



EFFECT OF *PHYLLANTHUS NIRURI* ON ALCOHOL AND POLYUNSATURATED FATTY ACID INDUCED OXIDATIVE STRESS IN LIVER

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ABSTRACT

Alcohol is a toxin associated with significant morbidity and mortality. It induces oxidative stress when taken at higher concentrations. The elevation in lipid peroxidative products and the loss of antioxidant defense potential are enhanced when alcohol is consumed along with polyunsaturated fatty acid (PUFA). The biological disturbances are intensified when the PUFA is thermally oxidized. Hence in our study we investigated the effects of the crude extract of *Phyllanthus niruri* (PN), a traditional medicinal herb used in India and China, on alcohol and heated PUFA induced hepatotoxicity. Male Albino Wistar rats were used for the study. The liver marker enzymes - Gamma-glutamyl transferase (GGT), Alkaline phosphatase (ALP), Lipid peroxidative indices - Thiobarbituric acid reactive substances (TBARS), Lipid hydroperoxides (LH); Antioxidants - vitamin C, vitamin E, reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) were assayed for estimating the antioxidant potential of *P. niruri*. Histopathology was done to confirm the results. Our results suggested that the crude extract of *P. niruri* effectively decreased the oxidative stress and associated damage.

Keywords: Alcohol, Polyunsaturated fatty acid, Liver, Antioxidants, Lipid peroxidation, Liver marker enzymes, *Phyllanthus niruri*

INTRODUCTION

The liver is the most important organ governing essential biochemical activities in the human body. It has great capacity to detoxify and synthesize useful substances, and therefore damage to the liver has grave consequences¹. Ethanol-induced oxidative stress is known to play a major role in causing liver injury. Many pathways have been suggested to contribute to the ethanol induced oxidative stress. Some of these include redox state changes, production of the reactive product acetaldehyde, damage to mitochondria, direct or membrane effects caused by hydrophobic ethanol, ethanol-induced hypoxia, effects on the immune system, altered cytokine production, induction of CYP2E1 and mobilization of iron².

The adverse effect of ethanol is further aggravated by intake of diet rich in polyunsaturated fatty acids (PUFA). Present day reports on dietary fats indicate that increased intake of PUFA is detrimental to health. A high PUFA n-6 content and a high n-6/n-3 ratio in the newer heart friendly oils like sunflower oil are considered to be dangerous³. Moreover the repeated heating of these oils changes the oil characteristics through thermal oxidation. During deep fat frying, many volatile and non-volatile products are produced, some of which are toxic, depending on the level of intake⁴. Consumption of such oils has devastating effects on the functions of many organs especially liver⁵. Many alcoholics as a custom take fried food items after a heavy binge of alcohol. This combination of alcohol and PUFA can aggravate the toxic effects especially by increasing the oxidative stress⁶.

Phyllanthus niruri (family Euphorbiaceae) is a plant possessing several pharmacological properties. It is an ayurvedic medicine used for treating gastric lesion⁷, urolithiasis⁸, urinary infections and diuretics⁹. This herb is used since ancient times for the treatment of jaundice and other liver related disorders. It is also known to possess anti-inflammatory property by decreasing the inflammation caused by several pathways¹⁰. The administration of the extract has been found to increase the life span of rats with hepatocellular carcinoma¹¹. It is shown to have effective hepatoprotective actions against paracetamol¹², carbon tetrachloride¹³, galactosamine¹⁴ and thioacetamide induced toxicity¹⁵.

Hence in the present study we tested the effect of crude extract of *Phyllanthus niruri* on alcohol and PUFA induced oxidative stress.

MATERIALS AND METHODS

Animals

Male albino rats of Wistar strain of body weight ranging 140-160g bred in Central Animal House, Rajah Muthiah Medical College, Tamil Nadu, India, fed on standard pellet diet (Agro Corporation Private Limited, Bangalore, India) were used for the study and water was given *ad libitum*. The standard pellet diet comprised 21% protein, 5% lipids, 4% crude fibre, 8% ash, 1% calcium, 0.6% phosphorous, 3.4% glucose, 2% vitamin and 55% nitrogen free extract (carbohydrates). It provides metabolisable energy of 3600Kcal.

