Chemical Constituents from *Diospyros discolor* Willd. and their Acetylcholinesterase Inhibitory Activity

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**ABSTRACT**

**Background:** *Diospyros discolor* is commonly known as ‘buah mentega’ and traditionally used to treat various diseases. Many compounds especially triterpenes in *Diospyros* sp. were reported to inhibit acetylcholinesterase (AChE) and butyrylcholinesterase enzymes *in vitro* and *in vivo*. *D. discolor* was reported to contain triterpenes, yet to be investigated for their AChE inhibitory activity. *D. discolor* leaves extract showed high (95.80 ± 1.57 %) AChE inhibitory activity at the concentration of 100 µg/mL. **Objective:** The aim of the present study is to identify chemical constituents from *D. discolor* and their AChE inhibitory activity. **Materials and Methods:** The leaves and stem barks of *D. discolor* were air dried, powdered and successively extracted using *n*-hexane, dichloromethane and methanol. The solvents were evaporated to obtain dried crude extracts. The compounds were purified using exhaustive chromatographic procedures and their structures were determined by analyses of spectral data. The AChE inhibitory activity was carried out using Ellman’s method. **Results:** A new flavonol, 7,4′-dihydroxy-5,3′,5′-trimethoxyflavonol (1), along with five known flavonoids (2-6) and six known triterpenes (7-13) were isolated from the leaves and stem barks of *D. discolor*. Selected compounds were evaluated for AChE inhibitory activity, in which stigmast-4-ene-3-one (7) showed the lowest inhibition concentration with an IC<sub>50</sub> value of 11.77 ± 2.11 µM. **Conclusion:** A new flavonol (1) and twelve known compounds were identified and characterized. Even though *D. discolor* extracts showed high percent inhibition against AChE enzyme, the isolated compounds showed moderate inhibition. **Keywords:** Ebenaceae, Triterpenes, Flavonoid, Acetylcholinesterase.

**INTRODUCTION**

*Diospyros discolor* Willd. (syn. *D. blancoi*) belongs to the family of Ebenaceae, and it is locally known as ‘buah mentega’. *D. discolor* is used traditionally to treat wounds, snakebites, spider bites, stomachache, diabetes, heart problems, hypertension, dysentery, diarrhea and eczema. *D. discolor* was reported to have free radical scavenging, anti-diarrheal, antimicrobial, analgesic and anti-inflammatory activities. *Diospyros* sp. are rich in naphthaquinones, triterpenes, followed by flavonoid, naphthalene and coumarin-based groups. The triterpenes found in *Diospyros* sp. were mostly of pentacyclic core especially lupane, ursane and oleanane skeletons. Many triterpenes in *Diospyros* sp. showed inhibition against acetylcholinesterase (AChE) and butyrylcholinesterase enzymes *in vitro* and *in vivo*. *D. discolor* was reported to contain triterpenes, yet to be investigated for their AChE inhibitory activity. *D. discolor* leaves extract showed high acetylcholinesterase (AChE) inhibitory activity with 95.80 ± 1.57 % inhibition during preliminary screening of selected medicinal plants from Taman Herba Perlis. Therefore, this study is warranted to investigate the chemical constituents from the leaves and stem barks extracts of *D. discolor* and their AChE inhibitory activity.

**MATERIALS AND METHODS**

**General experimental procedures**

1H-NMR and APT-NMR spectra were recorded at 500 or 600 MHz and 125 or 150 MHz, respectively, using Bruker 500 Ultrashield Plus (Bruker, Switzerland) and Bruker Ascend 600 (Bruker, Switzerland). FTIR-ATR spectra were recorded on FTIR Spectrometer INVENIO (Bruker, Switzerland). The mass spectra were recorded using LC/MS/MS QTOF Agilent Technologies 6520 (Agilent, Santa Clara, USA). The absorbance for *in-vitro* analysis was obtained by Spectrostar Nano spectrometer (BMG Labtech, Germany). The solvents used for extraction and isolation were of analytical grade solvents. The silica gel used were silica gel 60 F<sub>254</sub> (1.07747), silica gel 60 (0.040-0.063 mm, 1.09385), silica gel 60 PF<sub>254</sub> (1.07749), and TLC silica gel 60 F<sub>254</sub> aluminium sheets (1.05554). The silica gel and TLC were purchased from Merck (Germany). All chemicals and reagents used for acetylcholinesterase inhibitory activity were purchased from Sigma Aldrich unless stated otherwise.

