

1 RESEARCH ARTICLE

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

5 YERRA RAJESHWAR, RAYEES AHMAD, A. SHYAM SUNDER, J. DEVILAL, MALAYA GUPTA and
6 UPAL KANTI MAZUMDER

7 For author affiliations, see end of text.

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10 ABSTRACT

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

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

26 There has been growing interest in the investigation 46 PN (family: Euphorbiaceae) is a perennial herb dis-
27 of the natural products from plants for the discovery of 47 tributed throughout the tropical and subtropical regions
28 new antimicrobial and antioxidant agents as well as an 48 of both hemispheres. In India, it is widespread in drier
29 alternative route for the substitution of synthetic chemi- 49 tropical areas of Andhra Pradesh, Tamil Nadu, Kerala
30 cals, side effects of which are always in question. For 50 and Karnataka states of South India. It is named the
31 this, the essential oils and the extracts of many plants 51 'stone breaker' by the indigenous people. Whole plant,
32 have been prepared and screened for their antimicrobial 52 fresh leaves and fruits are used to treat various ailments
33 and antioxidant activities leading to the accumulation of 53 like dysentery, influenza, vaginitis, tumors, diabetes,
34 a large number of reports in the literature concerning the 54 diuretics, jaundice, kidney stones, dyspepsia, antihepa-
35 above-mentioned properties of plants [1-5]. Much atten- 55 tototoxic, antihapatitis-B, antihyperglycemic and also as
36 tion has been paid to the plant extracts and the isolated 56 antiviral and antibacterial [8]. Antitumor and anticar-
37 compounds because of their less side effects and the 57 cinogenic activities of *Phyllanthus amarus* have also
38 strong resistance towards various microorganisms [6]. 58 been reported [9]. Other medicinal properties such as
39 Plant-based antimicrobials represent a vast untapped 59 hypolipidemic [10] and antiviral [11, 12] activities of
40 source for medicines and further exploration of plant 60 *Phyllanthus niruri* have also been shown. Several bioac-
41 antimicrobials is needed as antimicrobials of plant ori- 61 tive molecules, such as lignans, phyllanthin, hypophyl-
42 gin have enormous therapeutic potential. They are effec- 62 lanthin, flavonoids, glycosides and tannins, have been
43 tive in the treatment of infectious diseases while simul- 63 shown to be present in the extracts of PN [9]. The phy-
44 taneously mitigating many of the side effects that are 64 tochemicals from PN and their pharmacological proper-
45 often associated with synthetic antimicrobials [7]. 65 ties were studied by Bagalkotkar *et al* [13]. Using a rat

66hepatocyte primary culture, Shamasundar *et al* [14] 121Biology, Kolkata and Mycology and Plant Pathology
67have shown that *phyllanthin* and *hypophyllanthin* pro- 122Laboratory, Calcutta University, Kolkata, India.
68tected cells against carbon tetrachloride cytotoxicity 123
69whereas *triacontanal* was protective against galactosa- 123 *In vitro lipid peroxidation*
70mine toxicity. PN is used as one of the components of a 124 Lipid peroxidation induced by Fe²⁺-ascorbate system
71multiherbal preparation for treating liver ailments [15]. 125in rat liver homogenate by the method of Bishayee and
72Liver damage is followed by complex disturbances in 126Balasubramaniyam [24] was estimated as thiobarbituric
73the lipolytic activity of the vascular space which often 127acid reacting substances (TBARS) by the method of
74appeared with hyperlipoproteinemia in patients [16]. 128Ohkawa *et al.* [25]. The reaction mixture contained rat
75Abnormalities with lipid metabolism have been reported 129liver homogenate 0.1ml (25% w/v) in Tris-HCl buffer
76in cholesteosis [17], alcoholism [18], chemical intoxica- 130(20mM, pH 7.0); KCl (30mM); FeSO₄(NH₄)₂SO₄.7H₂O
77tion [19] and hepatitis [20]. The plant is also useful in 131(0.06mM); and various concentrations of PN extract in a
78treating viral and bacterial diseases [21]. 132final volume of 0.5 ml. The reaction mixture was incu-
79 Previously, we reported the antihyperglycemic [22] 133bated at 37°C for 1 h. After the incubation period, 0.4
80activity of MEPN. In the present study, we have tested 134ml was removed and treated with 0.2 ml sodium dode-
81the *in vitro* lipid peroxidation and antimicrobial activity 135cyl sulphate (SDS) (8.1%); 1.5 ml thiobarbituric acid
82(against various Gram positive and Gram negative bac- 136(TBA) (0.8%); and 1.5 ml acetic acid (20%, pH 3.5).
83teria) of the methanol extract of PN. 137The total volume was made up to 4.0 ml with distilled
138water and then kept in a water bath at 95 to 100°C for 1
139h. After cooling, 1.0ml of distilled water and 5.0 ml of
140*n*-butanol and pyridine mixture (15:1 v/v) were added to
141the reaction mixture, shaken vigorously and centrifuged
142at 4000 rpm for 10 min. The butanol-pyridine layer was
143removed and its absorbance at 532 nm was measured to
144quantify TBARS. Inhibition of lipid peroxidation was
145determined by comparing the optical density (OD) of
146treatments with that of the control. Quercetin and L-
147ascorbic acid were used as the standard controls. The %
148inhibition of lipid peroxidation was calculated by using
149the following formula:

