

# Antioxidant Activity, Total Phenolic Content and Total Flavonoid Content of Water and Methanol Extracts of *Phyllanthus* species from Malaysia

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## ABSTRACT

**Aims:** The effects of 2 types of solvents, water and methanol were investigated to determine the presence of antioxidant activity, total phenolic content (TPC) and total flavonoid content (TFC) from three *Phyllanthus* species namely, *Phyllanthus urinaria*, *Phyllanthus niruri* and *Phyllanthus debilis*. **Material and Methods:** The antioxidant activities were measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays. The chemical contents of the *Phyllanthus* sp. were presented as total phenolic content (TPC) and total flavonoid content (TFC). **Statistical analysis:** All statistical analysis was conducted using SPSS for Windows, Version 22. All data were presented as mean  $\pm$  standard deviation. **Results:** Our result showed that *P. urinaria* showed higher TPC, followed by *P. debilis* and *P. niruri* for both methanol and water extracts. Similarly, *P. urinaria* showed higher TFC than *P. debilis* and *P. niruri*. The antioxidant activity by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay showed EC<sub>50</sub> of samples ranged from 15.8 to 29.3  $\mu$ g/mL for methanol extract and 33.5 to 73.0  $\mu$ g/mL for water extract. The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay showed EC<sub>50</sub> ranged from 11.2 to 26.0  $\mu$ g/mL for methanol extract and 13.5 to 37.4  $\mu$ g/mL for water extract. **Conclusion:** Methanol extract showed higher TPC, TFC value and lower EC<sub>50</sub> values for antioxidant activities when compared to water extract. In both methanol and water extracts, *P. urinaria* had higher TPC and TFC value and lower EC<sub>50</sub> for both DPPH and ABTS assay followed by *P. debilis* and *P. niruri*.

**Key words:** *Phyllanthus*, Phenolic, Flavonoid, DPPH, ABTS.

**Key Messages:** This paper compared water and methanol extracts between *Phyllanthus* species in which there is lack of publication available on the effect of different solvents (water and methanol) on the physicochemical profiles (antioxidant, total phenolic and total flavonoid contents) between the species. Our study adds further data information of *Phyllanthus debilis*, in which this species was not well-researched. We found out that *Phyllanthus urinaria*, either water or methanol extract, gave better physicochemical profiles compared with *P. debilis* and *P. niruri*. Methanolic extract of *P. urinaria* showed better physicochemical profiles compared with water extract and its biological activity should be further studied.

## INTRODUCTION

Pokok 'dukung anak' is the local name in Malaysia for a few species of herbaceous *Phyllanthus* plants.<sup>1</sup> They are known as 'dukung anak', which means 'to carry a child', referring to the fruits at the bottom of each leaf petiole.<sup>2</sup> It originated from India, but can be found in the tropical region worldwide.<sup>3</sup> Recently, the species have become of research interest due to their use in traditional medicine and were shown to have strong medicinal properties.<sup>4</sup>

*Phyllanthus* species such as *Phyllanthus acidus*, *Phyllanthus debilis*, *Phyllanthus pulcher*, *Phyllanthus reticulatus*, *Phyllanthus urinaria*, *Phyllanthus niruri* and *Phyllanthus myrtifolius* are traditionally used for treatment of several diseases in many tropical countries. Whole

plant or parts of it were used for the treatment of kidney stones, intestinal infections, diabetes, hepatitis B and neonatal jaundice.<sup>5,6</sup> There are 3 common *Phyllanthus* species that were traditionally used for treatment in Malaysia. The species are *Phyllanthus niruri*, *Phyllanthus debilis* and *Phyllanthus urinaria*. *P. niruri* is the most common *Phyllanthus* species used as medicine in most of tropical countries.<sup>7</sup> It has diverse medicinal qualities where it has been used for treatment ranging from kidney problems, diarrhoea, fever, diabetes, colic, anti-lipid and analgesic.<sup>8,9</sup> The analysis of its extracts showed many bioactive compounds, which has diverse pharmacological and therapeutic potentials such as lignans,

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phyllanthin, hypophyllanthin, tannins, triterpenes, phenyl propanoids, ricinolic acid, niruriside and phyltetralin.<sup>5,10-14</sup> Bioactive compounds such as lignans and terpenes were shown to have an excellent hepatoprotective effects, anti-cancer and anti-microbial properties.<sup>11</sup> Other active compounds, niruriside has antiviral activity that can inhibit the replication of HIV virus.<sup>15</sup>