**Plant materials**

The leaves and stem barks of *D. discolor* (syn. *D. blancoi*) were collected from Kuala Nerang, Kedah, Malaysia in March 2016. The plant sample was identified by Dr Shamsul Khamis of Universiti Kebangsaan Malaysia and the voucher specimen.
(PID 210517-13) was deposited at Forest Research Institute Malaysia (FRIM), Kepong, Selangor, Malaysia.

**Extraction and isolation**

The fresh plant samples (3.5 kg) were air dried and ground into powder using a hammer mill. The ground samples were extracted successively using n-hex, CHCl₃ and MeOH at room temperature. The filtrates were concentrated using rotary evaporator. n-Hex stem bark extract (3.38 g) was fractionated by VLC eluted with n-hex-CHCl₃-CH₂Cl₂-MeOH (9:12) was further fractionated using CC eluted with CHCl₃-MeOH (97:3) to obtain ursolic acid (10:0, 9:1). Fraction E3-10 was further purified by PTLC developed with n-hex-acetone (9:1) yielded stigmaster (9:1) and seven known compounds. Fraction A (8-11) was further fractionated using CC eluted with CH₂Cl₂-MeOH (9:1) was fractionated by CC eluted with CH₂Cl₂-MeOH (9:1). Further purification of B5-8 by using RC eluted with CH₂Cl₂-MeOH (2.9 mg). Fraction G (18-19) fractionated again with CC by isocratic elution CH₂Cl₂:MeOH (9:1→0:10) (25 mg).

CH₂Cl₂ stem bark extract (5 g) was fractionated by VLC eluted with n-hex-CH₂Cl₂-MeOH (n-hex:CH₂Cl₂, 1:9, 0:10, CH₂Cl₂:MeOH, 98:2→91:9) yielded 15 fractions. Fraction B (5-7) was further fractionated using CC eluted with n-hex-CHCl₃-CH₂Cl₂-MeOH (n-hex:CHCl₃, 1:9→1:9, CH₂Cl₂:MeOH, 10:0→7:3) of which purification of A19-25 using PTLC developed with n-hex-acetone (9:1) yielded stigmaster (9:1) and 7,4′-dihydroxy-5,3′,5′-trimethoxyflavonol (0.8 mg) (12) as (+)-epicatechin (2), kaempferol (3), astragalin (4), hyperin (5), isoorientin (6), stigmaster-3-O-glucopyranoside (10), betulin (11), betulinic acid (12), and ursolic acid (13) (20). A new flavonol (1) was obtained along with compound (2) as a mixture in the form of brown powder. A molecular formula of C_{36}H_{30}O_{14} was derived from LC-MS QTOF with its [M+H]^+ at m/z 361.3136 (cald 361.3179 for C_{36}H_{30}O_{14}). The FTIR-ATR spectrum showed a broad peak of hydroxyl (O-H) at 3384 cm⁻¹, a strong peak of carboxyl (C=O) at 1719 cm⁻¹, medium peak of aromatic (C=C) at 1609 cm⁻¹ and a strong peak of C=O stretch at 1719 cm⁻¹.

The 1H-NMR spectrum revealed three aromatic proton signals. A pair of meta-coupled signals resonated at δ_H 6.89 (1H, d, J=1.8 Hz) and 7.05 (1H, d, J=1.8 Hz, H-8) was assigned as H-6 and H-8 of ring A. A singlet aromatic proton signal at δ_H 7.45 assigned for two protons was assigned as H-2′ and H-6′ of ring B. A singlet appeared at δ_H 3.90 integrated for two protons assigned as H-2′ of ring B which β-sitosterol-3-O-glucopyranoside (10) (361.3179 for C_{36}H_{30}O_{14}). The FTIR-ATR spectrum showed a broad peak of hydroxyl (O-H) at 3384 cm⁻¹, a strong peak of carboxyl (C=O) at 1719 cm⁻¹, medium peak of aromatic (C=C) at 1609 cm⁻¹ and a strong peak of C=O stretch at 1719 cm⁻¹.

The 1H-NMR spectrum revealed three aromatic proton signals. A pair of meta-coupled signals resonated at δ_H 6.99 (1H, d, J=1.8 Hz) and 7.05 (1H, d, J=1.8 Hz, H-8) was assigned as H-6 and H-8 of ring A. A singlet aromatic proton signal at δ_C  152.1. The chemical shift for C-3 was not detected. The crude MeOH stem bark extract of *D. discolor* was dissolved in MeOH and subjected to LLE with EtO to reduce the tannin. The crude MeOH stem bark extract of *D. discolor* yielded a new flavonol (1), five known flavonoids (2-6) and seven known triterpenes (7-13) (Figure 1). The known compounds were identified as (+)-epicatechin (2), kaempferol (3), astragalin (4), hyperin (5), isoorientin (6), stigmaster-3-O-glucopyranoside (10), betulin (11), betulinic acid (12), and ursolic acid (13) (16-19).

