Extraction and Phytochemical Characterization of Phyllanthus niruri Leaves and their Evaluation for Ethanol Intoxicated Hepatotoxic Albino Rats

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ABSTRACT
Liver is the most important organ, which plays a pivotal role in regulating various physiological processes in the body. Living in a world of inadequately controlled environment, pollution and expanding therapy with potent drugs, it is continuously exposed to variety of xenobiotics and therapeutic agents resulting in its structural or functional damage. In an attempt to establish its pharmacological potential, we studied the Hepatoprotective activity of methanolic extract of Phyllanthus niruri obtained via extraction of leaves. The methanolic extract of P. niruri leaves was evaluated on serum glutathione, protein, serum marker enzymes, serum bilirubin, and thiobarbutiric acid reactive substances against ethanol induced liver damage in rats has been studied to find out the possible mechanism of hepatoprotection. Pre and post-treatment with extract showed a dose-dependent reduction of ethanol induced rats were elevated levels of enzyme activity with parallel increase in total protein and bilirubin, indicating the extract could preserve the normal functional status of the liver. The weight of the organs such as liver, heart, lung, spleen and kidney in ethanol induced experimental animals administered with P. niruri showed an increase over ethanol control group.

Keywords: Hepatoprotective activity, Phyllanthus niruri, hepatotoxicity, enzyme activity, Biochemical studies.

INTRODUCTION
Liver diseases are a serious health problem. In the absence of reliable liver protective drugs in allopathic medical practices, herbs play an important role in the management of various liver disorders. Numerous medicinal plants and their formulations are used for liver disorders in ethnomedical practices and in traditional system of medicine in India. Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effect. The Liver is the largest organ in the body carrying out most of the biochemical synthesis and secretary functions. It is involved in several vital functions, such as metabolism, secretion and storage. It has great capacity to detoxicate toxic substances and synthesize useful principles[1]. Liver functions as a centre of metabolism of nutrients such as carbohydrates, proteins and lipids and excretion of waste metabolites. Additionally, it also handles the metabolism and excretion of drugs and other xenobiotics from the body thereby providing protection against foreign substances by detoxifying and eliminating those[2]. Liver diseases have become a global problem. The principal causative factor for the liver diseases in developed countries is chronic alcoholism, while in the developing countries
the most frequent causes are malnutrition, anemia, infection and availability of hepatotoxic drugs over the counter. Hepatotoxicity manifest as necrosis and cirrhosis. Hardly any effective measures are available for the treatment of liver diseases viz, corticosteroids, anti-viral and immunosuppressant agents are sometimes inadequate and may lead to serious adverse effects.

This organ plays a major role in metabolism and has a number of functions in the body, including glycogen storage, decomposition of red blood cells, plasma protein synthesis, hormone production, and detoxification. It produces bile, an alkaline compound which aids in digestion, via the emulsification of lipids. The liver's highly specialized tissues regulate a wide variety of high-volume biochemical reactions, including the synthesis and breakdown of small and complex molecules, many of which are necessary for normal vital functions [3].

The liver is a vital organ present in vertebrates and some other animals. The liver is a reddish brown organ with four lobes of unequal size and shape. A human liver normally weighs between 1.4–1.6 kg (3.1–3.5 lb) and is a soft, pinkish-brown, triangular organ. It is both the largest internal organ (the skin being the largest organ overall) and the largest gland in the human body. Liver is the vital organ of metabolism and excretion. About 20,000 deaths are found every year due to liver disorders. Hepatocellular carcinoma is one of the ten most common tumors in the world with over 2,50,000 new cases each year. In India, about 40 polyherbal commercial formulations reputed to have hepatoprotective action are being used. It has been reported that 160 phytoconstituents from 101 plants have hepatoprotective activity [5].

Liver protective herbal drugs contain a variety of chemical constituents like phenols, coumarins, lignans, essential oil, monoterpenes, carotinoids, glycosides, flavonoids, organic acids, lipids, alkaloids and xanthines. Plant extracts of many crude drugs are also used for the treatment of liver disorders. Extracts of different plants of about 25 plants have been reported to cure liver disorders [6].

