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Phytochemical and Xanthine Oxidase Inhibitory Activity in *Nypa fruticans* Wurmb. Fruit Extracts

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Abstract

Thai wisdom knows the benefits of mangrove palm fruits to be boiled in water and drink as herbal medicines for healing gout. However, there is no scientific paper to confirm the relief of gout symptoms by mangrove palm, this is the first report. The mangrove palm: exocarp/mesocarp, endocarp, endosperm, and the whole of fruit in the ripe stage were extracted with water and ethanol. The crude extracts were studied for the phytochemical and inhibitory activity of xanthine oxidase, one of the causes of gout. Most of the crude extracts showed similar phytochemicals as, phenolic compounds, flavonoids, terpenoids, and steroids, but there was no alkaloid, anthraquinone, and iridoid glycoside. The endocarp extracted with ethanol showed the highest phenolic content (102.85±5.73 mg GAE/g), flavonoid content (531.02±10.46 mg QUE/g), and strongest antioxidant activity (26.79±0.57 mg VCE/g). The ethanolic and water extract (heating at 60 °C) of endocarp showed the strongly inhibited xanthine oxidase activity with IC_{50} of 0.029±0.001 mg/mL and 0.14±0.04 mg/mL, respectively. Therefore, this research has already confirmed that the fruit of mangrove palm could inhibit xanthine oxidase enzyme. It would be useful for further development as an herbal medicine or supplementary food for gout likely in the form of hot brewed teas or products that are safe for consumers. Moreover, it also adds value to the peel of the leftovers from consumption.

Keywords: *Nypa fruticans* Wurmb., Fruit, Xanthine oxidase, Uric acid, Gout, Herbal

Introduction

Nypa fruticans Wurmb. is a native palm species found along the coastline in the mangrove forest area. It belongs to Arecaceae family and has a common name: Nypa, Atap palm, Nipa palm, Mangrove palm. The trunk grows beneath the ground, while the leaves and flower stalk grow upwards above the surface. Each part of *Nypa fruticans* Wurmb. has many benefits for local community such as various parts are used as resources for fuels and chemicals [1], fruits are used for cooking and have a great potential source of the natural antioxidant [2-4], leaves are used to construct the roof. In addition, the extracted of *Nypa fruticans* Wurmb. showed a significant antidiabetic by the inhibition of α-amylase [5,6].

Gout is a type of arthritis caused by the accumulation of uric acid crystals in the joints. This disorder is caused by an excess of xanthine oxidase and many foods that produce high content of purines. Xanthine oxidase (XOD) oxidized the hypoxanthine to xanthine and xanthine to uric acid that is the last two steps of purine catabolism in human. The XOD-inhibitors are substances that can inhibit the activity of XOD to reduce the production of uric acid. Thus, XOD-inhibitor could be used for the treatment of gout. Recently, several modern medicines which inhibit XOD (allopurinol, oxypurinol, phytic acid, tisopurine, etc.) are introduced for hyperuricemia treatment and related medical conditions including gout. Allopurinol, one of the synthetic drugs, was widely used to treat gout. But the most common adverse effects of allopurinol are gastrointestinal distress, hypersensitivity reactions, and skin rash. Therefore, a use of these drugs requires careful monitoring [7]. Because of drug side effects, most people are interested in treating gout with herbs. Various natural products have been found to inhibit XOD as well,

such as *Leucas zeylanica* (whole plant) [8], leaves of *Tephrosia purpurea* Linn. and *Cyclocarya paliurus* [9,10], *Carissa opaca* roots [11] and *Plumeria rubra* flowers [12].

