Assessment of *In vitro* antioxidant, antibacterial and immune activation potentials of aqueous and ethanol extracts of *Phyllanthus niruri*

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Abstract

BACKGROUND: Recently much attention has been paid to biologically active plants because of their low production cost and fewer adverse effects compared with chemical drugs. In the present investigation the bioactivity of *Phyllanthus niruri* ethanol and aqueous extracts was evaluated *in vitro*.

RESULTS: The ethanol extract of *P. niruri* showed a high level of flavonoid content (123.9 ± 0.002 mg g⁻¹), while the aqueous extract showed the highest 2,2-diphenyl-1-picrylhydrazyl (DPPH; IC₅₀ 6.85 ± 1.80 µmol L⁻¹) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; 46.44 ± 0.53 µmol L⁻¹) free radical scavenging activities with high phenol content (376 ± 0.02 mg g⁻¹) and elevated levels of ferric reducing antioxidant power (FRAP; 23 883 ± 0.019 mmol g⁻¹) with excellent antibacterial activity against *Staphylococcus aureus* (20 mm inhibition zone) and *Streptococcus agalactiae* (12 mm inhibition zone), respectively, in addition to the best immune activation potential of human peripheral blood mononuclear cells (450.5%).

CONCLUSIONS: It is clear from our results that both extracts of *P. niruri* has excellent bioactivity roles via elevated levels of antibacterial, antioxidant and percentage of peripheral blood mononuclear cell proliferation, which could lead to the development of medications for clinical use.

Keywords: *Phyllanthus niruri*; antioxidant; PBMC; antibacterial

INTRODUCTION

Herbal-derived medications are an important resource in eliminating serious diseases in developing countries. About 60–85% of the world’s inhabitants depend on traditional medicine for the treatment of common diseases. Many Malaysian plants have been investigated for their beneficial use as antioxidants or source of antioxidants using currently available techniques.¹ The antioxidant capacity and total phenolic content of selected Malaysian underutilized fruits have been evaluated, and methanolic extracts of seven Malaysian medicinal plants have been screened for antioxidant and nitric oxide inhibitory activities by Saha *et al.*² In addition, Habshah *et al.*³ screened the dichloromethane and methanol extracts of 13 Zingiberaceae species from the *Alpinia*, *Costus* and *Zingiber* genera for antimicrobial and antioxidant activities. *Phyllanthus niruri* is a well-known plant in Malaysian traditional medicine locally known as dukung anak and has been reported to exhibit lipid-lowering activity,⁴ anti-diabetic,⁵ antihyperglycemic⁶ and analgesic effects.⁷ Recently it has attracted the attention of researchers because of its hepatoprotective properties and its clinical efficacy in viral hepatitis B.⁸ The present investigation aimed to compare the antioxidant, antibacterial and immune activation properties of ethanol and aqueous extracts of *P. niruri* *in vitro*.

MATERIALS AND METHODS

Plant extraction techniques

*Phyllanthus niruri* whole plant was obtained from Ethno Resources Sdn Bhd, Selangor, Malaysia. For preparation of ethanol extract 100 g fine powder was soaked in 1000 mL of 95% ethanol for 3 days. The mixture was then filtered using Whatman No. 1 filter paper and extracted under compact pressure in a rotating evaporator (Buchi, Switzerland). Conversely, the aqueous extract was prepared by soaking 100 g fine powder in 2000 mL distilled water then shaken for 4 h in a water bath. Subsequently, the filtered mixture was stored in a freezer to make small ice blocks.
and the plant extract was obtained by using a freeze-drying machine (Labconco, Kansas, USA). All extracts were kept at −20 °C until the tests were performed.

**Antioxidant activity**
2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The scavenging activity of stable DPPH free radical was determined according to literature methods with slight modifications. Plant extracts (1 mg mL−1) and the reference standard (ascorbic acid) were prepared as stock solutions, and a series dilution with five varying concentrations were tested. 5 µL of samples/standards were loaded, followed by 195 µL DPPH reagent. The mixtures were then mixed vigorously and incubated in the dark at room temperature for 2 h, and the absorbance was measured spectrophotometrically at 515 nm. The percentage of DPPH free radical scavenging activity was calculated as: DPPH (%) = ([absorbance of blank – absorbance of sample]/absorbance of sample) × 100. The results were expressed as (IC50 value) the concentration of extract that was required to reduce 50% of the hydroxyl radical produced.

