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Chemical Constituents from the Leaves and Stems of *Piper ornatum* N.E.Br (Piperaceae) and their Antioxidant Activity

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Plants of the genus *Piper* have long been used as medicinal herbs. The chemistry of *Piper* species has been widely investigated, and phytochemicals investigations led to the isolation of a number of physiologically active compounds. This study was carried out to investigate the phytochemicals isolated from the leaf and stem extracts of *Piper ornatum*. Cold extraction of the dried powdered leaf and stem was employed to extract the phytochemicals according to the polarity gradient of hexane, ethyl acetate, and methanol. The phytochemicals were obtained using chromatography techniques (column chromatography and prep-TLC) and their structures were confirmed by spectroscopic analysis (IR, NMR and MS) and comparison with literature. The isolation was successfully led to the identification of 5,7-dimethoxyflavone (**1**), 4',7-dihydroxy-3',5',5-trimethoxyflavone (**2**), 4'-hydroxy-3',5',5,7-tetramethoxyflavone (**5**) and 3',4',5,5',7-pentamethoxyflavone (**6**), together with piperine (**3**) and β-sitosterol (**4**). Compounds (**2**) and (**3**) showed significant activity towards DPPH radical scavenging (concentration 1,000 μg/mL) with percentage inhibition of 47.5% and 41.5%, respectively. This study may provide valuable and useful information and indications for further exploring the potential nutraceutical and pharmaceutical applications of the *Piper* species.

Keywords: Piperaceae; *Piper ornatum*; constituent; flavonoid; amide

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Antioxidants are crucial in preventing chronic diseases caused by oxidative stress by the accumulation of free radicals in the body, which can lead to oxidative damage and health problems. Antioxidant refers to neutralizing free radicals to reduce damage and protect against diseases. They work by inhibiting the oxidation of molecules, neutralizing free radicals, and preventing or repairing damage caused by oxygen. Cells produce free radicals that cause oxidative damage to proteins with high reactivity. Studies show flavonoids' antioxidant properties can prevent oxidative stresses, making them potential drugs for preventing oxidative stress. Flavonoids, including flavones and catechins, are potent antioxidants, protecting the body against reactive oxygen species [1].

Piperaceae is a family of herbs, shrubs, small trees, and hanging vines with woody stems, aromatic leaves, and fragrant flowers. Its pulpy fruit, stamens, and small drupes indicate its ecological niche and provide food resources for herbivores. They are widespread in warm tropical and subtropical regions and are especially common in South and Central America, Asia, and India. It contains roughly 3600 species in 13 genera, mainly distributed into two main genera: *Piper* (2000 species) and *Peperomia* (1600 species) [2]. *Piper* is the largest genus in the Piperaceae family. This pantropical genus is estimated to contain 2000 species dispersed widely in American and Asian tropics, including Indian, Indonesian, and Malaysian tropical rainforests. Plants from the genus *Piper* have been used for many practical applications, like remedies in traditional medicinal systems such as traditional Chinese medicine, the Indian Ayurvedic system, and folklore medicines of Latin America and the West Indies. Chemical studies carried out on *Piper* species have revealed the presence of several compounds, including alkaloids, amides, propenylphenols, lignans, neolignanas, terpenes, steroids, kawapyrones, piperolides, chalcones, dihydrochalcones, flavones, and flavanones, with various biological activities, such as antioxidant, antimicrobial, antifungal, antityrosinase, anticholinesterase, antituberculosis, antiplasmodial, anti-inflammatory, anti-leishmanial, and insecticidal activities [3-8]. Moreover, many studies on essential oils from *Piper* species have documented monoterpenes, sesquiterpenes,

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and phenylpropanoids with significant biological effects [9-14].