The animals were housed in plastic cages under controlled condition of 12 h light/ 12 h dark cycles, 50% humidity and at 30 ± 2°C. The animals used in the present study were maintained in accordance with the guidelines of the National Institute of nutrition, Indian Council for Medical Research, Hyderabad, India and approved by the Animal Ethical Committee, Pondicherry University.

Materials

Ethanol Absolute ethanol (AR) was obtained from Private Limited, England.

Thermally oxidized Sunflower oil (Gold Winner) was subjected to heating at PUFA (Δ PUFA) 180° C twice to produce thermally oxidized PUFA.

***Phyllanthus niruri* (PN)** The fresh leaves and stems of the young plants were homogenized in 50mM sodium phosphate buffer, pH7.2 at 40°C and centrifuged at 12,000 g for 30 minutes to remove unwanted debris. 3 ml of the supernatant was used for the treatment.

All other chemicals and solvents used in this experiment were purchased from Himedia Pvt Ltd, India.

Experimental design

The animals were divided into 4 groups of 6 rats each.

At the end of the experimental period (45 days), the rats were sacrificed after an overnight fast by cervical dislocation and the liver was collected, cleared off blood and immediately transferred to ice cold containers containing 0.9% NaCl for various biochemical estimations.

Table 1: Treatment schedule

Groups	Diet followed
Group 1 (Normal)	Standard pellet diet
Group 2 (Alcohol + ΔPUFA)	20% ethanol orally using intragastric tube + 15% heated sunflower oil mixed with the diet
Group 3 (Alcohol + ΔPUFA+PN)	20% ethanol + 15% heated sunflower oil mixed with the diet + 3ml of crude extract of <i>Phyllanthus niruri</i>
Group 4 (<i>Phyllanthus niruri</i>)	3ml of crude extract of <i>Phyllanthus niruri</i>

Preparation of plasma

Blood was collected in heparinised tubes and plasma was separated by centrifugation at 1000g for 15 minutes for the estimation of GGT and ALP.

Preparation of liver homogenate

Known amount of the liver tissue was weighed and homogenized in appropriate buffer for the estimation of lipid peroxidative indices and enzymic and non-enzymic antioxidants.

Biochemical investigations

The activity of plasma Gamma-glutamyl transferase (GGT) was assayed by the method of Fiala et al.¹⁶. The activity of alkaline phosphatase (ALP) was assayed by the method of King and Armstrong using a reagent kit¹⁷. The concentration of Thiobarbituric acid reactive substances (TBARS) was estimated by the method of Niehaus and Samuelsson¹⁸ and Lipid hydroperoxides (LH) by the method of Jiang et al.¹⁹. Ascorbic acid was estimated by the method of Roe and Kuether²⁰ and vitamin E by the method of Baker and Frank²¹. Reduced glutathione (GSH) content in the tissue was determined by the method of Ellman²². The activity of

glutathione peroxidase (GPx) was assayed by the method of Rotruck et al.²³, superoxide dismutase (SOD) by the method of kakkar et al.²⁴ and catalase (CAT) by the method of Sinha²⁵.

For histopathological study, two animals from each group were perfused with formalin (10%) and the tissues were separated and stored in 10% formalin. They were latter sectioned using a microtome, dehydrated in graded alcohol, embedded in paraffin section, and stained with hemotoxylin and Eosin (H & E).

Statistical analysis

Statistical analysis was done by analysis of variance (ANOVA) followed by Tukey's test. The values were considered statistically significant when $p \leq 0.05$.

RESULTS

Figure 1 and 2 show the changes in the activities of GGT and ALP. The activities of these liver marker enzymes were increased significantly in plasma of alcohol + ΔPUFA group when compared to normal. Co-administration of PN significantly reduced their activity. PN control group showed no significant change in the activities when compared to normal.

