Investigation of the n-hexane extract of Melodorum fruticosum stem

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ABSTRACT

Introduction: Melodorum fruticosum is distributed widely in ASEAN countries. Although this species has been used in folk medicine to cure some diseases, its chemical constituents and biological activity have not been systematically studied. This paper reports the isolation and structural elucidation of seven compounds from the n-hexane extract as well as their cytotoxicity against Jurkat and MCF-7-cell lines. Method: Dried powder of Melodorum fruticosum stem was macerated in ethanol at room temperature to prepare the crude extract. This crude was separated into n-hexane, ethyl acetate, and the remaining aqueous extracts by liquid–liquid partition method. Then, the organic compounds were isolated by chromatographic methods and structurally elucidated by modern spectroscopic techniques. Cytotoxicity assays were performed using the sulforhodamine B method (SBR assay). Results: The n-hexane extract showed potential activity against the MCF-7-cell line with an IC₅₀ value of 14.37 ± 3.18 μg/mL. From this extract, seven compounds consisting of techtochrysin (1), pinostrobin (2), flavokavin B (3), 2′-hydroxy-3′,4′,6′-trimethoxychalcone (4), 2′-hydroxy-4′,5′,6′-trimethoxychalcone (5), stigmast-4-ene-3-one (6), and lupeol (7) were isolated and chemically elucidated. Conclusion: It is necessary to continue to isolate and perform a bioassay of organic compounds from this extract to determine the bioactive components.

Key words: Melodorum fruticosum, flavonoid, chalcone, steroid, terpenoid, cytotoxicity

INTRODUCTION

Melodorum fruticosum Bour. MERR. (a synonym name, Rauwenhoffia siamensis Schefl) belongs to the Annonaceae family. It is distributed in Vietnam, Laos, Cambodia, Thailand, Malaysia, and Indonesia. In Vietnamese folk medicine, leaves of M. fruticosum were used to treat digestive ailments and breast swelling, and roots were used to treat abdominal distension and blood tonic for postpartum women¹. Flowers and bark of M. fruticosum possess antiviral, antioxidant, and cytotoxic activities². Some extracts and compounds isolated from M. fruticosum revealed significant cytotoxicity against some cancer cell lines, such as A-549, MCF-7, HT-29, SK-MEL-5, Malme-3 M, 9-PS, and KBMRI³⁴. Previously, phytochemical studies reported the presence of flavonoids, alkaloids, steroids, heptenoids, and tannins from leaves, stem bark, and flowers of M. fruticosum⁵.⁶. This paper showed the cytotoxicity against the Jurkat and MCF-7 cancer cell lines of the n-hexane extract of the M. fruticosum stem and the chemical elucidation of some compounds isolated from this one.

MATERIALS AND METHODS

General experimental procedures

The NMR spectra were registered on Bruker Avance at 500 MHz for ¹H-NMR and 125 MHz for ¹³C-NMR. HR-ESI-MS spectra were recorded on a Bruker MicroOTOF-QII. The optical specific rotation was measured on a Kruss (Germany) polarimeter with a tube length of 0.5 decimetres.

Plant material

The stem of Melodorum fruticosum L. was collected at Di Linh Ward, Lam Dong Province, Vietnam, in July 2017. The scientific name of this species was authenticated by Dr. Dang Van Son, Institute of Tropical Biology, Vietnam Academy of Science and Technology. A voucher specimen (No US-A012) was deposited in the herbarium of the Department of Organic Chemistry, University of Science, Vietnam National University - Ho Chi Minh City.

Extraction and isolation

The dried powder of M. fruticosum stem (90 kg) was macerated in ethanol at room temperature, and then the filtrated solution was evaporated at reduced pressure to give a crude extract (6 kg). The liquid–liquid
partition method with n-hexane and ethyl acetate, in turn, was used to separate the crude extract into different fractions.

