Bioactive Compounds and *In Vitro* Evaluation of *Phyllanthus niruri* Extract as Antioxidant and Antimicrobial Activities

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Abstract

Phyllanthus niruri grows easily in Indonesia and is widely used in traditional medicine. Differences in growing conditions, methods of preparation, and harvest time greatly affect the phytochemical contents, affecting the efficacy and toxicity of medicinal plants. This study aims to identify the phytochemical contents and toxicity and determine the antioxidant and antimicrobial activities of *Phyllanthus niruri extract* (PNE) commonly used by people in Aceh. Phytochemical analysis was carried out qualitatively and semi-quantitatively with GC-MS. A toxicity test was carried out with the brine shrimp lethality test (BSLT). The antioxidant activity was assessed by measuring IC_{50} against the DPPH system. Antimicrobial assays against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* were determined based on the disc diffusion test. The results of this study indicate that PNE contains phenolics, flavonoids, tannins, steroids, saponins, and alkaloids. Based on the semiquantitative phytochemical analysis using GC-MS, the major phytochemical component of PNE is 2,3 dihydro-3-5-dihydroxy-6-methyl-4H-pyran-4-one, hexadecanoic acid, triazole derivates, linolenic acid, benzaldehyde 3,4-dimethoxy, hexahydroanthracene derivatives, and phytol. The LC_{50} PNE is 532.96 ppm. The IC_{50} value for PNE in inhibiting DPPH is 56.72 ppm. PNE has antimicrobial activity against *S. aureus*, directly proportional to the increase in concentration. PNE obtained in community yards in Aceh has a low level of toxicity with potent antioxidant and antimicrobial activity against *S. aureus*.

Keywords: *Phyllanthus*, BSLT, Antioxidant, Antimicrobial, Flavonoid, IC_{50}, DPPH

Introduction

Medicinal plants have long been used to heal and solve the daily health problems of societies in developing countries [1]. The bioactive compounds found in medicinal plants mediate their therapeutic effect. The plants are processed through soaking, boiling, or extraction to generate a vicious dosage form that can extract the plants' secondary metabolite. Differences in the processing techniques and growing landscape significantly affect their phytochemical contents [2]. Indonesia has prominently known for consuming medicinal plants, such as *Jamu,* using various processing methods. The plant biodiversity of Indonesia, the 3rd rank in the world, supports the use of medicinal plants as a community self-medicate technique [3]. Therefore, scientific research is recommended to support the evidence for the toxicity and effectiveness of medicinal plants in overcoming various diseases.

Phyllanthus niruri, known as meniran in Indonesia, grows easily and wildly in Indonesia and is predominantly used for medical treatments. This plant, from the *Phyllanthaceae family*, has a unique aroma traditionally processed for boosting immunity, reducing fever symptoms, cough, toothache, gastrointestinal tract disorder, and skin diseases, as well as an additional treatment in patients with malignancy [4-6] The extract of *P. niruri* has been reported to have biological activities such as antimicrobial, anti-inflammatory, anti-viral, anti-mutagenic, and immunomodulatory activity. Various studies showed that *P. niruri* contains flavonoids, alkaloids, tannins, polyphenols, saponins, terpenoids, and lignans [7,8]. Some studies have also shown differences in the phytochemical content from different growing conditions, processing methods, and harvest times [2,9]. While several studies have substantiated the advantageous properties of *P. niruri* extract, the outcomes obtained exhibit notable variability. Consequently, this investigation was undertaken to establish the safety of the extract through toxicity assessments and to assess its *in vitro* antioxidant and antimicrobial activities.

Materials and methods

This study used the leaves of *P. niruri* gathered from Aceh, Indonesia. The plant identity was confirmed by a taxonomist from the Biology laboratory of the University Syiah Kuala, Aceh. The voucher specimen of the plant was kept for future reference. (No. 612/TA.00.01/2021). Next, 1,1-diphenyl-2-picrylhydrazyl (DPPH), L-ascorbic acid, gallic acid, quercetin, Folin-Ciocalteu reagent, Tween 80 were generated from Sigma-Aldrich®. For the sake of the analysis, this study also added other analytical-grade chemicals and reagents.