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

151 where A_{blank} is the absorbance of the blank reaction
152 and A_{test} is the absorbance in the presence of the sam-
153 ple of the extracts.

154 *Determination of antimicrobial activity*

155 Antimicrobial activity was measured using the stan-
156 dard method of disc diffusion plates on agar [26]. Then
157 0.1 ml of each culture of bacteria was spread on agar
158 plate surfaces. For antibacterial assays, all bacterial
159 strains were grown in Mueller Hinton Broth medium
160 (Merck) for 24 h at 37°C. The concentration of bacterial
161 suspensions was adjusted to 10⁸ colony forming units
162 (10⁸cfu/ml) in Mueller Hinton Agar. Paper discs (6 mm
163 in diameter) were impregnated on the agar to load 10µl
164 of each sample. The impregnated discs were placed on
165 the medium suitably spaced apart and the plates were
166 incubated at 5°C for 1 h to permit good diffusion and
167 then transferred to an incubator at 37°C for 24 h. The
168 results were recorded by measuring the zones of growth
169 inhibition surrounding the disc. Clear inhibition zones
170 around the discs indicate the presence of antimicrobial
171 activity. All data on antimicrobial activity are the aver-
172 age of triplicate analyses. In order to determine the anti-
173 bacterial effect of the MEPN, chloramphenicol
174 (10µg/ml/disc) were used as positive control. Inhibition
175 diameters were measured after incubation for 24 h at
176 37°C.

84 MATERIALS AND METHODS

85 *Chemicals*

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

91 *Extraction procedure*

92 The plant PN was obtained from the tribal area of
93 Karimnagar District, Andhra Pradesh, India. The plant
94 was identified taxonomically by Dr. Alok Bhattacharya
95 of the Botanical Survey of India (BSI), Shibpur, Kol-
96 kata, India. A voucher specimen (No. GPS-2) has been
97 preserved in our laboratory for future purposes. For the
98 extract, the whole plant was dried in shade and pow-
99 dered in a mechanical grinder. The powder of PN was
100 initially defatted with petroleum benzene (60-80°C)
101 followed by 1 liter of methanol by using a Soxhlet ex-
102 tractor for 72 h at a temperature not exceeding the boil-
103 ing point of the solvent [23]. The extract was filtered
104 using Whatman filter paper (No. 1) and then concen-
105 trated in vacuum and dried. The methanol extract was
106 used in the assay of lipid peroxidation inhibitory and
107 antimicrobial activity.

108 *Previously isolated classes of compounds*

109 The phytochemical study revealed that the MEPN
110 contained alkaloids, flavonoids, saponins and cou-
111 marins, polyphenols, tannins, terpenoids, lipids and lig-
112 nans [13].

113 *Microorganisms utilized for antimicrobial activity*

114 Microorganisms (Staphylococcus aureus 8531,
115 Staphylococcus aureus ML 174, Staphylococcus aureus
116 ML 152, Bacillus pumillus 8241, Bacillus cereus, Es-
117 cherichia coli 51, Escherichia coli 54B, Vibrea cholera
118 14035, Vibrea cholera 1353, and Vibrea cholera
119 226101) were obtained from the stock culture of Central
120 Drugs Laboratory, Kolkata; Indian Institute of Chemical

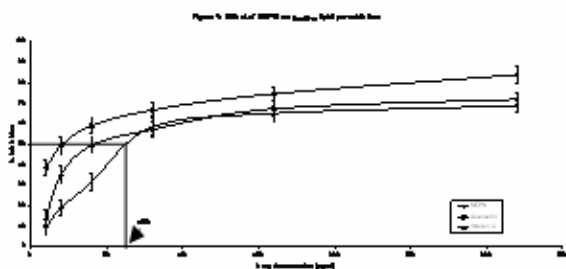


Fig 1. Effect of methanol extract of *Phyllanthus niruri* (MEPN) on lipid peroxidation.

177 Statistical Analysis

178 All treatments were performed in triplicate. Statisti-
179 cal analysis was performed using Graphpad prism, 3.0
180 version (Graphpad Software Inc., San Diego, CA,
181 USA). The statistical significance of a treatment effect
182 was evaluated by student's *t*-test and ANOVA. The val-
183 ues were expressed as mean \pm SD. IC₅₀ values for all the
184 above experiments were determined by linear regres-
185 sion. Probability limit was set at $p < 0.05$.