In this work, the fruit of *Nypa fruticans* Wurmb. was boiled and the water extract was used to treat gout. This procedure is from the local knowledge of people in Trat, an eastern province of Thailand with currently no research data about this fruit on the XOD inhibition. In this research, various parts of the *Nypa fruticans* Wurmb. fruit were studied in preliminary phytochemicals, total phenolic and total flavonoids. The samples were extracted with water and ethanol. Antioxidant activity was evaluated for scavenging ability on 1,1-diphenyl-2-picrylhydrazyl (DPPH) and xanthine oxidase inhibitory activity was also evaluated by using allopurinol as a positive control. This screening uses an easy operation to obtain the extracts without isolation or any purification procedures to get the traditional supplementary products approachable for any local communities. This work can be a guideline for the development of herbal medicines for treating gout and adding value to the products from local plants.

Materials and methods

Chemicals and reagents

All chemicals used were of analytical reagent grade. The deionized water used to prepare all solutions was produced by the water purification system (Millipore, Sweden). Phosphate buffer solution (PBS), 0.05 M, pH 7.5 was prepared by dissolving 1.15 g of $NaH_2PO_4.H_2O$ (Ajax Finechem, Australia) and 2.36 g of Na_2HPO_4 (La Jota, Spain) in 500 mL water and adjusted to pH 7.5 at 25 °C. An 0.1 unit/mL XOD enzyme solution was prepared by dissolving 5 unit XOD (5 unit, Sigma-Aldrich, USA) in cold PBS solution. A 0.015 M xanthine solution was prepared by dissolving 0.057 g of xanthine (Sigma-Aldrich, USA) with an addition of a few drops of 1 M NaOH and adjusted to a volume of 25.00 mL with PBS solution. A 0.15 mM xanthine solution was freshly prepared by diluting the stock solution with PBS solution. A stock solution of 1,000 mg/L allopurinol solution was prepared by dissolving 0.025 g of allopurinol (Laboratory Reagent Grade, USA) with an addition of a few drops of 1 M NaOH and adjusted to a volume of 25.00 mL with PBS solution. Working solution of allopurinol was freshly prepared by diluting the stock solution with PBS solution.

Fruit samples

Fresh *Nypa fruticans* Wurmb. fruits (**Figure 1(a)**) at the 4th month maturity were collected from the swamp area in Khlong Khwang Subdistrict, Muang District, Trat Province, Thailand. The fruits were washed under running tap water and dried at room temperature for 4 h. The whole fresh fruit was assigned as sample 1 and endosperm, endocarp, exocarp-mesocarp (**Figure 1(b)**) were assigned as samples 2 - 4, respectively. The 4 samples were separated and cut into small pieces for the extraction procedure.

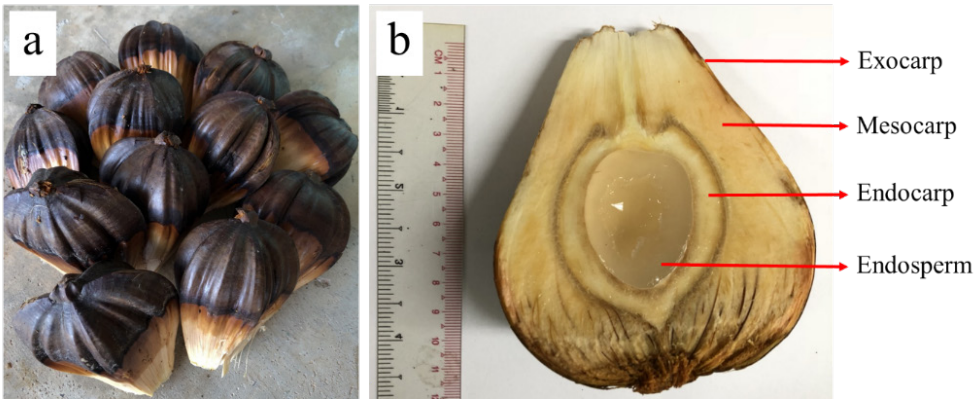

Figure 1 Fresh *Nypa fruticans* Wurmb. fruits (a) and cross-section of individual fruit (b).