This study was conducted in 4 steps: Extraction, qualitative and semiquantitative phytochemical analysis, antioxidant activity assay, and antimicrobial activity assay. The *in vitro* experiments were carried out upon the approval of the Ethical Clearance Committee of the Faculty of Medicine, Universitas Syiah Kuala (letter No. 103/EA/FK-RSUDZA/2020).

Plant extraction

Phyllanthus niruri leaves (250 g) were obtained from Aceh, Indonesia, in the dry season. They were collected, cleaned, and dried without direct sunlight exposure. The dried plants were ground using a grinder, and the dried powder was macerated in 70 % ethanol. Whatman No.1 filtered the extracts and concentrated them into dry mass using reduced pressure on a rotary evaporator at 50 °C, and the result was stored for the next steps. Next, it was labeled with PNE (*P. niruri* extract). Furthermore, the phytochemical analysis was undertaken qualitatively following the standard methods, and semiquantitative analysis was done using gas chromatography-mass spectroscopy (GC-MS). The component of *P.niruri* extract was determined by GC-MS using a Shimadzu - QP2010 Ultra (Shimadzu Corporation, Kyoto, Japan) [10].

Determination of total phenol content (TPC) and total flavonoid content (TFC)

The TPC of the extract was examined using the Folin-Ciocalteu reagent [11]. The mixture consisted of 100 μL of extracts, 500 μL of the Folin-Ciocalteu reagent, and 1.5 mL of 20 % sodium carbonate. Pure water was added to reach 10 mL. The absorbance at 765 nm was measured after 2 h of reaction and continued calculating the phenolic using the gallic acid standard. (Sigma-Aldrich, St. Louis, MO, USA).

The TFCs were evaluated using a modified version of the previously described spectrophotometric technique. Briefly, an aliquot extract (500 μL) was diluted with water (3.2 mL). Next, 5 % $NaNO_2$ solution (150 μL) was put into each sample, and 10 % $AlCl_3$ (150 μL) was added 5 min later. Six min later, it was mixed with 1.0 M NaOH (1 mL). The absorbance was identified at 510 nm, followed by calculating the total flavonoid using the standard of quercetin. The TPC was presented in a unit of mg quercetin equivalent/g of dry extract.

Brine shrimp lethality test (BSLT)

The toxicity test with BSLT refers to the previous method [12]. In short, Brine shrimp (*Artemia salina*) eggs were hatched in seawater. The extract (20 mg) was dissolved in Tween 80 (1 mL), yielding a solution stock of 2,000 ppm. Salt water was used for serial dilution to get a test solution ranging from 10 to 1,000 ppm. Each concentration was tested in Duplo. The test tubes were then observed, and after 24 h, the number of dead larvae and shrimps in each bottle was tallied. Statistical analysis determined the death percentage Eq. (1), and lethal concentration (LC_{50}) was determined according to probit analysis.

$$\text{Percentage of death (\%)} = \frac{\text{Total naupii} - \text{Alive naupii}}{\text{Total naupii}} \times 100 \% \tag{1}$$

Antioxidant activity test

The DPPH (1,1-diphenyl-2-picrylhydrazyl) method used by previously studies was employed to test the antioxidant activity [13]. The positive control was the ascorbic acid solution, with 4, 6, 8, and 10 ppm concentrations. The sample was dissolved in distilled water at a concentration of 1.2 mg/mL (1,200 ppm). Next, the sample was put into the 96 well microplates with the following proportions: 160 μL of sample solution with 40 μL of DPPH solution (sample), 160 μL of sample solution with 40 μL of methanol (control sample), 160 μL of methanol with 40 μL of DPPH solution (negative control), and 160 μL methanol with 40 μL methanol (blank). The microplate was left to stand for 30 min at room temperature in a dark room. Furthermore, the sample's absorbance was measured using a spectrophotometer at 517 nm. Antioxidant activity was expressed as the percentage of resistance obtained from the absorbance value of the sample. The ability to capture free radicals (inhibition) was calculated using the following equation.

$$\% \text{ inhibition} = [(\text{control abs} - \text{sample abs})/\text{control abs}] \times 100 \% \tag{2}$$

The IC_{50} value was determined by creating a curve between the inhibition percentage and concentration to obtain the regression equation. The regression equation determined the amount of extract concentration that could inhibit DPPH free radical activity by 50 %.

