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RESEARCH ARTICLE

In Vitro Lipid Peroxidation Inhibitory and Antimicrobial Activity of *Phyllanthus niruri* (Euphorbiaceae) Extract

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ABSTRACT

The present work was designed to evaluate the in vitro lipid peroxidation inhibitory and antimicrobial activities of the methanol extract of Phyllanthus niruri (MEPN) (Family: Euphorbiaceae). Lipid peroxidation was measured by the optical density of the prepared solutions (10-320 µg/ml) and then the percent inhibition was calculated. Ascorbate/FeSO4-induced peroxidation was inhibited by standard antioxidants such as L-ascorbic acid, quercetin and MEPN. Moreover, the percent inhibition of the methanol extract was increased in a concentration-dependent manner. IC50 value for the MEPN, L-ascorbic acid and quercetin for lipid peroxidation was found to be 62.5 µg/ml, 41 µg/ml and 19.75 µg/ml respectively. The antimicrobial activity of MEPN was determined by disc diffusion method with various gram-positive and gram-negative microorganisms. The MEPN showed strong antimicrobial activity against Bacillus pumillus 8241, Bacillus cereus, Escherichia Coli 54B and Vibrae Cholera at a concentration of 750 µg/ml/disc. However, its activity against Staphylococcus aureus ML 152 and Vibrae cholera 14035 was less significant. The antimicrobial activity of the extract was compared with the standard drug, chloramphenicol at a concentration of 10µg/ml/disc. The results obtained in the present investigation clearly suggest that MEPN can be a potential source of natural antioxidant and antimicrobial agent.

Keywords: *Phyllanthus niruri, In vitro lipid peroxidation inhibitory activity, Antimicrobial activity*

There has been growing interest in the investigation of the natural products from plants for the discovery of new antimicrobial and antioxidant agents as well as an alternative route for the substitution of synthetic chemicals, side effects of which are always in question. For this, the essential oils and the extracts of many plants have been prepared and screened for their antimicrobial and antioxidant activities leading to the accumulation of a large number of reports in the literature concerning the above-mentioned properties of plants [1-5]. Much attention has been paid to the plant extracts and the isolated compounds because of their less side effects and the strong resistance towards various microorganisms [6]. Plant-based antimicrobials represent a vast untapped source for medicines and further exploration of plant antimicrobials is needed as antimicrobials of plant origin have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials [7].

PN (family: Euphorbiaceae) is a perennial herb distributed throughout the tropical and subtropical regions of both hemispheres. In India, it is widespread in drier tropical areas of Andhra Pradesh, Tamil Nadu, Kerala and Karnataka states of South India. It is named the 'stone breaker' by the indigenous people. Whole plant, fresh leaves and fruits are used to treat various ailments like dysentery, influenza, vaginitis, tumors, diabetes, diuretics, jaundice, kidney stones, dyspepsia, antihepatotoxic, antihapatitis-B, antihyperglycemic and also as antiviral and antibacterial [8]. Antitumor and anticarcinogenic activities of *Phyllanthus amarus* have also been reported [9]. Other medicinal properties such as hypolipidemic [10] and antiviral [11, 12] activities of *Phyllanthus niruri* have also been shown. Several bioactive molecules, such as lignans, phyllanthin, hypophyllanthin, flavonoids, glycosides and tannins, have been shown to be present in the extracts of PN [9]. The phytochemicals from PN and their pharmacological properties were studied by Bagalkotkar *et al* [13]. Using a rat

hepatocyte primary culture, Shamasundar *et al* [14] have shown that *phyllanthin* and *hypophyllanthin* protected cells against carbon tetrachloride cytotoxicity whereas *triacontanal* was protective against galactosamine toxicity. PN is used as one of the components of a multiherbal preparation for treating liver ailments [15]. Liver damage is followed by complex disturbances in the lipolytic activity of the vascular space which often appeared with hyperlipoproteinemia in patients [16]. Abnormalities with lipid metabolism have been reported in cholesteosis [17], alcoholism [18], chemical intoxication [19] and hepatitis [20]. The plant is also useful in treating viral and bacterial diseases [21].

Previously, we reported the antihyperglycemic [22] activity of MEPN. In the present study, we have tested the *in vitro* lipid peroxidation and antimicrobial activity (against various Gram positive and Gram negative bacteria) of the methanol extract of PN.

MATERIALS AND METHODS

Chemicals

L-ascorbic acid, quercetin and thiobarbituric acid (TBA) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All other chemicals and reagents used were purchased from SD-Fine Chem, Hyderabad (A.P), India.