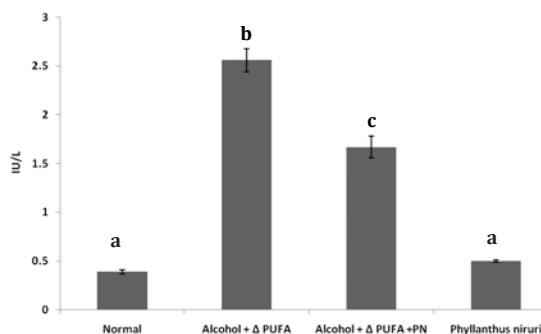


Fig 1: Changes in the activities of GGT in plasma. values are mean ± S.D (n=6) Values sharing a common superscript do not differ significantly at $P \leq 0.05$

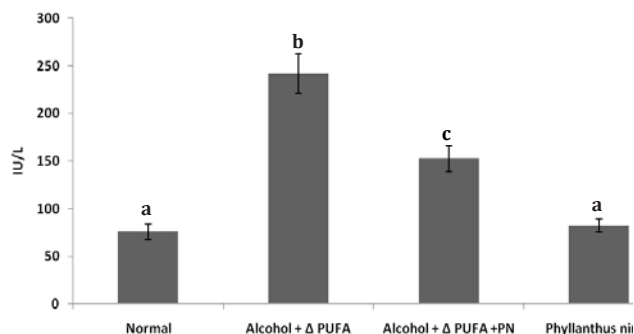


Fig 2: Changes in the activities of ALP in plasma. Values are mean ± S.D (n=6) Values sharing a common superscript do not differ significantly at $P \leq 0.05$

Figure 3 and 4 show the changes in the levels of TBARS and lipid hydroperoxides in liver. The levels of TBARS and HP were increased significantly in alcohol + ΔPUFA group when compared to normal. Treatment with PN significantly decreased their levels. PN control group did not show any significant change in the levels when compared to normal.

The changes in the levels of vitamin C, vitamin E and GSH in liver are given in Figure 5, 6 and 7 respectively. There was a significant decrease in the levels of vitamin C, vitamin E and GSH in alcohol +

ΔPUFA groups when compared to normal. The levels were significantly increased in PN treated groups. PN control group showed no significant change in their levels when compared to normal

The activities SOD (Figure 8), CAT (Figure 9), GPx (Figure 10) showed a significant decrease in liver of alcohol + ΔPUFA group. Treatment with PN significantly increased their activities. Administration of PN alone did not alter the activities of these enzymic antioxidants.

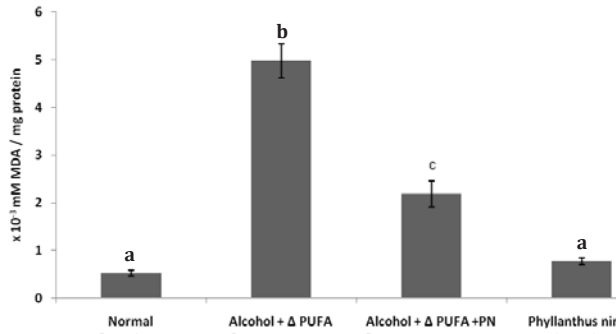


Fig. 3: Levels of TBARS in liver tissue. Values are mean ± S.D (n=6). Values sharing a common superscript do not differ significantly at P ≤ 0.05

