catalase and GSH levels

Activities of the antioxidant enzymes superoxide dismutase, catalase and reduced glutathione showed a significant decrease in ethanol administered rats, when compared with pair fed controls. In ethanol + NMAE treated rats, the activities of these enzymes and reduced glutathione concentration increased significantly in a dose dependant manner. The activities were comparable to silymarin in NMAE 500 mg/kg treated rats (Figures 4 and 5).

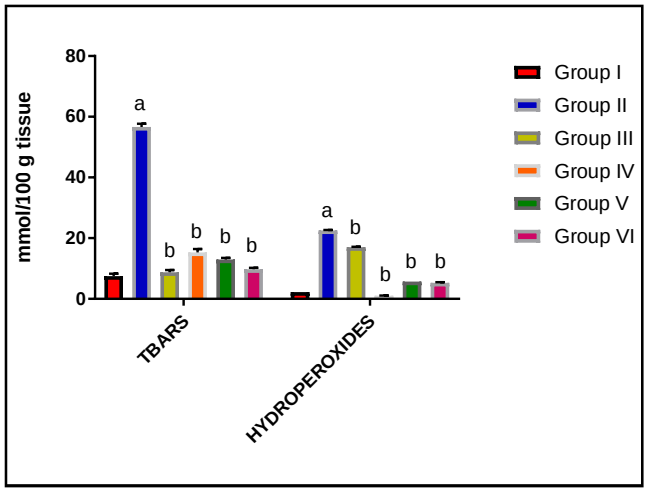

Figure 2: Effect of NMAE on TBARS and hydroperoxides levels.

Values are expressed as mean ± SD for six animals in each group. a = p<0.05 when compared to normal group.

b = p<0.05 when compared to ethanol administered group (Group II).

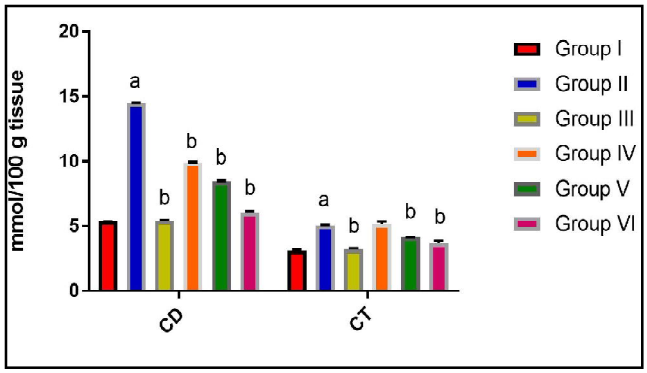

Figure 3: Effect of NMAE on CD and CT levels.

Values are expressed as mean ± SD for six animals in each group. a = p<0.05 when compared to normal group.

b = p<0.05 when compared to ethanol administered group (Group II).

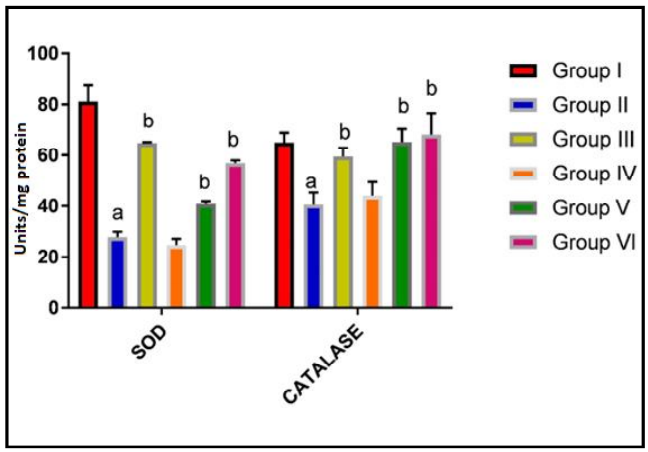

Figure 4: Effect of NMAE on the activities of SOD and catalase.

Values are expressed as mean ± SD for six animals in each group. a = p<0.05 when compared to normal group.

b = p<0.05 when compared to ethanol administered group (Group II).