The n-hexane extract (250 g) was subjected to normal-phase silica gel column chromatography. The eluent solvent systems n-hexane-ethyl acetate (stepwise, 99:1, 98:2, 95:5, 90:10, 80:20, 50:50, 0:100) were used to afford 11 fractions (H1–H11). Fraction H3 (20.0 g) was further separated into 6 subfractions (H3.1–H3.6) by normal-phase silica gel column chromatography eluted with n-hexane-ethyl acetate (stepwise, 95:5, 90:10, 80:20). Subfraction H3.2 (4.48 g) was subjected to silica gel column chromatography and eluted with solvent systems of n-hexane-ethyl acetate (95:5, 90:10, in turn) to give compound 1 (10.0 mg). The same method was applied to subfraction H3.3 (2.58 g) to afford 3 (8.9 mg) and 5 (7.0 mg). A silica gel column chromatography eluted with n-hexane-ethyl acetate (95:5, 90:10, in turn) was applied to subfraction H3.5 (4.36 g) to afford four subfractions (H3.5.1–H3.5.4). Compounds 6 and 7 (7.0 mg) and 16 (9.6 mg) were isolated from subfraction H3.5.1 (0.20 g), while compound 2 (12.0 mg) was obtained from subfraction H3.5.3 (1.02 g) via silica gel column chromatography eluted with n-hexane-ethyl acetate (stepwise, 95:5, 90:10). Finally, compound 4 (7.4 mg) was isolated from fraction H3.6 (4.05 g) through silica gel column chromatography, eluted with n-hexane-ethyl acetate (95:5, 90:10, in turn), further purified by Sephadex LH-20, and eluted with methanol.

### Cytotoxic assay

The cytotoxicity activities against Jurkat and MCF-7 cancer cell lines of the n-hexane extract and compounds 1 and 2, using the sulforhodamine B method (SRB assay) and camptothecin as a positive control, were determined as described in a previous report. This assay was performed at the Molecular Biology Laboratory, Faculty of Biology, University of Science, Vietnam National University-Ho Chi Minh City.

### RESULTS

From the n-hexane extract of *Melodorum fruticosum* collected in Di Linh Ward, Lam Dong Province, seven compounds, 1 (10.0 mg), 2 (12.0 mg), 3 (8.9 mg), 4 (7.4 mg), 5 (7.0 mg), 6 (7.0 mg), and 7 (9.6 mg), were isolated. Their physical properties and spectroscopic data were obtained as follows.

#### Technochrysin (1): Yellow powder. HR-ESI-MS: m/z 291.0628 [M+Na]+ (calcd. for C_{10}H_{12}O_{3}Na, 291.0633). H-NMR data (DMSO-d_6) (J in Hertz): δ_H (s, H-3), 6.40 (d, 2.0, H-6), 6.82 (d, 2.0, H-8), 8.11 (dd, 8.0, 1.5, H-2′/H-6′), 7.59 (m, H-3′/H-5′), 7.62 (m, H-4′), 3.88 (s, 7-OCH_3), and 12.81 (s, 5-OH). 13C-NMR (DMSO-d_6): δ_C 163.5 (C-2), 105.3 (C-3), 182.0 (C-4), 161.1 (C-5), 98.1 (C-6), 156.1 (C-7), 92.8 (C-8), 157.4 (C-9), 104.9 (C-10), 130.6 (C-1′), 126.4 (C-2′/C-6′), 129.1 (C-3′/C-5′), 132.1 (C-4′) and 56.1 (7-OCH_3). Selected HMBC correlations: see Figure 1.

#### Pinostrobin (2): Yellow powder. [α]_{D}^{25} = 0 (c 0.10, acetone) (Pinostrobin, not mentioned). HR-ESI-MS: m/z 293.0784 [M+Na]+ (calcd. for C_{16}H_{14}O_{4}Na, 293.0790). H-NMR data (DMSO-d_6) (J in Hertz): 5.63 (dd, 13.0, 3.0, H-2), 2.83 (dd, 17.0, 3.0, H-3a), 3.31 (dd, 17.0, 13.0, H-3b), 6.10 (d, 2.5, H-6), 6.15 (d, 2.5, H-8), 7.52 (dd, 8.5, 1.0, H-2′/H-6′), 7.43 (m, H-3′/H-5′), 7.39 (m, H-4′), 3.80 (s, 7-OCH_3), and 12.10 (s, 5-OH). 13C-NMR data (DMSO-d_6): δ_C 78.5 (C-2), 42.1 (C-3), 196.4 (C-4), 163.2 (C-5), 94.7 (C-6), 167.4 (C-7), 93.8 (C-8), 162.6 (C-9), 102.6 (C-10), 138.5 (C-1′), 126.6 (C-2′/C-6′), 128.5 (C-3′/C-5′), 128.5 (C-4′) and 55.8 (7-OCH_3). Selected HMBC correlations: see Figure 1.