In spite of tremendous strides in modern medicine, there are hardly any drugs that stimulate liver function, offer protection to the liver from damage or help regeneration of hepatic cell. There are however, members of drugs employed in traditional system of medicine for liver affections [8]. Many formulations containing herbal extracts are sold in the Indian market for liver disorders. But management of liver disorders by a simple and precise herbal drug is still an intriguing problem. Several Indian medicinal plants have been extensively used in the Indian traditional system of medicine for the management of liver disorder. Some of these plants have already been reported to strong antioxidant activity [9].

Alcohol administration causes accumulation of reactive oxygen species, which in turn causes lipid peroxidation of cellular membranes and proteins and DNA oxidation resulting in hepatocyte injury [10]. Alcohol treatment of rats is known to cause the translocation of fat from the peripheral adipose tissue to liver, kidney and brain for accumulation [11]. 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 iron.

India has a rich heritage of traditional knowledge and is home to several important time-honored systems of health care like Ayurveda, Siddha and Unani. It has been estimated that the proportion of medicinal plants in India (7,500 of the 17,000 higher plant species are medicinal plants) is higher than any country of the world with respect to the existing flora of that respective country [12-14].

Phyllanthus niruri are originated in India, usually occurring as a winter weed throughout the hotter parts.
The Phyllanthus genus contains over 600 species of shrubs, trees, and annual or biennial herbs distributed throughout the tropical and subtropical regions of hot hemispheres. Unfortunately, there remains a great deal of confusion among scientists regarding plant identification and many cases, plant misidentification make evaluation of published information difficult. *P. amarus* and *P. sellowianus* are often considered a variety of *P. niruri*, or no distinction is made among these three species in published clinical research. Often time’s one name is indicated tube synonymous with another and, sometimes, both names are used interchangeably as if referring to one plant. It became so confusing that, in the 1990s, a major reorganization of the Phyllanthus genus was conducted (which classified *P. amarus* as a type of *P. niruri*). *Phyllanthus niruri* is an herb of Euphorbiaceae family that grows upto 60 cm. Phyllanthus means “leaf and flower” because the flower, as well as the fruit, seems to become one with the leaf. *Phyllanthus niruri* is a common kharif (rainy season) weed found in both cultivated fields and wastelands. *Phyllanthus niruri* is an annual herb belonging to the family Euphorbiaceae grows 50 to 70 centimeters tall and bears ascending herbaceous branches. The bark is smooth and light green. It bears numerous pale green flowers which are often flushed with red. The fruits are tiny, smooth capsules containing seeds. It produces phyllanthid branches with the presence of flowers and fruits at the base of each leaf, one of the identification characteristics of this plant. *Phyllanthus niruri* is used in the treatment of various ailments like jaundice, diabetes, kidney stones and liver disorders and for treatment of Hepatitis B viral infection. Therefore, in the present study was aimed to investigate whether injection of ethanol induces oxidative stress and if so, *Phyllanthus niruri* reduces the ethanol intoxicated oxidative stress and hepatic damage in the liver of albino rats.

**MATERIALS AND METHODS**

For the present study, the mature green leaves of *Phyllanthus niruri* belongs to family Euphorbiaceae were collected from in and around area of Thanjavur District, Tamil Nadu, South India. The plant was identified with the help of manual of Tamil Nadu and Karnatic flora [15-16] with standard references [17].

**Preparation of plant extract**

The *Phyllanthus niruri* was collected, washed, cut into small pieces and dried at room temperature (28±1°C) for two weeks and made into powder for further analysis. The aerial parts were washed under tap water, air dried, homogenized to fine powder and stored in airtight bottles. Ten grams of dried powder was first defatted with petroleum ether and then extracted with methanol by using Soxhlet apparatus. The solvent was evaporated to dryness and the dried crude extract was stored in air tight bottle at 4°C. The percentage yield of methanol extract was 36%. The methanol extract of *Phyllanthus niruri* was used for the entire study.