Preparation of extracts

The ratio of the sample by the solvent at 1:3 was used for extraction with 3 conditions: (1) water at room temperature, (2) water at 60 °C, the extraction time of 30 min, and (3) ethanol at the extraction time of 24 h in the dark. The water extract solutions were filtered through a filter paper and were freeze-dried. The ethanolic extract solution was filtered through a filter paper and was vacuum evaporated.

Phytochemical screening

Phytochemical screening procedures were modified from Ayoola [13], Farnsworth [14] and Sithara [15]. The extracts were screened for polyphenols, alkaloids, flavonoids, anthraquinones, terpenoids, saponins, cardiac glycosides and iridoid glycosides.

Analysis of antioxidant activity

The antioxidant activity was evaluated by using the DPPH procedure according to the method reported by Gastaldi *et al.* [16] with modification. Briefly, the 0.1 mL of extract solution was mixed with 0.1 mL of DPPH (0.2 mmoL/L, in ethanol) in 0.250 mL 96-well plate. The mixture was left in the dark for 30 min before reading the absorbance at 490 nm by using the microplate reader (Metertech, Taiwan M965+). Ascorbic acid (vitamin C) was used as a reference standard, which was calibrated against the absorbance. The milligram vitamin C equivalent per gram of extract (mg VCE/g) was reported for the evaluation of the radical scavenging activity. Each extract was done in 3 replications.

Determination of total phenolic content

Total phenolic content was determined by using the Folin-Ciocalteu (FC) colorimetric method [17]. The 0.1 mL of each extract were mixed with 0.1 mL of 10 % (v/v) FC reagent and 0.9 mL of water. After 5 min, 1 mL of 7 % (w/v) Na_2CO_3 and 0.4 mL of water were added. The mixture was left in the dark for 30 min before measuring the absorbance using a UV-visible spectrophotometer (G10S, China) at 765 nm. Total phenolic content was expressed as milligram gallic acid equivalent per gram of extract (mg GAE/g). The extract was measured in 3 replications.

Determination of total flavonoid content

Total flavonoid content was determined using a colorimetric method of aluminum trichloride reagent [17]. Each extract (1 mL) was mixed with 0.3 mL of 5 % (w/v) $NaNO_2$. After 5 min, 0.5 mL of 2 % (w/v) $AlCl_3$ was added. A sample was mixed and left for 6 min before adding the 0.5 mL of 1 M NaOH. The mixture was kept in the dark for 10 min and then absorbance was measured at 510 nm using a UV-visible spectrophotometer (G10S, China). A calibration curve was constructed using quercetin and results were expressed as milligram of quercetin equivalent per gram of extract (mg QUE/g). Absorbance was measured in 3 replications.

Evaluation of XOD inhibitory activity

The XOD inhibitory activity was evaluated by spectrophotometric measurement of the uric acid produced from xanthine-XOD reaction. This method was modified from Owen [18] and Havlik [19], the allopurinol was used as a positive control. The assay mixture consisted of 0.100 mL of sample solution, 0.550 mL of 0.05 M PBS (pH 7.5) and 0.300 mL of 0.15 mM xanthine solution. The reaction was initiated by adding 0.050 mL of XOD solution (0.1 unit/mL), and the mixture was subjected to absorbance measurement immediately, then a change in absorbance was recorded at 295 nm for 5 min at room temperature. The XOD inhibitory activity was expressed as percent inhibition of XOD, calculated as follow:

$$\%\text{Inhibition} = \frac{(\Delta A_{\text{control}} - \Delta A_{\text{sample}})}{\Delta A_{\text{control}}} \times 100$$

where $\Delta A_{control}$ is a change in an absorbance of the assay without the sample at the time of 0 - 5 min, ΔA_{sample} is a change in an absorbance of the assay with the sample at the time of 0 - 5 min.

The IC_{50} (50 % inhibitive concentration) value of the XOD inhibitor was then determined by plotting % inhibition (y) versus an inhibitor concentration (x) at the % inhibition close to 50 %, i.e., more than 3 points at lower and higher than 50 % inhibition, respectively.