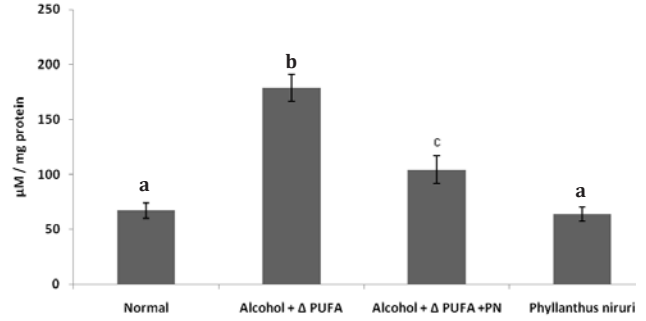


Fig. 4: Levels of Hydroperoxides in liver tissue. Values are mean ± S.D (n=6) Values sharing a common superscript do not differ significantly at P ≤ 0.05

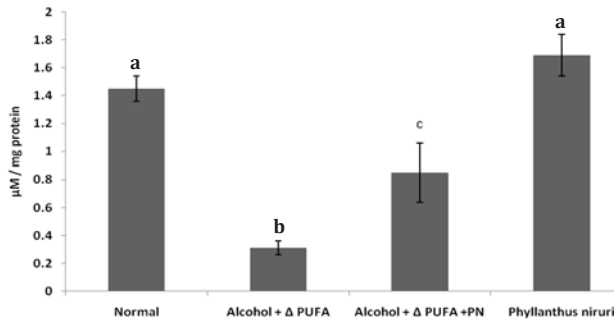


Fig. 5: Levels of Vitamin C in liver tissue. Values are mean ± S.D (n=6) Values sharing a common superscript do not differ significantly at P ≤ 0.05

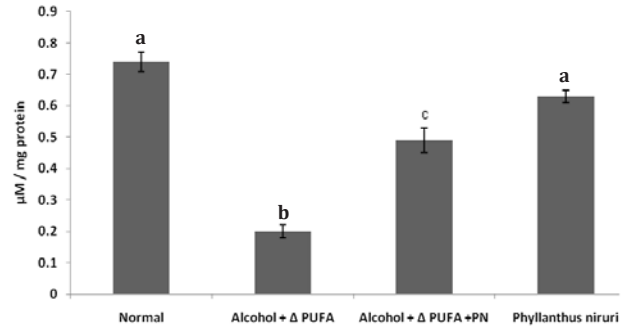


Fig. 6: Levels of Vitamin E in liver tissue. Values are mean ± S.D (n=6) Values sharing a common superscript do not differ significantly at P ≤ 0.05

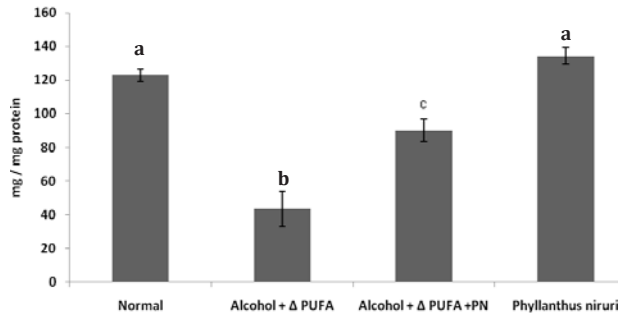


Fig.7: Levels of Reduced Glutathione in liver tissue. Values are mean ± S.D (n=6) Values sharing a common superscript do not differ significantly at P ≤ 0.05

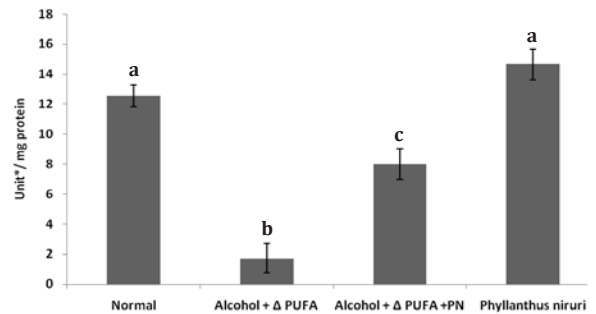


Fig. 8: Activities of Superoxide Dismutase in liver tissue. Values are mean ± S.D (n=6) Values sharing a common superscript do not differ significantly at P ≤ 0.05

