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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; IC_{50} 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.
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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, Habsah *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] antidiabetic,[5] antihyperglycemic[6] 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

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and the plant extract was obtained by using a freeze-drying machine (Labconco, Kansas, USA). All extracts were kept at $-20\,^{\circ}$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[9,10] 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 (IC$_{50}$ 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*.[11] 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 (IC$_{50}$ 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[12] 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 (Na_2CO_3) 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 miligrams 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.[13,14] Briefly, 0.5 mL of extract solutions (1 mg mL^{-1}) was added to a separate test tube and mixed with1.5 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.[15] The reaction mixture contained 300 mmol L^{-1} acetate buffer, 10 mmol L^{-1} 2,4,6-tripyridyl-*s*-triazine (TPTZ) in 40 mmol L^{-1} of HCl and 20 mmol L^{-1} of $FeCl_3.6H_2O$. The working FRAP reagent was prepared freshly by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution and 2.5 mL FeCl3.6H_2O. 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 25923 (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 $\geq$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) = $157 \times g$) 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 = $157 \times g$ for 10 min three times and resuspended with RPMI media supplemented with 2 mM Glutamine and $NaHCO_3$ (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,

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

	Vitamin C	BHT	PN/A	PN/E	Standard curve equation
DPPH (IC_{50})	3.346 ± 1.20	–	6.85 ± 1.80	11.07 ± 1.37	$y = 2.4335x - 1.319$ ($R^2 = 0.997$)
ABTS (IC_{50})	21.368 ± 0.12	–	46.44 ± 0.53	53.34 ± 1.97	$y = 27.448x - 0.8469$ ($R^2 = 0.999$)
TPC (mg GA eq. g^{-1})	–	477.33 ± 0.01	376 ± 0.02	270 ± 0.003	$y = 0.0013x - 0.0032$ ($R^2 = 0.987$)
TFC (mg Q eq. g^{-1})	–	–	34.6 ± 0.001	123.9 ± 0.002	$y = 0.004x + 0.0085$ ($R^2 = 0.992$)
FRAP (mmol g^{-1})	–	57 300 ± 0.01	23 883 ± 0.019	7755 ± 0.015	$y = 0.0007x - 0.0038$ ($R^2 = 0.983$)

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

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:

$$\% \text{ cell viability} = (\text{absorbance of extract sample} - \text{absorbance of control}/\text{absorbance of control}) \times 100$$

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). IC_{50} 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 of (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.

Table 2. Disk diffusion method results

	E. coli	*K. pneumoniae*	*S. agalactiae*	*S. aureus*
CTRL	27 ± 1.4	31 ± 0.6	16 ± 0.6	12 ± 0.3
PN/A	N	N	**12 ± 0.6**	**20 ± 1.2**
PN/E	N	N	6 ± 0.3	6 ± 0.2

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.

Immune activation potential

As mentioned, the ethanol and aqueous extracts of the studied plant were tested for their immune activation potential on human peripheral blood mononuclear cells (PBMC) by the MTT assay. Results shown Fig. 1 show that cell proliferation was observed for the tested plant. The *P. niruri* aqueous extract was the most statistically significant immune activator on PBMC (450.5%), while its ethanol extract was less active among the studied extracts (272.3%) but it still twice the value of the control.

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.*,[16] 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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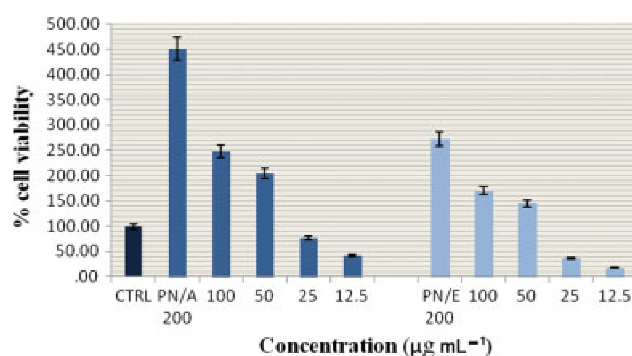

Figure 1. immune activation potential on human PBMC. CTRL, distilled water; PN/A, *P. niruri* aqueous extract; PN/E, *P. niruri* ethanol extract.

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 coumarins[22] 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.