Results and discussion

The characteristics of water extract at 27±1, 60±1 °C and ethanolic extract of the exocarp-mesocarp, endocarp, and the whole of fruits gave similar red-brown color and viscous. While the endosperm part gave white color and powdery. The weight and yields of all extracts were shown in **Table 1**.

Table 1 Weight and yields of crude extracts.

Crude extracts	Solvents	Weight (g)	Yields (% w/w (wet))
Exocarp-mesocarp	Water (60 °C)	0.37	0.74
	Water (Room temp.)	0.54	1.08
	Ethanol	0.91	1.81
Endocarp	Water (60 °C)	1.19	2.38
	Water (Room temp.)	0.61	1.21
	Ethanol	3.70	7.40
Endosperm	Water (60 °C)	1.98	3.95
	Water (Room temp.)	1.77	3.54
	Ethanol	1.19	2.38
Whole of fresh fruits	Water (60 °C)	2.17	1.42
	Water (Room temp.)	0.59	0.39
	Ethanol	3.62	2.76

From **Table 1**, the yields of all extracts were in the range of 0.39 - 7.40 % (w/w (wet)). The mostly ethanolic extract presented the yield more than the water extracts, especially the endocarp part showed the highest yield of 7.40 %.

Phytochemical screening

The crude extracts obtained from various conditions were studied for the phytochemicals such as phenolic compounds (Ph), saponins (Sa), alkaloids (Al), flavonoids (Fl), terpenoids (Te), anthraquinones (An), iridoid glycosides (Ir), steroids (St), and deoxy sugars (De). The results of phytochemical screening carried out for our mangrove palm are presented in **Table 2**.

Table 2 Phytochemicals of *Nypa fruticans* Wurmb. fruit extracts.

Crude extracts	Solvents	Phytochemicals								
		Ph	Sa	Al	Fl	Te	An	Ir	St	De
Exocarp-mesocarp	Water (60 °C)	✓	×	×	✓	✓	×	×	✓	×
	Water (Room temp.)	✓	✓	×	✓	✓	×	×	✓	×
	Ethanol	✓	×	×	✓	✓	×	×	✓	✓
Endocarp	Water (60 °C)	✓	×	×	✓	✓	×	×	✓	×
	Water (Room temp.)	✓	✓	×	✓	✓	×	×	✓	×
	Ethanol	✓	×	×	✓	✓	×	×	✓	✓
Endosperm	Water (60 °C)	×	×	×	×	×	×	×	×	×
	Water (Room temp.)	×	×	×	×	×	×	×	×	×
	Ethanol	×	×	×	×	×	×	×	×	×
Whole of fresh fruits	Water (60 °C)	✓	×	×	✓	✓	×	×	✓	×
	Water (Room temp.)	✓	✓	×	✓	✓	×	×	✓	×
	Ethanol	✓	×	×	✓	✓	×	×	✓	✓

Key: Ph = Phenolics, Sa = Saponins, Al = Alkaloids, Fl = Flavonoids, Te = Terpenoids, An = Anthraquinones, Ir = Iridoid glycosides, St = Steroids, De = Deoxy sugars.

In the crude water and ethanolic extracts of the exocarp-mesocarp, endocarp, and the whole of fruits have similar phytochemicals. The phenolics, flavonoids, terpenoids, and steroids were detected, while alkaloids, anthraquinones, and iridoid glycoside were not found. In the case of saponin, this group is normally soluble in water due to similar polarity. Therefore, it was detected in the water crude extract of both the exocarp-mesocarp and endocarp without boiling, which was not found in the ethanolic extract. Due to the hydrolysis of saponin were increase at the high temperature [20], the boiling method may cause the saponins to be destroyed, which is not detectable in the extract after boiling. The deoxy sugar is detected only in crude extracts of ethanol due to it may contain aglycone moiety. Thus, these extracts are dissolved well in ethanol. This result corresponded to Chigayo *et al.* [21] report that found cardenolide deoxy sugar in methanol and acetone solvents but not found in water.

