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Isolation of new monoterpene coumarins from *Micromelum minutum* leaves and their cytotoxic activity against *Leishmania major* and cancer cells

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ABSTRACT

On the basis of a leishmanicidal assay-guided isolation, two new monoterpene coumarins, minutin A and minutin B, were purified from *Micromelum minutum* leaves together with four known coumarins, 8,4″-dihydroxy-3″,4″-dihydrocapnolactone-2′,3′-diol, 8-hydroxyisocapnolactone-2′,3′-diol, 8-hydroxy-3″,4″-dihydrocapnolactone-2′,3′-diol, and clauslactone E. Among these compounds, minutin A, minutin B, 8-hydroxyisocapnolactone-2′,3′-diol and clauslactone E showed a significant cytotoxic activity against *Leishmania major* with IC_{50} values of 26.2, 20.2, 12.1, and 9.8 μM, respectively, while 8,4″-dihydroxy-3″4″-dihydrocapnolactone-2′,3′-diol and 8-hydroxy-3″,4″-dihydrocapnolactone-2′,3′-diol were not active. However, all these compounds exhibited some inhibitory activity against one or more lung adenocarcinoma (SBC3 and A549) and leukaemia (K562, and K562/ADM) cell lines. Amongst these, clauslactone E, minutin B and 8-hydroxyisocapnolactone-2′,3′-diol possessed the strongest cytotoxic activity against SBC3, A549, K562, and K562/ADM cell lines, with IC_{50} values of 3.7, 10.4, 12.1, and 10.8 μM for clauslactone E; 9.6, 17.5, 8.7 and 6.7 μM for minutin B; 8.8, 10.1, 16.9, and 10.1 μM for 8-hydroxyisocapnolactone-2′,3′-diol, respectively.

1. Introduction

Lung cancer is now the leading cause of cancer deaths in industrialised as well as in developing countries (Jemal et al., 2011). Dietary chemoprevention using vegetables and fruits as an economical, practical, and effective approach for reducing the risk of cancers has been reported (Pan & Ho, 2008; Tan & Spivack, 2009; Vainio & Weiderpass, 2006). Also protection against lung cancer by having a higher dietary intake of vegetables and fruits has been reported in several epidemiological studies (Riboli & Norat, 2003).

Leishmaniasis, a zoonotic protozoan disease caused by *Leishmania*, is still considered a major health problem in the rural areas of the Middle East, Africa, Asia, Europe and Central and South America. Treatment of leishmaniasis remains problematic in developing countries, where it is most often found. Many of the available drugs against the disease are expensive and in certain cases parasite drug resistance has developed (El-Ona, Ozer, Gopas, Sneir, & Golan-Goldhirsh, 2009; Ozer, El-On, Golan-Goldhirsh, & Gopas, 2010; Santos et al., 2008). The development of a new, cheap, effective anti-leishmanial treatment would be extremely beneficial for the treatment and control of the disease.

There is a huge biodiversity in Thai vegetables used in foods and it is possible that some could be a great resource for detecting new anticancer and anti-leishmanial agents. *Micromelum minutum* (G. Forst.) Wight and Arn. (Rutaceae) is an edible plant belonging to the family Rutaceae. In Thailand, it is commonly known as "Mui" and its shoots are often used as fresh vegetables. The leaves of this plant are also traditionally used for the treatment of fever and dizziness (Bulkill, 1966). It has also been reported that the leaves of *M. minutum* contain two coumarin derivatives, 2′,3′-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2′,3′-diol with strong cytotoxic activity against cancer cells (Susidarti et al., 2009; Tan, Alitheen, Yeap, Ali, & Mawardi, 2009). Many other chemicals, some with potential biological activities, have also been isolated, including five other coumarin derivatives, 3″,4″-dihydrocapnolactone, 8-hydroxy-3″,4″-dihydrocapnolactone-2′,3′-diol, 8,4″-dihydroxy-3″,4″-dihydrocapnolactone-2′,3′-diol, 8-methoxycapnolactone; two triterpenes and stigmasterol (Rahmani et al., 2003; Susidarti et al., 2006, 2007, 2009). In a previous investigation we reported antibacterial activity for a methanol extract of *M. minutum* leaves against *Helicobacter pylori*, *Escherichia coli*, *Salmonella typhimurium*, *Salmonella typhi* and *Shigella sonnei* (Sakunpak & Panichayupakaranant, 2012).

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In this study, we report a phytochemical study of *M. minutum* leaves based on a leishmanicidal assay-guided isolation, and also report on the cytotoxic activity of some isolated compounds against lung adenocarcinoma (A549 and SBC3) and leukaemia (K562 and K562/ADM) cell lines.

