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SHORT COMMUNICATION

CHEMICAL CONSTITUENTS AND LIPOXYGENASE INHIBITORY ACTIVITY OF *PIPER STYLOSUM* MIQ.

Wan Mohd Nuzul Hakimi Wan Salleh[1*], Nur Athirah Hashim[2] and Shamsul Khamis[3]

[1]Department of Chemistry, Faculty of Science and Mathematics, Universiti Pendidikan Sultan Idris, 35900 Tanjung Malim, Perak, Malaysia
[2]Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia
[3]School of Environmental and Natural Sciences, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia

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ABSTRACT. Chemical constituents and lipoxygenase inhibitory activity of the aerial part of *Piper stylosum* have been studied. Fractionation and purification of the extracts afforded five lignans, identified as yangambin (**1**), sesamin (**2**), syringaresinol (**3**), pinoresinol (**4**), and medioresinol (**5**), together with other constituents; 4-allyl resorcinol, β-sitosterol, β-sitostenone, taraxerol, vanilin, and vanilic acid. The structures of these compounds were established by analysis of their spectral data, as compared to that of reported compounds. The lipoxygenase inhibitory activity of the extracts and isolated lignans were also evaluated.

KEY WORDS: Piperaceae, *Piper*, *Piper stylosum*, Lignan, Lipoxygenase

INTRODUCTION

The genus *Piper* consists of over 1000 species distributed mainly in tropical regions of the world. It has high ethnobotanical potential worldwide and diverse application in pharmaceutical botany, traditional medicine, aromatic industries, foods, and landscape decoration [1-3]. *Piper stylosum* is known as '*kaduk hutan*' in Malay, is a sprawling and upright herb with little height to 20 cm tall and creeping stem. The leaves are used as vegetable and seasoning, while the root is used medicinally as poultice or decoction [4]. We have recently reported the chemical compositions and biological activities of the essential oils from this species [5]. GC and GC–MS analysis of the leaves and stems oils of *P. stylosum* resulted in the identification of 50 (89.2%) and 45 (88.8%) components, respectively. The major components were aromadendrene (leaves 26.6%; stems 18.8%), sabinene (leaves 13.8%; stems 6.7%), and β-caryophyllene (leaves 11.5%; stems 17.9%). The essential oils showed low antioxidant, phenolic content, and tyrosinase inhibitory activities. The essential oils also reported moderate antimicrobial activity against *Bacillus cereus* and *Staphylococcus aureus*, both with minimum inhibitory concentration value of 125 μg/mL. Meanwhile, the extracts from this species displayed weak inhibitory activity against tyrosinase and cholinesterase enzymes [6].

As part of our continuing investigation on Malaysian Piperaceae species, herein, we report the detailed phytochemicals study of *P. stylosum* and their lipoxygenase inhibitory activity. To the best of our knowledge, there is no report on their phytochemicals study.

*Corresponding author. E-mail: wmnhakimi@fsmt.upsi.edu.my

EXPERIMENTAL

Plant material. Sample of *Piper stylosum* (SK1963/11) was collected from Kuala Berang, Terengganu in June 2011. This species was identified by Shamsul Khamis and the voucher specimen deposited at the Universiti Putra Malaysia.

General experimental procedures. Solvents systems used in the chromatographic method were; *n*-hexane, ethyl acetate (EtOAc), chloroform ($CHCl_3$), dichloromethane (DCM) and methanol (MeOH). Soxhlet extraction technique was applied to extract the phytochemicals from the dried sample. Vacuum liquid chromatography (VLC) was performed on Merck silica gel 60 (230-400 mesh) while column chromatography (CC) on Merck silica gel 60 (70-230 mesh) as the stationary phase. Thin layer chromatography (TLC) analysis was performed on Merck pre-coated silica (SiO_2) gel F_{254} plates with 0.2 mm thickness to detect and monitor compounds present in the samples. The spots were visualized under UV light at 254 and 365 nm, and spraying reagent vanillin-sulphuric acid in methanol followed by heating. Melting points were measured using melting point apparatus equipped with a microscope, Leica Gallen III and were uncorrected. The ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Bruker Avance 400 Spectrophotometer. Chemical shifts were reported in ppm and $CDCl_3$ as the solvent. Residual solvent was used as an internal standard. The IR spectra were recorded on Perkin Elmer ATR and 1600 spectrophotometer series as KBr disc or thin film of NaCl discs. Mass spectral data were obtained from Mass Spectrometry Service, National University of Singapore (NUS), Singapore.

