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Short communication

Determination of Antioxidant Compounds, Proximate Compositions and Assessment of Free Radical Scavenging Activities of *Nypa Fruticans* Wurmb. Sap

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ABSTRACT

Nipa palm sap (NPS) as source of medicine traditionally used to treat various diseases. This study identified good radical scavenging activity in NPS with the IC_{50} value of 33.36 µg/mL using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. NPS comprises of moisture (72.44%), ash (1.04%), protein (7.04%), carbohydrate (19.48%), fat (0%), and energy level (106 kcal). Glucose (0.3%) and fructose (1.8%) were detected using high-performance liquid chromatography. Maleic acid, cinnamic acid, chlorogenic acid, and kaempferol were the predominant compounds revealed by ultra-high-performance liquid chromatography. Overall, NPS has the potential antioxidants sources with significant health benefits and values for commercialisation.

Keywords: Antioxidant, DPPH, nipa palm sap, proximate analysis, UHPLC

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INTRODUCTION

From prehistoric time, global population relies primarily on traditional medicine like plants and animals to alleviate various ailments (Ekor, 2014). This approach persisted in Asia and America Latin populations because of their historical facts, cultural evidence, and beliefs (Ekor, 2014). The use of natural antioxidants from natural plants have gained considerable scientific interests because of their natural origin and lower detrimental side effects (Lourenço et al., 2019).

There is also increased consumption of exogenous antioxidants by the public, for example, ascorbic acid (i.e., Vitamin C), tocopherol-net (i.e., Vitamin E), carotenoids and polyphenols that are present in fruits, vegetables, cereals, beverages, and other food products. This is due to the belief that these products could support the antioxidative defence system (Lourenço et al., 2019).

Nipa palm (*Nypa fruticans* Wurmb.) is a palm species that can found at the coastlines and estuarine habitats distributed all over Malaysia. In Peninsular Malaysia, Kelantan is one of the states with broad coverage of nipa palm vegetation besides Perak, Selangor, and Terengganu (Hamdan et al., 2012). Hamdan et al. (2012) reported that nipa palm was grown and located in Kota Bharu and Tupat, Kelantan covering 204.575 ha of land area. The nipa palm contains the infructescence that can be tapped to yield abundant of sap called nipa palm sap (NPS) (Hafizi et al., 2018). Alternatively, NPS can be freshly consumed or fermented into wine (Phetrit et al., 2020).

Preliminary studies of NPS showed that it exhibits high phenolic and flavonoid content, as well as antioxidant activity (Hafizi et al., 2018; Phetrit et al., 2020; Sukairi et al., 2019). Based on the local's belief, NPS has the medicinal potential to treat fever, gout, kidney stone and metabolic syndrome such as diabetes and hypertension (Mohd et al., 2011; Sukairi et al., 2019). This drink is well-known among the elderly citizens of some countries such as Malaysia, Philippines, Indonesia, Thailand, Sri Lanka, and India, where they believed NPS could be as an energy booster, remedy for fever, helps in the digestion process and as a medication for certain chronic diseases (Tamunaidu et al., 2013, Yusoff et al., 2015; Hafizi et al., 2018; Phetrit et al., 2020). However, these postulations required further investigations.

Although NPS have shown good antioxidant activities with high concentration of phenolic and flavonoid contents, the study that reports on individual phenolic and flavonoid compounds in NPS remains unnoticed. The information of antioxidants compounds derived from NPS is essential to determine the quality of the sap and to explore the ability of its antioxidants compound to produce the therapeutic effect that leads to health improvements of local consumers in Malaysia. Hence, the present study aims to determine the antioxidant activity, proximate compositions, and individual antioxidant compounds of NPS produced in Kg Pulau Gajah, Kota Bharu, Kelantan.