**Statistical analysis**

The AChE inhibitory activity data were expressed as mean ± standard deviation. All the data were subjected to one-way analysis of variance (ANOVA) completed with Tukey’s post hoc test and p<0.05 was considered as statistically significant using IBM SPSS Statistic version 20. The IC₅₀ was obtained by plotting nonlinear-regression curve of percentage AChE inhibitory activity against logarithm of compound concentration using GraphPad Prism statistical software version 6.01.

**RESULTS AND DISCUSSION**

Phytochemical study on the leaves and stem barks of *D. discolor* yielded a new flavonol (1), five known flavonoids (2-6) and seven known triterpenes (7-13) (Figure 1). The known compounds were identified as (+)-epicatechin (2), kaempferol (3), astragalin (4), hyperin (5), isoorientin (6), stigmaster (8) and stigmasterol (9), β-sitosterol-3-O-glucopyranoside (10), betulin (11), betulinic acid (12), and ursolic acid (13) (16-19). A new flavonol (1) was obtained along with compound (2) as a mixture in the form of brown powder. A molecular formula of C_{36}H_{30}O_{14} was derived from LC-MS QTOF with its [M+H]^+ at m/z 361.3136 (cald 361.3179 for C_{36}H_{30}O_{14}). The FTIR-ATR spectrum showed a broad peak of hydroxyl (O-H) at 3384 cm⁻¹, a strong peak of carboxyl (C=O) at 1719 cm⁻¹, medium peak of aromatic (C=C) at 1609 cm⁻¹ and a strong peak of C=O stretch at 1719 cm⁻¹.

The 1H-NMR spectrum revealed three aromatic proton signals. A pair of meta-coupled signals resonated at δ_H 6.99 (1H, d, J=1.8 Hz) and 7.05 (1H, d, J=1.8 Hz) was assigned as H-6 and H-8 of ring A. A singlet aromatic proton signal at δ_C  152.1. The chemical shift for C-3 was not detected. The absence of typical singlet aromatic proton signal assignable to C-3 as well as chemical shift value for C-2 suggesting this compound is of flavonol moiety (18).
The assignment of H-8 of ring A was confirmed based on its HMBC correlations to C-7 and C-10 while the placement of H-6 was determined based of its HMBC correlations to C-5, C-7 and C-10. The hydroxyl group is located at C-7 based on HMBC cross peaks between H-6 and H-8 with C-7. Meanwhile the methoxy group was assigned to C-5 based on correlations observed between H-6 and C-5. The singlet proton signal of H-8 with C-7. Meanwhile the methoxy group was assigned to C-5 based of its HMBC correlations to C-5, C-7 and C-10. The hydroxyl group at C-4' was confirmed based on 'f' correlations of H-2' and H-6' with C-4. Even though no HMBC correlation was observed to confirm the assignment of C-4 and C-9 at ring C, their chemical shift values are quite typical of flavonol moiety. Close inspection of all spectroscopic data confirmed that compound 1 is 7,4'-dihydroxy-5, 3',5'-trimethoxyflavonol.

A flavonoid (4) and six triterpenes (7-12) from the leaves and stem barks of Diospyros discolor were examined for AChE inhibitory activity. All the compounds exhibited positive AChE inhibitory activity at 10 µM concentration, but only stigmast-4-en-3-one (7) showed inhibition of more than 50% (Table 1). When evaluated for AChE inhibitory activity...
in dose-dependent manner, it gave an IC₅₀ value of 11.77 ± 2.11 μM. Some of the compounds isolated in the present study showed moderate inhibition concentration against AChE. 20,21 while kaempferol (3) and β-sitosterol-3-O-glucopyranoside (10) were previously reported to have low inhibition concentration against AChE. 20,21,23

**CONCLUSIONS**

*D. discolor* (syn. *D. blancoi*) was found to inhibit AChE during random screening. Phytochemical study on the leaves and stem bark of *D. discolor* yielded a new flavonol, 7,4'-dihydroxy-3',5,3'-trimethoxyflavonol (1) along with five known flavonoids and six known triterpenes. The compounds examined for AChE inhibitory activity showed moderate inhibition concentration except for stigmasterol-3-O-glucopyranoside (10) & stigmasterol (β-sitosterol) (8) of *D. discolor* (syn. *D. blancoi*). It is postulated that the AChE inhibitory activity of the extract of *D. discolor* is due to synergistic effect of the phytochemicals collectively.

**CONFLICTS OF INTEREST**

None.

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GRAPHICAL ABSTRACT

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