Any phytochemicals were not found from the endosperm extracts, while the previous research has reported the positive results [2]. The phytochemicals and antioxidant capacity from *Nypa fruticans* Wurmb. were investigated from endosperms extracts (unripe and ripe fruits). The unripe fruits showed higher content of phenolics, flavonoids and antioxidant capacity as compared to ripe fruits. Chlorogenic acid, protocatechuic acid, and kaempferol were found in the extract which are important phenolic compounds. Thus, unripe endosperm extract could be used as a natural antioxidant. The ripe fruit contains less important substances than the unripe fruit [2]. In our research, due to the different extraction method from the above previous report, the results of endosperm phytochemicals contents may have less concentration than detection limit of our screening method. This result could be confirmed in the part of antioxidant activity, total phenolic and flavonoid content, which showed low activity and content.

Antioxidant activity, total phenolic and flavonoid content

From the results of phytochemical screening, the phenolic and flavonoids were found in most of the crude extracts. Therefore, antioxidant activity, total phenolic and flavonoid content were investigated in all of the extracts, the results were shown in **Table 3**. The content of antioxidants (VCE), phenolic (GAE) and flavonoids (QUE) were reported in mg/g of plant material. In each of crude extracts, the endocarp extracts presented higher content of phenolic, flavonoid and antioxidant activity compared to the others. Especially, the endocarp extracted with ethanol displayed the strongest antioxidant activity (26.79±0.57 mg VCE/g), the highest phenolic (102.85±5.73 mg GAE/g) and flavonoid content (531.02±10.46 mg QUE/g). The second is the endocarp extracted with water (boiling) showed the antioxidant activity of 5.96±0.11 mg VCE/g, the phenolic content of 25.77±0.48 mg GAE/g, and flavonoid content of 86.06±0.74 mg QUE/g. All of the endosperm extracts showed the lowest phenolic, flavonoid content and weakly antioxidant activity, which corresponded to the results in the section of the phytochemical screening.

The phenolic (such as ellagic acid, gallic acid, and ferulic acid) and flavonoid (such as catechin, apigenin, quercetin, morin, myricetin, kaempferol, naringenin) have been reported a high potential for inhibition of XOD, and high antioxidant activity [22-24]. Therefore, the endocarp (which gave the highest yield ethanol extract, phenolic and flavonoid content) showed the corresponding highest antioxidant. These results revealed that the endocarp is an important part of *Nypa fruticans* Wurmb. fruit. It has a high potential source of natural antioxidant to be a good source of phytochemical compounds for use as new food supplements.

Table 3 Content of antioxidants, phenols and flavonoids in extracts.

Part of plants	Extracts	mg VCE/g	mg GAE/g	mg QUE/g
Exocarp-mesocarp	Water (60 °C)	1.93±0.05	5.09±0.20	16.34±0.49
	Water (Room temp.)	2.93±0.07	5.56±0.33	23.45±0.77
	Ethanol	1.46±0.19	2.65±0.21	14.05±0.45
Endocarp	Water (60 °C)	5.96±0.11	25.77±0.48	86.06±0.74
	Water (Room temp.)	2.70±0.04	8.30±0.19	25.09±0.23
	Ethanol	26.79±0.57	102.85±5.73	531.02±10.46
Endosperm	Water (60 °C)	0.25±0.03	1.39±0.08	1.42±0.12
	Water (Room temp.)	0.089±0.011	0.14±0.03	1.03±0.08
	Ethanol	ND	0.33±0.34	0.19±0.05
Whole of fresh fruits	Water (60 °C)	3.26±0.04	7.93±0.29	27.27±0.35
	Water (Room temp.)	0.97±0.02	2.90±0.04	6.87±0.16
	Ethanol	7.46±1.20	12.61±0.56	52.97±1.88

Key: VCE = Vitamin C equivalents, GAE = Gallic acid equivalents, QUE = Quercetin equivalents, ND = Not detection.