MATERIALS AND METHODS

Chemicals

All chemicals used in this study were purchased from the Sigma–Aldrich (Chemie, Steinheim, Germany), Merck (Darmstadt, Germany) and Nacalai-Tesque (Kyoto, Japan) manufacturers. All reagents were used as follows: 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid (99.93%, analytical grade), kaempferol (≥97%, HPLC grade), gallic acid (98%, analytical grade), coumaric acid (≥ 95%, HPLC grade), quercetin (≥ 95%, HPLC grade), caffeic acid (99%, HPLC grade), cinnamic acid (99%, analytical grade), chlorogenic acid (≥ 95%, HPLC grade), maleic acid (97%, analytical grade), protocatechuic acid (≥ 98%, HPLC grade), vanillic acid (≥ 95%, analytical grade), rutin (≥9 %, HPLC grade), methanol (99%), distilled water, formic acid (85%, analytical grade), and acetonitrile (99%, HPLC grade) .

Sample Collection

Fresh samples of NPS were obtained from Kampung Pulau Gajah, Kelantan, Malaysia in April 2019. The nipa palm with matured fruit was randomly selected to yield sap twice per day. The long stalks containing infructescence were cut and tapped carefully to yield the sap, with a sterile plastic bag being attached to the end of the stalk for sap collection.

After the collection process of approximately 5-6 hrs, the collected sap was immediately filtered, stored into 500 mL Schott bottle, and packed in boxes with ice. All samples were immediately transported back to the laboratory and kept at -20ºC for a few days before sample preparation. The species identity of the sample was verified by Dr. Shamsul Khamis, a plant biologist from Universiti Kebangsaan Malaysia (UKM). A Voucher Specimen of the sample (PIIUM 0314) was stored at the Herbarium (UKMB) Faculty of Science and Technology.

Sample Preparation

The collected sap was transferred into a labelled 500 mL round conical flask and incubated in -80°C freezer (SANYO ultra-low temperature freezer, model MDF-U53V) for deep freezing and then being transferred to freeze-dryer (CHRIST Christ, Beta 2-8 LDplus). The samples were freeze-dried for one week. The freeze-dried yield was 105.3 g/500mL of sap. The dried samples were then kept at 4°C for further experiments. The sample preparation steps were conducted carefully to preserve the amounts and activity of antioxidant compounds in the samples.

Determination of Antioxidant Capacities

DPPH Radical Scavenging Activity. In this study, the 2,2-diphenyl-1-picrylhydrazyl assay was modified from Prasad et al. (2013) and Yusoff et al. (2015) methods using 96

wells microplates. Briefly, 100 µL of DPPH (i.e., 0.5mM dissolve in methanol) was mixed with different sample concentrations (i.e., 1.95–62.5 µg/mL) in a 96-well plate. For the blank assay, DPPH solution was added with 100 µL of methanol. The prepared plate was immediately inserted into a spectrophotometer (SpectraMax Plus 384). The absorbance value at 517 nm was noted after 30 min incubation in the dark at room temperature. The ascorbic acid dissolved in distilled water was used as the standard. The change of colour of the DPPH solution from violet to pale yellow at λ=517 nm reveals the DPPH radical-scavenging activity. The measurement was conducted in triplicates, and the percentage of DPPH inhibition (%) was obtained using the following Equation 1.

$$(\%\ \text{DPPH inhibition}) = \left[\frac{Actr - As}{Actr}\right] x\ 100 \qquad [1]$$

Where Actr and As denote the absorbance of the control (blank) and sample or standard, respectively. IC_{50} values obtained from the graphical plot of percentage inhibition against concentration were used to determine the free radical scavenging activity of the sample.

Proximate Analysis

The proximate composition of NPS includes moisture, ash, energy, protein, carbohydrate, and fat contents were measured using the standard protocols set by the Association of Official Analytical Chemists (2000). The Kjeldahl method was used to determine the protein content of the sample. Briefly, the percentage of nitrogen (% Nitrogen) was multiplied with the factor of 6.25 to obtain the percentage of protein (% Protein) in the samples. Total moisture in the sample was determined using oven drying method at 100°C for 3 hrs until the constant weight was obtained and ash content in the sample was determined by heating in the furnace at 550°C to constant mass. Fat was determined by Soxhlet extraction with petroleum ether as a solvent. The carbohydrate content (%) and energy value (kcal) were determined by calculation as follows, respectively.