**ABTS assay**

The total antioxidant capacity assay was carried out using an improved method described by Pellegrini et al. Briefly, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation is generated by reacting 7 mmol L−1 ABTS and 2.45 mmol L−1 potassium persulfate via incubation at room temperature, in the dark for 12–16 h. The ABTS solution was diluted with methanol to an absorbance of 0.700 ± 0.020 at 734 nm and equilibrated at 30 °C, while plant extracts were diluted with distilled water. To 1 mL of diluted ABTS, 10 µL of each plant extract solution was added and mixed thoroughly. The reactive mixture was allowed to stand at room temperature for 15 min and the absorbance was recorded immediately at 734 nm. The percentage of ABTS free radical scavenging activity was calculated as ABTS (%) = ([absorbance of blank – absorbance of sample]/absorbance of sample) × 100. The results were expressed as (IC50 value) the concentration of extract that was required to reduce 50% of the free radical produced.

**Total phenolic content (TPC) assay**

TPC was determined using Folin–Ciocalteu reagent following the method of Singleton and Rossi using gallic acid as a standard. 10 µL of extract solution (1 mg mL−1) was added in a test tube, followed by 0.5 mL of 1:10 Folin–Ciocalteu reagent. The mixture was incubated at room temperature for 5 min. Then, 0.35 mL of 115 mg mL−1 sodium carbonate (Na2CO3) was added and mixed thoroughly. The mixture was then allowed to stand at room temperature for 2 h. Absorbance readings were taken spectrophotometrically at 765 nm and all determinations were done in triplicate. The total phenolic content was expressed as milligrams of gallic acid equivalent to grams of dried plant material, and butylated hydroxytoluene (BHT) was used as positive control.

**Total flavonoid content (TFC) assay**

TFC of each sample was determined using the aluminium chloride colorimetric method. Briefly, 0.5 mL of extract solutions (1 mg mL−1) was added to a separate test tube and mixed with 1.5 mL of 95% ethanol, 0.1 mL of 1 mol L−1 potassium acetate, 0.1 mL aluminium chloride and 2.8 mL distilled water. The mixtures were then incubated for 30 min at room temperature. The absorbance readings were taken spectrophotometrically at 415 nm. Total flavonoid content was expressed as milligrams of quercetin equivalent to grams of dried plant material.

**Ferric reducing antioxidant power (FRAP) assay**

The ferric reducing activity of the plant extracts was estimated using the method developed by Benzie and Strain. The reaction mixture contained 300 mmol L−1 acetate buffer, 10 mmol L−1 2,4,6-tripryidyl-s-triazine (TPTZ) in 40 mmol L−1 of HCl and 20 mmol L−1 of FeCl3.6H2O. The working FRAP reagent was prepared freshly by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution and 2.5 mL FeCl3.6H2O. The freshly prepared mixture was incubated at 37 °C in a water bath for 5 min and then a blank reading was taken spectrophotometrically at 593 nm. After that, 30 µL extract or standard and 90 µL distilled water were added to 900 µL of the working FRAP reagent. Absorbance was measured at 0 min immediately upon addition of the working FRAP reagent after vortexing with a Genie-2 vortex (Scientific Industries Inc., Bohemia, NY, USA). Thereafter, an absorbance reading was taken after 4 min. All results were expressed as millimoles of ferric reducing activity of the extract per gram of dried weight based on three experiments and BHT was used as the positive control.

**Antibacterial activity**

The strains of bacteria and their reference antibiotics used in this study were Escherichia coli ATCC 25922 (gentamicin 30 µg), Klebsiella pneumoniae ATCC 1937000 (gentamicin 30 µg), Staphylococcus aureus ATCC 25293 (vancomycin 5 µg) and Streptococcus agalactiae (laboratory isolates obtained from the Molecular Bacteriology Laboratory, Molecular Medicine Department, Faculty of Medicine, University of Malaya).

The Kirby–Bauer method was used, in which sterile 6.0 mm diameter blank disks were used to saturate 50 µL (100 mg mL−1) of the plant extracts (5 mg per disk). The extracts, sterile dimethyl sulfoxide (DMSO), sterile distilled-water impregnated disks and the commercially available antibiotic disks were placed on Muller–Hinton agar and incubated at 37 °C for 18–20 h. Antibacterial activity was then indicated by clear zones of inhibition (millimeters). Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) methods were relevant to the extracts that verified their effectiveness to bacteria by the disk diffusion method (inhibition zone ≥8 mm). In brief, each plant extract was subjected to a serial dilution by using sterile Muller–Hinton broth. 10 µL of bacteria (in log phase) was inoculated to each dilution separately, then incubated at 37 °C for 18–20 h. The higher dilution of the plant’s extracts that showed an absence of turbidity of bacteria is evidenced as the MIC of the extract. The MBC was identified as the lowest concentration of the plant needed to kill the microorganisms. This was established by culturing the sample dilution on fresh Muller–Hinton agar medium and incubating further at 37 °C for 18–20 h.