*enzyme reaction which gives 50% inhibition of NBT reduction / minute

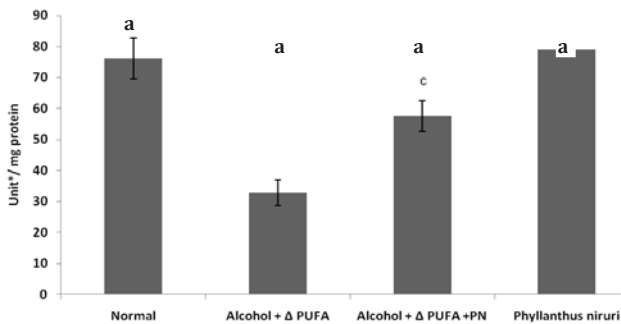


Fig. 9: Activities of Catalase in liver tissue. Values are mean ± S.D (n=6) Values sharing a common superscript do not differ significantly at P ≤ 0.05

* μm of H₂O₂ liberated / minute.

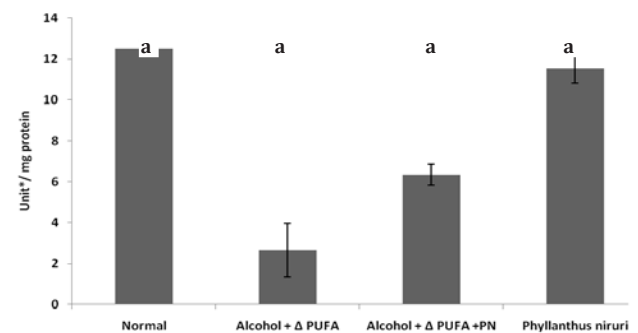


Fig. 10: Activities of Glutathione Peroxidase in liver tissue. Values are mean ± S.D (n=6) Values sharing a common superscript do not differ significantly at P ≤ 0.05

*mmoles of glutathione liberated / minute.

In histopathological observations (Figure 11), the liver of alcohol + heated PUFA treated rats showed fibrosis (Fig B), and *Phyllanthus niruri* treated rats showed only sinusoidal dilatation (Fig C). The

histology of liver was not affected in both normal (Fig A) and *Phyllanthus niruri* control (Fig D) rats.

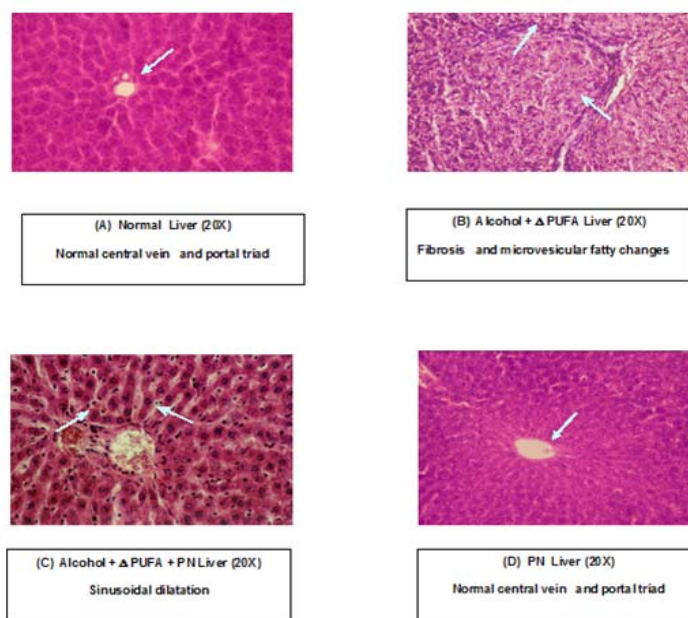


Fig. 11: Histopathological observations

DISCUSSION

Oxidative stress is defined as the imbalance between the prooxidant and antioxidant status. It results primarily due to increased production of reactive oxygen species or decreased levels of antioxidant defense in several tissues and organs. This may finally lead to irreversible organ and tissue damage when the production of these free radicals is outsized. The liver is the major site for toxicity of xenobiotics and drugs since most of the orally ingested drugs and chemicals are metabolized into toxic intermediates. Hence increased ingestion of alcohol, mainly through its metabolism, produces severe injury to the liver. Due to this injury, the biomembranes of the hepatocytes are damaged. This causes the increase in the activities of liver marker enzymes in the plasma.