#### Flavokavin B (3): Yellow powder. HR-ESI-MS: m/z 285.1180 [M+H]^+ (calcd. for C_{17}H_{14}O_{4}H^+, 285.1127). H-NMR data (CDCl_3) (δ_H in Hertz): 7.61 (dd, 8.0, 1.5, H-2/H-6), 7.41 (m, H-3/H-5), 7.40 (m, H-4), 7.74 (d, 16.0, H-7), 7.90 (d, 16.0, H-8), 6.11 (d, 2.5, H-3′), 5.97 (d, 2.5, H-5′), 3.92 (s, 4′-OCH_3), and 3.84 (s, 6′-OCH_3). 13C-NMR data (CDCl_3): δ_C 135.6 (C-1), 128.4 (C-2/C-6), 128.9 (C-3/C-5), 130.1 (C-4), 142.3 (C-7), 127.6 (C-8), 192.7 (C-9), 106.4 (C-1′), 166.3 (C-2′), 93.9 (C-3′), 168.4 (C-4′), 91.3 (C-5′), 126.2 (C-6′), 55.9 (4′-OCH_3) and 55.6 (6′-OCH_3). Selected HMBC correlations: see Figure 1.

#### 2′-Hydroxy-3′,4′,6′-trimethoxycalcone (4): Yellow powder. HR-ESI-MS: m/z 337.1054 [M+Na]^+ (calcd. for C_{16}H_{18}O_{5}Na, 337.1052). H-NMR data (DMSO-d_6) (δ_H in Hertz): 7.72 (dd, 80, 1.5, H-2/H-6), 7.45 (m, H-3/H-5), 7.44 (m, H-4), 7.63 (d, 16.0, H-7), 7.66 (d, 16.0, H-8), 12.63 (s, 2′-OCH_3), 3.61 (s, H-5′), 3.65 (s, 3′-OCH_3), and 3.92 (s, 4′-OCH_3/6′-OCH_3). 13C-NMR data (DMSO-d_6): δ_C 134.7 (C-1), 128.4 (C-2/C-6), 128.9 (C-3/C-5), 134.7 (C-4), 142.4 (C-7), 127.6 (C-8), 192.7 (C-9), 107.1 (C-1′), 157.7 (C-2′), 130.0 (C-3′), 157.6 (C-4′), 88.5 (C-5′), 157.2 (C-6′), 60.0 (3′-OCH_3), 56.3 (4′-OCH_3) and 56.0 (6′-OCH_3). Selected HMBC correlations: see Figure 1.

#### 2′-Hydroxy-4′,5′,6′-trimethoxycalcone (5): Yellow powder. HR-ESI-MS: m/z 337.1048 [M+Na]^+ (calcd. for C_{18}H_{18}O_{5}Na, 337.1052). H-NMR data (CDCl_3) (δ_H in Hertz): 7.64 (dd, 80, 1.5, H-2/H-6), 7.43 (m, H-3/H-5), 7.39 (m, H-4), 7.84 (d, 15.5, H-7), 6.82 (d, 2.0, H-8), 8.11 (dd, 8.0, 1.5, H-2/H-6′), 7.59 (m, H-3′/H-5′), 7.62 (m, H-4′), 3.88 (s, 7-OCH_3), and 12.81 (s, 5-OH).
7.93 (d, 15.5, H-8), 13.64 (s, 2'-OH), 6.29 (s, H-3'), 3.84 (s, 4'-OCH$_3$), 3.90 (s, 6'-OCH$_3$), and 3.94 (s, 6'-OCH$_3$). 13C-NMR data (CDCl$_3$): $\delta$C 135.3 (C-1), 128.4 (C-2/C-6), 129.0 (C-3/C-5), 130.3 (C-4), 143.2 (C-7), 126.6 (C-8), 193.0 (C-9), 108.8 (C-1'), 162.7 (C-2'), 96.6 (C-3'), 155.0 (C-4'), 135.4 (C-5'), 160.2 (C-6'), 61.9 (4'-OCH$_3$), 61.3 (5'-OCH$_3$), and 56.1 (6'-OCH$_3$). Selected HMBC correlations: see Figure 1.