**Immune activation potential**

For isolating human peripheral blood mononuclear cells (PBMC) 10 mL of whole blood was drawn from a healthy donor and diluted with the same volume of Histopaque. The mixed solution was centrifuged under at (relative centrifugal force (RCF) = 157 × g) for 30 min using a Kubota 2010 centrifuge (Kubota, Tokyo, Japan). The mononuclear layer was carefully transferred out and washed, then pelleted down with 30 mL phosphate-buffered saline (PBS) and centrifuged at RCF = 157 × g for 10 min three times and resuspended with RPMI media supplemented with 2 mM Glutamine and NaHCO3 (Sigma-Aldrich, Gillingham, UK) with addition of 100 g dL−1 fetal bovine serum (FBS). Counting of cells was done using a Neubaur hemocytometer (Weber, Teddington, UK).
UK) to ascertain the PBMC cell number with the same volume of trypan blue. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Merck, Darmstadt, Germany) assay was used to study the effect of the extract on cell viability. Briefly, 100 µL of cells suspended in RPMI media (100 g dl⁻¹ FBS) was added to the 96-well plate and incubated in an IR jacketed incubator (NuAire Laboratory Equipment Supply, Plymouth, MN, USA) for 24 h at 37 °C. Then, 10 µL of the extracts was added and further incubated for 24 h. After the corresponding period 10 µL MTT reagent (5 mg mL⁻¹ PBS) was added to each well and further incubated for 4 h. 100 µL DMSO was added and shaken for 20 min to solubilize and extract the formazan crystals. Finally, the plate was read at 595 nm using a PowerWave X 340 ELISA (enzyme-linked immunosorbent assay) plate reader (BioTek Instruments, Winooski, VT, USA). All extract samples and controls were tested in triplicate in three independent experiments. The percentage cell viability was calculated using the following formula:

\[
\% \text{ cell viability} = \left( \frac{\text{absorbance of extract sample} - \text{absorbance of control}}{\text{absorbance of control}} \right) \times 100
\]

**Statistical analysis**

Data were articulated as mean ± standard deviation and statistically analyzed using PASW Statistics version 18.0 software (SPSS Inc, Chicago, USA).

**RESULTS**

**Antioxidant activity**

The aqueous extract of *P. niruri* showed the highest DPPH and ABTS free radical scavenging activities, with high phenol contents and elevated levels of FRAP, while the ethanol extract showed higher total flavonoid content (Table 1). IC₅₀ values of DPPH and ABTS were 6.85 ± 1.80 and 11.07 ± 1.37 µmol L⁻¹, and 46.44 ± 0.53 and 53.34 ± 1.97 µmol L⁻¹, for aqueous and ethanol extracts, respectively. Pearson’s correlation coefficient results for both plants showed that the highest positive correlation was between ABTS and DPPH (r = 0.921), followed by TPC and FRAP (r = 0.826). Generally, the exhibited antioxidant activity was comparable to the references (BHT and vitamin C).

**Antibacterial activity**

Results found in our study revealed that by the disk diffusion method the aqueous extracts of *Phyllanthus niruri* showed significant activity against Gram-positive bacteria only: *Staphylococcus aureus* (20 mm inhibition zone) and *Streptococcus agalactiae* (12 mm inhibition zone), respectively (Table 2). No activities were observed toward Gram-negative bacteria: *Escherichia coli* and *Klebsiella pneumoniae*. The MIC and MBC results showed that *Staphylococcus aureus* and *Streptococcus agalactiae* had MIC values (0.5 mg mL⁻¹ and 1 mg mL⁻¹), respectively. The MBC values were next to MIC values in all cases. The lower MIC and MBC values for the bacteria are indications of the efficacy of the plant extract.

