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Original Article

Antioxidant, Antimicrobial and Cytotoxic Activities of Amides and Aristolactams from *Piper wallichii* (Miq.) Hand.-Mazz. Stems

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Abstract: Four amides, namely, piperine (**1**), pellitorine (**2**), piperiline (**5**) and *N-trans-p*-coumaroyltyramine (**7**), and three aristolactams, i.e., piperolactams D (**3**), B (**4**) and A (**6**), were isolated from the methanol extract of *Piper wallichii* stems. Piperiline, piperolactams B and D were obtained from this plant for the first time. Compounds **1**, **3**, **5**, **6** and **7** were evaluated for their antioxidant, antimicrobial and cytotoxic activities. Piperine (**1**) displayed the highest antioxidant activity in scavenging DPPH radicals with an IC_{50} value of 94.51 ± 11.91 µM. Piperolactams D (**3**) and A (**6**) showed antibacterial activity against Gram-positive bacteria (*B. subtilis* and *S. aureus*) with MICs of between 500-1000 µM. All test compounds were cytotoxic to breast cancer (MCF-7) cells, while the aristolactams were more toxic to colon cancer (Caco-2) cells than the amides. Compounds **1**, **3**, **6** and **7** were moderately cytotoxic to the doxorubicin-resistant MCF-7 subline (MCF-7/DOX). All compounds were non-toxic to normal human fibroblast (NIH/3T3) cells.

Keywords: *Piper wallichii*, amides, aristolactams, antioxidant, antimicrobial, cytotoxicity.

Introduction

Approximately 2,000 Piper species (Piperaceae) are distributed worldwide, mostly in tropical forests, and more than 40 of these plants have been recorded in Thailand [1,2]. Many of them, including black pepper (*Piper nigrum*), Javanese long pepper (*P. retrofractum*) and betel vine (*P. betle*), are aromatic climbing plants widely utilized as culinary ingredients and herbal medicine. These *Piper* plants contain various terpenoids, alkaloids, flavonoids, lignans and phenolic compounds which display biological activities such as antiproliferative/anticancer, anti-inflammatory and neuropharmacological activities [3,4]. Several *Piper* species that have been used traditionally as cancer remedies contain amide-type alkaloids,

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including the pungent-tasting piperine, as their active constituents [5]. Another distinct type of alkaloids called aristolactams (or piperolactams) found in a number of *Piper* species [6] has been demonstrated to possess anti-platelet aggregation [7], anti-inflammatory [8], antileishmanial [9] and antitumor activities [10].

The stem of *Piper wallichii* (Miq.) Hand.-Mazz. is a herbal material listed in Thai Herbal Pharmacopoeia for its carminative and anti-inflammatory effects [11]. The plant is a woody climber usually found in semi-evergreen and rainy deciduous forests of South China, South Asia and Southeast Asia [12-13]. Its distribution in Thailand is mainly in the northern and northeastern regions [14]. In China, *P. wallichii* stems are used to treat rheumatoid arthritis, inflammatory diseases, cerebral infarction and angina [3,15-16]. Local healers in northeastern Thailand also use this plant to stimulate blood circulation and to treat influenza and asthma [14]. Previous phytochemical studies on *P. wallichii* stems have revealed the presence of several amides (dihydropiperlonguminine, futoamide, guineensine, *N*-isobutyl-2*E*,4*E*-octadecadienamide, *N*-*trans*-*p*-coumaroyltyramine, *N*-*trans*-feruloyltyramine, pellitorine, piperine and piperlonguminine) [15-18], aristolactams (aristolactams AII and AIIIa, cepharanone B, goniothalactam, piperolactam A and stigmalactam) [15,19-20], dioxoaporphine alkaloids (cepharadione A and piwallidione) [18], diketopiperazine alkaloid (neoechinulin A) [21], lignans [16-17,20,22] and phenolic compounds [17,20-21]. The last two groups of compounds might be responsible for the antithrombotic activity of this plant [20]. Both dioxoaporphine alkaloids displayed antibacterial activity against three pathogenic Gram-positive bacteria [18]. In addition, *P. wallichii* extracts were able to inhibit the proliferation of human tongue squamous cell carcinoma Tca83 cells [16]. The present study reports the amide- and aristolactam-type alkaloidal constituents of the methanol extract and their *in vitro* antioxidant, antimicrobial and cytotoxic activities against two human cancer cell lines and two drug-resistant sublines.