Piper ornatum or *celebes pepper* in Indonesia, is a small shrub native to tropical western South America and Southeast Asia. Its leaves have various spots and colors, with glossy, heart-shaped leaves and olive green leaves with pink and silver mottling. The leaves produce cystolyths and black pepper specs [15]. Recently, we have reported the chemical constituent of the leaf oil of *P. ornatum* [16]. Analysis of the essential oil revealed the presence of twenty-seven components, accounting for 79.6% of the total oil. The major components of essential oil are caryophyllene oxide (31.5%), sphatulenol (5.9%), aromadendrene (4.9%), and β-caryophyllene (4.5%). The essential oil showed significant inhibitory activity on acetylcholinesterase (I%: 70.2%) and butyryl-cholinesterase (I%: 75.8%) activities.

As part of the continuation of our search for bioactive compounds from *Piper* species, we have investigated the phytochemicals present in the stem and leaves of *P. ornatum*. To our knowledge, no report exists on their phytochemical studies and their anti-oxidant activity.

EXPERIMENTAL

Plant Material

The leaves and stems of *P. ornatum* were collected from Muar, Johor, and identified by Dr. Shamsul Khamis from Universiti Kebangsaan Malaysia (UKM). The voucher specimen (SK05/08/22) was deposited at UKM Herbarium. All samples were washed to remove excess sand particles and shed and dried at room temperature.

General Experimental Procedures

A cold extraction technique was applied to extract the phytochemicals from the dried leaf and stem using different polarity solvents (*n*-hexane, ethyl acetate, and methanol). Column chromatography (CC) was performed using Merck silica gel 60 (70-230 mesh) as the stationary phase. Thin layer chromatography (TLC) analysis was performed on Merck precoated silica (SiO_2) gel F_{254} plates (0.22 mm thickness) to detect and monitor the presence of compound samples. The spots were visualized under UV light (254 and 365 nm) and included with spraying reagent vanillin sulphuric acid in MeOH followed by heating. Melting points were measured by comparing them with other literature. The ^{1}H-NMR (500 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded on a Bruker Avance 500 Spectrometer. Chemical shifts were reported in ppm and $CDCl_3$ as solvent. The residual solvent was used as an internal standard. The IR spectra were recorded on Perkin Elmer ETR and 1600 spectro-photometer series as KBr discs or thin film of NaCl discs. Mass spectral data were obtained from Orbitrap Exploris 240 Mass Spectrometer.

Extraction and Isolation

The dried stem (150 g) and leaves (150 g) parts of *P. ornatum* were extracted consecutively by soxhlet extraction with *n*-hexane (5 L), EtOAc (5 L), and MeOH (5 L), respectively. The *n*-hexane extract (POSH - 2.4 g), EtOAc (POSE - 1.6 g), and MeOH (POSM - 0.7 g) of stems were fractionated by CC on silica gel 70-230 mesh. According to the TLC profile, there are similarities in spots; hence, the POSH and POSE extracts were combined as POSHE (4.0 g). The POSHE was purified by CC to give six major fractions (POSHE1-6). The fraction POSHE2 was purified by preparative thin layer chromatography (prep-TLC) to afford compound (**1**) (9.2 mg, 0.23%) ($CHCl_3$:MeOH 98:2). Meanwhile, the fraction POSHE4 was purified by CC followed by washing with diethyl ether to afford compound (**2**) (9.8 mg, 0.25%) and (**3**) (9.1 mg, 1.30%). However, no compounds were found in the methanol extract, POSM. The *n*-hexane extract (POLH - 1.3 g), EtOAC (POLE - 16.2 g), and MeOH (POLM - 1.6 g) of leaves were fractionated using CC on silica gel 70-230 mesh. According to the TLC profile, there are similarities in spots. Hence, the POLE and POLM extracts were combined as POLEM (17.8 g). The POLEM was purified by CC to give nine major fractions (POLEM1-13). Fractions POLEM5 and POLEM 9 were purified by CC, followed by washing with diethyl ether to yield compounds (**5**) (50.7 mg) and (**6**) (100.2 mg), respectively. Meanwhile, POLH extract was purified by CC to give six fractions (POLH1-6). The fraction POLH4 was purified by re-CC followed by washing with diethyl ether to afford compound (**4**) (11.2 mg).