**Experimental Animals**

Adult Wistar albino rats weighing of 200 - 220 gm breed in the Central Animal House, Department of Pharmacology, Periyar College of Pharmaceutical Sciences, Trichy - 21, were used in this study. They were housed in Tarson’s polypropylene cages with metal grill tops and provided with food and water ad libitum. They were maintained in a controlled environment under standard conditions of temperature and humidity with alternating light/dark (LD 12:12) cycle. In the laboratory, rats were fed with standard rat pellet diet (Lipton India Ltd., Bangalore). The animals used in the present study were approved by the ethical committee, Department of Pharmacology, Periyar College of Pharmaceutical Sciences, Trichy - 21, India (The Registration Number is CPCSEA/265) and were in accordance with the guidelines of the National Institute of Nutrition (NIN), Indian Council of Medical Research (ICMR), Hyderabad, India.

**Acute Toxicity Study**

Acute oral toxicity study was performed as per OECD-404 guidelines (1987). 10 rats/group (5 males and 5 females) were used for the study. Group 1 was control group and other three groups were that of WFM at different doses (500, 2000, and 4000 mg/kg body weight).
Single dose of the extract was administrated orally to each animal. Signs of toxicity, body weight, feed and water consumption of each animal was observed every day for 14 days.

**Experimental design:**
The animals were randomly divided into four groups, each containing three animals. Four groups (Group I, Group II, Group III and Group IV) of rats, three rats in each group were taken. The *Phyllanthus niruri* extract at a fixed dose (100 mg/kg) that was daily fed for ten days to one group (Group III) of rats and ethanol (20%) was administered on seven days after 15th day’s administration of the extract. The ethanol treated group (Group II) received normal saline in place of plant extract. After 48h of ethanol feeding rats were sacrificed by cervical dislocation for estimation of blood biochemical parameters and serum marker enzymes were analyzed following standard methods.

**Biochemical parameters** like serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvate transaminase (SGPT) by the methods of Reitman & Frankel [18], alkaline phosphatase [19], total bilirubin [20] and protein [21] were analyzed. Reduced glutathione (GSH) was estimated using DTNB [22]. The blood glutathione was estimated by the method of Beutler et al. [23]. The concentration of Thiobarbutiric acid reactive substances (TBARS) was measured in liver using the method of Ohkawa et al. [24].

**RESULTS**
The treatment with the extract did not decrease water and food consumption. The body weight of the rats treated with methanolic extract once a day during 14 days (sub-acute treatment) did not show any significant change when compared with the control group, although had a tendency to decrease body weight (100 mg/kg of *Phyllanthus niruri*). This decrease can be associated with the decrease of liver weight at the dose of 100 mg/kg in comparison with the control group without any concomitant alteration in the activity of alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase. Estimation of the serum activity of total bilirubin, protein, reduced glutathione, TBARS, alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase is one of the most widely used means of measuring hepatocellular injury (Table 1). The macroscopic analysis of the target organs of the treated rats (liver, lung, heart, spleen & left kidney) did not show significant changes in colour and texture when compared with the control group (Table 2).

**DISCUSSION**
A lot of medicinal plants, traditionally used for thousands of years, by the Indian traditional health care system (ayurvedic) named ‘Rasayana’ for their antioxidative properties. *Phyllanthus niruri* was a very good antioxidant and hepatoprotective agent [2,25]. *P. niruri* (100-200 mg/kg) increased cell viability of rat hepatocytes being treated with ethanol intoxicated rats. The present study was carried out to evaluate the hepatoprotective activity of *P. niruri* against ethanol induced hepatocellular degenerative in albino rats. The effectiveness of this medicinal plant was screened by assessing biochemical changes of different groups of experimental animals. *P. niruri* possessed very high levels of alkaloids and flavonoids and are employed in medicinal uses. The plants studied here can be seen as a potential source of useful drugs. The results of biochemical parameters revealed the elevation of enzyme level in ethanol treated group, indicating that ethanol induces damage to the liver (Table 1). Liver tissue rich in both transaminases increased in acute hepatic diseases SGPT, which is slightly elevated by cardiac necrosis is a more specific indicator of liver disease [26,27]. A significant reduction \((P < 0.005)\) was observed in SGPT, SGOT, ALP, total bilirubin and protein levels in the groups treated with silymarin and extract of *P. niruri*. The results confirmed that the enzyme levels were almost restored to the normal levels [28,12].