Extraction procedure

The plant PN was obtained from the tribal area of Karimnagar District, Andhra Pradesh, India. The plant was identified taxonomically by Dr. Alok Bhattacharya of the Botanical Survey of India (BSI), Shibpur, Kolkata, India. A voucher specimen (No. GPS-2) has been preserved in our laboratory for future purposes. For the extract, the whole plant was dried in shade and powdered in a mechanical grinder. The powder of PN was initially defatted with petroleum benzene (60-80^{o}C) followed by 1 liter of methanol by using a Soxhlet extractor for 72 h at a temperature not exceeding the boiling point of the solvent [23]. The extract was filtered using Whatman filter paper (No. 1) and then concentrated in vacuum and dried. The methanol extract was used in the assay of lipid peroxidation inhibitory and antimicrobial activity.

Previously isolated classes of compounds

The phytochemical study revealed that the MEPN contained alkaloids, flavonoids, saponins and coumarins, polyphenols, tannins, terpenoids, lipids and lignans [13].

Microorganisms utilized for antimicrobial activity

Microorganisms (Staphylococcus aureus 8531, Staphylococcus aureus ML 174, Staphylococcus aureus ML 152, Bacillus pumillus 8241, Bacillus cereus, Escherichia coli 51, Escherichia coli 54B, Vibrea cholera 14035, Vibrea cholera 1353, and Vibrea cholera 226101) were obtained from the stock culture of Central Drugs Laboratory, Kolkata; Indian Institute of Chemical Biology, Kolkata and Mycology and Plant Pathology Laboratory, Calcutta University, Kolkata, India.

In vitro lipid peroxidation

Lipid peroxidation induced by Fe^{2+}-ascorbate system in rat liver homogenate by the method of Bishayee and Balasubramaniyam [24] was estimated as thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa *et al.* [25]. The reaction mixture contained rat liver homogenate 0.1ml (25% w/v) in Tris-HCl buffer (20mM, pH 7.0); KCl (30mM); $FeSO_4(NH_4)_2SO_4.7H_2O$ (0.06mM); and various concentrations of PN extract in a final volume of 0.5 ml. The reaction mixture was incubated at 37^{o}C for 1 h. After the incubation period, 0.4 ml was removed and treated with 0.2 ml sodium dodecyl sulphate (SDS) (8.1%); 1.5 ml thiobarbituric acid (TBA) (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The total volume was made up to 4.0 ml with distilled water and then kept in a water bath at 95 to 100^{o}C for 1 h. After cooling, 1.0ml of distilled water and 5.0 ml of *n*-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The butanol-pyridine layer was removed and its absorbance at 532 nm was measured to quantify TBARS. Inhibition of lipid peroxidation was determined by comparing the optical density (OD) of treatments with that of the control. Quercetin and L-ascorbic acid were used as the standard controls. The % inhibition of lipid peroxidation was calculated by using the following formula:

$$\%inhibition = \frac{[A_{blank} - A_{test}]}{A_{blank}} \times 100$$

where A_{blak} is the absorbance of the blank reaction and A test is the absorbance in the presence of the sample of the extracts.

Determination of antimicrobial activity

Antimicrobial activity was measured using the standard method of disc diffusion plates on agar [26]. Then 0.1 ml of each culture of bacteria was spread on agar plate surfaces. For antibacterial assays, all bacterial strains were grown in Mueller Hinton Broth medium (Merck) for 24 h at 37°C. The concentration of bacterial suspensions was adjusted to 10^8 colony forming units (10^8cfu/ml) in Mueller Hinton Agar. Paper discs (6 mm in diameter) were impregnated on the agar to load 10µl of each sample. The impregnated discs were placed on the medium suitably spaced apart and the plates were incubated at 5°C for 1 h to permit good diffusion and then transferred to an incubator at 37°C for 24 h. The results were recorded by measuring the zones of growth inhibition surrounding the disc. Clear inhibition zones around the discs indicate the presence of antimicrobial activity. All data on antimicrobial activity are the average of triplicate analyses. In order to determine the antibacterial effect of the MEPN, chloramphenicol (10µg/ml/disc) were used as positive control. Inhibition diameters were measured after incubation for 24 h at 37°C.

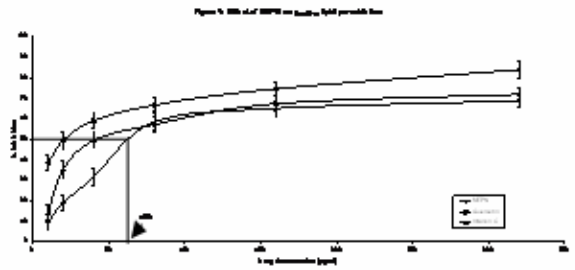

Fig 1. Effect of methanol extract of *Phyllanthus niruri* (MEPN) on lipid peroxidation.