5,7-Dimethoxyflavone (1): white solid (9.2 mg); m.p. 98-99°C; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.95 (3H, s, 7-OCH_3), 3.99 (3H, s, 5-OCH_3), 6.42 (1H, d, J = 2.2 Hz, H-8), 6.62 (1H, d, J = 2.2 Hz, H-6), 6.42 (1H, s, H-3), 7.53 (3H, m, H-3′/H-4′/H-5′), 7.92 (2H, dd, J = 7.6 and 2.0 Hz, H-2′/H-6′); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 55.9 (5-OCH_3), 56.5 (7-OCH_3), 96.6 (C-8), 107.9 (C-3/C-4a), 126.2 (C-2′/C-6′), 129.0 (C-3′/C-5′), 131.0 (C-4′), 160.1 (C-2/C-5), 161.0 (C-7), 164.8 (C-8a), 177.8 (C=O); EIMS: *m/z* 283 [M^+, $C_{17}H_{14}O_4$].

4',7-Dihydroxy-3',5',5-trimethoxyflavone (2): white solid (9.8 mg); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.95 (3H, s, 5-OCH_3), 3.99 (6H, s, 3′/5′-OCH_3), 6.43 (1H, d, J=1.4 Hz, H-8), 6.66 (1H, m, H-6), 6.99 (1H, s, H-3), 7.85 (1H, br.s, 7-OH), 7.87 (1H, br s, 4′-OH); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 55.9 (3′-OCH_3), 56.4 (5′-OCH_3), 61.1 (5-OCH_3), 92.9 (C-8), 96.3 (C-6), 103.5 (C-2′/C-6′), 108.8 (C-3), 109.2 (C-4a), 126.8 (C-1′), 153.6 (C-3′/C-5′), 159.9 (C-8a), 160.6 (C-5), 160.9 (C-2), 164.2 (C-7), 165.9 (C-4′), 177.6 (C=O); EIMS: *m/z* 343 [M^+, $C_{18}H_{16}O_7$].

Piperine (3): light yellow needle crystal (9.1 mg); m.p. 128-130°C; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.60 (2H, m, H-3/H-5), 1.66 (2H, m, H-4), 3.59 (2H, s, H-2/H-6), 5.97 (2H, s, H-2''), 6.44 (1H, d, *J* = 14.6 Hz, H-2'), 6.72 (1H, d, *J* = 15.5 Hz, H-5') 6.78 (1H, d, *J* = 8.0 Hz, H-7''), 6.89 (1H, dd, *J* = 8.0 and 1.6 Hz, H-6''), 7.42 (1H, dd, *J* = 14.7 and 10.2 Hz, H-3′); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 24.2 (C-6), 24.6 (C-4), 26.1 (C-3/C-5), 26.2 (C-2), 101.3 (C-2''), 105.7 (C-4''), 108.5 (C-7''), 119.9 (C-2'), 122.5 (C-6''), 125.3 (C-4'), 138.4 (C-5'), 142.7 (C-3'), 148.1 (C-7a), 148.2 (C-4a), 165.5 (C-1'); EIMS: *m/z* 285 [M^+, $C_{17}H_{19}NO_3$].

β-Sitosterol (4): white crystalline needles (11.2 mg); m.p 133-134°C; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.67 (1H, s, H-18), 0.80 (3H, d, *J* = 6.9 Hz, H-27), 0.82 (3H, d, *J* = 6.8 Hz, H-26), 0.83 (3H, d, *J* = 6.9 Hz, H-29), 0.91 (3H, d, *J* = 6.5 Hz, H-21), 1.00 (3H, s, H-19), 1.02-2.35 (29H, m, overlapping CH and CH_2), 3.52 (1H, m, H-3), 5.34 (1H, d, *J* = 5.2 Hz, H-6); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 12.0 (C-18/C-29), 18.8 (C-21), 19.0 (C-27), 19.8 (C-19/C-26), 21.1 (C-11), 23.1 (C-28), 24.3 (C-15), 29.2 (C-2/C-25), 31.7 (C-7), 31.9 (C-8), 34.0 (C-22), 36.1 (C-20), 36.5 (C-10), 37.3 (C-1), 39.8 (C-12), 42.3 (C-4), 50.1 (C-9), 56.0 (C-17), 56.8 (C-14), 71.8 (C-3), 121.7 (C-6), 140.8 (C-5); EIMS: *m/z* 415 [M^+, $C_{29}H_{50}O$].