Materials and methods

General experimental procedures

Thin-layer chromatography (TLC) detection was performed on pre-coated silica gel 60 F_{254} plates (E. Merck, Darmstadt, Germany) and visualized by spraying with 10% sulfuric acid and heated. ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were obtained on a Bruker DPX-300 FT-NMR spectrometer. ESI-MS spectra were recorded on a Bruker Daltonics microTOF mass spectrometer (Bruker Corp., Billerica, MA, USA). Absorbances were measured on a Wallac 1420 Victor 3 microplate reader (PerkinElmer Inc., Massachusetts, USA). All solvents used were of commercial grade and were redistilled prior to use. All chemicals were of analytical grade and were purchased from Sigma Aldrich Inc. (St. Louis, MO, USA).

Plant material

Dried *P. wallichii* stems (3 kg) were purchased from a traditional drugstore in Bangkok, Thailand, in July 2016 and authenticated by one of the authors (R. Suttisri). A voucher specimen of this plant (No. RS16071) has been deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

Isolation of alkaloids from methanol extract

Dried, powdered *P. wallichii* stems (3 kg) were macerated in MeOH (3 × 10 L, 3 days each). The combined extract was filtered through cotton wool and concentrated under reduced pressure in a rotary evaporator at 45°C to obtain MeOH extract (100 g), which was subjected to silica gel column chromatography (CC) eluted with a gradient of *n*-hexane/acetone (7:3 to 3:7) to obtain 6 fractions (A-F). Gel filtration of fraction A on Sephadex LH-20 columns eluted with MeOH and CH_2Cl_2/MeOH (1:1), respectively, gave a mixture of β-sitosterol and stigmasterol [23] (22.5 mg). Fraction B (14 g) was purified by precipitation in MeOH to yield compound **1** (4.2 g). The rest of this fraction (9.8 g) was loaded on a Sephadex LH-20 column eluting with CH_2Cl_2/MeOH (1:1) to afford 3 subfractions (B21-B23). Subfraction B23 was further separated by silica gel CC, eluting with CH_2Cl_2/acetone (9:1), into 6 subfractions (B231-B236). A mixture (12.3 mg) of compound **2** and a fatty acid was obtained from subfraction B234. Silica gel CC of fraction

C (4.3 g), eluted with CH_2Cl_2/acetone (9:1), yielded 5 subfractions (C1-C5). Subfraction C3 (0.6 g) was further separated by silica gel CC, eluted with CH_2Cl_2, into 5 subfractions (C31-C35). Subfraction C34 was a 9:1 mixture of compounds **3** and **4** (2.4 mg), whereas subfraction C35 yielded **3** (5.6 mg) as a pure compound. Sephadex LH-20 CC of subfraction C33 (0.2 g), washed down with CH_2Cl_2/MeOH (1:1), afforded 4 subfractions (C331-C334). Compound **5** (13.2 mg) was obtained from subfraction C332 (70 mg) by silica gel CC with CH_2Cl_2/acetone (9:1) as the mobile phase. Subfraction C4 (1.0 g) was separated by Sephadex LH-20 CC, eluted with CH_2Cl_2/MeOH (1:1), into 4 subfractions (C41-C44). Silica gel CC of subfraction C44 (0.1 g), washed down with CH_2Cl_2/acetone (18:1), gave 4 subfractions (C441-C444). Subfraction C442 (57 mg) was further separated on a silica gel column, eluted with CH_2Cl_2/acetone (40:1), into 3 subfractions (C4421-C4423). Repeated Sephadex LH-20 CC of subfraction C4423 (32 mg) eluting with CH_2Cl_2/MeOH (1:1) afforded compound **6** (12.4 mg). Sephadex LH-20 CC of fraction D (2.1 g) eluting with CH_2Cl_2/MeOH (1:1) gave 4 subfractions (D1-D4). Subfraction D3 (0.7 g) was further separated on a silica gel column, using CH_2Cl_2/MeOH (9:1) as mobile phase, into 4 subfractions (D31-D34). Compound **7** (36.3 mg) was obtained after Sephadex LH-20 CC of subfraction D32 (90 mg) with CH_2Cl_2/MeOH (1:1) as an eluent.

a) Piperine (**1**)