Antimicrobial activity of PNE

The microorganism isolates used were *E. coli* ATCC 8739, *S. aureus* ATCC 29213 and *C. albicans* ATCC 10231, bred at the Microbiology Laboratory of the Faculty of Medicine, Syiah Kuala University. The standard approach was used to determine the antimicrobial activity to ensure the appropriateness of disc diffusion. The 1st step was creating bacterial suspension by taking colonies of 1 loop *of E. coli* and *S. aureus* bacteria in nutrient agar (NA) media. It was then diluted using sterile 0.9 % NaCl and measured for the bacterial suspension density using spectrophotometry at a wave of 625 nm and absorbents of 0.08 - 0.10 to obtain a standard speed of bacterium at $1 - 2 \times 10^8$ CFU/mL. Inoculation of test microorganisms was performed using sterile cotton swabs on the entire surface to match the 60 ° angle 3 times.

In the 2nd step, an empty disc was dripped with 25 µL green of the extracted liquid with the concentrations of 10, 20, 40 and 80 %. Subsequently, 4 discs were placed with different concentrations, 1 disc soaked in 70 % ethanol (control negative) and 1 positive control disc, with a 24 mm distance between discs on Mueller Hinton agar containing test bacteria. Petri disc incubation was undertaken at 37 °C for 24 h. If the extract can inhibit the growth of test microorganisms, an inhibitory zone (transparent/clear area) will appear around the disc. The given positive controls were ciprofloxacin 5 µg (as an *E. coli* control), amoxicillin 10 µg (as an *S. aureus* control), and 100 IU nystatin (as a *C. Albicans* control). Measurement of the inhibitory zone on the disc was conducted using a caliper.

Statistical analysis

All analyses were carried out in triplo. The data diameter of inhibitory zone was expressed in mean ± standard deviation (SD). Data were analyzed by one-way ANOVA to examine the mean difference between groups. The amounts of polyphenols, flavonoids, and antioxidant activity were calculated from the regression analysis's best-fit line. The probity analysis approach proposed by Finney [14] was used to calculate the median lethal concentration (LC_{50}) and 95 % confidence intervals of the test samples as a gauge of the plant extract's toxicity.

Results and discussion

Characteristics and contents of the extract

The phytochemical characteristics in the PNE showed the presence of phenolics, flavonoids, tannins, steroids, saponins, and alkaloids. Based on the semiquantitative phytochemical analysis using GC-MS, the major phytochemical component of PNE is 2,3 dihydro-3-5-dihydroxy-6-methyl-4H-pyran-4-one, hexadecanoic acid, triazole derivates, linolenic acid, benzaldehyde 3,4-dimethoxy, hexahydroanthracene derivates, and phytol (**Figure 1**). Detected compounds, retention time, and composition of metabolite (in percent) were presented in **Table 1**.

Table 1 The compound of PNE based on GC-MS.