Extraction and isolation. The dried and powdered aerial part of *P. stylosum* (1 kg) was extracted consecutively by Soxhlet extractor with hexane, EtOAc and MeOH. Evaporation of the respective solvents gave hexane (5.5 g), EtOAc (9.5 g) and MeOH (12.2 g) extracts. The hexane extract was subjected to vacuum liquid chromatography (VLC) on SiO_2 60 (230-400 mesh) using hexane and $CHCl_3$ in 5% increasing polarity to give 9 fractions (PSH1-9). The combined fractions of PSH1-3 were purified by column chromatography (CC) on silica gel 70-230 mesh to afford 4-allyl resorcinol (50 mg), β-sitosterol (15 mg) and β-sitostenone (20 mg). The combined fractions of PSH4-6 were purified by CC on silica gel 70-230 mesh to afford compounds **1** and **2**, while the combined fractions of PSH7-9 afforded taraxerol (7 mg). The crude EtOAc was fractionated by VLC on SiO_2 70-230 mesh, using hexane and EtOAc in 10% increasing polarity to give 10 fractions (PSE1-10). The combined fractions PSE5-9 were purified and recrystallised in hexane:$CHCl_3$ to yield compounds **3** and **4**, while the combined fractions PSE1-3 afforded vanilin (5 mg). The crude MeOH was fractionated by VLC on SiO_2 70-230 mesh, using $CHCl_3$:MeOH in 10% increasing polarity to give fractions (PSM1-5). The combined fractions PSM3-4 were purified by CC to yield **5** and vanilic acid (7 mg).

Lipoxygenase inhibitory activity. The reagents were prepared according to the standard protocol (Lipoxygenase inhibitor screening assay kit, Item No. 760700 Cayman Chemicals Co) [7-9]. Stock solutions of samples were prepared so as to obtain various concentrations (extracts: 1000-62.5 µg/mL; compounds 100−6.25 µM) in the respective wells. The prepared solutions were then introduced onto 96 well plates where the cells were distributed as blanks 1A-2A-1D (triplicate), positive control 1B-2B (duplicate), and 100% initial activity wells 1C-2C-2D (triplicate). The remaining wells were designated for inhibitor (tested samples) solutions in duplicate. The addition of the reagents was done according to the standard protocol. Aliquot (100 µL) of assay buffer was added to the blank wells and 90 µL of lipoxygenase (15-LOX) enzyme and 10 µL of assay buffer were added to positive control wells. For the 100% initial activity wells, 90 µL of lipoxygenase enzyme and 10 µL of solvent (DMSO) were added. The inhibitor (tested samples) wells were charged with 90 µL of lipoxygenase enzyme and 10 µL of respective stock (tested samples) solution. The reaction was initiated by adding 10 µL of the

substrate (AA) to all wells. The plate was then shaken for 5 min on an orbital shaker. Ultimately, 100 µL of chromogen solution (prepared according to standard protocol) was added to each well to stop the enzyme activity. The plate was incubated for 30 min and was read at 500 nm. Quercetin was used as a standard and known as a competitive inhibitor of LOX [6]. The percentage inhibitions (I%) of the tested sample were calculated using the following equation:

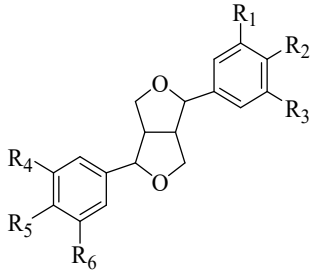
$$I\% = [\ A_{\text{initial activity}} - A_{\text{inhibitor}} / A_{\text{initial activity}}\ ] \times 100$$

where $A_{\text{initial activity}}$ is the absorbance of 100% initial activity wells without sample and $A_{\text{inhibitor}}$ is the absorbance of sample/reference. The sample concentration providing 50% inhibition (IC_{50}) was calculated by plotting inhibition percentages against concentrations of the sample. All tests were carried out in triplicate and IC_{50} values were reported as mean ±SD of triplicates.

Statistical analysis. Data obtained from biological activities are expressed as mean values. Statistical analyses were carried out by employing one way ANOVA ($p > 0.05$). A statistical package (SPSS version 11.0) was used for the data analysis.