Yellow needles, ESI-MS *m/z* calcd. for $C_{17}H_{20}NO_3$ $[M+H]^+$ 286.1443, observed- 286; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 7.38 (1H, ddd, J = 14.7, 7.2, 2.7 Hz, H-3′), 6.95 (1H, d, J = 1.7 Hz, H-4″), 6.86 (1H, dd, J = 8.1, 1.7 Hz, H-6″), 6.75 (1H, d, J = 8.1 Hz, H-7″), 6.72 (1H, m, H-5′), 6.71 (1H, m, H-4′), 6.41 (1H, d, J = 14.7 Hz, H-2′), 5.94 (2H, s, H-2″), 3.61 (2H, br s, H-6), 3.50 (2H, br s, H-2), 1.63 (2H, m, H-4), 1.57 (4H, m, H-3/H-5); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 165.4 (C-1′), 148.2 (C-3a″), 148.1 (C-7a″), 142.4 (C-3′), 138.2 (C-5′), 131.0 (C-5″), 125.3 (C-4′), 122.5 (C-6″), 120.1 (C-2′), 108.4 (C-7″), 105.6 (C-4″), 101.3 (C-2″), 46.9 (C-6), 43.2 (C-2), 26.7 (C-5), 25.6 (C-3), 24.6 (C-4). The data were in agreement with the reported literature values [24].

b) Pellitorine (**2**)

Colorless needles; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 7.16 (1H, dd, J = 15.0, 9.5 Hz, H-3), 6.10 (1H, dd, J = 9.5, 6.0 Hz, H-4), 6.04 (1H, m, H-5), 5.88 (1H, s, NH), 5.78 (1H, d, J = 15.0 Hz, H-2), 3.11 (2H, t, J = 6.6 Hz, H-1′), 2.09 (2H, m, H-6), 1.76 (1H, m, H-2′), 1.37 (2H, m, H-7), 1.26 (4H, m, H-8, H-9), 0.87 (6H, d, J = 6.6 Hz, H-3′, H-4′), 0.85 (3H, t, H-10). The data were in agreement with the reported literature values [25].

c) Piperolactam D (**3**)

Orange needles, ESI-MS *m/z* calcd. for $C_{17}H_{12}NO_4$ $[M-H]^-$ 294.0766, observed- 294; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 9.21 (1H, dd, J = 7.2, 2.1 Hz, H-5), 7.93 (1H, br s, 4-OH), 7.86 (1H, dd, J = 7.2, 2.1 Hz, H-8), 7.58 (2H, m, H-6, H-7), 7.45 (1H, s, NH), 7.22 (1H, s, H-9), 4.56 (3H, s, 2-OCH_3), 4.15 (3H, s, 3-OCH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 167.2 (C-1), 152.8 (C-4), 152.4 (C-2), 138.3 (C-3), 133.1 (C-9a), 132.9 (C-8a), 128.5 (C-8), 127.1 (C-5), 126.8 (C-4b), 126.5 (C-9b), 126.3 (C-7), 125.6 (C-6), 111.3 (C-1a), 106.0 (C-9), 105.3 (C-4a), 63.0 (2-OCH_3), 61.9 (3-OCH_3). The data were in agreement with the reported literature values [26].

d) Piperolactam B (**4**)

Orange needles; ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 9.20 (1H, m, H-5), 7.98 (1H, br s, 2-OH), 7.84 (1H, m, H-8), 7.60 (2H, m, H-6, H-7), 7.45 (1H, s, NH), 7.23 (1H, s, H-9), 4.15 (3H, s, 3-OCH_3), 4.10 (3H, s, 4-OCH_3). The data were in agreement with the reported literature values [26].

e) Piperiline (**5**)

Yellow needles, ESI-MS *m/z* calcd. for $C_{16}H_{18}NO_3$ $[M+H]^+$ 272.1287, observed- 272; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 7.39 (1H, dd, J = 14.7, 9.8 Hz, H-3′), 6.97 (1H, d, J = 1.4 Hz, H-4″), 6.88 (1H, dd, J = 8.1, 1.4 Hz, H-6″), 6.79 (1H, m, H-5′), 6.74 (1H, d, J = 8.1 Hz, H-7″), 6.72 (1H, m, H-4′), 6.24 (1H, d, J = 14.7 Hz, H-2′), 5.95 (2H, s, H-2″), 3.55 (2H, t, J = 3.5 Hz,