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REFERENCES

1 Ikram E, Eng K, Jalil A, Ismail A, Idris S, Azlan A, *et al*, Antioxidant capacity and total phenolic content of Malaysian underutilized fruits. *J Food Compos Anal* **22**:388–393 (2009).
2 Saha K, Lajis N, Israf D, Hamzah A, Khozirah S, Khamis S, *et al*, Evaluation of antioxidant and nitric oxide inhibitory activities of selected Malaysian medicinal plants. *J Ethnopharmacol* **92**:263–267 (2004).
3 Habsah M, Amran M, Mackeen M, Lajis N, Kikuzaki H, Nakatani N, *et al*, Screening of Zingiberaceae extracts for antimicrobial and antioxidant activities. *J Ethnopharmacol* **72**:403–410 (2000).
4 Khanna A, Rizvi F and Chander R, Lipid lowering activity of *Phyllanthus niruri* in hyperlipemic rats. *J Ethnopharmacol* **82**:19–22 (2002).
5 Bavarva J and Narasimhacharya A, Comparative antidiabetic, hypolipidemic, and antioxidant properties of *Phyllanthus niruri* in normal and diabetic rats. *Pharm Biol* **45**:569–574 (2007).
6 Mazunder U, Gupta M and Rajeshwar Y, Antihyperglycemic effect and antioxidant potential of *Phyllanthus niruri* (Euphorbiaceae) in streptozotocin induced diabetic rats. *Eur Bull Drug Res* **13**:15–23 (2005).
7 Santos A, Analgesic effects of callus culture extracts from selected species of *Phyllanthus* in mice. *J Pharm Pharmacol* **46**:755–759 (1994).
8 Harish R and Shivanandappa T, Antioxidant activity and hepatoprotective potential of *Phyllanthus niruri*. *Food Chem* **95**:180–185 (2006).
9 Lim Y and Murtijaya J, Antioxidant properties of *Phyllanthus amarus* extracts as affected by different drying methods. *LWT – Food Sci Technol* **40**:1664–1669 (2007).
10 Dastmalchi K, Damien Dorman H, Kosar M and Hiltunen R, Chemical composition and *in vitro* antioxidant evaluation of a water-soluble Moldavian balm (*Dracocephalum moldavica* L.) extract. *LWT – Food Sci Technol* **40**:239–248 (2007).
11 Re R, Pellegrini N, Proteggente A, Pannala A, Yang M and Rice-Evans C, Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic Biol Med* **26**:1231–1237 (1999).
12 Singleton V and Rossi J Jr, Colorimetry of total phenolics with phosphomolybdic–phosphotungstic acid reagents. *Am J Enol Vitic* **16**:144–158 (1965).
13 Chang C, Yang M, Wen H and Chern J, Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J Food Drug Anal* **10**:178–182 (2002).
14 Harborne J and Williams C, Advances in flavonoid research since 1992. *Phytochemistry* **55**:481–504 (2000).
15 Benzie I and Strain J, The ferric reducing ability of plasma (FRAP) as a measure of 'antioxidant power': the FRAP assay. *Anal Biochem* **239**:70–76 (1996).
16 Delgado-Andrade C, Rufian-Henares J and Morales F, Assessing the antioxidant activity of melanoidins from coffee brews by different antioxidant methods. *J Agric Food Chem* **53**:7832–7836 (2005).
17 Wang K, Pan Y, Wang H, Zhang Y, Lei Q, Zhu Z, *et al*, Antioxidant activities of *Liquidambar formosana* Hance leaf extracts. *Med Chem Res* **19**:166–176.
18 Thaipong K, Boonprakob U, Crosby K, Cisneros-Zevallos L and Hawkins Byrne D, Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *J Food Compos Anal* **19**:669–675 (2006).
19 Wong S, Leong L and Koh JW, Antioxidant activities of aqueous extracts of selected plants. *Food Chem* **99**:775–783 (2006).
20 Yin S, Fan CQ, Wang Y, Dong L and Yue JM, Antibacterial prenylflavone derivatives from *Psoralea corylifolia*, and their structure–activity relationship study. *Bioorg Med Chem* **12**:4387–4392 (2004).
21 Chitravadivu C, Manian S and Kalaichelvi K, Antimicrobial studies on selected medicinal plants, Erode Region, Tamilnadu, India. *Middle-East J Sci Res* **4**:147–152 (2009).
22 Bagalkotkar G, Sagineedu S, Saad M and Stanslas J, Phytochemicals from *Phyllanthus niruri* Linn. and their pharmacological properties: a review. *J Pharm Pharmacol* **58**:1559–1570 (2006).
23 Lee CY, Peng WH, Cheng HY, Chen FN, Lai MT and Chiu TH, Hepatoprotective effect of *Phyllanthus* in Taiwan on acute liver damage induced by carbon tetrachloride. *Am J Chin Med* **34**:471–482 (2006).
24 Mellinger CG, Carbonero ER, Noleto GR, Cipriani TR, Oliveira MBM, Gorin PAJ, *et al*, Chemical and biological properties of an arabinogalactan from *Phyllanthus niruri*. *J Nat Prod* **68**:1479–1483 (2005).

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