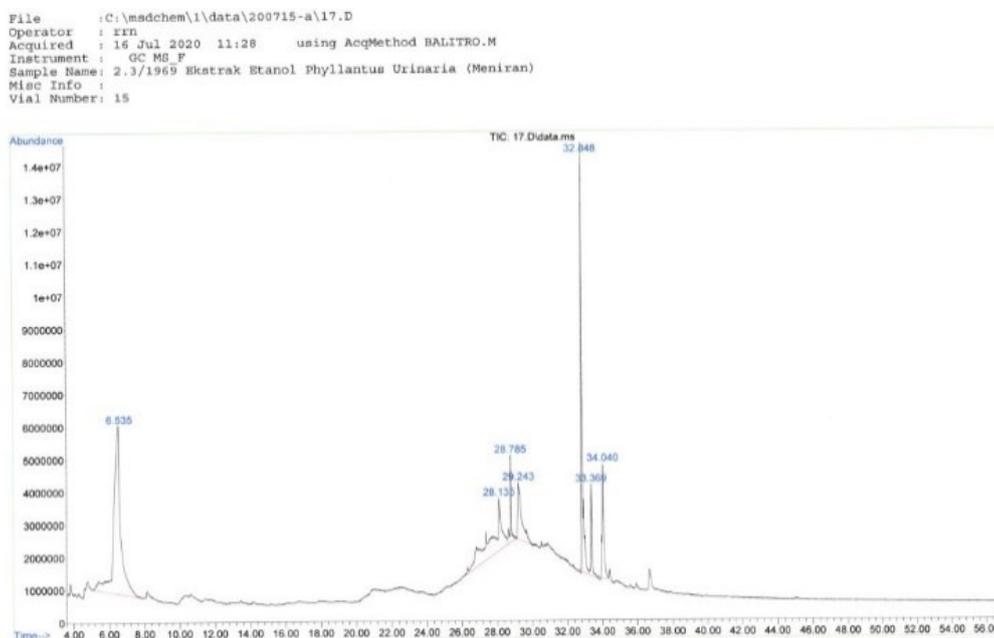| Compound	Retention time (min)	Percentage Composition (%)
2,3 dihydro-3-5-dihydroxy-6-methyl-4H-pyran-4-one	6.535	42.83
Hexadecanoic acid	28.135	19.08
Triazole derivates	28.785	3.29
Linolenic acid	29.243	7.86
Phytol	32.848	16.35
Hexahydroanthracene derivates	33.369	3.47
Benzaldehyde 3,4-dimethoxy	34.040	7.12

Figure 1 GC-MS Spectral of *Phyllanthus niruri* extract.

BSLT

The toxicity test data with BSLT were determined based on the number of dead shrimp larvae at various extract concentrations (**Table 2**). The data probit analysis showed the LC_{50} value of 532.96 ppm.

Table 2 LC_{50} of PNE.

PNE	Response (number of dead shrimp larvae)															LC_{50} (ppm)
	Control			10 ppm			100 ppm			500 ppm			1,000 ppm			
	0	0	0	0	0	1	3	0	2	7	6	8	9	10	10	532.96

Data ini triplo, PNE *P. niruri* extract, LC lethal concentration.

Total phenol and flavonoid contents and antioxidant activity test

Table 2 shows the PNE's phenolic and flavonoid contents. The phenolic contents were presented as mg/g gallic acid equivalent with the standard curve equation: $y = 0.0138x – 0.0248$, R2 = 0.983. The y value represents the absorbance at 765 nm, and the x represents the phenolic content in PNE in mg/g. The flavonoid contents were expressed as mg/g quercetin equivalent with the standard curve equation: $y = 0.0102x – 0.0062$, $R^2 = 0.9956$. The value of the antioxidant activity was obtained from equivalence to the standard, namely ascorbic acid. The IC_{50} values were generated from the average inhibitory using logarithmic regression analysis. The curve equation used was $y = 23.71\ln(x) – 46.054$, $R^2 = 0.9954$ and $y = 23.44\ln(x) – 44.346$, $R^2 = 0.9933$ and IC_{50} PNE was 56.72 ppm. Ascorbic acid acted as the positive control and had an IC_{50} of 3.32 ppm. The results are presented in **Table 3**.

Table 3 Total Phenolic and flavonoid contents with antioxidant activity of PNE.

PNE	Phenolic contents mg GAE/g extract	Flavonoid contents mg quercetin/g extract	Antioxidant activity IC_{50} DPPH (ppm)
	25.73	195.50	56.72

PNE *P. niruri* extract, GAE Gallic acid equivalent, IC inhibitory concentration

Extract antimicrobial effect

Table 4 shows the diameter of the inhibitory zone on the disc, indicating the positive relationship between the inhibition zone formed and the concentration of PNE. The higher concentration of PNE, the larger inhibition zone formed. **Table 4** shows that PNE can inhibit the growth of *S. aureus* starting from PNE 10 %. The inhibition increased with the increasing concentration. PNE 40 and 80 % inhibited *S. aureus*, which was not significantly different from 10 μg amoxicillin ($p > 0.05$).

Table 4 The average inhibition zone of PNE in inhibiting microbial growth.