**Stigmaster-4-ene-3-one (6):** White powder. HR-ESI-MS: m/z 435.3555 [M+Na]$^+$ (calcd. for C$_{29}$H$_{48}$ONa, 435.3603). $^1$H-NMR data (CDCl$_3$) ($\delta$H, J in Hertz): 5.72 (s, H-4), 0.71 (s, H-18), 1.18 (s, H-19), 0.92 (d, 6.5, H-21), 0.84 (d, 7.5, H-26), 0.81 (d, 7.5, H-27), and 0.85 (t, 7.5, H-29). 13C-NMR data (CDCl$_3$): $\delta$C 35.7 (C-1), 34.0 (C-2), 199.6 (C-3), 123.7 (C-4), 171.8 (C-5), 33.0 (C-6), 32.1 (C-7), 35.7 (C-8), 53.9 (C-9), 38.6 (C-10), 21.0 (C-11), 39.7 (C-12), 42.4 (C-13), 56.0 (C-14), 24.2 (C-15), 28.2 (C-16), 56.0 (C-17), 12.0 (C-18), 17.4 (C-19), 36.1 (C-20), 18.7 (C-21), 34.0 (C-22), 26.1 (C-23), 45.9 (C-24), 29.2 (C-25), 20.0 (C-26), 19.0 (C-27), 23.1 (C-28), and 11.9 (C-29). Selected HMBC correlations: see Figure 1.

**Lupeol (7):** White powder. $^1$H-NMR data (CDCl$_3$) ($\delta$H, J in Hertz): $\delta$H 3.19 (dd, 11.5, 5.0, H-3), 2.39 (ddd, 11.5, 11.5, 6.9, H-19), 0.97 (s, H-23), 0.76 (s, H-24), 0.83 (s, H-25), 1.03 (s, H-26), 0.94 (s, H-27), 0.79 (s, H-28), 4.68 (d, 2.5, H-29a), 4.56 (m, H-29b), and 1.68 (bs, H-30). 13C-NMR data (CDCl$_3$): $\delta$C 38.8 (C-1), 27.5 (C-2), 79.0 (C-3), 38.9 (C-4), 55.4 (C-5), 18.4 (C-6), 34.3 (C-7), 41.0 (C-8), 50.5 (C-9), 37.2 (C-10), 21.0 (C-11), 25.2 (C-12), 38.1 (C-13), 42.8 (C-14), 27.5 (C-15), 35.6 (C-16), 43.2 (C-17), 48.3 (C-18), 48.3 (C-19), 151.0 (C-20), 29.9 (C-21), 40.0 (C-22), 28.0 (C-23), 15.4 (C-24), 16.1 (C-25), 16.0 (C-26), 14.6 (C-27), 18.0 (C-28), 109.3 (C-29), and 19.3 (C-30). Selected HMBC correlations: see Figure 1.