H-5), 3.52 (2H, t, *J* = 3.5 Hz, H-2), 1.96 (2H, m, H-4), 1.86 (2H, m, H-3); ^{13}C NMR (75 MHz, $CDCl_3$) *δ* ppm 165.2 (C-1′), 148.4 (C-3a″), 148.3 (C-7a″), 142.1 (C-3′), 139.0 (C-5′), 131.1 (C-5″), 125.3 (C-4′), 122.8 (C-6″), 121.5 (C-2′), 108.7 (C-7″), 105.9 (C-4″), 101.5 (C-2″), 46.7 (C-2), 46.1 (C-5), 26.3 (C-3), 24.5 (C-4). The data were in agreement with the reported literature values [27].

f) Piperolactam A (6)

Pale yellow amorphous powder, ESI-MS *m/z* calcd. for $C_{16}H_{12}NO_3$ $[M+H]^+$ 266.0795, observed- 266; ^{1}H NMR (300 MHz, $CDCl_3$) *δ* ppm 9.25 (1H, m, H-5), 7.85 (1H, m, H-8), 7.79 (1H, s, H-2), 7.60 (2H, m, H-6, H-7), 7.31 (1H, s, NH), 7.13 (1H, s, H-9), 4.15 (3H, s, 3-OCH_3); ^{13}C NMR (75 MHz, $CDCl_3$) *δ* ppm 169.5 (C-1), 148.5 (C-4), 147.1 (C-3), 134.2 (C-9a), 133.8 (C-8a), 128.8 (C-8), 128.6 (C-4b), 128.4 (C-9b), 127.1 (C-5), 127.1 (C-7), 125.8 (C-6), 116.4 (C-1a), 114.7 (C-4a), 107.4 (C-2), 105.8 (C-9), 57.3 (3-OCH_3). The data were in agreement with the reported literature values [28].

g) *N-trans-p*-coumaroyltyramine (7)

White amorphous powder, ESI-MS *m/z* calcd. for $C_{17}H_{18}NO_3$ $[M+H]^+$ 284.1287, observed- 284; ^{1}H NMR (300 MHz, CD_3OD) *δ* ppm 7.45 (1H, d, *J* = 15.6 Hz, H-7), 7.40 (2H, d, *J* = 8.4 Hz, H-2/H-6), 7.06 (2H, d, *J* = 8.2 Hz, H-2′/H-6′), 6.80 (2H, d, *J* = 8.4 Hz, H-3/H-5), 6.73 (2H, d, *J* = 8.2 Hz, H-3′/H-5′), 6.39 (1H, d, *J* = 15.6 Hz, H-8), 3.47 (2H, t, *J* = 7.2 Hz, H-7′), 2.76 (2H, t, *J* = 7.2 Hz, H-8′); ^{13}C NMR (75 MHz, CD_3OD) *δ* ppm 169.2 (C-9), 160.5 (C-4′), 156.9 (C-4), 141.8 (C-7), 131.3 (C-1′), 130.7 (C-2′/C-6′), 130.6 (C-2/C-6), 127.7 (C-1), 118.4 (C-8), 116.7 (C-3′/C-5′), 116.2 (C-3/C-5), 42.6 (C-8′), 35.8 (C-7′). The data were in agreement with the reported literature values [29].

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Antioxidant activity of the isolated amides and aristolactams was evaluated using the DPPH radical scavenging assay [30]. Different concentrations (25-1000 μM) of the alkaloids and standard were prepared in MeOH. Each sample solution (100 μL) was mixed with an equal volume of 0.3 mM DPPH in MeOH in a 96-well microplate. The reaction mixture was incubated in the dark at room temperature for 2 h, then its absorbance was measured at 517 nm. The percentage of DPPH radical scavenging activity was calculated as follows:

DPPH scavenging activity (%) = $[(A_{control} - A_{sample})/ A_{control}] \times 100$

where $A_{control}$ is the absorbance of 0.3 mM DPPH solution and A_{sample} is the absorbance of sample or standard solution. Ascorbic acid was used as antioxidant standard. The results were reported as IC_{50} (in μM) calculated from concentration-response curve.

Antimicrobial assay

Antimicrobial activity of the alkaloids was assayed against two Gram-positive bacteria (*Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 25923), two Gram-negative bacteria (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) and one pathogenic yeast (*Candida albicans* ATCC 10231). Minimum inhibitory concentrations (MICs), which were the lowest concentrations of samples that could prevent visible microbial growth, were determined using broth dilution method [31]. Clarithromycin, tetracycline and ketoconazole were used as positive controls against Gram-positive bacteria, Gram-negative bacteria and yeast, respectively.