Groups	The diameter of the inhibition zone against microbes (in mm)		
	E. coli	*S. aureus*	*C. albicans*
Negative control	6 ± 0.00	6 ± 0.00	6 ± 0.00
PNE 10 %	6.53 ± 0.81[b]	13.91 ± 1.86[a,b]	7.37 ± 0.01[a,b]
PNE 20 %	7.52 ± 1.18[a,b]	15.93 ± 1.89[a,b]	7.30 ± 0.01[a,b]
PNE 40 %	8.68 ± 0.17[a,b]	19.11 ± 1.19[a]	7.81 ± 0.40[a,b]
PNE 80 %	10.12 ± 0.25[a,b]	20.19 ± 0.59[a]	8.53 ± 0.11[a,b]
Positive control	34.23 ± 1.01	20.45 ± 1.89	20.43 ± 0.02

PNE *P. niruri* extract, data in mean ± SD (n = 4 cycles). [a]$p < 0.05$ VS negative control, [b]$p < 0.06$ VS positive control.

The ability of PNE to inhibit *E. coli* and *C. albicans* is not as good as in inhibiting *S. aureus*. Only PNE 80 % was able to inhibit the growth of *E. coli* and *C. albicans*, which were significantly different from the negative control. In addition, the diameter of the inhibitory zone on the disc containing PNE 80 % was significantly different from the positive control (ciprofloxacin 5 μg and 100 IU nystatin).

Discussion

This study presents an investigation encompassing the phytochemical contents of *Phyllantus niruri,* its toxicity profile, and its potential as an antioxidant and antimicrobial agent. The employed extraction method, involving the use of 70 % ethyl alcohol for *P. niruri* leaf extraction, yielded secondary metabolite compounds, aligning with previous research that has reported the presence of phenolics, flavonoids, tannins, steroids, saponins, and alkaloids in *P. niruri* extracts (hereafter referred to as PNE). Additionally, prior studies have corroborated the existence of polyphenols, flavonoids, saponins, triterpenoids, tannins, and lignins within PNE [15,16]. Further analysis found that the active compound content of *P. niruri* leaves consists of hypophyllanthin, nirtetralin, quercetin, niruriflavone, ellagic acid, and several other compounds [7,8]. It is notable, however, that our study utilizing Gas Chromatography-Mass Spectrometry (GC-MS) analysis did not yield more specific identification of these bioactive compounds. It is imperative to recognize that the production of diverse secondary metabolites in plants is contingent upon various factors, such as the specific environmental conditions, the constituents of the plant, and the techniques employed during the extraction process [17,18]. Additionally, the choice of solvent for the extraction process exerts a significant impact on the composition of active compounds in leaf extracts. Specifically, the polar nature of the solvent plays a pivotal role, with more polar solvents attracting higher concentrations of active compounds. In the present study, a semi-polar ethanol solvent (70 %) was employed for the extraction process.

The composition of primary and secondary metabolite compounds *P. niruri* is highly susceptible to environmental factors associated with its growth habitat. This susceptibility is contingent upon a complex interplay of intrinsic and extrinsic determinants, encompassing genetic factors, structural attributes, and the availability of precursors essential for the biosynthesis of secondary metabolites [17]. Variations in the phytochemical content of *P. niruri* can be ascribed to variations in exposure to environmental parameters such as light intensity, temperature, humidity, pH levels, soil nutrient composition, solar radiation, and the elevation of the plant's habitats [18]. Furthermore, the active compounds identified in the present study are in accordance with previous research findings, suggesting a degree of consistency in the phytochemical profile of PNE. It is postulated that the observed antioxidant and antibacterial effects in this study are mediated by the intricate phytochemical composition inherent to this extract.

The regression equation shows that the IC_{50} value for PNE is 56.72 ppm. For DPPH free radical scavenger activation, the IC_{50} value is defined as the extract concentration, decreasing the DPPH

concentration by 50 % from the initial concentration [19]. Previous research revealed that the *P. niruri* leaf extract has excellent antioxidant effects. The content of secondary metabolite mediates its antioxidant activity. Plant flavonoid levels are strongly related to their antioxidant activity [20]. In the present study, the notably high flavonoid content within PNE underscores its likely role as a key mediator of its antioxidative effects.