4'-Hydroxy-3',5',5,7-tetramethoxyflavone (5): white solid (50.7 mg); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.89 (3H, s, 7-OCH_3), 3.93 (3H, s, 5-OCH_3), 3.95 (6H, s, 3′/5′-OCH_3), 6.38 (1H, d, *J* = 2.2 Hz, H-8), 6.50 (1H, d, *J* = 2.2 Hz, H-6), 6.60 (1H, s, H-3), 7.08 (2H, s, H-2′/H-6′); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 56.3 (3′/5′-OCH_3), 56.8 (7-OCH_3), 61.1 (5-OCH_3), 92.8 (C-6), 96.1 (C-8), 103.9 (C-2′/C-6′), 104.9 (C-3), 105.7 (C-4a), 125.9 (C-1′), 142.8 (C-4′), 153.7 (C-3′/C-5′), 160.7 (C-7), 160.9 (C-5), 162.3 (C-2), 165.7 (C-8a), 182.3 (C=O); EIMS: *m/z* 359 [M^+, $C_{19}H_{18}O_7$].

3',4',5,5',7-Pentamethoxyflavone (6): white solid (100.2 mg); m.p 197°C; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.94 (3H, s, 7-OCH_3), 3.96 (3H, s, 5-OCH_3), 3.98 (6H, s, 3′/5′-OCH_3), 3.99 (3H, s, 4′-OCH_3), 6.42 (1H, m, H-6), 6.60 (1H, d, *J* = 2.1 Hz, H-8), 6.67 (1H, s, H-3), 7.11 (2H, s, H-2′/H-6′); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 55.8 (7-OCH_3), 56.4 (3′/5′-OCH_3), 61.0 (4′-OCH_3), 92.9 (C-8), 96.2 (C-6), 103.4 (C-2′/C-6′), 108.8 (C-3), 109.2 (C-4a), 126.7 (C-1′), 140.9 (C-4′), 153.5 (C-3′/C-5′), 159.8 (C-8a), 160.9 (C-2/C-5), 164.1 (C-7), 177.6 (C=O); EIMS: *m/z* 373 [M^+, $C_{20}H_{20}O_7$].

Antioxidant Activity

The DPPH free radical scavenging assays of phytochemicals were investigated as a previous method [12] with slight modifications. The DPPH solution was freshly prepared in MeOH. The samples were in MeOH (200 µL) with a 1000 µg/mL concentration and mixed with the DPPH solution (3.8 mL). The mixture was allowed to stand for 30 min at room temperature in the dark, and then the absorbance was recorded at 517 nm. The percentage inhibition of DPPH (%) was calculated using the following formula;

$$I\% = [A_{blank} - A_{sample} / A_{blank}] \times 100$$

where A_{blank} is the absorbance value of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance value of the test compounds. Ascorbic acid was used as a standard and diluted to the same concentration as the samples.