Cell cultures

Human colon cancer (Caco-2), breast cancer (MCF-7) and normal fibroblast (NIH/3T3) cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute 1640 (RPMI-1640) medium, fetal bovine serum (FBS) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Gibco Life Technologies (Grand Island, NY, USA). Caco-2 and NIH/3T3 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM), whereas the MCF-7 cell line

and its doxorubicin-resistant and mitoxantrone-resistant sublines (MCF-7/DOX and MCF-7/MX) [32-33] were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium. These cell cultures were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and kept at 37ºC in a humidified atmosphere with 5% CO_2.

Cytotoxicity assay

In vitro cytotoxic activity of the amide- and aristolactam-type alkaloids against Caco-2, MCF-7 and NIH/3T3 cells, as well as two chemotherapy-resistant MCF-7 sublines (MCF-7/DOX and MCF-7/MX), was evaluated by MTT assay [34]. The cells were seeded onto 96-well plates at a density of 5×10^3 cells/well and cultured for 24 h. Then, they were incubated with different concentrations of the test compounds at 37 °C for 72 h. After treatment, the cells were washed and further incubated in serum-free medium containing 0.83 mg/ml MTT solution for 3 h. The formazan crystals in these cells were dissolved with dimethyl sulfoxide (DMSO) and the absorbance was measured at 570 nm with a microplate reader. Percentage inhibition of cell growth was calculated as follows:

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

where $A_{control}$ is the absorbance of control solution (without test compound) and A_{sample} is the absorbance of the solution with test compound. Vinblastine was used as positive control. The results were reported as IC_{50} values (in μM) calculated from linear regression analysis of the concentration-response curve.

Results and discussions

The chromatographic separation of methanol extract of *P. wallichii* stems led to the isolation of seven amide- and aristolactam-type alkaloids (**Figure 1**), as well as a mixture of β-sitosterol and stigmasterol. They were characterized by comparison of their spectral data (Supplementary Figures S1-S16) with those reported in the literature and identified as piperine (**1**), pellitorine (**2**), piperolactam D (**3**), piperolactam B (**4**), piperiline (**5**), piperolactam A (**6**) and *N-trans-p*-coumaroyltyramine (**7**).

Compound **1** was the most abundant constituent of *P. wallichii* stems isolated in this study. Its molecular formula of $C_{17}H_{19}NO_3$ was determined from ESI-MS data. ^{1}H NMR and

