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Original Article

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

Norhafizoh Abdul Somat[1,2], Zaini Yusoff[3], Che Puteh Osman[1,2,*]

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_{50} 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.

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INTRODUCTION

Diospyros discolor Willd. (syn. *D. blancoi*) belongs to the family of Ebenaceae, and it is locally known as 'buah mentega'[1]. *D. discolor* is used traditionally to treat wounds, snakebites, spider bites, stomachache, diabetes, heart problems, hypertension, dysentery, diarrhea and eczema[1]. *D. discolor* was reported to have free radical scavenging, anti-diarrheal, antimicrobial, analgesic and anti-inflammatory activities[1-5]. *Diospyros* sp. are rich in naphthoquinones, triterpenes, followed by flavonoid, naphthalene and coumarin-based groups[6,7]. The triterpenes found in *Diospyros* sp. were mostly of pentacyclic core especially lupane, ursane and oleanane skeleton[6]. 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[8]. 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

^{1}H-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 LCMS/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 PF_{254} (1.07747), silica gel 60 (0.040-0.063 mm, 1.09385), silica gel 60 PF_{254} containing gypsum (1.07749), and TLC silica gel 60 F_{254} 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

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(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, CH_2Cl_2 and MeOH at room temperature. The filtrates were concentrated using rotary evaporator. *n*-Hex stem bark extract (3.38 g) was fractioned by VLC eluted with *n*-hex-CH_2Cl_2-EtOAc (*n*-hex:CH_2Cl_2, 10:0→1:9, CH_2Cl_2: EtOAc, 10:0→1:9) afforded 23 fractions. Fraction A (8-11) was further fractionated using CC eluted with *n*-hex-CH_2Cl_2-EtOAc (*n*-hex: CH_2Cl_2, 9:1 →1:9, CH_2Cl_2: EtOAc, 10:0→7:3), of which purification of A19-25 using PTLC developed with *n*-hex-acetone (9:1) yielded stigmast-4-en-3-one (**7**) (15 mg).

CH_2Cl_2 stem bark extract (5 g) was fractionated by VLC eluted with *n*-hex-CH_2Cl_2-MeOH (*n*-hex:CH_2Cl_2, 1:9, 0:10, CH_2Cl_2: MeOH, 98:2→91:9) yielded 15 fractions. Fraction B (5-7) was further fractionated using CC eluted with *n*-hex-$CHCl_3$-EtOAc (*n*-hex: $CHCl_3$: EtOAc, 40:55:5→20:75:5) afforded 31 fractions. Betulin (**11**) (50 mg) was obtained from the purification of B10 by using RC (*n*-hex: CH_2Cl_2, 1:9). Further purification of B5-8 by using RC eluted with *n*-hex-$CHCl_3$- EtOAc (28.5:70:1.5) yielded a mixture of β-sitosterol (**8**) and stigmasterol (**9**) (5.4 mg).

The crude MeOH stem bark extract of *D. discolor* was dissolved in MeOH and subjected to LLE with Et_2O to reduce tannin. The tannin clumped together, and the remaining solution was filtered and dried. MeOH stem bark extract (14 g) was fractionated by VLC eluted with *n*-hex-CH_2Cl_2-EtOAc-MeOH (*n*-hex:CH_2Cl_2, 1:9, 0:10, CH_2Cl_2: EtOAc, 9:1→10:0, EtOAc: MeOH, 9:1, 8:2) yielded 33 fractions. Fraction C (9-12) was further fractionated using CC eluted with *n*-hex-CH_2Cl_2-EtOAc-MeOH (*n*-hex: CH_2Cl_2, 4:6→0:10, CH_2Cl_2: EtOAc, 9:1→0:10, EtOAc: MeOH, 9:1→8:2). The purification of fraction C46 using PTLC eluted with *n*-hex-$CHCl_3$-EtOAc (1:8:1) yielded betulinic acid (**12**) (40 mg).