*P. urinaria* has similar uses as *P. niruri* in traditional practises. It is commonly used for treatment of fever, improving eyesight, urinary problems, and liver diseases and to detoxify poison from the body.<sup>9,8</sup> In China, *P. urinaria* was mainly used for treatment of cancer.<sup>16,17</sup> Some compounds such as 7'-hydroxy-3',4',5,9,9'-pentamethoxy-3,4-methylene dioxy lignan isolated from *P. urinaria* extracts was shown to exhibit anticancer activity.<sup>18</sup> Other bioactive compounds found in the *P. urinaria* extract such as coumarin, ellagitannin and sterol can act as antioxidant, antiviral and anti-inflammatory.<sup>19</sup>

*P. debilis*, which is a less known *Phyllanthus* species, is usually used as substitute to other popular *Phyllanthus* species such as *P. amarus*.<sup>8</sup> *P. debilis* is commonly used for treatment such as jaundice, diarrhea, ulcers, sores, ringworms and scabies.<sup>20</sup> Although known as a substitute for *P. amarus*, *P. debilis* has been found to have a better hepatoprotective activity than *P. amarus*.<sup>21</sup> It is also showing a strong anti-inflammatory<sup>22</sup> and antihyperglycemic properties.<sup>20</sup> Bioactive compound found in *P. debilis* such as debelactone acts as antihepatotoxic whereas phytoesterols such as  $\beta$ -sitosterol act as analgesic and anti-inflammatory.<sup>19</sup>

This study was aimed to compare physicochemical profiles (TPC, TFC and antioxidant activity) of methanol and water extracts in 3 common Malaysian *Phyllanthus* species.

## MATERIALS AND METHODS

### Herbal specimen

Three *Phyllanthus* species were collected from local collection at Tasek Gelugor, Penang, Malaysia. These species were identified by a botanist, Dr. Rahmad Zakaria from the School of Biological Sciences, Universiti Sains Malaysia. The voucher specimens (*Phyllanthus debilis*: 11623, *Phyllanthus niruri*: 11624 and *Phyllanthus urinaria*: 11625) were deposited at the USM herbarium.

### Sample extraction

#### Water/methanol extraction

The sample extraction method was done according to Huang et al., (2008)<sup>23</sup> with slight modification. Briefly, the sample was dried in the oven of 50°C for 3 days. Dried samples were ground and prepared in powder form. Five gram of sample was extracted with 100 mL of deionized water or methanol (Fisher Chemical) in ultrasonic bath (Power Sonic 405) for 20 min and then filtered. The process was repeated twice with the remaining residual extract. The extracts were dried using freeze dryer for water extract and rotary evaporator (Buchi Rotary Evaporator RII) for methanol extract. Dried extracts were stored at -20°C prior to use.

### Total phenolic content

Total phenolic concentration in the extracts was determined based on the method of Sahu and Saxena, (2013).<sup>24</sup> with slight modification. Briefly, 2 mg/mL of sample was diluted in methanol or ultrapure water. Fifty microliters of Folin-Ciocalteu's reagent (50% v/v) (Merck) was then added to 10  $\mu$ L of sample solution and incubated for 5 min incubation at room temperature. Forty microliters of 20% (w/v) sodium carbonate (Bio-basic) was added to the mixture which was then incubated for 30 min. The absorbance was read at 765 nm using microplate reader (FLUOstar omega). The total phenolic was expressed in gallic acid equivalent (GAE) per g dry weight (DW) of extract (mg GAE/g DW).

The standard curve of gallic acid was constructed using seven serial concentrations (1.563 to 50  $\mu$ g/mL). All samples were assays in triplicate. Water and methanol were used as negative control.

### Total flavonoid content

Total flavonoid content (TFC) of *Phyllanthus* samples was determined based on the method of Sahu and Saxena, (2013),<sup>24</sup> with some modifications. Briefly, sample solution was prepared using 2 mg/ml of extract, diluted using methanol or ultrapure water. Hundred  $\mu$ L of sample solution (2 mg/mL) was then mixed with 100  $\mu$ L of 2% aluminium chloride ( $AlCl_3$ ) (Merck) in 96 microwell plate. A flavonoid-aluminium complex was formed after 10 min of incubation time at 25°C. The formation of the complex was measured at 415 nm using microplate reader (FLUOstar omega). Total flavonoid content was measured as quercetin equivalent (QE) per g dry weight (DW) of extract (mg QE/g DW). The standard curve of quercetin was constructed using seven serial concentrations (1.563 to 50  $\mu$ g/mL). All assays were carried out in triplicate. Water and methanol were used as blank and negative control.