RESULTS AND DISCUSSION

Investigation on the chemical constituents of the aerial part of *P. stylosum* has led to the isolation of eleven compounds, characterized as lignans, triterpenes, steroids, and phenylpropanoid. These metabolites were identified by analyzing their spectroscopic data and comparing with the literature data. Five lignans have been isolated and elucidated as yangambin (**1**) [10], sesamin (**2**) [10], syringaresinol (**3**) [10], pinoresinol (**4**) [11], and medioresinol (**5**) [11], together with other constituents; 4-allyl resorcinol [12], β-sitosterol [10], β-sitostenone [10], taraxerol [13], vanillin [14] and vanilic acid [14] (Figure 1). The isolated lignans were characterised as furofuran lignans. Many of these furofuran lignans were isolated previously from various *Piper* species such as *P. terminaliflorum* [15], *P. boehmeriifolium* [16], *P. wallichii* [11], and *P. cubeba* [17]. Lignans are a large class of natural products that have been isolated from many plants. They reveal diverse biological activities, especially antiviral and antitumor properties. From *P. cubeba*, lignans of several classes can be isolated from the roots, rhizomes, stems, leaves, seeds, and fruits. Among its various chemical constituents, (-)-cubebin and (-)-hinokinin are found in significant quantities. Although they have been known for some time, during the last few decades their biological properties have been studied by several researchers [18]. The secondary metabolism in most Piperaceae species appears to be restricted to the production of only a few classes of compounds. In the case of *P. regnellii*, only benzofuran lignans are biosynthesized and accumulate, together with other phenylpropanoids [19]. For *P. solmsianum* several benzofuran lignans have been reported [20], while in *P. wightii* [21] and *P. clarkia* [22] a species accumulation of tetrahydrofuran lignans was investigated.



(**1**) $R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = OCH_3$
(**2**) $R_1 = R_6 = H$; $R_2 = R_3 = R_4 = R_5 = OCH_2O$
(**3**) $R_1 = R_3 = R_4 = R_6 = OCH_3$; $R_2 = R_5 = OH$
(**4**) $R_1 = R_4 = H$; $R_2 = R_5 = OH$; $R_3 = R_6 = OCH_3$
(**5**) $R_1 = R_3 = R_4 = OCH_3$; $R_2 = R_5 = OH$; $R_6 = H$

Figure 1. Lignans isolated from *Piper stylosum*.

Yangambin. White solid (50.1 mg); m.p 125-126 °C; IR (KBr) ν_{max} cm^{-1}: 3071, 2952, 1590, 1511, 1237; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.12 (2H, ddd, *J* = 9.2, 7.2 and 4.4 Hz, H-8/H-8′), 3.86 (6H, s, 4/4′-OCH_3), 3.89 (12H, s, 3/5/3′/5′-OCH_3), 3.96 (2H, dd, *J* = 9.2 and 3.6 Hz, H-9b/H-9′b), 4.34 (2H, dd, *J* = 9.2 and 7.2 Hz, H-9a/H-9′a), 4.76 (2H, d, *J* = 4.4 Hz, H-7/H-7′), 6.59 (4H, s, H-2/H-6/H-2′/H-6′); EIMS: *m/z* 446 [M^+, $C_{24}H_{30}O_8$] [10].

Sesamin. Colourless needles (10.5 mg); m.p 117-119 °C; IR (KBr) ν_{max} cm^{-1}: 3080, 2851, 1500, 1443, 1251; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.07 (2H, ddd, *J* = 9.2, 6.8 and 4.4 Hz, H8/H-8′), 3.90 (2H, dd, *J* = 9.2 and 3.6 Hz, H-9b/H-9′b), 4.26 (2H, dd, *J* = 9.2 and 6.8 Hz, H-9a/H-9′a), 4.74 (2H, d, *J* = 4.4 Hz, H-7/H-7′), 5.97 (4H, s, OCH_2O), 6.81 (2H, d, *J* = 8.0 Hz, H-5/H-5′), 6.83 (2H, d, *J* = 1.2 Hz, H-2/H-2′), 6.87 (2H, dd, *J* = 8.0 and 1.2 Hz, H-6/H-6′); EIMS: *m/z* 354 [M^+, $C_{20}H_{18}O_6$] [10].