Similar method was used to reduce tannin for MeOH leaves extract of *D. discolor* to give 27 g extract. The extract was fractionated by VLC eluted with *n*-hex-CH_2Cl_2-EtOAc-MeOH (*n*-hex: CH_2Cl_2, 10:0 → 1:9, CH_2Cl_2: EtOAc, 10:0→1:9, EtOAc: MeOH, 10:0→8:2) yielded 27 fractions (E-H). Fraction E (11-12) was fractionated using Sephadex LH 20 eluted with MeOH to obtain 4 fractions. Fraction E3 was fractionated by CC eluted with *n*-hex-CH_2Cl_2-MeOH (*n*-hex: CH_2Cl_2, 4:6→1:9, CH_2Cl_2: MeOH, 10:0, 9:1). Fraction E3-10 was further purified by PTLC developed with $CHCl_3$: MeOH (97:3) to obtain ursolic acid (**13**) (11 mg). Fraction F (13-16) was further fractionated using CC packed with sephadex LH-20 and eluted with MeOH. Purification of F5 by RC eluted with CH_2Cl_2-EtOAc (10:0→7:3) yielded kaempferol (**3**) (2.9 mg). Fraction G (18-19) was fractionated again with CC by isocratic elution CH_2Cl_2: MeOH (9:1) afforded 17 fractions. Purification of G15 by PTLC developed with CH_2Cl_2: MeOH (9:1) yielded a mixture of (+)-epicatechin (**2**) and 7,4'-dihydroxy-5,3',5'-trimethoxyflavonol (0.8 mg) (**1**). Fraction H (25-27) was further fractionated using VLC eluted with CH_2Cl_2-MeOH (10:0→8:2) afforded 18 fractions, in which β-sitosterol-3-*O*-glucopyranoside (**10**) (14 mg) was obtained by recrystallization of H7-9 from $CHCl_3$ and MeOH. Fraction H15 was subjected to RC eluted with CH_2Cl_2: MeOH (9:1, 8:2) to afford astragalin (**4**) (30 mg). Further purification of H16-18 using RC eluted with CH_2Cl_2-MeOH (9:1→7:3) followed by reverse phase PTLC developed with H_2O-MeOH (6:4) yielded a mixture of hyperin (**5**) and isoquercitrin (**6**) (20 mg).

7,4'-dihydroxy-5,3',5'-trimethoxyflavonol (***1***), brown powder. UV (MeOH) λ_{max} (log ε) 284 nm; FTIR-ATR (solid) V_{max}: 1214, 1515, 1609, 1719, 3384 cm^{-1}. ^{1}H-NMR (600 MHz, CD_3OD) δ_H 6.99 (1H, d, J_m= 1.8 Hz, H-6), 7.05 (1H, d, J_m= 1.8 Hz, H-8), 7.45 (2H, s, H-2' & H-6'), 3.90 (6H, s, 3'/5'-OCH_3), 3.81 (3H, s, 5-OCH_3). APT-NMR (150 MHz, CD_3OD) δ_C 143.0 (C-2), 172.4 (C-4), 149.1 (C-5), 106.4 (C-6), 167.5 (C-7), 112.0 (C-8), 152.1 (C-9), 40.8 (C-10), 120.2 (C-1'), 108.8 (C-2'/6'), 149.2 (C-3'/5'), 166.2 (C-4'), 56.9 (3'/5'-OCH_3), 56.7 (5-OCH_3).; HRESIMS m/z 361.3316 $[M + H]^+$ (cald 360.31004 for $C_{18}H_{16}O_8$).

Acetylcholinesterase inhibitory activity

The extracts and compounds were dissolved in DMSO and phosphate buffers (0.1 M, pH 7.4). The reaction mixture consisted of phosphate buffer (140 µL), sample (20 µL) and AChE enzymes (20 µL, 0.09 U/mL) was pre-incubated at room temperature for 15 minutes. Then, 5,5-dithiobis (2-nitrobenzoic) acid (10 µL, 10 mM) and acetylthiocholine iodide (10 µL, 14 mM) were added and incubated again for further 15 minutes. The formation of coloured product was measured at 412 nm using 96-well microplate reader. Compounds having more than 50% inhibition were further evaluated at different concentrations to determine its half maximal inhibitory concentration (IC_{50})[9].

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 IC_{50} 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**), isoquercitrin (**6**), stigmast-4-en-3-one (**7**), mixture of β-sitosterol (**8**) and stigmasterol (**9**), β-sitosterol-3-*O*-glucopyranoside (**10**), betulin (**11**), betulinic acid (**12**), and ursolic acid (**13**)[10-17].

A new flavonol (**1**) was obtained along with compound (**2**) as a mixture in the form of brown powder. A molecular formula of $C_{18}H_{16}O_8$ was generated from LC-MS QTOF with its $[M+H]^+$ at *m/z* 361.3316 (cald 361.3179 for $C_{18}H_{16}O_8$). The FTIR-ATR spectrum showed a broad peak of hydroxyl (O-H) at 3384 cm^{-1}, a strong peak of carbonyl (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 1214 cm^{-1}.