RESULTS AND DISCUSSION

In this study, we successfully isolated six compounds from the stem and leaves of *P. ornatum*, which were characterized as flavones, amides, and sterols. They were all identified by analyzing their spectroscopic data and comparing them with the reported literature. Their chemical structures are shown in Figure 1. Compound (**1**) was identified as 5,7-dimethoxyflavone, it exhibited distinctive signals in its NMR spectra, confirming its identity. The presence of methoxy groups at positions C-5 and C-7 was established by the characteristic chemical shifts at δ 3.95 and 3.99, respectively, in the ^{1}H NMR spectrum. The presence of these methoxy groups was further confirmed by the corresponding carbon signals in the ^{13}C NMR spectrum at δ 55.9 and 56.5, respectively. Additionally, the presence of the flavone core structure was evident from the carbon signals at δ 160.1 (C-2/C-5) and 161.0 (C-7) in the ^{13}C NMR spectrum. Compound (**2**) was identified as 4',7-dihydroxy-3',5',5-trimethoxyflavone, it displayed characteristic NMR signals, indicating the presence of two methoxy groups at positions C-3', C-5', and C-7, and hydroxy groups at positions C-4' and C-7. The distinctive signals at δ 3.95 (3H, s, 5-OCH_3), 3.99 (6H, s, 3′/5′-OCH_3), 7.85 (1H, br.s, 7-OH), and 7.87 (1H, br. s, 4′-OH) in the ^{1}H NMR spectrum, along with the corresponding carbon signals in the ^{13}C NMR spectrum, validated the presence of these functional groups. Compound (**3**) was identified as piperine, exhibited characteristic signals in the NMR spectra, confirming its structure. The methoxy groups at C-2' and C-6' were evidenced by the signals at δ 3.59 (2H, s, H-2/H-6) and the corresponding carbon signals in the ^{13}C NMR spectrum at δ 101.3 and 105.7, respectively. The presence of double bonds and the piperidine ring was verified by the distinctive signals in both ^{1}H and ^{13}C NMR spectra. Compound (**4**) was identified as β-sitosterol, displaying characteristic signals in its NMR spectra, confirming its identity. The ^{1}H NMR spectrum exhibited signals corresponding to the aliphatic chain and the double bond, whereas the ^{13}C NMR spectrum displayed signals for the various carbon atoms present in the sterol structure. The distinctive signal at δ 5.34 (1H, d, J = 5.2 Hz, H-6) in the ^{1}H NMR spectrum confirmed the presence of the double bond, while the signals at δ 0.67 (1H, s, H-18) and 0.80-0.91 (various CH_3 and CH_2) validated

the aliphatic chain. Compound (**5**) was identified as 4'-hydroxy-3',5',5,7-tetramethoxyflavone, exhibited characteristic signals in its NMR spectra, confirming the presence of four methoxy groups at positions C-3', C-5', C-7, and C-3. The signals at δ 3.89 (3H, s, 7-OCH_3), 3.93 (3H, s, 5-OCH_3), and 3.95 (6H, s, 3′/5′-OCH_3) in the ^{1}H NMR spectrum, along with the corresponding carbon signals in the ^{13}C NMR spectrum, provided evidence for the presence of these moieties. Compound (**6**) was identified as 3',4',5,5',7-pentamethoxyflavone, displaying characteristic signals in its NMR spectra, confirming the presence of five methoxy groups at positions C-3', C-5', C-4', C-7, and C-3. The signals at δ 3.94 (3H, s, 7-OCH_3), 3.96 (3H, s, 5-OCH_3), 3.98 (6H, s, 3′/5′-OCH_3), and 3.99 (3H, s, 4′-OCH_3) in the ^{1}H NMR spectrum, along with the corresponding carbon signals in the ^{13}C NMR spectrum, provided clear evidence for the presence of these moieties.

Compound (**1**) was isolated previously from the aerial part of *P. abbreviatum* [5] and the leaves of *P. caninum* [8]. In addition, compound (**3**) was isolated previously from the leaves of *P. chaba*, *P. sarmentosum*, and *P. interruptum* [17], *P. longum*, and *P. nigrum* [18]. Compound (**4**) was also reported from the leaves of *P. maingayi* [19], *P. abbreviatum* [5], and *P. erecticaule* [6], whereas compound (**6**) from the roots of *P. alatabaccum* [20]. Interestingly, compounds (**2**) and (**5**) were reported for the first time from the genus *Piper*.

2,2-diphenyl-1-picrylhydrazyl (DPPH) is an affordable approach, quick and easy for the measurement of antioxidant properties as it uses free radicals to assess the potential of substances. Free radicals are molecules or fragments of independent existence that contain an unpaired electron in the atomic orbital, which are generated by cells during cell-mediated immune and respiration. Radicals are unstable and highly reactive due to the presence of an unpaired electron. Oxygen-containing free radicals, such as hydroxyl, superoxide anion, and hydrogen peroxide, are important in many disease states [21]. In the current study, the selected isolated compounds from *P. ornatum* were subjected to DPPH radical scavenging activity, and the results are shown in Table 1.