### Antioxidant activity: DPPH free radical scavenging assay

The antioxidant activity was measured based on the free radical scavenging activity of the *Phyllanthus* extracts against the stable 2, 2-diphenyl-1-picrylhydrazil (DPPH) free radical.<sup>25</sup> A total 250  $\mu$ L of a reaction mixture was prepared in 96 microwell plates. The reaction mixture consisted of 50  $\mu$ L of extracts and 200  $\mu$ L of 0.2 mM DPPH (Sigma-Aldrich). The solution was incubated at room temperature for 30 min before measuring the absorbance value at 517 nm using microplate reader (FLUOstar omega). Different concentrations of the extracts were measured and  $EC_{50}$  concentration (concentration required to inhibit 50% of DPPH radicals) was determined. All assays were carried out in triplicate. Water and methanol were used as negative controls. Obtained absorbance value was converted into the percentage of radical scavenging activity using the following equation:

$$\text{Radical scavenging activity(\%)} = 100 - \frac{AS}{AC} \times 100$$

Where AS refers to: absorbance of the sample;

AC: absorbance of the negative control (methanol/ water).

### Antioxidant activity: ABTS scavenging activity

Antioxidant activity was also measured using ABTS 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (Roche Diagnostic). The ABTS radical cation  $ABTS^+$  solution was prepared through the reaction of 7 mM ABTS with 2.45 mM potassium persulphate (Sigma-Aldrich). The mixture was incubated at room temperature for 16 hours in the dark. The  $ABTS^+$  solution was then diluted with 99% ethanol (R&M Chemical) to obtain an absorbance of  $0.7 \pm 0.02$  at 734nm. A total of 200 $\mu$ L of reaction mixture was prepared in 96 well plates.

The reaction mixture consists of 10 $\mu$ L of extracts and 190 $\mu$ L of diluted  $ABTS^+$ . The mixture was incubated for 6 min at the room temperature before measuring the absorbance value at 734 nm using microplate reader (Powerwave XS bio-tek). Different concentrations of the extracts were measured and  $EC_{50}$  concentration (concentration required to inhibit 50% of ABTS) was determined. All assays were carried out in triplicate. Water and methanol were used as negative controls.

Obtained absorbance value was converted into the percentage of radical scavenging activity using the following equation:

$$\text{Radical scavenging activity(\%)} = 100 - \frac{AS}{AC} \times 100$$

Where AS: absorbance of the sample;

AC: absorbance of the negative control (methanol/ water).

### Statistical analysis

All statistical analysis was conducted using SPSS for Windows, Version 22.

All data were presented as mean  $\pm$  standard deviation.

## RESULTS AND DISCUSSION

The total phenolic content of the whole plant of *P. niruri*, *P. urinaria* and *P. debilis* was presented in Table 1. For methanol extract, *P. urinaria* had a higher total phenolic content (308.71  $\pm$  0.04 mg GAE/g DW) followed by *P. debilis* (197.09  $\pm$  0.03 mg GAE/g DW) and *P. niruri* (159.13  $\pm$  0.02 mg GAE/g DW). Similarly, for water extract, *P. urinaria* showed the highest phenolic content (219.83  $\pm$  0.01 mg GAE/g DW), followed by *P. debilis* (172.09  $\pm$  0.01 mg GAE/g DW) and *P. niruri* (107.09  $\pm$  0.01 mg GAE/g DW). The plant phenolic compounds constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators. This is due to their redox properties, which play an important role in adsorbing and neutralising free radicals, quenching singlet or triplet oxygen and decomposing peroxides.<sup>26</sup> Our results showed that the methanol extract of *P. urinaria* exhibited the highest TPC, followed by *P. debilis* and *P. niruri*. Similar observation was previously reported<sup>27</sup> which found that *P. urinaria* had higher TPC content compared to *P. debilis* and *P. amarus*. However, TPC for methanol extract of *P. urinaria* in our study was higher than TPC content in methanol extract of *P. urinaria* found in Eldeen *et al.* (2011)<sup>27</sup> study (308.0  $\pm$  0.01 mg GAE/g DW vs 205.0  $\pm$  21.3 mg GAE/g DW). The water extract of *P. urinaria* showed lower TPC content when being compared to methanol extract. When being compared with the study by Cheah and Radu, (2011),<sup>28</sup> the water extracts of *P. urinaria* and *P. niruri* from our samples were showing higher TPC values (219.83  $\pm$  0.01 mg GAE/g DW vs 41.19  $\pm$  0.689 mg GAE/g DW for *P. urinaria* and 107.09  $\pm$  0.01 mg GAE/g DW vs 55.38  $\pm$  0.496 mg GAE/g DW for *P. niruri*). The higher TPC value found in our samples may be due to different drying process in which our samples were dried at 50°C whereas in Eldeen *et al.*, (2011),<sup>27</sup> their samples were dried in an oven at 60°C. This may suggest that the drying temperature of more than 50°C may not be suitable for extracting the phenolic compounds. Exposing the samples to direct sunlight or relevant high temperature may cause some phenolic compounds to degrade rapidly.<sup>29</sup>