Moreover, the membranes are mostly made up of lipid conjugates. The polyunsaturated fatty acids are highly susceptible to lipid peroxidation. The increased intake of dietary polyunsaturated fatty acid increases the degree of unsaturation of biomembranes and thus the lipid peroxidation is intensified. Hence the membrane becomes leaky to the liver marker enzymes, which is evidenced by the increase in the levels of GGT and ALP in our study. The increase in the lipid peroxidation which is implied by the increase in the measured TBARS and hydroperoxides in the alcohol and PUFA is also indicative of the hepatic damage.

The constant production of these free radicals must be counteracted by the enzymic and non-enzymic antioxidants. But the uncontrollable increase in the free radicals directs the increased exploitation of the antioxidants leading to their depletion. In our study, the levels of non-enzymic antioxidants reduced glutathione, vitamin C and vitamin E were significantly reduced in the alcohol and Δ PUFA treated rats because of their complete utilization due to oxidative stress. The activities of enzymic antioxidants like glutathione peroxidase, catalase and superoxide dismutase were also decreased in our study. The high amount of H_2O_2 and lipid hydroperoxides, $O_2^{\cdot-}$, 1-hydroxy ethyl radical, produced during alcohol and PUFA ingestion, might have contributed to their inactivation and depletion.

Phyllanthus niruri, a widely distributed herb is in use in herbal medicine systems and in clinical research over years. It is effective against toxicity caused by several hepatotoxicants. It is used for treating liver, brain and kidney diseases due to iron overload. *P. niruri* can potentially inhibit the transcription of pro inflammatory cytokines (TNF α) produced by the kupffer cells. Thus it could have acted as an anti-inflammatory agent, curing the inflammation caused by alcohol and PUFA. The hepatoprotective nature of *P. niruri* can be attributed to the presence of several bio-active compounds such as lignans, alkaloids, terpenoids and tannins especially phyllanthin and hypophyllanthin, quercetin, astragaline, gallic acid, ellagic acid and corilagin. It also possesses glycosides, flavonoids, flavonols, polyphenols, phenylpropanoids, which are known antioxidants^{26,27,28,29}. The presence of all these phenolic compounds may contribute for the high free radical scavenging activity of *P. niruri*³⁰. Phenolic compounds, by virtue of their hydrogen donating ability, forming aryloxy radicals, act as free radical scavengers and quench the lipid peroxidation. Moreover *P. niruri* extract has been found to inhibit many drugs metabolizing P450 enzymes³¹. This could also be one of the reasons for the increased protection of hepatocytes during *P. niruri* treatment.

The protective effect is further confirmed by the histopathological analysis, in which the severe pathological change fibrosis, which was observed in alcohol+ heated PUFA rats, was altered by *Phyllanthus niruri* treatment. The *Phyllanthus niruri* treated rats showed only sinusoidal dilatation which is the preliminary state of damage. Administration of *Phyllanthus niruri* to normal rats did not alter the histology of the liver.

Administration of the crude extract of *P. niruri* targeted a drop in the lipid peroxidation, enhanced the antioxidant status and thereby prevented the damage to the liver and leakage of enzymes GGT and ALP. Previous studies have shown that the crude extract has very high potency in scavenging DPPH radical than all other extracts³². This is indicative of its high radical scavenging property and hepatoprotective activity. Such a high antioxidant property has been shown only by few other plants³³. All these properties make *P. niruri*, a novel herb for treating oxidative stress and associated toxicity.

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