Secondary Metabolites from *Aquilaria subintegra* Leaves and their Radical Scavenging Activity

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The present study reports on the isolation, purification and antioxidant activity evaluation of the dichloromethane extract of *Aquilaria subintegra* Ding Hou leaves. Four compounds were successfully isolated and purified from the dichloromethane extract using column chromatography. Purification was monitored by thin layer chromatography, and four isolated compounds were identified by spectroscopic analyses such as NMR and mass spectrometry. These compounds were 5-hydroxy-7,4′-dimethoxyflavone, luteolin-7,3′,4′-trimethyl ether, β-sitosterol and friedelin. Their antioxidant activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH). All compounds exhibited weak antioxidant activity, with inhibition values of less than 30 % at the highest tested concentration (500 µg/mL). To the best of our knowledge, the current study is the first to report on the DPPH radical scavenging activity of isolated secondary metabolites from *A. subintegra* leaves.

Keywords: *Aquilaria subintegra*; leaves; flavonoids; steroid; terpene; antioxidant activity

Received: July 2023; Accepted: September 2023

Aquilaria species are widely distributed in Asia and usually found in tropical forests [1]. The genus *Aquilaria* comes from the tribe *Aquilarieae* of the Thymelaeaceae family. About twenty-one of these species, including *A. subintegra*, produce fragrant, aromatic and scented wood known as agarwood or gaharu [2, 3]. These species are traditionally used as herbal medicine and in cultural and religious practices. The *Aquilaria* species are rich sources of many bio-active compounds including phenolic acids, flavonoids, terpenoids, steroids, xanthonoids, benzophenones, and lignans [1, 4, 5].

The biological properties of *Aquilaria* species have been demonstrated in several studies. In a study on the ethanolic extract of *A. sinensis* leaves, Ito et al. found that mangiferin and genkwanin 5-O-β-prime-veroside possessed laxative properties [6]. Similarly, Kakino et al. found that *A. sin*ensis leaves had laxative properties that did not cause diarrhoea [7]. Other biological properties reported included anti-obesity activity through inhibition of fatty acid production by pancreatic lipase [8,9], and enhanced fertility rates [10]. *A. subintegra* also showed promising potential in Alzheimer's therapy [11] and exhibited cytotoxic properties against breast cancer cell lines [12, 13].

One of the most interesting biological properties of the *Aquilaria* sp. is its antioxidant activity. Two studies have shown that extracts of *A. malaccensis* leaves possessed significant antioxidant activity [14, 15]. Further investigation of *A. crassna* revealed a polyphenolic compound, mangiferin, that was also a promising antioxidant [16]. In addition, a recent study showed that 5-hydroxy-7, 3', 4'- trimethoxyflavone was an effective radical scavenger [17].

An oxidant donates a proton or hydrogen to an oxidizing agent [18]. An oxidant or free radical results from a reaction of triplet oxygen, water and unsaturated fatty acids [14]. Accumulation of free radicals causes oxidative stress and cellular damage in body nucleic acids, proteins and lipids, which may ultimately lead to various degenerative diseases including cancer, diabetes, atherosclerosis and gastric ulcer [14, 18, 19]. Recent research has been focused on exploring natural antioxidants from medicinal plants to replace synthetic drugs, which have a high potential to cause harmful effects [20]. Only three species of *Aquilaria*, namely *A. malaccensis*, *A. sinensis* and *A. crassna*, have been reported in the literature in terms of their phytochemical screening and antioxidant activity. No prior studies on *A.*

subintegra's antioxidant properties have been found to date. Therefore, in the present study, we report on the phytochemical constituents of the dichloromethane extract of *A. subintegra* Ding Hou leaves and their antioxidant activity.

EXPERIMENTAL

Plant Material and Instrumentation

The leaves of *A. subintegra* were collected from Kajang, Selangor, in July 2014. Collected samples were identified by a botanist from the Malaysian Timber Industry Board (MTIB) and a voucher specimen was deposited at the Herbarium of Biology Department, Universiti Pendidikan Sultan Idris (UPSI), Tanjung Malim, Perak, Malaysia, with voucher number AMWNI-M6002/1.

Mass spectral analysis was performed using an LTQ Orbitrap Liquid Chromatograph-Mass Spectrometer (Thermo Scientific, San Jose, USA). Nuclear Magnetic Resonance spectra (^{1}H and ^{13}C NMR) were obtained using a JEOL ECX (500 MHz) NMR. Column chromatography was performed using silica gel 60, 200-400 mesh. Aluminium-supported silica gel 60 F254 plates were utilised for thin-layer chromatography (TLC) and TLC spots were sprayed with *p*-anisaldehyde reagent and visualized under ultraviolet light (254 and 365 nm).