Total Carbohydrate (%) = [100 − % Moisture - % Protein - % Fat - % Ash]

Total energy = (% Protein × 4) + (% Fat × 9) + (% Carbohydrate × 4)

Determination of Sugar Profiles

The sugar profile was determined according to the method of Association of Official Analytical Chemists (2000). Jasco high-performance liquid chromatography (HPLC) was used with a carbohydrate column (Prevail™ Carbohydrate ES, 250 x 4.6 mm, 5 µm size)

and an evaporative light scattering detector (ELSD). The working temperature was fixed at 35°C. The acetonitrile solution and deionised H_2O (83:17) were used as the mobile phase, with a flow rate of 1.0-1.3 mL/min and injection volume ranging from 10-20 µL. The total run time was 10 min. Before HPLC analysis, the solutions were filtered using a 0.45 µm syringe filter (nylon) to eliminate particulates.

The sugar standard consisting of D-glucose, D-fructose, sucrose, and maltose were diluted in acetonitrile and deionised H_2O (1:1). The identification of sugar compounds was performed by comparing retention times with the standard. All the detection of samples and standards repeated in triplicate. The concentration of the sugar compounds, expressed in gram per 100 mL, was calculated by comparing their peak areas to a calibrated standard curve.

Identification and Quantification of Phenolic Compounds Using Ultra-High-Performance Liquid Chromatography (UHPLC-MS/MS)

The chromatographic separation was performed using Agilent 1100 series HPLC system together with Sciex 3200 hybrid trap triple quad tandem mass spectrometer to identify phenolic compounds of NPS. The method used was conducted according to Kong et al. (2012) with a slight modification. Phenolic compounds were chromatographically separated using a column Phenomenex Synergy RP C18, 100A (100 mm x 3 uM x 2.0 mm) maintained at 40 °C. Twenty microliters of the sample were injected into the system and ran using a specific elution program. Briefly, 0.1 (% v/v) formic acid with water was used for the mobile phase A while 0.1 (% v/v) formic acid with acetonitrile was used for the mobile phase B. The flow rate was maintained at 0.6 mL/min. The mobile phase composition was modified following gradient solvent system: 0 min, 1% B; 0.5 min, 1% B; 16.00 min, 35% B; 18.00 min, 100% B; 20.00 min, 1% B. The total runtime was 54.5 min.

The standards used were kaempferol, gallic acid, coumaric acid, quercetin, caffeic acid, cinnamic acid, chlorogenic acid, and maleic acid. Each of the standard (0.01-10 ppm) and the sample were filtered through a 0.45 µm pore size membrane filter before injecting with the aliquots (20 µL).

The phenolic compounds were identified by comparing the retention times with available standards, which is based on the bases of their peak areas and expressed in part per million (ppm). The analysis was carried out in triplicate determinations.

Statistical Analysis

The data were presented as mean±SD of three replicate and analysed by using Microsoft Excel 2016.

RESULTS

Antioxidant Capacities

DPPH Radical Scavenging Activity. The DPPH radical scavenging activities obtained from the antioxidant activities of the NPS, and ascorbic acid are shown in Table 1, which corresponded to the three analytical replicates. DPPH radical scavenging activity of NPS and ascorbic acid increased with the increase in the concentration (Table 1). The IC_{50} value of NPS was 33.36±0.96 µg/mL. However, ascorbic acid showed the highest antioxidant activity with an IC_{50} value of 21.29±0.74 µg/mL.

Table 1
Percentage of DPPH radical scavenging activity at different concentrations of Nipa palm sap

Concentration of sample (µg/mL)	Percentage of inhibition (%)		IC_{50} (µg/mL)	
	Nipa palm sap*	Ascorbic acid*	Nipa palm sap*	Ascorbic acid*
1.95	21.99±2.82	43.71±1.45	-	-
3.9	24.5±3.57	45.01±0.54	-	-
7.81	27.76±2.57	47.85±0.11	-	-
15.63	40.41±0.48	51.43±1.62	-	21.29±0.74
31.25	50.87±0.67	52.39±1.41	-	-
62.5	60.01±0.45	62.03±2.30	33.36±0.96	-

Note. All values are expressed as mean ± SD of triplicate measurement.