In India, more than 87 medicinal plants are used in different combinations in the preparation of 33 patented herbal formulations [29-31]. Some of the polyherbal formulations are verified for their hepatoprotective action against chemical induced liver damage in experimental animals [32-33].
In most of these studies, marginal or moderated levels of hepatoprotective activities were observed. It is believed that efficacy is not sufficient enough to use these agents as effective drugs [34].

The results of biochemical parameters revealed the elevation of enzyme level in ethanol treated group, indicating that ethanol induces damage to the liver (Table 1). Liver tissue rich in both transaminases increased in patients with acute hepatic diseases, SGPT, which is slightly elevated by cardiac necrosis, is a more specific indicator of liver disease [28-29]. A significant reduction ($P < 0.001$) was observed in SGPT, SGOT, ALP, total bilirubin and protein levels in the groups treated with silymarin and alcoholic extract of $S. brevistigma$. The enzyme levels were almost restored to the normal [12,25]. The present study was observed that $Phyllanthus niruri$ has a significant hepatoprotective effect in ethanol administrated rats that hepatocellular degenerative and necrotic changes are slight without advanced fibrosis and cirrhotic process in $Phyllanthus niruri$ treated group. However, Ramamurthy and Raveendran [3] found that $Nigella sativa$ L can prevent liver fibrosis and cirrhosis, suggesting that $Nigella sativa$ L protects liver against fibrosis possibly through immunomodulator and antioxidant activities.

### Table 1. Effect of $Phyllanthus niruri$ extracts on some biochemical and serum marker enzyme parameters in ethanol intoxicated albino rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Ethanol treated</th>
<th>$P. niruri$ treated</th>
<th>Silymarin (2.5 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin (mg/dl)</td>
<td>$0.82 \pm 0.15$</td>
<td>$2.80 \pm 0.72$</td>
<td>$1.15 \pm 0.13$</td>
<td>$1.08 \pm 0.27$</td>
</tr>
<tr>
<td>Protein (g/dl)</td>
<td>$8.20 \pm 0.17$</td>
<td>$5.15 \pm 0.12$</td>
<td>$7.17 \pm 0.22$</td>
<td>$7.55 \pm 0.38$</td>
</tr>
<tr>
<td>TBARS (n moles/ml)</td>
<td>$2.50 \pm 0.11$</td>
<td>$6.10 \pm 0.23$</td>
<td>$3.80 \pm 0.27$</td>
<td>$3.25 \pm 0.13$</td>
</tr>
<tr>
<td>GSH (µ mole/g of tissue)</td>
<td>$8.12 \pm 0.12$</td>
<td>$5.15 \pm 0.16$</td>
<td>$7.50 \pm 0.25$</td>
<td>$7.12 \pm 0.22$</td>
</tr>
<tr>
<td>SGOT (IU/L)</td>
<td>$140 \pm 0.31$</td>
<td>$225 \pm 0.13$</td>
<td>$160 \pm 0.18$</td>
<td>$150 \pm 0.15$</td>
</tr>
<tr>
<td>SGPT (IU/L)</td>
<td>$40.3 \pm 0.15$</td>
<td>$95.5 \pm 0.12$</td>
<td>$52.1 \pm 0.25$</td>
<td>$48.7 \pm 0.33$</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>$132 \pm 0.12$</td>
<td>$252 \pm 0.26$</td>
<td>$155 \pm 0.16$</td>
<td>$143 \pm 0.42$</td>
</tr>
</tbody>
</table>

Results are mean of three observations $\pm$ SD.