**Table 1: Total phenolic content of three *Phyllanthus* species in mg GAE/g DW.**

Species	Methanol extract	Water extract
<i>P. niruri</i>	159.13 $\pm$ 0.02	107.09 $\pm$ 0.01
<i>P. urinaria</i>	308.71 $\pm$ 0.04	219.83 $\pm$ 0.01
<i>P. debilis</i>	197.09 $\pm$ 0.03	172.09 $\pm$ 0.01

Values are mean  $\pm$  standard deviation (n=3)

**Table 2: Total flavonoid content of three *Phyllanthus* species in mg QE/g DW.**

Species	Methanol extract	Water extract
<i>P. niruri</i>	22.08 $\pm$ 0.04	7.07 $\pm$ 0.01
<i>P. urinaria</i>	35.86 $\pm$ 0.04	17.27 $\pm$ 0.01
<i>P. debilis</i>	25.64 $\pm$ 0.16	8.19 $\pm$ 0.01

Values are mean  $\pm$  standard deviation (n=3)

The total flavonoid content of three *Phyllanthus* species is presented in Table 2. For methanol extract, *P. urinaria* had a higher total flavonoid content (35.86  $\pm$  0.04 mg QE/g DW) followed by *P. debilis* (25.64  $\pm$  0.16 mg QE/g DW) and *P. niruri* (22.08  $\pm$  0.04 mg QE/g DW). For water extracts, *P. urinaria* shows the highest total flavonoid content (17.27  $\pm$  0.01 mg QE/g DW), followed by *P. debilis* (8.19  $\pm$  0.01 mg QE/g DW) and *P. niruri* (7.07  $\pm$  0.01 mg QE/g DW).

Flavonoids are commonly found in natural products and one of the most important natural phenolics.<sup>7</sup> For methanol and water extracts, *P. urinaria* exhibited the highest TFC compared to *P. debilis* and *P. niruri*. Methanol extracts showed higher TFC value when being compared to water extract. Our result was in agreement with Kumaran and Karunakaran (2007),<sup>7</sup> which showed that *P. urinaria* exhibited higher TFC value compared with other *Phyllanthus* species such as *P. debilis* and *P. amarus*.

### Antioxidant assay

Two assays which were 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) scavenging activity were used to evaluate antioxidant activity. Table 3 presents the EC<sub>50</sub> values (concentration required to inhibit 50% of DPPH radicals) of three *Phyllanthus* species. Lower EC<sub>50</sub> value indicates higher radical scavenging activity and higher antioxidant activity. The highest radical scavenging activity was shown by methanol extract of *P. urinaria* with EC<sub>50</sub> of 15.8  $\pm$  0.01  $\mu$ g/mL. The radical scavenging activity of the methanol extracts decreased in the following order; *P. urinaria* (15.8  $\pm$  0.01  $\mu$ g/mL) > *P. debilis* (26.3  $\pm$  0.01  $\mu$ g/mL) > *P. niruri* (29.3  $\pm$  0.01  $\mu$ g/mL). For water extract, *P. urinaria* (33.5  $\pm$  0.04  $\mu$ g/mL) > *P. debilis* (45.0  $\pm$  0.02  $\mu$ g/mL) > *P. niruri* (73.0  $\pm$  0.03  $\mu$ g/mL).

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule.<sup>30</sup> The reduction capability of DPPH radical is determined by the decrease in absorbance at 517 nm induced by antioxidants. In general, methanol extracts of all *Phyllanthus* sp. showed lower EC<sub>50</sub> values when compared with water extracts. In both methanol and water extracts, *P. urinaria* showed the highest DPPH scavenging activity, followed by *P. debilis* and *P. niruri*. A study<sup>27</sup> also reported a similar finding where the DPPH inhibition activity of methanol extract of *P. urinaria* was found to be higher than other *Phyllanthus* spp. such as *P. debilis*.