Proximate Composition and Sugar Type

Proximate compositions of NPS are shown in Table 2. The compositional analysis of NPS indicated the contents of moisture, ash, protein, carbohydrate, and fat as 72.44%, 1.04%, 7.04%, 19.48%, and 0%, respectively, with an energy value of 106 kcal (Table 2). The type and concentration of sugar in NPS are also presented in (Table 2). The sugar components of NPS consisted of glucose (0.3 g/100mL) and fructose (1.8 g/100mL). The total concentration of sugar in nipa palm sap was 2.1 g/100mL.

Table 2
Proximate compositions and sugar profile of nipa palm sap expressed in g/100mL

Composition	Sap
Moisture	72.44±0.005
Ash	1.04±0.001
Energy (kcal)	106±0.094
Protein	7.04±0.001
Carbohydrate	19.48±0.024
Fat	0.00±0.00
Total Sugar	2.1±0.000
Glucose	0.3±0.000
Fructose	1.8±0.000
Sucrose	ND
Maltose	ND

Note. ND = non-detectable. Values are expressed as mean ± SD from three determinations.

Identification and Quantification of Phenolic Compounds

There are several phenolic and flavonoid compounds such as kaempferol, quercetin, gallic acid, cinnamic acid, caffeic acid, coumaric acid, maleic acid and chlorogenic acid that can

Table 3
Phenolic and flavonoids compounds of nipa palm sap

Compounds	Retention time (min)	[M-H-]/ Fragment (m/z)	Nipa palm sap (ppm)
Kaempferol	4.25	285/93	0.0407±0.0011
Quercetin	4.11	301/151	0.0251±0.0041
Gallic Acid	0.58	169/125.1	0.0093±0.0012
Cinnamic acid	1.64	147/103	0.0741±0.0029
Caffeic acid	1.74	179/135	0.0169±0.0008
Coumaric acid	2.8	163/119.1	0.0250±0.0026
Maleic acid	0.56	115/71	0.0745±0.0096
Chlorogenic acid	1.55	353/191	0.0494±0.0001
Protocatechuic acid	-	-	ND
Vanillic acid	-	-	ND
Rutin	-	-	ND

Note. ND = Not detected

be found in NPS. The maleic acid, cinnamic acid, and chlorogenic acid were identified to be the major compounds present in NPS. Among the identified phenolic compounds, the content of maleic acid was the highest in NPS (0.0745±0.0096 ppm) while the lowest content was gallic acid (0.0093±0.0012 ppm) (Table 3). Following the phenolic compounds in NPS, cinnamic acid was identified as the second most abundant with 0.0741±0.0029 ppm, followed by chlorogenic acid (0.0494±0.0001 ppm) and kaempferol (0.0407±0.0011 ppm). The coumaric acid, quercetin and caffeic acid were detected in a minor amount (<0.03 ppm).

DISCUSSION

The moisture content value of NPS was 72.44% indicated that the NPS might has a shorter life span due to the growth of microorganisms that degrade the constituents and compounds (Olalude et al., 2015). Phetrit et al. (2020) reported that the NPS cultivated in southern parts of Thailand contained approximately 77.69% moisture, 95.92% of total sugar (dw), 0.49% protein (dw), and 3.32% ash (dw). In contrast to the present study, the NPS collected from eastern parts of Malaysia had lower moisture (72.44%) and sugar contents (10.1% dw) but had higher protein (33.43% dw) and ash contents (4.95% dw). These discrepancies of the various chemical constituents may be related to harvest date, time, location, and year-to-year growing period (Sharp et al., 2014). Recent study conducted by Cavender et al. (2019) reported that harvested period had a greatly effect to influence the variations of sugar contents.

The amount of protein content in NPS at 7.04% indicated that it could be used as a protein supplement for muscle development and maintaining human health (Wakili et al.,

2015). Likewise, the fat content was 0% and indicated that the NPS is a food source with a low cholesterol level (Olalude et al., 2015). Furthermore, the ash content was 1.04% and indicated that the NPS contains low mineral composition (Wakili et al., 2015). Besides that, the NPS can be classified as carbohydrate-rich juice due to its high carbohydrate content of 19.48%. The high carbohydrate contents of NPS indicated that it can be used as a good energy source for the body (Olalude et al., 2015) and is suitable to be processed as raw materials for the juice production.