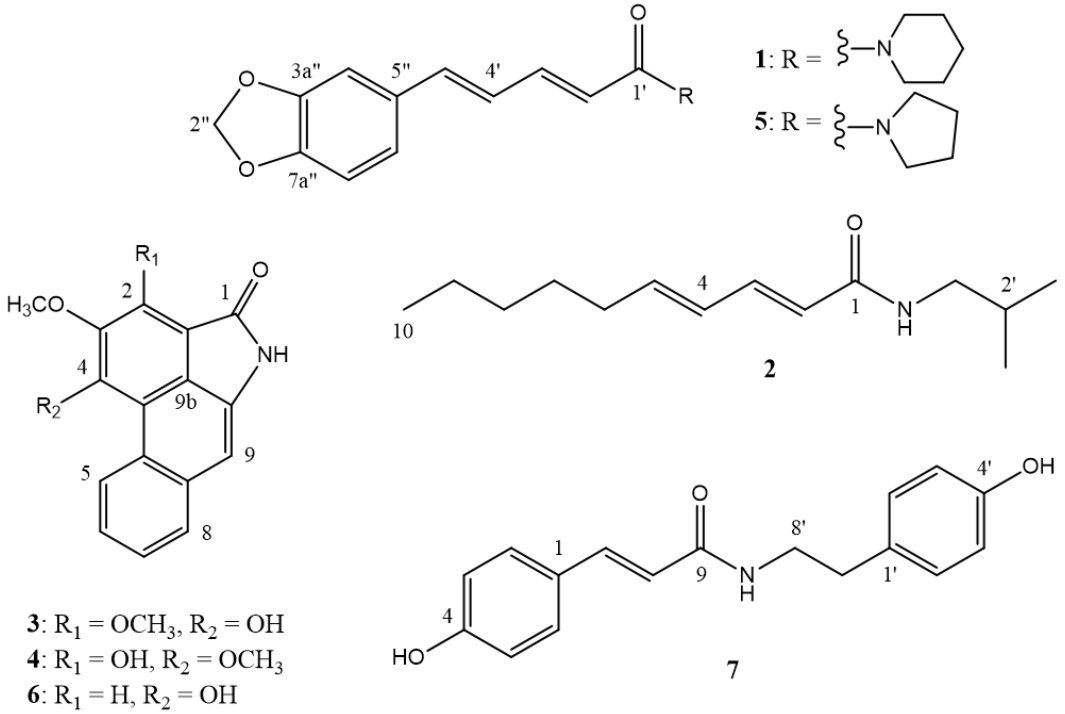

Figure 1. Chemical structures of compounds 1-**7**

^{13}C NMR spectra of compound **1** showed the presence of a piperonyl ring (at δ_H 5.94, 6.75, 6.86 and 6.95 ppm), an acyl piperidine group (at δ_C 24.6, 25.6, 26.7, 43.2, 46.9 and 165.4 ppm) and a butadiene chain. Based on these spectral data and comparison with reference standards available in the laboratory, the compound was identified as piperine [24], a well-known amide responsible for the pungent taste of this herbal material and its use as a spice in some Asian locales [13]. Compound **2**, which was obtained as part of a mixture with an unidentified fatty acid, displayed the presence of a secondary amide (NH) proton at δ 5.88 ppm, an isobutyl group at δ 0.87 (6H, t, *J* = 6.6 Hz), 1.76 (1H, m) and 3.11 (2H, t, *J* = 6.6 Hz) and a nonadiene chain in its ^{1}H NMR data. Comparison of the data with literature helped tentatively identify this compound as pellitorine [25]. Compound **5** was similar to piperine in its appearance but its molecular weight of 271 was lower by 14 mass units, indicating one less methylene group. Its ^{1}H and ^{13}C NMR data showed evidence for a piperonyl ring and a butadiene chain similar to compound **1**. The difference was the presence of an acyl pyrrolidine group (at δ_C 24.5, 26.3, 46.1, 46.7 and 165.2 ppm) instead of an acyl piperidine, as in piperine. Based on these spectral data and comparison with literature values, compound **5** was confirmed as piperiline [27]. Molecular formula $C_{17}H_{17}NO_3$ of the fourth amide, compound **7**, was deduced from its pseudo-molecular ion $[M+H]^+$ peak at *m/z* 284 in the ESI mass spectrum. In addition to an amide carbonyl resonance at δ_C 169.2 ppm, its NMR spectra also displayed signals of two *para*-substituted benzene rings at δ_H 6.73 (2H, d, *J* = 8.2 Hz), 7.06 ppm (2H, d, *J* = 8.2 Hz) and 6.80 (2H, d, *J* = 8.4 Hz), 7.40 ppm (2H, d, *J* = 8.4 Hz), a *trans* double bond (*J* = 15.6 Hz) at δ_H 6.39 and 7.45 ppm, and two methylene triplets (*J* = 7.2 Hz) at δ_H 2.76 and 3.47 ppm. Therefore, compound **7** was identified as *N-trans-p*-coumaroyltyramine [29].

The molecular formula of compound **3**, which was found to be $C_{17}H_{13}NO_4$ based on its ESI-MS data, equals to 12 degrees of unsaturation. The yellow colour of this compound, its degrees of unsaturation and the presence in its NMR spectra of NH proton singlet at δ 7.45 ppm and an amide carbonyl at δ_C 167.2 ppm indicated a phenanthrene moiety and a lactam ring of aristo lactam-type alkaloid. Its ^{1}H NMR spectrum revealed five coupled aromatic resonances, one of which appeared most downfield at 9.21 ppm (H-5) characteristic of this ring system. Two methoxy singlets (at δ 4.15 and 4.56 ppm) and one broad hydroxy singlet (at δ 7.93 ppm) were also observed. The notable downfield shift (4.56 ppm) of one methoxy signal suggested that this methoxy substituent was at C-2 and was therefore deshielded by the *peri*-carbonyl group of the lactam ring, whereas another methoxy group and a hydroxy group were located at C-3 and C-4, respectively. These spectral data were in agreement with the reported values for piperolactam D [26]. Compound **4**, which is an isomer of compound **3**, displayed similar NMR data except its methoxy signals were at δ_H 4.10 and 4.15 ppm, indicating their difference in the 2,3,4-substitution pattern whereby methoxy groups in this compound were at C-3 and C-4. Comparison of its NMR data with literature led to its tentative identification as piperolactam B [26]. Compound **6** exhibited its pseudo-molecular ion $[M+H]^+$ at *m/z* 266, consistent with molecular formula of $C_{16}H_{11}NO_3$. Its ^{1}H NMR spectrum showed six aromatic protons, one methoxy (at δ 4.15 ppm) and one NH singlet (at δ 7.31 ppm). The sharp H-2 singlet at δ_H 7.79 ppm and the shielded H-5 signal at δ_H 9.25 ppm indicated that its methoxy and hydroxy groups were located at C-3 and C-4, respectively, of this aristolactam molecule. Finally, a comparison of these data with previous reports revealed compound **6** as piperolactam A [28].