The ^{1}H-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) assignable as H-6 and H-8 of ring A. A singlet aromatic proton signal at δ_H 7.45 integrated for two protons was assigned as H-2' and H-6' of ring B. A singlet appeared at δ_H 3.90 integrated for six protons is assigned to two methoxy protons at C-3' and C-5' of ring B while another singlet signal at δ_H 3.81 is attributed to methoxy protons at C-5 of ring A. The integration of methoxy protons and aromatic protons of ring B suggesting these protons are arranged in symmetrical manner. The APT-NMR spectrum displayed 14 carbon signals including two signals of methoxy carbon at δ_C 56.7 (5-OCH_3) and 56.9 (3'/5'-OCH_3), three signals of methine carbon at δ_C 106.4 (C-6), 112.0 (C-8) and 108.8 (C-2'/6') and nine signals of quaternary carbons. Carbonyl carbon was observed at δ_C 172.4 (C-4) while C-9 was observed at δ_C 152.1. The chemical shift for C-3 was not detected. The absence of typical singlet aromatic proton signal assignable at C-3 as well as chemical shift value for C-2 suggesting this compound is of flavonol moiety[18].

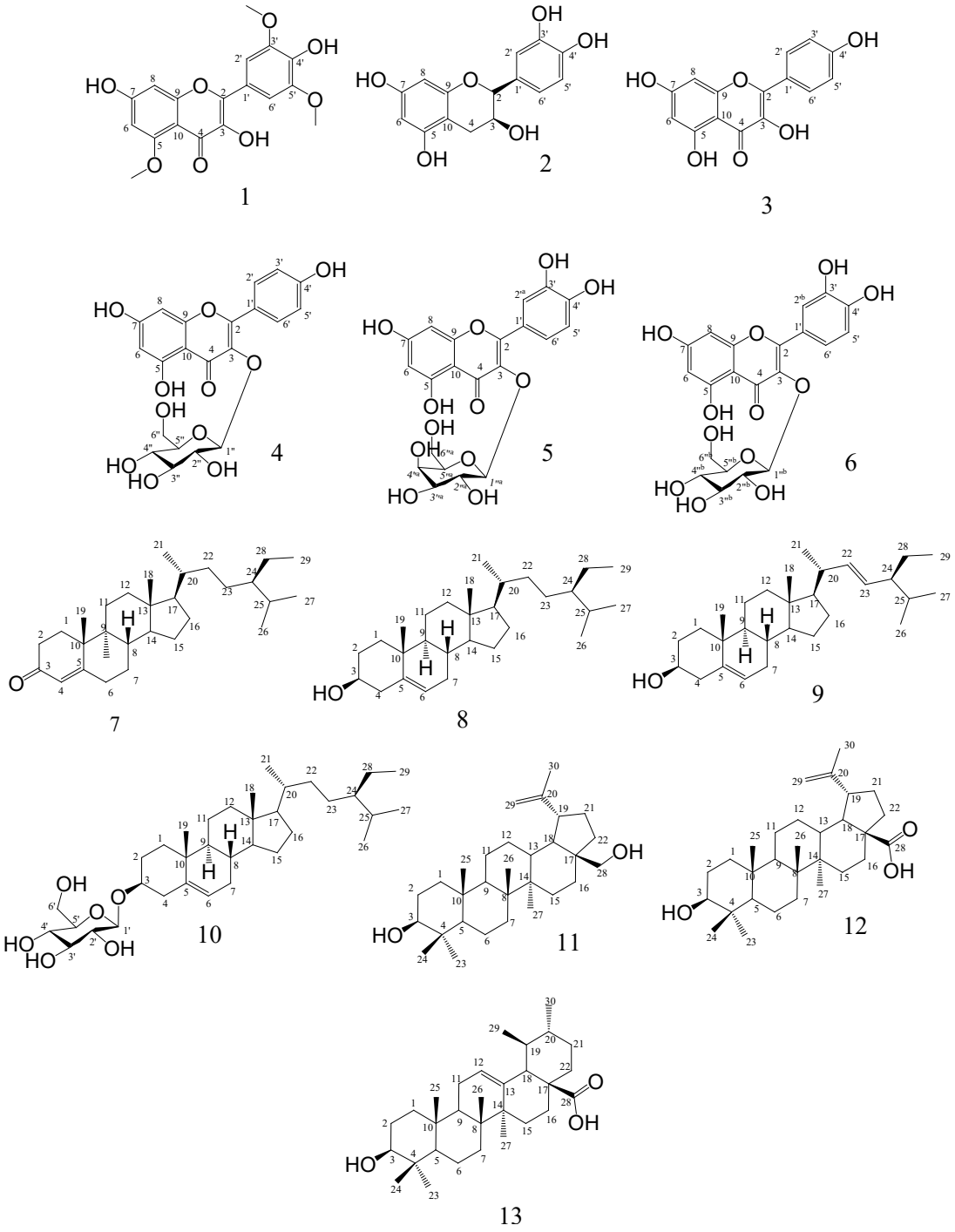

Figure 1: Chemical structures from *D. discolor.*

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-2'/6' showed HMBC cross peaks with C-1', C-2, C-3'/5' and C-4' which confirmed their placement at ring B. The connection of ring B to ring C was deduced based on HMBC correlations of H-2'/6' to C-1' and C-2. Two methoxy groups were assigned at C-3' and C-5' by observing HMBC cross peaks of H-2' and H-6' with C-3' and C-5'. The location of hydroxyl group at C-4' was confirmed based on ^{3}J 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[19]. Close inspection of all spectroscopic data confirmed that compound **1** is 7,4'-dihdroxy-5, 3',5'-trimethoxyflavonol.