Our findings from the standard antioxidant assay using the DPPH molecules showed that the NPS exhibited good antioxidant activity with the value of IC_{50} 33.36 µg/mL, which is higher than those studies reported by Hafizi et al. (2018) and Sukairi et al. (2019). The antioxidant activity of the sap reported in this study was also higher than nipa palm of different plant structures/products, such as nipa palm fruits and nipa palm vinegar with the IC_{50} values of 7.78 mg/L and 36 µg/mL, respectively (Prasad et al., 2013; Yusoff et al., 2015). According to Sowndhararajan and Kang (2013), a lower the IC_{50} value indicates the higher antioxidant activity.

Further investigation for this study was focused on the bioactive compounds present in NPS to understand the physiological functions in giving therapeutics effects due to the antioxidant potency. Based on the data presented in Table 3, it can be concluded that our results were corroborated with Prasad et al. (2013) that reported chlorogenic acid and kaempferol as among the major compounds found in the nipa palm fruit cultivated in the north part of Malaysia. In addition, cinnamic acid, quercetin, gallic acid were also detected in nipa palm fruit.

However, Prasad et al. (2013) also reported that the nipa palm fruits comprised protocatechuic acid and rutin. In contrast, the protocatechuic acid and rutin compounds were not detected in the NPS extract in this study. Similarly, another study conducted by Pherit et al. (2020) has detected the presence of vanillic acid but not the rutin compound. In comparison with this study, neither of these two compounds were detected. Based on Ahmed et al. (2015), these contradictory results for phenolics and flavonoids might be due to the use of different extraction solvents as well as the distribution of both compounds varied and not consistent among different plant samples.

Previous studies reported that NPS have antidiabetic effects by inhibiting the α-amylase and α-glucosidase activities, the two key enzymes linked to type-2 diabetes (Hafizi et al., 2018; Phetrit et al., 2020). Consistent with the literature, this research has discovered a high amount of phenolic compounds and flavonoids that may contribute to the biological and medicinal properties of the NPS. Several animal experiments and epidemiological studies have demonstrated that chlorogenic acid and kaempferol exerted antidiabetic activity by delaying carbohydrate digestion and glucose absorption in rats and may have antihypertensive effects in human (Al-Numair et al., 2015; Zhao et al., 2012).

In a biological system, the phenolic and flavonoid compounds act as free radical-trapping agents with antioxidant, antimicrobial and antifungal activities (Lourenço et al., 2019). *In vivo* studies indicated that phytonutrients such as chlorogenic acid and kaempferol might be vital in protecting the biological systems from oxidative stress (Al-Numair et al., 2015; Zhao et al., 2012). Therefore, the beneficial effects of bioactive compounds present in the NPS may possess antioxidant activity gives protective elements from chronic diseases.

The NPS consumption has been attributed to the ability to promote good health and provide some degree of protection towards chronic diseases due to different bioactive compounds. The findings of this study will lead to the following research, which is a community trial study to investigate the potential of NPS as a health supplement in improving health status. Thus, these data play a vital role and will be part of the larger study in future that could be commercialised as a healthy drink.

CONCLUSION

In conclusion, the NPS showed a good antioxidant activity with a recorded IC_{50} value of 33.36 µg/mL. The proximate compositions of NPS indicated that moisture, ash, protein, carbohydrate, fat, and energy were presented at 72.44%, 1.04%, 7.04%, 19.48%, 0%, and 106 kcal, respectively. In addition, the NPS comprised of two types of sugars, which is fructose (0.3g/100mL) and sucrose (1.8g/100mL). Besides that, maleic acid, cinnamic acid, chlorogenic acid and kaempferol were significant antioxidant compounds present in the NPS. It can be postulated that the antioxidant compounds found in the NPS can combat the free radicals in our body system and have great potential to be commercialised as a healthy drink with scientific evidence and validation.

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