XOD inhibitory activity in *Nypa fruticans* Wurmb. fruit extracts

Twelve crude extracts from each part of *Nypa fruticans* Wurmb. fruit were investigated for XOD inhibitory activity, the IC_{50} values were shown in **Table 4**.

Table 4 IC_{50} of various crude extracts in different solvents.

Part of plants	Extracts	IC_{50} (mg/mL)
Exocarp-mesocarp	Water (60 °C)	0.35±0.05
	Water (Room temp.)	0.15±0.05
	Ethanol	0.12±0.01
Endocarp	Water (60 °C)	0.14±0.04
	Water (Room temp.)	% inhibition < 50
	Ethanol	0.029±0.001
Endosperm	Water (60 °C)	NI
	Water (Room temp.)	NI
	Ethanol	NI
Whole of fresh fruits	Water (60 °C)	0.40±0.04
	Water (Room temp.)	0.71±0.10
	Ethanol	0.24±0.06
Allopurinol	Water	0.0081±0.0001
	Ethanol	0.0024±0.0001

Key: NI = No inhibition

From **Table 4**, most of the crude extract of the exocarp-mesocarp, endocarp, and the whole of fruits extracted with water and ethanol presented strongly XOD inhibitory activity (IC_{50} of 0.029 - 0.71 mg/mL). In the case of endosperm extract, no inhibition of XOD was observed, despite the increased concentration of the extracts. For the extraction between water and ethanol, it was found that the ethanolic extracts gave better inhibitory values than those of the water extracts. The crude extract of ethanol from endocarp gave the highest inhibition with IC_{50} of 0.029±0.001 mg/mL. When compared with the standard allopurinol in ethanol solvent (positive control, IC_{50} value 0.0024±0.0001 mg/mL) it gave a lower inhibitory value of approximately 12 times. In the case of water extraction, most of the boiled water extracts showed better inhibitory activity than those of without boiled water. It can be concluded that heat promotes the extraction to get the important substances that show a good inhibitory effect on XOD. The endocarp extracted with boiled water provided the high inhibitory activity with the IC_{50} value of 0.14±0.04 mg/mL. However, it gave a lower inhibitory activity than the standard allopurinol in water (IC_{50} value 0.0081±0.0001 mg/mL) of approximately 17 times.

The inhibitory effect on XOD of the exocarp-mesocarp, endocarp, and the whole of fruits extracted with water and ethanol were related to phytochemicals, such as phenolic compound, flavonoids, terpenoid, and steroids. According to various research reported the phenolic and flavonoid group could be inhibited the XOD [24-26]. The antioxidants, phenols, and flavonoids in those extracts were also found high content (**Table 3**), that affects to be a good inhibitory of XOD (**Table 4**). Especially, ethanol extracts from endocarp provide the highest contents of the antioxidants, phenols, and flavonoids. It also has the strongest XOD inhibition. In part of the endosperm extracted with water and ethanol, all phytochemicals was not found in the screening method. It presented lower content of antioxidants, phenols, and flavonoids that affect not inhibited the XOD.

The fruit of manage palm are a source of phytochemicals and antioxidants with its high activity toward XOD inhibition. It can be developed to be an herbal medicine or supplementary food for healing gout, such as hot brewed teas.

Conclusions

In this work, the antioxidant of *Nypa fruticans* Wurmb. fruit was investigated. The ethanolic extract of endocarp gave the highest activity that is consistent with the highest content of phenolics and flavonoids which are well known as the significant antioxidant substances. Moreover, this extract also showed the highest XOD inhibition activity. The *Nypa fruticans* Wurmb. fruit is the natural source of gout herbal medicine and antioxidant supplement. However, toxicity evaluations are required to make convinced to be safe for food further.

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