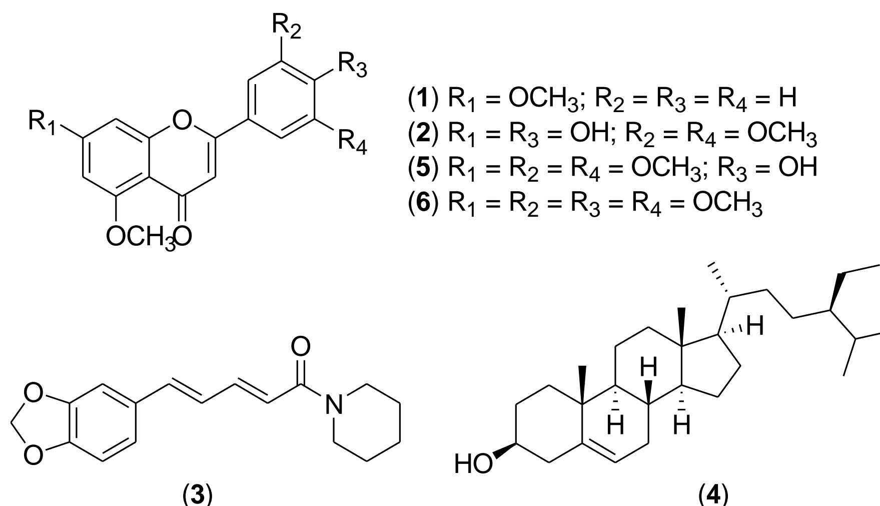

Figure 1. Chemical structures of isolated compounds from *P. ornatum*

Table 1. DPPH radical scavenging of the selected phytochemicals.

Compounds	**Percentage inhibition (%) at 1000 µg/mL**
4',7-Dihydroxy-3',5',5-trimethoxyflavone (**2**)	47.5
Piperine (**3**)	41.5
β-Sitosterol (**4**)	33.3
3',4',5,5',7-Pentamethoxyflavone (**6**)	31.6
Ascorbic acid	95.1

Compound (**2**) showed the highest antioxidant activity with a percentage inhibition of 47.5%, followed by compound (**3**), which inhibited 41.5%. Meanwhile, the other compounds showed moderate activity ranging from 33.3-31.6% inhibition. The activity could be due to the presence of phenolic and hydroxyl groups. In light of previous research, our study's findings on the antioxidant activity of the isolated compounds gain substantial support. Specifically, compound (2) exhibited heightened activity due to its possession of two hydroxyl groups, aligning with the existing literature emphasizing the role of highly hydroxylated flavones in oxidation. However, it is important to note that establishing a definitive structure-activity relationship for flavonoids remains intricate due to their extensive chemical structure diversity. Some studies have underscored the significance of methoxyl groups and sugar moieties, while others have highlighted different substituent positions contributing to potent antioxidant activity, thereby highlighting the intricate nature of these relationships [22].

Additionally, the moderate inhibition demonstrated by piperine (**3**) can be attributed to its amino-substituted phenol aromatic amines. Prior investigations have linked piperine's antioxidant effects to the inhibition of oxidative damage levels, reducing free radicals and reactive oxygen species. Piperine's antioxidative properties are closely associated with its carbon-oxygen five-membered rings and amide structure, inhibiting lipid peroxidation and enhancing glutathione synthesis or transport [23][24].

CONCLUSION

In the present study, the phytochemical investigation of the stem and leaf parts of *P. ornatum* yielded four flavones together with one amide and one sterol. This study is the first report of the occurrence of flavones from this species. Additionally, the isolation and identification of these compounds may have a comprehensive understanding to reveal variants of flavonoid compounds from this species, which might be used as an antioxidant agent. This study also provides useful and valuable information and indications for further research on nutraceutical and pharmaceutical applications of the genus *Piper*.

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