### Table 2. Effect of $Phyllanthus niruri$ extracts on body and organs weight in ethanol intoxicated rats

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Control</th>
<th>Ethanol treated</th>
<th>$P. niruri$ treated</th>
<th>Silymarin (25 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body (g)</td>
<td>$221 \pm 0.12$</td>
<td>$197 \pm 0.22$</td>
<td>$213 \pm 0.31$</td>
<td>$218 \pm 0.15$</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>$1.80 \pm 0.25$</td>
<td>$1.48 \pm 0.15$</td>
<td>$1.68 \pm 0.45$</td>
<td>$1.72 \pm 0.25$</td>
</tr>
<tr>
<td>Heart (g)</td>
<td>$0.170 \pm 0.12$</td>
<td>$0.140 \pm 0.27$</td>
<td>$0.150 \pm 0.18$</td>
<td>$0.160 \pm 0.24$</td>
</tr>
<tr>
<td>Lungs (g)</td>
<td>$0.220 \pm 0.15$</td>
<td>$0.180 \pm 0.12$</td>
<td>$0.210 \pm 0.14$</td>
<td>$0.215 \pm 0.22$</td>
</tr>
<tr>
<td>Spleen (g)</td>
<td>$0.180 \pm 0.22$</td>
<td>$0.130 \pm 0.25$</td>
<td>$0.150 \pm 0.13$</td>
<td>$0.170 \pm 0.31$</td>
</tr>
<tr>
<td>Kidney (g)</td>
<td>$0.880 \pm 0.23$</td>
<td>$0.560 \pm 0.12$</td>
<td>$0.780 \pm 0.22$</td>
<td>$0.840 \pm 0.51$</td>
</tr>
</tbody>
</table>

Mean values of 3 animals $\pm$ S.D.
Liver is the most important and main part of the animal body. It is highly affected primarily by toxic agents and that is why the above-mentioned parameters have been found to be of great importance in the assessment of liver damage. The abnormal high level of serum ALT, AST, ALP and bilirubin observed in our study (Table 1) are the consequence of ethanol induced liver dysfunction and denotes the damage to the hepatic cells. Treatment with *Phyllanthus niruri* reduced the enhanced level of serum ALT, AST, ALP and bilirubin, which seem to offer the protection and maintain the functional integrity of hepatic cells.

Liver plays an important role in the protein synthesis. It is considerably affected when there is a disturbance in protein metabolism. The site-specific oxidative damage of some of the susceptible amino acids of proteins is now recorded as the major cause of metabolic dysfunctions during pathogenesis [35]. Decrease in serum bilirubin after treatment with the extract in liver damage indicated the effectiveness of the extract in normal functional status of the liver. In the present study the lowered level of total proteins and bilirubin recorded in blood sample of ethanol treated rats reveals the severity of hepatopathy. In the *Phyllanthus niruri* treated group, the protein and bilirubin level of animal was almost normal. This result is support by stimulations of protein synthesis have been advanced as a contributory hepatoprotective mechanism, which accelerates the regeneration process and the protection of liver cell [28].

Reduced glutathione is tripeptide consisting of glutamate, cysteine and glycine. It acts as antioxidant both intracellular and extracellular. Glutathione reductase is a major enzyme in GSH regeneration which produces hydroxyl radical by reaction with $H_2O_2$ [26,12]. In the present study the GSH level was decreased in ethanol induced animals, while treatment with *Phyllanthus niruri* extract clearly enhanced the GSH levels. The restoration of GSH indicates hepatoprotective effect of herbal extract [35,3].

ALT and AST are the specific markers to assess hepatocellular damage leading to liver cell necrosis. In present study ALT and AST activities were assessed as it is the more specific index of liver cell damage. High level of SGOT indicates liver damage such as due to cellular damage. SGPT catalyses the conversion of alanine to pyruvate and glutamate and is released in a similar manner. Therefore SGPT is more specific to the liver and a better parameter for detecting liver damage [12]. In the present study ethanol injection significantly increased serum ALT and AST indicating induction of hepatic damage. Methanol extracts of *Phyllanthus niruri* at the dose of 100 mg/kg decreased the levels of both SGOT and SGPT. In these investigations, it was observed that serum SGOT, SGPT and ALT levels were significantly reduced in animals receiving *Phyllanthus niruri* and ethanol than those given ethanol alone indicating that the degree of hepatic cell damage was lesser magnitude in treated groups.

In conclusion, the results of present study demonstrate that methanol extracts of *Phyllanthus niruri* has potent hepatoprotective activity against ethanol induced liver damage in rats. Hence our present investigation reveals that the *Phyllanthus* species possess the hepatoprotectivity activity. The extract is nontoxic even at relatively high concentrations. The hepatoprotectivity activity is probably due to the presence of flavanoids. These finding shows that *Phyllanthus niruri* extract have the ability to rectify hepatic damage or toxicity. Hence it is advised that if one happens to take ethanol in overdose they can consume *Phyllanthus niruri* extract as a hepatoprotective agent. Thus always have in mind that “Prevention is better than cure”.

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