A flavonoid (**4**) and six triterpenes (**7**-**12**) from the leaves and stem barks of *D. 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

Table 1: Acetylcholinesterase (AChE) Inhibitory Activity of Isolated Compounds.

Compounds	% AChE inhibition at 10 µM*	IC_{50} µM**
Astragalin (4)	34.57 ± 1.42^{c}	n.d.
Stigmast-4-en-3-one (**7**)	51.77 ± 1.05^{b}	11.77 ± 2.11
β-sitosterol (**8**) & stigmasterol (**9**)	$20.52 \pm 1.43^{d,e}$	n.d.
β-sitosterol-3-O-glucopyranoside (**10**)	15.58 ± 3.21^{e}	n.d.
Betulin (**11**)	$30.13 \pm 0.46^{c,d}$	n.d.
Betulinic acid (**12**)	$32.48 \pm 1.79^{c,d}$	n.d.
Physostigmine	100 ± 0.24^{a}	0.008 ± 0.004

Data were expressed as mean ± standard deviation where (*n=3 or **n=9), n.d. = not determined. Data with different superscript lower letter are significantly different ($p<0.05$).

in dose-dependent manner, it gave an IC_{50} 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 ß-sitosterol-3-*O*-glucopyranoside (**10**) were previously reported to have low inhibition concentration against AChE[20,22,23].

CONCLUSIONS

D. discolor (syn. *D. blancoi*) was found to inhibit AChE during random screening. Phytochemical study on the leaves and stem barks of *D. discolor* yielded a new flavonol, 7,4'-dihydroxy-5,3',5'-trimethoxyflavonol (**1**) along with five known flavonoids and six known triterpenes. The compounds examined for AChE inhibitory activity showed moderate inhibiton concentration except for stigmast-4-en-3-one (**7**). 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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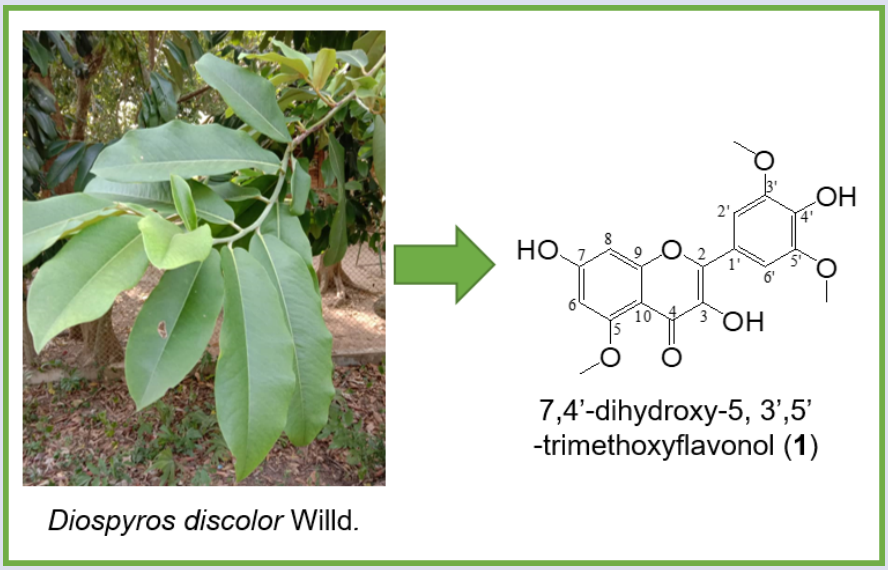
GRAPHICAL ABSTRACT



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