PMP11

CHEMICAL CONSTITUENTS AND BIOLOGICAL ACTIVITIES FROM BRANCHES OF *Mitragyna diversifolia* Havil.

องค์ประกอบทางเคมีและฤทธิ์ทางชีวภาพจากกิ่งกระทุ่มนา (*Mitragyna diversifolia* Havil.)

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ABSTRACT

Chromatographic separation of the hexane, EtOAc, MeOH and alkaloid extracts of air-dried branches of *Mitragyna diversifolia* Havil. or "Krathum na" yielded two triterpenoids (**1** and **4**), one phenolic acid (**3**), one cumarin (**2**) and one alkaloid (**5**) and their structures were determined based on the spectroscopic methods. The bioactivity assays found that compound **3** exhibited cytotoxicity against NCI-H187 cell lines with IC_{50} value of 25.24 µg/ml.

บทคัดย่อ

การแยกด้วยเทคนิคโครมาโทกราฟีของส่วนสกัดหยาบ เฮกเซน เอทิลอะซิเตท เมทานอล และ อัลคาลอยด์จากกิ่งกระทุ่มนา (*Mitragyna diversifolia* Havil.) ซึ่งการพิสูจน์โครงสร้างของสารอาศัยเทคนิคทางสเปกโทรสโกปีได้พบสารทั้งหมด 5 สาร คือสาร **1** และ สาร **4** เป็นสารในกลุ่มเทอร์ปีน สาร **2** เป็นสารในกลุ่มคูมาริน สาร **3** เป็นสารในกลุ่มของกรดฟีนอลริก และ สาร **5** เป็นสารในกลุ่มอัลคาลอยด์ จากการทดสอบฤทธิ์ทางชีวภาพพบว่า สาร **4** เป็นพิษต่อ เซลล์มะเร็งปอด (NCI-H187) ที่ค่า IC_{50} เท่ากับ 25.24 µg/ml

Key Words : *Mitragyna diversifolia* Havil., alkaloid
คำสำคัญ : กระทุ่มนา อัลคาลอยด์

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Introduction

Health problem becomes one of the serious problems around the world. There are a lot of diseases occurring long time ago but still cause death of human such as malaria, tuberculosis and cancer. People in many countries suffered from these diseases due to drug resistant behavior. To solve this problem, therefore, the search for substituted drugs is a good alternative. Bioactive natural products are our important source for new candidate drugs developed to treat these fatal diseases (Natural Product drug Discovery, 2002).

On our search for bioactive compounds from Thai plants, hexane extract from branches of *Mitragyna diversifolia* Havil. showed antimycobacterial activity against *Mycobacterium Tuberculosis* (MIC 12.5 μg/ml) and cytotoxicity against Human Breast Cancer cell (IC_{50} 20.02 μg/ml).

Morphology

The genus *Mitragyna* belongs to the family Rubiaceae and is found in swampy territory in the tropical and sub-tropical regions of Africa and Asia (*Mitragyna, n.d*).

Mitragyna diversifolia Havil. (Figure 1) is found in the Northeastern part of Thailand and commonly known as "Krathum na". It is the medium size tree with 8-15 m tall (*Mitragyna diversifolia*, 2006).

Figure 1 *Mitragyna diversifolia* Havil.

Experimental

Materials and chemicals

The branches of *M. diversifolia* Havil. were collected on the campus of Khon Kaen Universitry in December 2006. The plant material was identified by Prof. Dr. Pranom Chantaranothai, Department of Biology, Khon Kaen University, Khon Kaen, Thailand, where the voucher specimen was deposited.

Column Chromatography and Flash Column Chromatography (CC and FCC) were carried out on MERCK silica gel 60 (less than 0.063 mm and 0.063-0.200 mm). Silica gel 60 PF_{254} mesh for Preparative Thin Layer Chromatography (PLC). Thin Layer Chromatography (TLC) were carrier out on silica gel 60 PF_{254} aluminium sheets (20x20 cm). The chromatograms were detected by Ultraviolet light (UV) were at 254 nm and 366 nm and further sprayied with anisaldehyde reagent. Solvents for extraction were hexane, dichloromethane, ethyl acetate, methanol, hydrochloric acid, ammonium hydroxide.