**DISCUSSION**

Reactive oxygen species (ROS) may adversely affect immune functions and they are responsible for damage to cellular biomolecules such as proteins, enzymes, nucleic acids, lipids and carbohydrates. Antioxidants interrupt the production of ROS and also play a key role in inactivating them. Although all human cells protect themselves against oxidative damage by certain antioxidant mechanisms, these sometimes are not sufficient to prevent the ROS damage totally. Different kinds of plant materials have already been reported as natural antioxidants. In this experiment, there was a positive correlation between the two total antioxidant assays, in which the aqueous extracts of *P. niruri* showed higher DPPH and ABTS free radical scavenging activity and also had stronger ferric ion reducing activity than the ethanolic extract; this result may be explained by the fact the plants contained more water-soluble substances. These findings agrees with Delgado-Andrade *et al.*, who assessed the antioxidant activity of melanoidins from coffee brews by different antioxidant methods, and they concluded that the highest antioxidant activity was found in the aqueous media.

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### Table 1. Free radical scavenging activities and phytochemical screening of *P. niruri*  

<table>
<thead>
<tr>
<th>Vitamin C</th>
<th>BHT</th>
<th>PN/A</th>
<th>PN/E</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH (IC₅₀)</td>
<td>3.346 ± 1.20</td>
<td>–</td>
<td>6.85 ± 1.80</td>
</tr>
<tr>
<td>ABTS (IC₅₀)</td>
<td>21.368 ± 0.12</td>
<td>–</td>
<td>46.44 ± 0.53</td>
</tr>
<tr>
<td>TPC (mg GA eq. g⁻¹)</td>
<td>–</td>
<td>477.33 ± 0.01</td>
<td>376 ± 0.02</td>
</tr>
<tr>
<td>TFC (mg Q eq. g⁻¹)</td>
<td>–</td>
<td>–</td>
<td>34.6 ± 0.001</td>
</tr>
<tr>
<td>FRAP (mmol g⁻¹)</td>
<td>–</td>
<td>57 300 ± 0.01</td>
<td>23 883 ± 0.019</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SEM for triplicates. BHT, butylated hydroxytoluene; PN/A, *P. niruri* aqueous extract; PN/E, *P. niruri* ethanol extract.

### Table 2. Disk diffusion method results

<table>
<thead>
<tr>
<th>E. coli</th>
<th>K. pneumoniae</th>
<th>S. agalactiae</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTRL</td>
<td>27 ± 1.4</td>
<td>31 ± 0.6</td>
<td>16 ± 0.6</td>
</tr>
<tr>
<td>PN/A</td>
<td>N</td>
<td>N</td>
<td>12 ± 0.6</td>
</tr>
<tr>
<td>PN/E</td>
<td>N</td>
<td>N</td>
<td>6 ± 0.3</td>
</tr>
</tbody>
</table>

Values are represented as mean inhibition zone (mm) ± SEM of triplicates; CTRL, antibiotic positive control; N, no inhibition zone; PN/A, *P. niruri* aqueous extract; PN/E, *P. niruri* ethanol extract.
It is obvious from Table 1 that the ethanolic extracts contain more flavonoids than the aqueous extracts; these results agree with investigations of Wang et al.,17 in that various extracts followed the trend of ethanolic extract → aqueous extract → acetone extract in the flavonoid contents. Also, the present findings seem to be consistent with those of Thaipong et al.18 Wong et al.19 that the FRAP technique showed high correlation with total phenolic contents.

The high antibacterial activity of aqueous extract of P. niruri suggests that the activity may be due to the high level of phenolic compounds. These results link with a prior study by Yin et al.,20 who concluded that the phenolic compounds isolated from Psoralea corylifolia exhibited in vitro antibacterial properties. Similarly, Chitravadivu et al.21 reported the antibacterial activity of the root and the leaf extracts of P. niruri against four species of bacteria. On the other hand, results of human PBMC proliferation demonstrate that both extracts induced the proliferation of white blood cells after 24 h incubation, which suggests that the extracts are not toxic to normal immune cells and therefore exhibit a potential to modulate the cellular immune system. The above-mentioned bioactivity of P. niruri may be due to the isolated phytochemicals, which had a wide range of pharmacological properties such as alkaloids, flavonoids, terpenoids, polyphenols, lignans, tannins, saponins and coumarins22 that have been identified to have different therapeutic properties.23,24

CONCLUSION

In our findings the elevated values of antioxidant, antibacterial and immune activation potentials revealed the strong medicinal bioactivities of P. niruri extracts, which could lead to the development of medications for clinical usage.

ACKNOWLEDGEMENTS

Support for this work was provided by the Molecular Medicine Department, Faculty of Medicine, University of Malaya (grant number PS 200/2010A).

REFERENCES


Figure 1. immune activation potential on human PBMC. CTRL, distilled water; PN/A, P. niruri aqueous extract; PN/E, P. niruri ethanol extract.