**DISCUSSION**

Compound 1 was isolated as a yellow powder. The high-resolution mass spectrum of 1 showed a sodiated molecular ion peak at m/z 291.0628 [M+Na]$^+$ (calcd. for C$_{16}$H$_{12}$O$_4$Na, 291.0633). The proton spectrum of 1 displayed a singlet signal at $\delta$H 12.81 of the hydroxy group at C-5 possessing an intramolecular hydrogen bond with the carbonyl group at C-4 in the flavonoid skeleton, as usual. Two doublet signals at $\delta$H 6.40 and 6.82 with the small coupling constant of 2.0 Hz were assigned to H-6 and H-8, respectively, of ring A. One singlet aromatic proton signal at $\delta$H 7.03 (s, H-3) was H-3 of ring C. Five aromatic protons at $\delta$H 8.11 (dd, 8.0, 1.5 Hz, H-2'/H-6'), 7.59 (m, H-3'/H-5'), and 7.62 (m, H-4') belonged to a phenyl group (ring C). In addition, at a high magnetic field, there was a singlet methoxy proton signal at $\delta$H 3.88 (s, 7-OCH$_3$). This information suggested that 1 was a flavone possessing a hydroxy group at C-5 and a methoxy group at C-7. This corresponded to the presence of fourteen carbon signals, including one methoxy carbon, two aromatic carbons at $\delta$C 126.4 (C-2'/C-6') and 129.1 (C-3'/C-5'), appearing in double intensity and possessing both HSQC and HMBC experiments with protons H-2'/H-6' and H-3'/H-5', respectively, of a symmetrical benzene B ring. This phenyl group, attached to C-2, was determined by the HMBC cross-peaks of the proton H-3 to carbon C-1' ($\delta$C 130.6) and of protons H-2'/H-6' to carbons C-2 and C-1' (Figure 1). The HMBC spectrum also revealed the cross-peaks of the hydroxy proton with carbons C-5, C-6, and C-10 and of the methoxy proton with carbon at $\delta$C 156.1 (C-7). Based on the compatibility of the NMR data of 1 with those in the literature, 1 was assigned to be techtochrysin.
Compound 2 was isolated as a yellow powder. The HR-ESI-MS spectrum of 2 displayed a quasi-molecular ion peak at m/z 293.0784 [M+Na]⁺ (calcd. for C16H14O4Na, 293.0790). The comparison of the NMR data of 1 and 2 showed that carbons C-2 and C-3 of 2 were saturated, which was confirmed by the presence of two methylene proton signals at δ_H 2.83 (dd, 17.0, 3.0 Hz, H-3a) and 3.31 (dd, 17.0, 13.0 Hz, H-3b) and one oxygenated methine proton at δ_H 5.63 (dd, 13.0, 3.0 Hz, H-2), instead of the singlet olefinic proton H-3 as in 1. This corresponded to the observation of one methylene carbon signal at δ_C 42.1 (C-3) and one oxygenated methine at δ_C 78.5 (C-2), where its HSQC spectrum showed correlations to protons H-3 and H-2, respectively. This feature was further confirmed by the HMBC cross-peaks of both protons H-2 and H-3 to a carbonyl carbon at δ_C 196.4 (C-4) and of proton H-2 to carbons C-1' and C-2' of C-6'. The positions of the two substituents (OH and -OCH₃) were confirmed by the HMBC correlations, as shown in Figure 1. Due to the null specific optical rotation and the good compatibility of its NMR data with those of 1, its HMBC experiment well supported the proposed structure with cross-peaks of the hydroxy proton at δ_H 13.64 (s, 2'-OH) to carbons at δ_C 108.8 (C-1'), 162.7 (C-2'), and 96.6 (C-3'), of the methine proton at δ_H 6.29 (s, H-3') to carbons at δ_C 108.8 (C-1'), 155.0 (C-4'), and 135.4 (C-5'), and of three methoxy protons with carbons C-4', C-5' and C-6'. Therefore, 2 was determined to be 2'-hydroxy-4',5',6'-trimethoxychalcone, showing good compatibility. Its HMBC experiment well supported the proposed structure with cross-peaks of the hydroxy proton at δ_H 13.64 (s, 2'-OH) to carbons at δ_C 108.8 (C-1'), 162.7 (C-2'), and 96.6 (C-3'), of the methine proton at δ_H 6.29 (s, H-3') to carbons at δ_C 108.8 (C-1'), 155.0 (C-4'), and 135.4 (C-5'), and of three methoxy protons with carbons C-4', C-5' and C-6'. Therefore, 2 was determined to be 2'-hydroxy-4',5',6'-trimethoxychalcone.