Statistical Analysis

All treatments were performed in triplicate. Statistical analysis was performed using Graphpad prism, 3.0 version (Graphpad Software Inc., San Diego, CA, USA). The statistical significance of a treatment effect was evaluated by student's *t*-test and ANOVA. The values were expressed as mean ± SD. IC_{50} values for all the above experiments were determined by linear regression. Probability limit was set at $p < 0.05$.

RESULTS

Effect of MEPN on lipid peroxidation

The effect of MEPN and commercially available antioxidants namely L-Ascorbic acid and quercetin on the *in vitro* inhibition of lipid peroxidation is shown in Fig 1. The generation of lipid peroxidase by Fe^{2+}-ascorbate in rat liver homogenate appears to be inhibited by MEPN with IC_{50} value of 62.5µg/ml. Though, the inhibitory activity was observed, but it was found not so remarkable when compared to L-Ascorbic acid and Quercetin. The percentage inhibition of lipid peroxidation of MEPN at 320 µg/ml was found to be 68.88% and for L-ascorbic acid and Quercetin the percentage inhibition was found to be 72.11% and 84.09%, respectively.

Effect of MEPN on antimicrobial activity

The data presented in Table 1 indicate that the methanol extract of *Phyllanthus niruri* (MEPN) inhibit the growth of some of the tested microorganisms (Gram positive and Gram negative) to various degrees. The MEPN at a concentration of 500 µg/ml/disc showed moderate activity and 750 µg/ml/disc exhibited moderate to strong antimicrobial activity against all the tested microorganisms. The extract showed strong antibacterial activity against *Bacillus pumillus* 8241, *Bacillus cereus*, *Escherichia Coli* 54B and *Vibrae Cholera*. However, its activity against *Staphylococcus aureus* ML 152 and *Vibrae cholera* 14035 was found to be less. The antimicrobial activity of the extract was compared with the standard Chloramphenicol at a concentration of 10µg/ml/disc.

DISCUSSION

PN has many effective traditional uses for a wide variety of diseases. Some of the medicinal usages have been proven in experimental models, which suggest that the extracts of the plant possess various pharmacological actions. Unsaturated lipids in liver tissue are very susceptible to peroxidation when they are exposed to reactive oxygen species (ROS). In the present investigation, we have incubated the liver tissue in presence of a ROS generating system, ascorbate/$FeSO_4$, and examined the effect on tissue homogenate by measuring the optical density (OD) at 532nm. The results of the investigations reveal that MEPN has no potent lipid peroxidation inhibition activity.

The antimicrobial activity of the MEPN was studied by the disc diffusion method against various microorganisms. Disc diffusion methods are used extensively to investigate the antibacterial activity of natural substances and plant extracts [27]. These assays are based on the use of discs as reservoirs containing solutions of the substances to be examined. In case the activity is low, higher concentrated solutions are used. Because of

Table 1: Effect of methanol extract of *Phyllanthus niruri* (MEPN) on selected microbial strains

Microorganism	10% DMSO/ ml/disc	***MEPN***		Chloramphenicol (10µg/ml/disc)
		500µg/ ml/disc	750µg/ ml/disc	
Staphylococcus aureus 8531	9	6	10	16
Staphylococcus aureus ML 174	6	6	11	19
Staphylococcus aureus ML 152	6	6	7	22
Bacillus pumillus 8241	7	7	23	21
Bacillus cereus	6	10	16	14
Escherichia coli 51	6	7	10	24
Escherichia coli 54B	6	12	15	17
Vibrea cholera 14035	6	7	9	22
Vibrea cholera 1353	6	11	16	11
Vibrea cholera 226101	6	10	14	21

6-9mm: low activity; 10-14mm: moderate activity; ≥15mm: strong activity.
All the values were the mean of three experiments.
The values given are the diameter of zone of inhibition (mm) including disc diameter of 6mm.

the limited capacity of discs, holes or cylinders are preferably used [27]. MEPN showed a broad spectrum of activity against all the microorganisms employed as shown in Table 1. Chloramphenicol at a concentration of 10µg/ml/disc was used as a positive control.

On the basis of the results obtained in the present investigation, it is revealed that MEPN has no *in vitro* lipid peroxidation inhibitory but has significant antimicrobial activity. The phytoconstituents responsible for the inhibition of lipid peroxidation may be due to the presence of flavonoids such as rutin, quercetin, quercitrin, etc. and the antimicrobial activity of MEPN may be due to the presence of p-cymene, a monoterpenoid, present in the plant extract [13]. P-cymene was also tested for antimicrobial properties using the paper disc diffusion method, in which it revealed a good antimicrobial activity [28]. More importantly, there have been no side effects or toxicity reports from many years on this plant. Although there has been extensive research on this plant, there is still a lot of scope for further research, especially towards the mechanism of biological activity of phytochemicals from this plant.

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