Five of these alkaloids (**1**, **3**, **5**, **6** and **7**), which were obtained in pure form, were assayed for their antioxidant, antimicrobial and cytotoxic activities.

All aerobic cells can produce reactive oxygen and nitrogen species (RONS) which play an important role in the pathogenesis of aging and various age-related diseases. Imbalance between RONS production and the antioxidant defense mechanisms of the body can cause oxidative stress. Antioxidant therapy, by supplying exo-

genous antioxidants such as ascorbic acid, may alleviate the progression of several diseases [35]. Therefore, plant-derived antioxidants, particularly polyphenols, can be useful in promoting human health [36]. Previous studies on *P. wallichii* showed that the methanol extracts of its fruits and leaves possessed antioxidant activity [13,37]. Two phenolic compounds, namely, vanillic acid and 4-hydroxy-3,5-dimethoxy-benzoic acid [19], and an amide derivative [15] found in its stem displayed scavenging activity against the DPPH radical. In this study, among the isolated alkaloids, piperine (**1**) displayed the best antioxidant activity (IC_{50} = 94.51 ± 11.91 μM) in the DPPH assay, although it was about two folds less potent than ascorbic acid (IC_{50} = 39.94 ± 0.14 μM) (Table 1). Several reports have reported the *in vitro* and *in vivo* antioxidant activity of piperine [38-39]. This amide alkaloid could also act as a bio-enhancer by potentiating the activities of other biologically active compounds and should be useful in drug formulation and design [1]. Another amide, *N-trans-p*-coumaroyltyramine (**7**), which displayed an IC_{50} value of 453.92 ± 10.64 μM, was a weak antioxidant. Piperiline (**5**) and both aristolactams (**3** and **6**) were inactive (IC_{50} values > 1000 μM).

Piper species are aromatic plants rich in volatile oils. Many of these oils exhibit antimicrobial properties and could be used as potential alternatives to synthetic compounds in the fight against resistance developed by pathogenic microorganisms [40]. Therefore, *Piper* plants could be used as natural antimicrobial agents in preserving food [3]. The volatile oil of *P. wallichii* has been analyzed and reported to contain many terpenoids [41]. Its fruit extract has been incorporated into antibacterial gold nanoparticles active against *P. aeruginosa* and *S. aureus* [42]. Moreover, two dioxoaporphine alkaloids isolated from its leaves and stems exhibited antibacterial activity against *Bacillus cereus, B. subtilis* and *S. aureus* [17]. In this study, piperolactams D (**3**) and A (**6**) were specifically active against *B. subtilis* and *S. aureus* (Table 2), in agreement with previous reports of antimicrobial activity of aristolactams against Gram-positive bacteria [13]. Compound **3** was more active (MIC = 500 μM) against both bacteria than compound **6** (MIC = 1000 μM). However, none of the test compounds was active against the representative Gram-negative bacteria and *C. albicans*.