Melting points were recorded in ºC on a Gallenkamp electrothermal melting point apparatus. IR spectra were recorded as KBr disk on Perkin Elmer Spectrum One FT-IR Spectrometer. NMR spectra were obtained from a Varian Mercury plus 400 spectrometer. Chemical shift were recorded in δ (ppm) scale using $CDCl_3$ and CD_3OD as the solvents and TMS as the internal standard. HRESITOFMS mass spectra were obtained using a Micromass LCT mass spectrometer, and the lock mass calibration was applied for the determination of accurate masses.

Methodology

Extraction and isolation

Air-dried branches (6.3 kg) of *M. diversifolia* Havil. were ground into powder and then extracted successively at room temperature with hexane (20 mL x3), EtOAc (20 mL x3) and MeOH (20 mL x3). The extracted solutions were evaporated in vacuum to give crude hexane (14.8 g), crude EtOAc (55.2 g) and crude MeOH (442.0 g) extracts. Crude MeOH 150.0 g was extracted with 5% HCl and CH_2Cl_2 to give a CH_2Cl_2 layer and an acidified aqueous layer (Pandy et al., 2006). The acidified aqueous layer was then basified with 10% NH_4OH and extracted with CH_2Cl_2 to yield CH_2Cl_2 layer. The CH_2Cl_2 layer was dried over anhy. Na_2SO_4 and concentrated under reduced pressure to give the crude alkaloid extract (CH_2Cl_2) 668 mg. The extraction scheme of branches of *M. diversifolia* Havil. is shown in Figure 2.

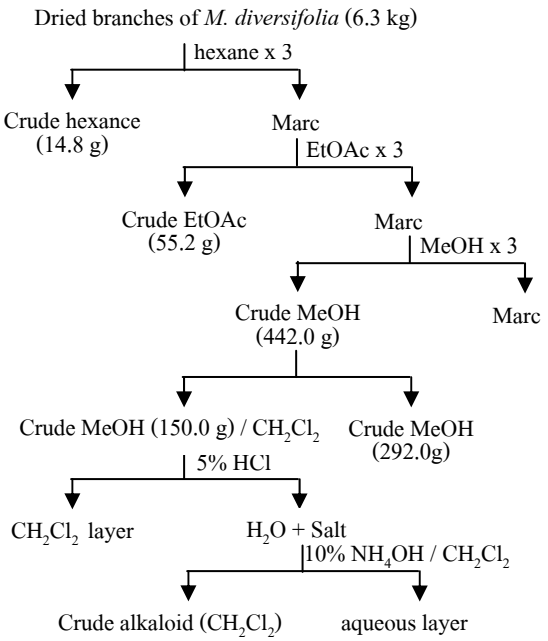

Figure 2 solvent extraction schemes of branches of *M. diversifolia*.

The crude hexane extract (14.8 g) was separated over silica gel FCC eluting with gradient of EtOAc:hexane, EtOAc, MeOH:EtOAc and MeOH, respectively. A 200 ml of eluent was collected for each fraction to give seventy-three fractions. According to TLC patterns, these fractions were combined to nine fractions designated as HF_1-HF_9. Fraction HF_4 was separated by FCC with gradient system of hexane and EtOAc by increasing polarity of solvents to give five subfractions, $HF_{4.1}$-$HF_{4.5}$. Fraction $HF_{4.2}$ was purified by PLC eluting with 55% CH_2Cl_2:hexane (developed four times) and recrystalized from CH_2Cl_2-hexane to give white amorphous solid of compound **1** (20.8 mg). Fraction HF_7 was dissolved in hexane and the precipitate was filtered out to obtain white solid of compound **4** (8.0 mg). The filtrate of fraction HF_7 and fraction HF_8 was separated by FCC to obtain five subfractions, $HF_{7.1}$-$HF_{7.5}$. Fraction $HF_{7.3}$ was further purified by FCC and PLC to give an additional amount of **1** (12.3 mg).

The crude CH_2Cl_2 extract (668 mg) was subjected to FCC, eluted with a gradient of CH_2Cl_2, MeOH:CH_2Cl_2 and MeOH, respectively. A 100 ml eluent was collected for each fraction to afford 21 fractions which were combined based on TLC patterns to yield 11 major fractions designated as CF_1 to CF_{11}. Fractions CF_3 was recrystalized with CH_2Cl_2-hexane to yield pale yellow needles of compound **2** (5.6 mg). Fractions CF_4 was separated by FCC to give three subfractions ($CF_{4.1}$-$CF_{4.3}$). Precipitation of combined fractions $CF_{4.2}$ and $CF_{4.3}$ from CH_2Cl_2/MeOH and hexane afforded a white amorphous solid of compound **5** (5.0 mg). Fraction CF_5 was separated by FCC to give three subfractions ($CF_{5.1}$-$CF_{5.3}$). Fraction $CF_{5.3}$ was then recrystallized with CH_2Cl_2/MeOH and

hexane to give an additional amount of compound **5** (20.4 mg).