Extraction and Isolation

Dried powdered leaves (3.3 kg) were extracted consecutively with hexane, dichloromethane (DCM) and methanol. Samples were soaked at room temperature, filtered and concentrated using a rotary evaporator to give 38.06 g, 43.34 g and 78.29 g of hexane, DCM and methanol crude extracts, respectively.

The DCM crude extract was analysed by silica gel column chromatography (CC). Twenty-two fractions (L1A-1 to L1A-22) were eluted with increasing concentrations of methanol in DCM, and fractions with similar TLC profiles were pooled. Fraction L1A-14 (2.22 g, 5.12 %) was subjected to repeated CC by elution with Hex, Hex/DCM mixtures, DCM, DCM/MeOH mixtures, and MeOH to give three compounds; **1** (10.0 mg, 0.45 %), **2** (15.0 mg, 0.68 %) and **3** (10.0 mg, 0.45 %). Fraction L1A-12 (2.03 g, 4.68 %) was re-chromatographed using multiple CC with a gradient system consisting of increasing concentrations of EtOAc in Hex (100:0 to 10:90, v/v) to yield compound **4** (10.0 mg, 0.49 %).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity

The antioxidant activity of an extract was determined using a DPPH-scavenging plate assay as previously described [21] but with slight modification. Briefly, 20 µL of each extract at a concentration range of 3.932 to 500 µg/mL was allowed to react with 180 µL of DPPH solution (150 µmol L^{-1}) in 80 % methanol. The solution was then incubated in the dark at room temperature for 40 min after which its absorbance (A) was measured at 515 nm using a microplate reader. Ascorbic acid was used as a positive control at 0.39 – 50 µg/mL and all samples were tested in triplicate. The DPPH radical scavenging activity was calculated as the percentage of DPPH decolouration relative to a negative control, using the following equation:

$$\text{Free-radical scavenging activity (\%)} = [\text{A (control) - A (extract)/A (control)}] \times 100 \quad (1)$$

RESULTS AND DISCUSSION

Chromatographic fractionation of the DCM extract of *A. subintegra* leaves yielded four compounds (Figure 1): two flavonoids identified as 5-hydroxy-7,4′-dimethoxy-flavone (**1**) [22] and luteolin-7,3′,4′-trimethyl ether (**2**) [22], a steroid, β-sitosterol (**3**) [23] and a triterpene, namely friedelin (**4**) [24]. These compounds were identified and characterized by 1D-NMR and MS spectroscopy. The spectral data for each isolated chemical was compared to the literature.

Compound **1** was isolated as yellow needles, and gave a molecular ion peak at *m/z* 299.088 $[M+H]^+$, consistent with the molecular formula $C_{17}H_{14}O_5$. Its ^{1}H NMR spectrum showed the presence of two methoxy resonances at δ 3.89 (C-7) and 3.88 (C-4′), and a broad singlet δ 12.82 which was assigned to a hydroxyl group. Seven aromatic protons were identified as one singlet at δ 6.58 attributed to H-3, two doublets at δ 6.37 (H-6) and 6.48 (H-8) characteristic of meta-protons, and four protons at δ 7.02 (H-3′, 5′) and 7.85 (H-2′, 6′) in an AA′XX′ coupling system. The ^{13}C NMR spectrum exhibited 17 carbon peaks consisting of two methoxy, one carbonyl, seven methine, and seven quaternary carbons which further support the molecular formula.

Compound **2** was obtained as a yellow amorphous solid. Its molecular formula was identified using ESI-MS as $C_{18}H_{16}O_6$ corresponding to the significant peak at *m/z* 329.099 $[M+H]^+$. The ^{1}H NMR spectrum showed the signals of methoxyl groups at δ 3.89, 3.97 and 3.98, attributed to C-7, C-3′ and C-4′ respectively. Six aromatic protons were identified: a singlet at δ 6.59 (H-3), three doublets with meta-proton characteristics at δ 6.38 (H-6), 6.50 (H-8) and 7.34 (H-2′), one doublet of doublets proton at δ 7.53 (H-6′) and a broad singlet of hydroxyl at δ 12.80. The ^{13}C NMR spectrum presented 18 carbon peaks including three methoxyl, one carbonyl, six methine and eight quaternary carbons.

Compound **3** was obtained as a white amorphous solid and its ESI-MS spectrum showed a molecular ion peak at *m/z* 415.302 $[M+H]^+$, corresponding to the

molecular formula $C_{29}H_{50}O$. Its ^{1}H NMR spectrum indicated 50 protons, with the presence of two methyl singlets at δ 1.00 and 0.67, and three methyl doublets at δ 0.92, 0.82, and 0.80, attributable to H-29, 28, 19, 26 and 27, respectively. In addition, one methyl triplet at δ 0.84 was assigned to H-24, and an olefinic proton at δ 5.34 was attributed to H-6.