Antioxidants are crucial for defending the body from the harm of free radicals, which may result from *in vivo* metabolic events. Oxidative stress is brought on by an imbalance in the levels of pro-oxidants and endogenous antioxidants [21]. It progresses metabolic diseases and triggers complications in microvascular and macrovascular organs. For example, hyperglycemia can trigger the production of ROS (reactive oxygen species) and nitrogen species [22]. Thus, antioxidant supplementation plays a role in preventing complications and protecting body tissues against further oxidative damage. Non-enzymatic defense mechanisms can be found in vitamin C, vitamin E, carotenoids, and polyphenols [21]. The antioxidant substances can act as metal-chelating agents, hydrogen atom donors, free radical scavengers, and quenchers of free radicals [23]. In this context, our study provides substantiating evidence of the advantageous antioxidant properties associated with PNE.

In addition to considerations of efficacy, the evaluation of the toxicity levels of herbs holds paramount importance in the development of herbal medicines. High-quality herbs are characterized by their effectiveness coupled with low levels of toxicity. Within the context of our study, we conducted toxicity assessments utilizing the Brine Shrimp Lethality Assay (BSLT), a cost-effective and straightforward bioassay employed for evaluating the bioactivity of phytochemicals present in plant extracts [24]. Numerous researchers have used the lethality of the test sample in a zoological creature like the brine shrimp (*Artemia salina*). It has proven to help screen various chemical compounds found in various bioactivities. Based on Clarkson's criteria, the toxicity of extracts based on the LC_{50} value is classified if the LC_{50} value above 1,000 μg/mL as non-toxic, LC_{50} 500 - 1,000 μg/mL is low toxicity, LC_{50} 100 - 500 μg/mL is medium toxic, while LC_{50} 0 - 100 μg/mL is highly toxic [25]. The LC_{50} value of PNE is 532.96 μg/mL with low toxicity.

This study presents that PNE exhibited potent antibacterial activity against S. aureus, and this activity displayed a dose-dependent pattern, where increasing the concentration of the extract correlated positively with heightened antibacterial efficacy. This is in line with previous research proving that *P. niruri* extract has a strong activity in inhibiting the growth of *S. aureus* [4,8,26,27]. This study also found that the antimicrobial activity of PNE against *E. coli* and *C. albicans* was lower than that of *S. aureus*. However, at 80 % concentration, the antimicrobial activity was not as effective as ciprofloxacin and nystatin. In accordance with theoretical principles, the mitigation of infections induced by Gram-positive bacteria is facilitated due to the relatively facile permeability of their bacterial cell walls by antibacterial agents. In contrast, Gram-negative bacteria exhibit a more intricate cellular architecture characterized by the presence of 3 additional complex cell layers, necessitating the possession of distinct properties by agents that aim to breach their formidable defensive barriers [28].

Phenolic compounds are secondary metabolite compounds with an aromatic ring with 1 or 2 hydroxy groups (OH). Phenolic compounds function as cell wall builders, color to flower pigments, growth controllers, and defenses [29]. Flavonoids are the largest group of phenolic compounds that are effective in inhibiting the growth of bacteria, fungi, and viruses. Flavonoids work as antibacterial by inhibiting the synthesis of nucleic acids and bacterial energy metabolism, disrupting bacterial wall synthesis and resulting in plasma leakage and cell lysis. In addition, flavonoids also have unpaired H^+ ions that trigger the withdrawal of phosphate groups on the phospholipid membranes of microorganisms, thereby interfering with the integrity of cell membranes [30]. Ibrahim *et al.* [31] also demonstrated that the antimicrobial effects of Phyllanthus niruri extract were attributed to the presence of phytochemicals, including phyllantin and hypophyllanthin (lignan compounds), quercetin (flavonoid compound), triterpenoids, and ellagitannins.

Conclusions

This study provides evidence of the presence of bioactive compounds in *Phyllanthus niruri* extract (PNE), including phenolics, flavonoids, tannins, steroids, saponins, and alkaloids, with notably elevated levels of flavonoids. Moreover, PNE exhibits robust antioxidant activity with concurrent low toxicity. Additionally, PNE demonstrates pronounced antimicrobial activity against the growth of S. aureus while exhibiting comparatively lower potency against E. coli and C. albicans. Consequently, it is conceivable that this extract may have utility in addressing diverse health-related concerns within the community.

Acknowledgments

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