SOD-One unit of enzyme activity is defined as the enzyme concentration required to decrease the OD at 560 nm of chromogen production by 50% per minute under the assay conditions

Catalase-One unit is defined as µmoles of H_2O_2 consumed/min/mg of protein sample

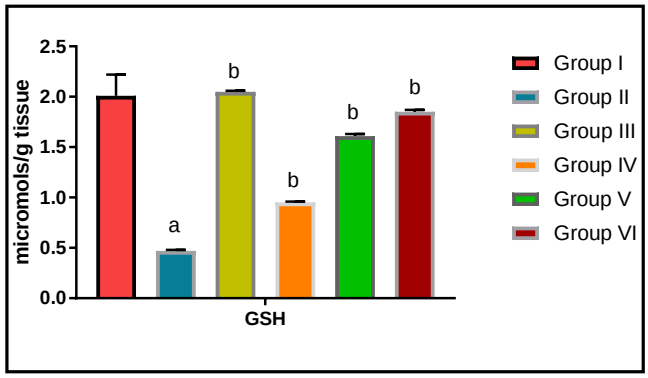

Figure 5: Effect of NMAE on GSH levels.

Values are expressed as mean± SD for six animals in each group. a=p<0.05 when compared to normal group.

b=p<0.05 when compared to ethanol administered group (Group II).

3.4 Effect of NMAE on the activities of GR, GPx and GST

The activities of GR and GPx were decreased significantly in ethanol administered rats, when compared with pairfed controls. In ethanol and NMAE treated rats, the activities of these enzymes increased in a dose dependent manner. However, the activity of glutathione S transferase was significantly higher in ethanol-treated rats, which was reduced to near normal values in NMAE treated rats. The activities were comparable to silymarin in NMAE 500 mg/kg treated rats (Figure 6).

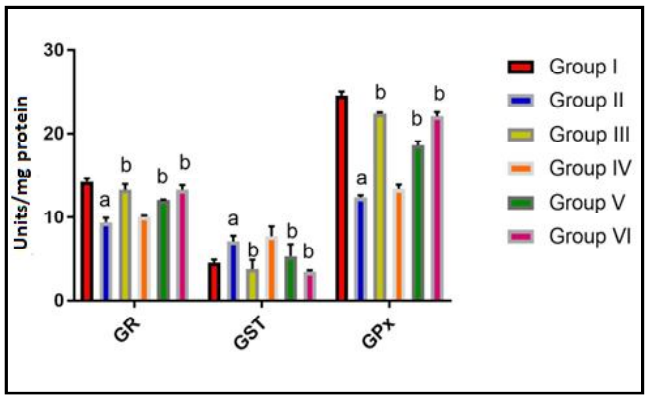

Figure 6: Effect of NMAE on the activities of GR, GPx and GST.

Values are expressed as mean ± SD for six animals in each group. a = p<0.05 when compared to normal group.

b = p<0.05 when compared to ethanol administered group (Group II).

GR-Glutathione reductase-The activity is expressed as micro moles of NADPH oxidized/min/mg protein.

GST-Glutathione S transferase-One unit of the enzyme is the amount required to conjugate 1 µmole of the substrate with glutathione in 1 min.

GPx-Glutathione peroxidase-Enzyme activity is defined as µmoles of NADPH oxidized/min/mg protein

4. Discussion

The fundamental mechanism underlying alcoholic liver disease is the overproduction of free radicals and alterations in the antioxidant status of the cell. Alcohol is primarily oxidised in the liver by ADH, a zinc containing cytosolic enzyme, to acetaldehyde. ADH is found in maximum amounts in the liver. Smaller amounts are present in the gastrointestinal tract, kidneys, nasal mucosa, testes, and uterus. CYP2E1, the isoform of cytochrome P450 which is a microsomal enzyme induced by ethanol also converts ethanol to acetaldehyde. Mitochondrial aldehyde dehydrogenase converts acetaldehyde to acetate which diffuses into the circulation. Activation of CYP2E1 by ethanol leads to the generation of hydroxyl ethyl, superoxide and hydroxyl free radicals and lipid peroxides and lowers the cellular antioxidant levels leading to oxidative stress and cellular damage. Increased production of NADH decreases the redox or the NAD^+/ NADH ratio within the hepatocyte favouring the reductive synthesis leading to triglyceride and fatty acid accumulation and hepatic steatosis (Ambade and Mandrekar, 2012; Osna *et al.*, 2017). In our study, ADH level was increased in the ethanol treated rats. In the NMAE and silymarin treated groups the ADH activity was found to be decreased.