186 RESULTS

187 Effect of MEPN on lipid peroxidation

188 The effect of MEPN and commercially available an-
189 tioxidants namely L-Ascorbic acid and quercetin on the
190 *in vitro* inhibition of lipid peroxidation is shown in
191 Fig 1. The generation of lipid peroxidase by Fe²⁺-
192 ascorbate in rat liver homogenate appears to be inhibited
193 by MEPN with IC₅₀ value of 62.5 µg/ml. Though, the
194 inhibitory activity was observed, but it was found not so
195 remarkable when compared to L-Ascorbic acid and
196 Quercetin. The percentage inhibition of lipid peroxida-
197 tion of MEPN at 320 µg/ml was found to be 68.88%
198 and for L-ascorbic acid and Quercetin the percentage
199 inhibition was found to be 72.11% and 84.09%, respec-

200 tively.

201 Effect of MEPN on antimicrobial activity

202 The data presented in Table 1 indicate that the
203 methanol extract of *Phyllanthus niruri* (MEPN) inhibit
204 the growth of some of the tested microorganisms (Gram
205 positive and Gram negative) to various degrees. The
206 MEPN at a concentration of 500 µg/ml/disc showed
207 moderate activity and 750 µg/ml/disc exhibited moder-
208 ate to strong antimicrobial activity against all the tested
209 microorganisms. The extract showed strong antibacte-
210 rial activity against *Bacillus pumillus* 8241, *Bacillus*
211 *cereus*, *Escherichia Coli* 54B and *Vibrae Cholera*.
212 However, its activity against *Staphylococcus aureus* ML
213 152 and *Vibrae cholera* 14035 was found to be less. The
214 antimicrobial activity of the extract was compared with
215 the standard Chloramphenicol at a concentration of
216 10 µg/ml/disc.

DISCUSSION

217 PN has many effective traditional uses for a wide va-
218 riety of diseases. Some of the medicinal usages have
219 been proven in experimental models, which suggest that
220 the extracts of the plant possess various pharmacologi-
221 cal actions. Unsaturated lipids in liver tissue are very
222 susceptible to peroxidation when they are exposed to
223 reactive oxygen species (ROS). In the present investiga-
224 tion, we have incubated the liver tissue in presence of a
225 ROS generating system, ascorbate/FeSO₄, and exam-
226 ined the effect on tissue homogenate by measuring the
227 optical density (OD) at 532nm. The results of the inves-
228 tigation reveal that MEPN has no potent lipid peroxida-
229 tion inhibition activity.

230 The antimicrobial activity of the MEPN was studied
231 by the disc diffusion method against various microor-
232 ganisms. Disc diffusion methods are used extensively to
233 investigate the antibacterial activity of natural sub-
234 stances and plant extracts [27]. These assays are based
235 on the use of discs as reservoirs containing solutions of
236 the substances to be examined. In case the activity is
237 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 (10 µg/ml/disc)
		500 µg/ ml/disc	750 µg/ ml/disc	
<i>Staphylococcus aureus</i> 8531	9	6	10	16
<i>Staphylococcus aureus</i> ML 174	6	6	11	19
<i>Staphylococcus aureus</i> ML 152	6	6	7	22
<i>Bacillus pumillus</i> 8241	7	7	23	21
<i>Bacillus cereus</i>	6	10	16	14
<i>Escherichia coli</i> 51	6	7	10	24
<i>Escherichia coli</i> 54B	6	12	15	17
<i>Vibrea cholera</i> 14035	6	7	9	22
<i>Vibrea cholera</i> 1353	6	11	16	11
<i>Vibrea cholera</i> 226101	6	10	14	21

6-9mm: low activity; 10-14mm: moderate activity; \geq 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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CURRENT AUTHOR ADDRESSES

Yerra Rajeshwar, S. R. College of Pharmacy, Ananthasagar, Hasanparthy, Warangal - 506 371 (A.P), India. (Corresponding author)
E-mail: yrajeshwar@yahoo.co.in

Rayees Ahmad, S. R. College of Pharmacy, Ananthasagar, Hasanparthy, Warangal - 506 371 (A.P), India.

A. Shyam Sunder, S. R. College of Pharmacy, Ananthasagar, Hasanparthy, Warangal - 506 371 (A.P), India.

J. Devilal, S. R. College of Pharmacy, Ananthasagar, Hasanparthy, Warangal - 506 371 (A.P), India.

Malaya Gupta, Department of Pharmaceutical Technology, Jadavpur University, Kolkata - 700 032 (W.B), India.

Upal Kanti Mazumder, Department of Pharmaceutical Technology, Jadavpur University, Kolkata - 700 032 (W.B), India.