Compound 6 was isolated as a white powder. The molecular formula of 6 was determined to be C29H48O via the sodiated molecular ion peak at m/z 435.3555 [M+Na]⁺ (calcd. for C29H48ONa, 435.3603). The 13C-NMR spectrum of 6 showed signals of 29 carbons, consisting of one carbonyl carbon at δ_C 199.6 (C-3) and two olefinic carbons at δ_C 123.7 (C-4) and 171.8 (C-5) of a conjugated ketone system. The remaining 26 carbons resonated at a high magnetic field from 11 to 56 ppm. These results suggested that 6 could be a sterol possessing a conjugated ketone group (>C=CH−CO−). Its 1H-NMR spectrum with two singlets, three doublets, and a triplet signal from 0.7 to 1.2 ppm was characterized for a stigmastane skeleton. In addition, the sole signal that resonated at the low magnetic field at δ_H 5.72 (s) belonged to the olefinic proton. The positions of two olefinic carbons, C-4 and C-5, were determined by the HMBC cross-peaks of the olefinic proton to carbons at δ_C 34.0 (C-2), 171.8 (C-5), 33.0 (C-6), and 38.6 (C-10). This meant that the position of the conjugated carbonyl carbon was suggested to be at C-3. In the comparison of the NMR data of 6 with published data, 6 was thus determined to be stigmast-4-ene-3-one.
Compound 7 was isolated as a white powder. The $^1$H and $^{13}$C-NMR data analysis of 7 suggested that it possessed a lupane skeleton characterized by signals of an isopropenyl unit at $\delta_H$ 4.68 (1H, d, 2.5 Hz, H-29a), 4.56 (1H, m, H-29b), and 1.68 (3H, brs, H-30) in the proton spectrum and two olefinic carbon signals at $\delta_C$ 151.0 (C-20) and 109.3 (C-29) in the $^{13}$C-NMR spectrum. In addition, 7 had a hydroxy group at C-3 as usual, which was confirmed by the HMBC cross-peaks of two singlet methyl proton signals at $\delta_H$ 0.97 (s, H-23) and 0.76 (s, H-24) to carbon C-3. Compound 7 was determined to be lupeol via the good compatibility of its NMR data with those published in the literature.

The n-hexane extract and compounds 1 and 2 were evaluated for their cytotoxicity against Jurkat and MCF-7 cancer cell lines. The results showed that the n-hexane extract possessed significant cytotoxicity against the MCF-7 cancer cell line with an IC$_{50}$ value of 14.37 ± 3.18 μg/mL. However, compounds 1 and 2 revealed weak activities against the two surveyed cancer cell lines. Therefore, the components that induced the toxicity of the n-hexane extract could be from other compounds in this extract. The isolation and bioassay of organic compounds from this extract should be continued.

**CONCLUSION**

From the n-hexane extract of *Melodorum fruticosum* stem, seven compounds were isolated consisting of two flavonoids (techtochrysin and pinosanol), three chalcones (flavokavin B, 2′-hydroxy-3′,4′,6′-trimethoxychalcone, and 2′-hydroxy-4′,5′,6′-trimethoxychalcone), a steroid (stigmast-4-ene-3β,7α), 4.56 (1H, d, 2.5 Hz, H-29a), and a triterpenoid (lupeol). Their chemical structures were elucidated based on NMR and HR-MS data analysis as well as comparison to published data. The n-hexane extract showed significant cytotoxicity against the MCF-7-cell line with an IC$_{50}$ value of 14.37 ± 3.18 μg/mL, whereas compounds 1 and 2 isolated from this extract were inactive. To the best of our knowledge, except for 1, six remaining compounds were isolated from *Melodorum fruticosum* for the first time.

**ABBREVIATIONS**

HR-ESI-MS: High resolution electrospray ionization-Mass spectrometry
$^1$H-NMR: Proton nuclear magnetic resonance
$^{13}$C-NMR: Carbon-13 nuclear magnetic resonance
HSQC: Heteronuclear single quantum coherence
HMBC: Heteronuclear multiple bond correlation
s: singlet
d: doublet
dd: doublet of doublets
$dd$: doublet of doublet of doublets
$t$: triplet
$m$: multiplet
calcd.: calculated
IC$_{50}$: Half-maximal inhibitory concentration
Jurkat cell: immortalized T-lymphocyte cell line
MCF-7 cell: breast cancer cell line

**COMPETING INTEREST**

The authors declare no competing financial interest.

**AUTHORS’ CONTRIBUTION**

Du T.T.X. and Nguyen T.M.H. contributed to conducting experiments and acquiring data. Nguyen N.K.V. and Nguyen K.P.P. interpreted NMR and MS data. Nguyen T.H.T. provided final approval of the manuscript to be submitted.

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