The crude EtOAc extract (55.2 g) was subjected to FCC, eluted with gradient system of CH_2Cl_2, MeOH:CH_2Cl_2 and MeOH, respectively. A 150 ml eluent was collected for each fraction to afford 8 major fractions combined based on TLC patterns designated as EF_1 to EF_8. Fraction EF_2 was separated by FCC to give five subfractions, $EF_{2.1}$-$EF_{2.5}$. Recrystalization of fraction $EF_{2.4}$ with CH_2Cl_2 and hexane gave an additional amount of compound **2** (23.8 mg). Fraction EF_5 was separated by FCC to give six subfractions $EF_{5.1}$-$EF_{5.6}$. Fraction $EF_{5.5}$ was recrystalized with CH_2Cl_2 and hexane to obtain an additional amount of compound **2** (19.8 mg). Fraction EF_8 was separated by FCC to give five fractions $EF_{8.1}$-$EF_{8.5}$. Fraction $EF_{8.4}$ was purified by PLC eluting with 7% MeOH:CH_2Cl_2 (developed twice) to give compound **3** (17.9 mg). All of the isolated compounds were tested for human diseases including *plasmodium flaciparum* (anti-malarial), *Mycrobacterium falciparum* (anti-TB) and anti cancer cell lines KB, BC1, NCI-H187.

Bioassays

The bioassay experiments were carried out at the National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand.

Antimalarial assay

Antimalarial activity was evaluated against the parasite *Plasmodium falciparum* (K1, multidrug-resistant strain), using the method of Trager and Jensen (Trager and Jensen, 1976). Quantitative assessment of malarial activity *in vitro* was determined by means of the microculture radioisotope technique based upon the method described by Desjardins (Desjardins et al., 1979). The inhibitory concentration (IC_{50}) represents the concentration that causes 50% reduction in parasite growth as indicated by the *in vitro* uptake of [^{3}H]-hypoxanthine by *P. falciparum*. The standard compound was artemisinin.

Cytotoxicity assay

Cytotoxic assays against human epidermoid carcinoma (KB), human breast cancer (BC1), and human small cell lung cancer (NCI-H187) cell lines were performed employing the colorimetric method as described by Skehan (Skehan et al., 1990). The reference substance was ellipticine.

Results and discussion

Compounds **1-5** isolated from hexane CH_2Cl_2 and EtOAc of the air-dried branches of *M. diversifolia* using a combination of silica gel column chromato-graphy and preparative TLC. The five isolated compounds were identified by spectroscopic data, including 1D and 2D NMR, IR and HRMS and by comparison with literatures.

For this study we have reported the structure elucidation of three known compounds **1, 2** and **3**.

Compound 1 was obtained as a white amorphous solid, mp 85-87 ºC. The IR spectrum showed the absorption at bands at 2947, 2859 cm^{-1} and 1459, 1379 cm^{-1} for C-H stretching and bending, respectively. The strong absorption band at 1679 cm^{-1} was characterized as a conjugate carbonyl, while the absorption band at 1622 cm^{-1} was assigned to C=C stretching. ^{1}H NMR spectral data exhibited resonance typical for steroidal skeleton. The ^{1}H NMR showed a singlet signal at δ 5.70 for olefinic proton, while the rest signals between δ 2.44-0.69 were in good agreement with those steroid moieties protons. The

signals at δ 1.16 (3H, s), 0.90 (3H, d, J = 6.5 Hz), 0.83 (3H, t, J = 7.5 Hz), 0.82 (3H, d, J = 7.7 Hz), 0.80 (3H, d, J = 6.9 Hz), 0.69 (3H, s) were characteristic of six methyl groups. The ^{13}C NMR spectrum exhibited of carbonyl ketone at δ 199.6, and the olefinic carbons at δ 171.7 and 123.7 as a quaternary and methane carbons, respectively, which revealed the presence of double bond conjunated to ketone group. The rest of signals between δ 55.9-11.9 were agreed well with those of steroid carbons skeleton. Compound **1** was identifined as sitost-4-en-3-one as shown in Figure 3 (Castola et al., 2002).