Compound **4** was isolated as colourless needles, and its ESI-MS spectrum showed a molecular ion peak at *m/z* 427.398 $[M+H]^+$ ($C_{30}H_{50}O$). Its ^{1}H NMR spectrum indicated the presence of 50 protons, including seven methyls as singlets at δ 0.72 (H-24), 0.86 (H-25), 0.99 (H-26), 1.04 (H-27), 1.17 (H-28), 0.95 (H-29) and 1.00 (H-30). A methyl doublet appeared at δ 0.88 (H-23), and multiplet and quartet signals were present at δ 2.37 (H-2) and δ 2.24 (H-4), respectively.

A DPPH assay is commonly used for the evaluation of the scavenging ability of antioxidant compounds toward 2,2-diphenyl-picrylhydrazyl (DPPH) radicals through donation of a hydrogen [17, 25]. Antioxidant activity is determined by measuring absorption at 515 nm and calculating their IC_{50} values, interpreted as the concentration of the sample (extract and isolated compound) required to scavenge 50 % of the DPPH free radicals [15].

The IC_{50} values of the crude extracts isolated from *A. subintegra* are presented in Table 1 and compared with three previous studies [14, 26, 27].

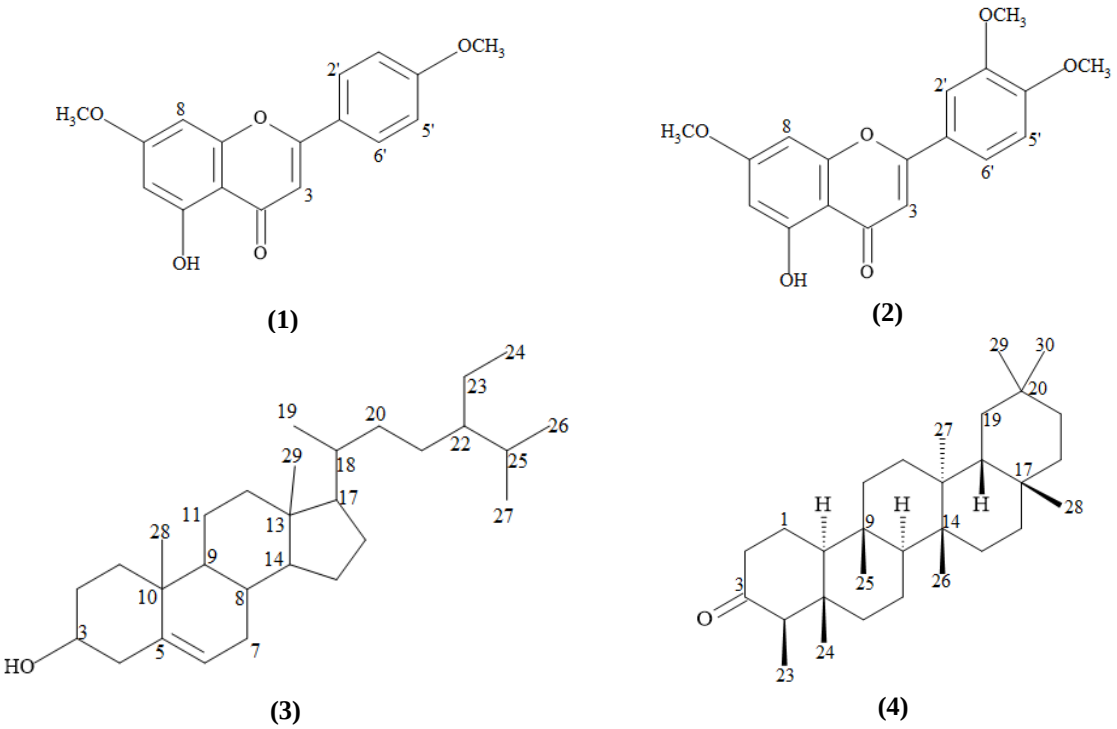

Figure 1. Compounds isolated from *A. subintegra* leaves.

Table 1. DPPH radical scavenging activity of *A. subintegra* crude extracts in comparison with previously reported results.

Crude extract	IC_{50} (µg/mL)			
	Present study	**[14]**	**[26]**	**[27]**
Hexane	305.83 ± 16.96	800	na	nd
DCM	356.68 ± 34.24	160	na	na
Methanol	29.56 ± 6.37	30	19.62±1.49	77.21±4.88
Control				
Ascorbic Acid	4.27 ± 0.74	na	2.12±0.38	na
Quercetin	na	3.33	na	10.65±0.25

na: not applicable; nd: not detected

It is noteworthy that some previous studies [26, 27] were not able to detect the IC_{50} value for the Hex and DCM extracts, while some references used quercetin as a control instead of ascorbic acid. The present study demonstrated that the MeOH extract had the strongest antioxidant activity, with an IC_{50} value of 29.56 ± 6.37. This result is in accordance with previous studies [14, 26, 27] which indicated that MeOH extracts possessed excellent antioxidant properties due to the presence of various active compounds, including phenolics, alcohols, sugars and glycosides [26].