In the present study, the concentration of TBARS, CD and CT and hydroperoxides, the indicators of oxidative stress significantly increased in the ethanol administered group. These are generated within the cell by the normal metabolic reactions. Their increased levels indicate the possibility of cellular damage. In NMAE administered groups, their levels were reduced significantly indicating protective effect. In NMAE 500 mg/kg treated group, the reduction was comparable to that of silymarin, the standard hepatoprotective drug.

SOD and catalase are the enzymatic antioxidants present in the cell. SOD which is present in the mitochondria and cytosol catalyses the dismutation of the superoxide anion into H_2O_2 and H_2O and molecular oxygen and peroxisomal catalase decomposes H_2O_2 into water and oxygen (Hemnani and Parihar, 1998). The increased activities of SOD and catalase upon administration of silymarin and NMAE indicates the reduction in the oxidative stress induced by ethanol.

Reduced glutathione, the low molecular weight non enzymatic antioxidant is involved in the detoxification by reacting with H_2O_2 and organic peroxides. It is essential for the maintenance of the normal structure of red blood cells and keeps haemoglobin in the ferrous state. GSH-Glutathione peroxidase system converts the lipid hydroperoxides to their corresponding alcohols and reduce H_2O_2 to water, thereby oxidising GSH to GSSG (Umamaheswari *et al.*, 2009). In our study the decreased levels of GSH and the GPx upon ethanol administration were significantly elevated on administration of NMAE and silymarin.

GR converts oxidised glutathione GSSG to its reduced form GSH. The GR activity was found to be reduced in the ethanol administered group. It was restored to near normal levels by silymarin and NMAE at the dose of 500 mg/kg b.wt/day.

GST catalyses the conjugation of glutathione to a wide variety of endogeneous and exogeneous electrophilic components. It is a phase II enzyme required for the detoxification of xenobiotics (Townsend and Tew, 2003). The increased activity of GST indicates toxicity in the ethanol administered groups. In silymarin and NMAE 500 mg/kg administered groups their values were reduced to normal.

Similar work on the protective effect of plant extracts and secondary metabolites on the oxidative damage induced by CCl_4 and cyclophosphamide has been reported by Jaswal and Shukla (2015), Mahmoodzadeh *et al.* (2017), Manoj Kumar *et al.* (2021) and Jyoti *et al.* (2022). Pyun *et al.* (2020) have studied the protective effects of *Ligularia fischeri* and *Aronia melanocarpa* extracts on alcoholic liver disease. ADH activity and CYP2E1 mRNA expression were found to be increased upon the administration of the plant extracts. Kumar *et al.* (2013) have reported that the hepatoprotective potential of the PHF, clearliv in thioacetamide-induced liver necrosis, DL-galactosamine induced liver injury, and in carbon tetrachloride induced hepatitis models, in wistar rats was due to an increase in the antioxidant levels. In our study also there was an increase in the antioxidant levels upon NMAE administration.

It can be summarised that ethanol administration generated oxidative stress in the cell due to which cellular damage was created. The increase in the levels of ALP, AST, ALT, GGT and bilirubin, the markers of hepatotoxicity, upon ethanol administration, observed in our previous study can be attributed to the damage of the hepatocytes due to oxidative stress. Administration of NMAE ameliorated the oxidative stress and thereby mitigated the toxic effects induced by ethanol.

The hyperlipidemia and the steatosis observed in the ethanol administered group in our previous study may be due to the decreased NAD^+/ NADH ratio upon ethanol administration. Treatment with NMAE and silymarin would have restored the redox ratio thereby decreasing the rates of reductive metabolic pathways and restoring the levels of lipids to normal.

Phytochemical analysis revealed that NMAE contained alkaloids (1.38), saponins (4.0), flavonoids(0.26) and phenols (6.14) mg/g of dry extract (Unpublished data). The synergistic effect of the secondary metabolites present in NMAE may be the reason for its protective effect.

5. Conclusion

NMAE reduced the oxidative stress induced by ethanol. The levels of lipid peroxides increased, and free radical scavengers decreased upon ethanol administration. The effect was reversed by NMAE. The effect of NMAE at the dose of 500 mg/kg b.wt/day was comparable to that of standard drug silymarin. NMAE may prove to be useful in treatment of diseases in which oxidative stress is involved. However, further studies are required to identify the compounds responsible for the protective action.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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