P. wallichii stem is an ingredient of the Benjakul formulation dispensed by practitioners of Thai traditional medicine to treat cancer [43]. *P. wallichii* stem extracts have been reported to display anti-proliferative effect against human tongue squamous cell carcinoma (Tca83) cells and their mechanism of action might be inhibition of telomerase activity [16]. Three aristolactams from this plant were demonstrated to be cytotoxic to human kidney (HK-2) cells [19]. All amides and aristolactams tested in this study were non-toxic to normal human fibroblast (NIH/3T3) cells. They were cytotoxic to breast cancer (MCF-7) cells (Table 3) but were less active than the positive control vinblastine. Piperolactams D (**3**) and B (**6**) were slightly more cytotoxic to MCF-7 cells than all three amides tested. They were also more cytotoxic to colon cancer (Caco-2) cells than piperiline (**5**) and *N-trans-p*-coumaroyltyramine (**7**), while piperine (**1**) was inactive against this cell line. Piperolactam D (**3**) (IC_{50} = 8.59 ± 0.57

Table 1. DPPH scavenging activity of amides and aristolactams from *P. wallichii* stems

Compound	IC_{50} (μM)
Piperine (**1**)	94.51±11.91
Piperolactam D (**3**)	>1000
Piperiline (**5**)	>1000
Piperolactam A (**6**)	>1000
N-trans-p-coumaroyltyramine (**7**)	453.92±10.64
Ascorbic acid	39.94±0.14

Values are mean ± SD (n = 3)

Table 2. Antimicrobial activity of amides and aristolactams from *P. wallichii* stems

Compound	MIC (μM)				
	B. subtilis	***S. aureus***	***E. coli***	***P. aeruginosa***	***C. albicans***
1	>1000	>1000	>1000	>1000	>1000
3	500	500	>1000	>1000	>1000
5	>1000	>1000	>1000	>1000	>1000
6	1000	1000	>1000	>1000	>1000
7	>1000	>1000	>1000	>1000	>1000
Clarithromycin	0.78	0.78	NT	NT	NT
Tetracycline	NT	NT	2.81	22.5	NT
Ketoconazole	NT	NT	NT	NT	62.5

NT = Not tested. MIC: minimum inhibitory concentration. Values are mean ± SD (n = 3)

Table 3. Cytotoxicity of amides and aristolactams from *P. wallichii* stems

Compound	IC_{50} (μM)				
	MCF-7	**MCF-7/DOX**	**MCF-7/MX**	**Caco-2**	**NIH/3T3**
1	52.95±0.39	43.54±0.60	>100	>100	>100
3	15.87±0.94	21.79±1.62	51.09±2.10	8.59±0.57	>100
5	40.64±0.82	>100	>100	97.05±1.38	>100
6	24.81±0.61	45.25±1.06	>100	20.68±0.71	>100
7	41.02±1.81	77.39±0.80	>100	49.04±1.12	>100
Vinblastine	2.47±1.48	>5	>5	5.32±0.88	>5

Values are mean ± SD (n = 3)

μM) was nearly as cytotoxic as vinblastine (IC_{50} = 5.32 ± 0.88 μM) against the Caco-2 cell line. All tested compounds, except piperiline (**5**), displayed moderate cytotoxic activity against doxorubicin-resistant MCF-7 cancer cells (MCF-7/DOX), whereas only piperolactam D (**3**) was active against mitoxantrone-resistant MCF-7 cancer cells (MCF-7/MX) with an IC_{50} of 51.09 ± 2.10 μM. In contrast with other test compounds, the cytotoxic effects of piperine (**1**) against non-resistant and doxorubicin-resistant MCF-7 cells were comparable. Piperine was known to be an inhibitor of both P-glycoprotein and several metabolizing enzymes [44-45] and its undiminished effect in some drug-resistant cancer cells might be due to this mechanism of action.

Conclusion

The phytochemical study of *P. wallichii* stems led to the purification of seven alkaloids including four amide-type (1, 2, 5, and 7) and three aristolactam-type ones (3, 4 and 6). Piperolactams D (**3**) and B (**4**) and piperiline (**5**) were isolated from *P. wallichii* for the first time. All compounds, except pellitorine (**2**) and piperolactam B (**4**), were assessed for their antioxidant, antimicrobial and cytotoxic activities. Piperine (**1**) showed the best antioxidant activity, while piperolactams D (**3**) and A (**6**) were active against the Gram-positive bacteria *B. subtilis* and *S. aureus*. Nearly all alkaloids tested were moderately cytotoxic to both Caco-2 and MCF-7 cancer cell lines, whereas some compounds were also active against two chemotherapy-resistant MCF-7 sublines. None of them was cytotoxic to normal fibroblast (NIH/3T3) cell line. The present study confirms the usefulness of this plant as an ingredient in Thai traditional remedy.

Declaration of conflict of interest

The authors report there are no competing interests to declare.

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