Isolated compounds from the DCM extract demonstrated a lower ability to scavenge DPPH radicals compared with ascorbic acid, as shown in Figure 2. Compound **1** showed the strongest antioxidative activity among the isolated compounds; however, its inhibitory ability was less than 30 %. IC_{50} values for all isolated compounds from the DCM crude extract were above 500 µg/mL. The isolated flavonoids (**1** and **2**) exhibited weak antioxidant properties, contrary to previous research that indicated flavonoids were free radical scavengers, where 5-hydroxy-7, 3', 4'-trimethoxyflavone was the strongest antioxidant [17]. Furthermore, β-sitosterol and friedelin (**3** and **4**) did not show significant antioxidant activity within the tested concentration range. This was consistent with a previous study that found non-significant anti-oxidant activity for fridelin and β-sitosterol from *Elaeocarpus floribundus* Blume [28]. It is possible that some compounds require higher concentrations to exhibit antioxidant properties, while the activity of the crude DCM extract may be due to synergistic interactions between phytochemicals in the extract [29].

CONCLUSION

This study reports on the isolation of flavonoids, terpenes and steroids from *A. subintegra* leaves. All isolated compounds exhibited weak DPPH scavenging activity, which indicated their potential as antioxidants. The higher antioxidant activity of the DCM crude extract may have resulted from synergistic interactions between phytochemicals present in the crude extract. To the best of our knowledge, the current study is the first to report on the DPPH radical scavenging activity of isolated secondary metabolites from *A. subintegra* leaves.

ACKNOWLEDGEMENTS

The authors acknowledge the support provided by the Department of Chemistry at Universiti Pendidikan Sultan Idris as well as the Kulliyyah of Pharmacy at the International Islamic University Malaysia and the Forest Research Institute of Malaysia for the sample analysis and laboratory facilities. The current work was supported by FRGS Grant 2014-0030-101-02 from the Ministry of Higher Education (MOHE).

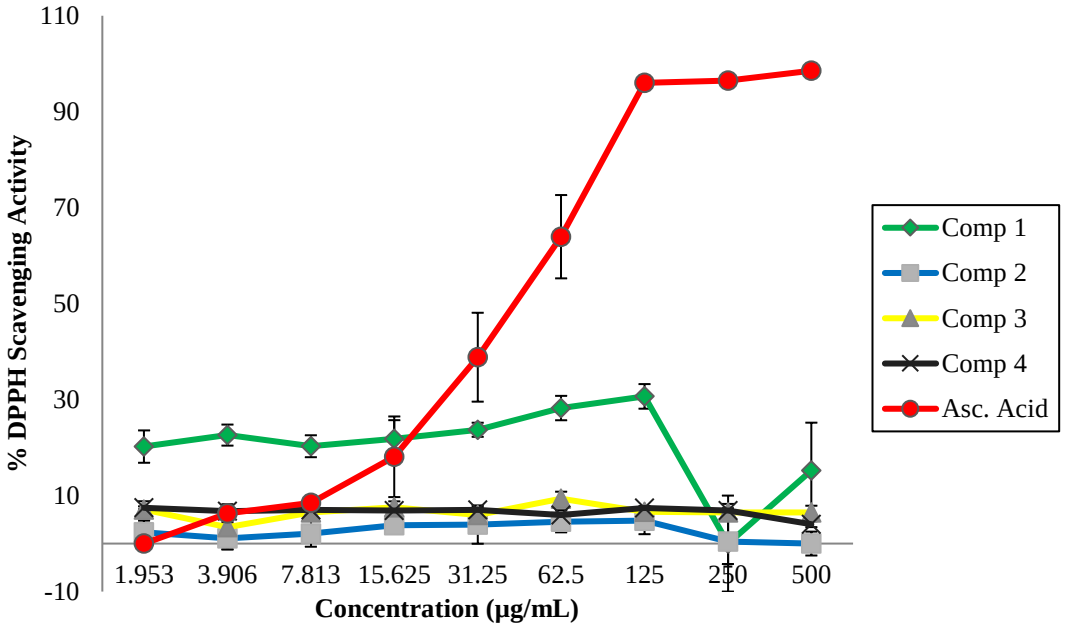

Figure 2. DPPH radical scavenging activity of isolated compounds from *A. subintegra*. Data are expressed as mean ± SD, n=3.

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