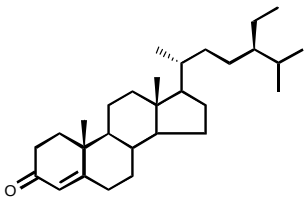

Figure 3 Stucture of sitost-4-en-3-one **1.**

Compound 2 was obtained as pale yellow needles and had the molecular formula $C_{10}H_8O_4$ as deduced by HRESITOFMS mass spectrum (observed *m/z* 193.0367 [M+ H]$^+$), mp 197-199 ºC. The IR spectrum showed characteristic absorption bands of hydroxyl group at 3337 cm^{-1}, aromatic ring at 3077 and 1566 cm^{-1}, chelating carbonyl lactone ring at 1703 cm^{-1}. The absorption band at 1290 cm^{-1} was assigned to C-O-C stretching. ^{1}H NMR spectrum showed signals of aromatic protons at δ 6.84 (1H, s) and δ 6.90 (1H, s). The resonance doublet signals at δ 7.59 (1H, J = 9.3 Hz) and δ 6.26 (1H, J = 9.3 Hz) were assigned to the cis olefinic protons, while a methoxy group was at δ 3.94 (3H, s). The ^{13}C NMR spectrum showed a resonance singles at δ 161.4, which was assigned to carbonyl lactone. Six carbons signals at δ 103.2, 107.5, 111.5, 144.0, 149.7 and 150.3 were characterized to aromatic carbons and two carbons signals at δ 113.4 and 143.3 were characterized to olefin carbons, while the methoxy carbon showed at δ 56.4.

The COSY spectrum showed correlation between H-3 and H-4 as well as the HMBC correlation of H-3 to C-2 and C-4a; H-4 to C-5 and C-8a; H-5 to C-4, C-6, C-7 and C-8a; H-8 to C-6, C-7, C-4a and C-8a. A methoxy proton to C-6 as well as hydroxyl protons to C-6, C-7 and C-8 also supported the connection of these groups. According to this spectral evidence, compound **2** was determined as scopoletin (Fujioka et al., 1999). The structure and selected HMBC correlation of scopoletin are shown in Figure 4.

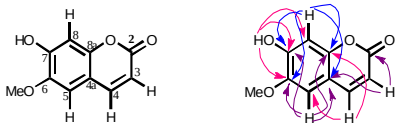

Figure 4 Stucture and selected HMBC correlation of scopoletin **2**.

Compound 3 was obtained as a pale brown amorphous solid, mp 172-174 ºC (decompose). The IR spectrum showed a broad absorption band of hydroxyl group at 3285 cm^{-1}. The strong absorption band at 1672 cm^{-1} was characterized as aromatic carboxylic acid group. The bands at 1605 and 1469 cm^{-1} represent skeletal vibrations of benzene ring. The absorption band at 1301 cm^{-1} were indicated C-O stretching. The ^{1}H NMR spectrum showed three aromatic protons at δ 7.4 (1H, brd, J = 6.2 Hz), 7.42 (1H, brs) and 6.8 (1H, d, J = 8.8 Hz). The ^{13}C NMR spectrum showed resonance signal carbonyl of

carboxylic group at δ 169.4. Six carbons signals at δ 149.7, 144.1, 123.3, 121.7, 116.5 and 114.6 were characterized to aromatic carbons. The above data suggested a phenolic skeleton. Comparison of physical and spectral data with those reported for know compound, compound **3** was identified as a protocatechuic acid which is shown in Figure 5 (Gerothanassis et al., 1998).

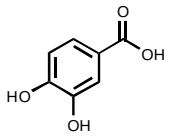

Figure 5 Stucture of protocatechuic acid **3.**

Conclusions

Chromatographic separation of crude hexane, CH_2Cl_2 and EtOAc extracts of *M. diversifolia* afforded five compounds. Among these, three compounds were known, sitost-4-en-3-one (**1**), scopoletin (**2**) and protocatechuic acid (**3**) while the structures of the other (**4** and **5**) are under investigations. The bioactivity assays found that compound **4** exhibited cytotoxicity against NCI-H187 cell lines with IC_{50} value of 25.24 µg/ml.

Acknowledgements

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