Table 4 presents the EC<sub>50</sub> values of ABTS inhibition of three *Phyllanthus* species. The highest radical scavenging activity of methanol extract was shown by methanol extract of *P. urinaria* with EC<sub>50</sub> of 11.2  $\pm$  0.01  $\mu$ g/mL, followed by *P. debilis* (16.2  $\pm$  0.03  $\mu$ g/mL) and *P. niruri* (26.0  $\pm$  0.02  $\mu$ g/mL). In water extract, *P. urinaria* also showed higher radical scavenging activity

**Table 3: EC50 in  $\mu$ g/ml of *Phyllanthus* species for DPPH radical scavenging activity.**

Species	Methanol extract	Water extract
<i>P. niruri</i>	29.3 $\pm$ 0.01	73.0 $\pm$ 0.03
<i>P. urinaria</i>	15.8 $\pm$ 0.01	33.5 $\pm$ 0.04
<i>P. debilis</i>	26.3 $\pm$ 0.01	45.0 $\pm$ 0.02

Values are mean  $\pm$  standard deviation (n=3)

**Table 4: EC50 in  $\mu$ g/ml of *Phyllanthus* species for ABTS scavenging activity.**

Species	Methanol extract	Water extract
<i>P. niruri</i>	26.0 $\pm$ 0.02	37.4 $\pm$ 0.02
<i>P. urinaria</i>	11.2 $\pm$ 0.01	13.5 $\pm$ 0.05
<i>P. debilis</i>	16.2 $\pm$ 0.03	23.0 $\pm$ 0.01

Values are mean  $\pm$  standard deviation (n=3)

with EC<sub>50</sub> of 13.5 ± 0.05 µg/mL, followed by *P. debilis* (23.0 ± 0.01 µg/mL) and *P. niruri* (37.4 ± 0.02 µg/mL).

For the antioxidant activity using ABTS scavenging activity, it was determined by the decolourisation of the ABTS by measuring the percentage of the reduction of absorbance at 734 nm and the results were presented as EC<sub>50</sub> concentration. Similar to what was observed in antioxidant activity of DPPH assay, *P. urinaria* exhibited the lowest EC<sub>50</sub>, followed by *P. debilis* and *P. niruri* in both methanol and water extracts.

Our data showed that the chemical contents and antioxidant activity of the methanol extract was consistently higher compared to water extract. We believed that the difference between methanol and water extracts was due to the solvent polarity difference. Lim and Murtijaya (2007)<sup>29</sup> had shown that methanol is better than water to degrade the cell wall, which results in greater amount of endocellular materials extracted. Furthermore, methanol is preferred than water to extract phenolic compounds in *Phyllanthus* species where major components, which are active hydrolysable tannins can be efficiently extracted using semi polar compounds.<sup>31</sup> A study by Cheah and Radu, (2011)<sup>28</sup> also showed that methanol extract had a better TPC and DPPH activity when compared to water extract. Plant antioxidant activity however, also depends on other non-polyphenolic compound such as vitamins, minerals and carotenoids. These non-polyphenolic compounds may exert synergistic effect with TPC and TFC, which could further enhance the antioxidants activities.<sup>7</sup>

## CONCLUSION

*Phyllanthus* species originated from Malaysia are high in antioxidant activities, total phenolic and flavonoid contents with possibilities of having high medicinal properties that can be potentially developed as medicine in future. *Phyllanthus* species especially *P. urinaria* showed higher amount of total phenolic and flavonoid contents and antioxidant activities when compared to *P. debilis* and *P. niruri*. Further studies are warranted for the isolation and identification of individual phenolic and flavonoid compounds and for better understanding of their biological mechanism.

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## ABBREVIATIONS

EC<sub>50</sub>: effective concentration; µg/mL: microgram per milliliter; HIV: Human Immunodeficiency Virus; °C: degree celcius; mg/mL : milligram per milliliter; v/v : volume per volume; µL: microliter; min: minutes; nm: nanometer; mg/g DW: milligram per gram dry weight; mM: miliMolar.

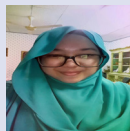
## CONFLICT OF INTERESE

Authors declare no conflict of interest.

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#### SUMMARY

- *P. urinaria* showed highest TPC, followed by *P. debilis* and *P. niruri* for both methanol and water extracts.
- *P. urinaria* also showed highest TFC than the other species.
- The antioxidant activity by using DPPH assay and ABTS assay showed *P. urinaria* has lowest EC50 values than *P. niruri* and *P. debilis*

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