Syringaresinol. Colurless needles (15.9 mg); m.p 183-184 °C; IR (KBr) ν_{max} cm^{-1}: 3340, 2923, 1567, 1462, 1262;^{1}H NMR (400 MHz, $CDCl_3$): δ 3.11 (2H, ddd, *J* = 9.2, 6.8 and 4.4 Hz, H8/H-8′), 3.91 (12H, s, 3/3′/5/5′-OCH_3), 3.93 (2H, m, H-9b/H-9′b), 4.30 (2H, dd, *J* = 9.2 and 6.8 Hz, H-9a/H-9′a), 4.75 (2H, d, *J* = 4.4 Hz, H-7/H-7′), 5.56 (2H, s, 4/4′-OH), 6.60 (4H, s, H-2/H-2′/H-6/H-6′); EIMS: *m/z* 418 [M^+, $C_{22}H_{26}O_8$] [10].

Pinoresinol. White needles (10.2 mg); m.p 153-154 °C; IR (KBr) ν_{max} cm^{-1}: 3345, 2925, 1565, 1460, 1260; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.08 (1H, m, H-2, H-5), 3.86 (4H, dd, *J* = 3.6, 9.0 Hz, H-3b, H6b), 3.94 (6H, s, 3',4'-OCH_3), 4.25 (4H, dd, *J* = 7.2, 9.0 Hz, H-3a, H-6a), 4.72 (2H, d, *J* = 4.2 Hz, H-1, H-4), 6.82 (2H, dd, *J* = 8.4, 1.8 Hz, H-6', H-6"), 6.87 (2H, d, *J* = 7.8 Hz, H-5', H-5"), 6.88 (2H, d, *J* = 1.8 Hz, H-2', H-2"); EIMS: *m/z* 358 [M^+, $C_{20}H_{22}O_6$] [11].

Medioresinol. White needles (12.2 mg); m.p 158-160 °C; IR (KBr) ν_{max} cm^{-1}: 3400, 2924, 1615, 1520, 1210, 1060; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.10 (2H, m, H-l, H-5),3.87 (2H, m, H-4b, H-8b), 3.90 (9H, s, 3', 5', 5"-OCH_3), 4.27 (2H, m, H-4a), 4.75 (2H, d, *J* = 4.2 Hz, H-2, H-6), 5.48 (1 H, s, 4', 4"-OH), 5.59 (1 H, s, 4', 4"-OH), 6.59 (2H, s, H-2', H-6), 6.82 (I H, dd, *J* = 8.2 and 1.8 Hz, H-2"), 6.89 (1H, d, *J* = 2.1 Hz, H-6"), 6.90 (1 H, d, *J* = 2.1 Hz, H-3); EIMS: *m/z* 388 [M^+, $C_{21}H_{24}O_7$] [11].

Table 1. Lipoxygenase inhibitory activity of extracts and lignans from *P. stylosum*.

Samples	IC_{50} (μM)
Hexane extract	85.2
EtOAc extract	82.8
MeOH extract	80.5
Yangambin (**1**)	22.4
Sesamin (**2**)	28.7
Syringaresinol (**3**)	24.0
Pinoresinol (**4**)	15.2
Medioresinol (**5**)	18.5
Quercetin	3.5

Data represent mean ± SD of three independent experiments.

Previous studies revealed that most of the lignans exhibited lipoxygenase (LOX) inhibitory effects [21]. Thus, the LOX inhibitory activities of compounds **1**–**5** were measured by slightly modifying the spectrophotometric method [5] and the results are shown in Table 1. All extracts showed significant activity with the IC_{50} values ranged from 80.5-85.2 μM. Meanwhile, compounds **4** and **5** showed good LOX inhibitory effects with IC_{50} values of 15.2 and 18.5 μM,

respectively. LOX is involved in arachidonic acid metabolism, generating various biologically active leukotrienes that play an important role in inflammation. Inhibition of leukotrienes synthesis on the lipoxygenase pathway may contribute toanti-inflammatory activity [24].

CONCLUSION

The present study is the first to report furofuran lignans isolated from *P. stylosum*, which have much chemotaxonomic importance within the genus *Piper*. Additionally, the isolation and identification of these compounds may have potential as anti-lipoxygenase inhibitors for suppression of inflammatory response.

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