2. Materials and methods

2.1. Plant materials

M. minutum leaves were collected from the Hat-Yai District, Songkhla Province, Thailand, in June 2008. Voucher specimens were deposited in the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. The leaves were dried at 50 °C for 24 h in a hot air oven and were then reduced to a coarse powder using a grinder, and the powder was passed through a sieve (No. 45).

2.2. Preparations of plant extracts

The dried leaf powder of *M. minutum* (1 kg) was extracted three times with methanol (3 L × 3) under reflux conditions for 1 h. The extracts were combined and concentrated under reduced pressure to produce the crude methanol extract (250 g).

2.3. Anti-Leishmania major activity

The anti-*Leishmania major* activity against promastigotes was determined by the colorimetric cell viability MTT assay (Macahig, Matsunami, & Otsuka, 2011). The promastigotes obtained from a culture in its logarithmic growth phase in M199 medium supplemented with 10% heat-inactivated foetal bovine serum and 100 μg/mL of kanamycin were used for the assay. In a 96-well plate, 1 μL of the sample solutions (concentration ranges from 0.5 to 100 μg/mL) and *L. major* cells (1×10^5 cells/well) in 100 μL of medium were added to each well and the plate incubated at 27 °C in a 5% CO_2 atmosphere for 48 h. A solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (100 μL) was then added to each well and the incubation was continued for a further 24 h. The formazan product of the MTT reduction was then dissolved in DMSO and an absorbance was measured using a Molecular Devices Versamax tunable microplate reader (Molecular Devices, LLC, Sunnyvale, CA). DMSO was used as a negative control and amphotericin B as a positive control. The experiment was performed in triplicate. The anti-*Leishmania major* activity was quantified as the percentage of the control absorbance of the reduced dye at 540 nm. The inhibitory activity was calculated as:

$$\% \text{ inhibition} = [1 - (A_{test} - A_{blank})/(A_{control} - A_{blank})] \times 100$$

where $A_{control}$ is the absorbance of the control (DMSO) well, A_{test} is the absorbance of the test wells and A_{blank} is the absorbance of the cell-free wells.

2.4. Cytotoxic activity against lung adenocarcinoma and leukaemia cells

The cytotoxic activity against lung adenocarcinoma and leukaemia cell lines was determined by the MTT colorimetric cell viability assay (Macahig et al., 2011). Four lung adenocarcinoma cell lines, A549, SBC3, K562, and K562/ADM, were kindly provided from the JCRB cell bank, Osaka, Japan. SBC3 and A549 cells were cultured in DMEM medium supplemented with 10% heat inactivated FCS, kanamycin (100 μg/mL) and amphotericin B (5.6 μg/mL), while the K562 and K562/ADM cells were cultured in RPMI-1690 medium supplemented with 10% heat-inactivated FCS, and kanamycin (100 μg/mL) and amphotericin B (5.6 μg/mL). In a 96-well plate, 1 μL of the sample solutions (concentration range from 0.5 to 100 μg/mL) and the cancer cells (5×10^3 cells/well) in 100 μL medium were added to each well and the plate incubated at 37 °C in a 5% CO_2 atmosphere for 72 h. One hundred microlitres of the MTT solution were then added to each well and incubation was continued for a further 1 h. The absorbance of each well was measured at 540 nm using a Molecular Devices Versamax tunable microplate reader. DMSO was used as a negative control and doxorubicin as a positive control. The cytotoxic activity was calculated as:

$$\% \text{ inhibition} = [1 - (A_{test} - A_{blank})/(A_{control} - A_{blank})] \times 100$$

where $A_{control}$ is the absorbance of the control (DMSO) well, A_{test} is the absorbance of the test wells and A_{blank} is the absorbance of the cell-free wells.

2.5. Bioassay guided isolation

The crude methanol extract of *M. minutum* leaves was subjected to silica gel vacuum chromatography. The extract (250 g), pre-adsorbed on silica gel, was applied to the top of the silica gel column (13 cm in diameter and 6 cm in height), and the column was subsequently eluted with 500 ml of solvent with the aid of a vacuum pump. Mixtures of hexane and ethyl acetate were used for column elution, using a step-gradient elution starting from 100% hexane to 100% ethyl acetate, followed by mixtures of ethyl acetate and methanol, starting from 100% ethyl acetate to 100% methanol, respectively. Based on the TLC chromatograms of each fraction (500 mL) fifteen pooled fractions (fractions 1–15) were obtained. The fractions were then tested by the leishmanicidal assay. The leishmanicidal active fraction (fractions 4 and 5) were subjected to further purification processes as follows.

Fraction 4 was purified by a reversed-phase ODS column eluted with mixtures of water and methanol, using a step-gradient elution starting from 10% methanol to 100% methanol, to give nine pooled fractions (fractions I–IX). Compounds **1** (21 mg) and **2** (15 mg) were obtained from the leishmanicidal active fraction (fraction VII) after being purified using a semi-preparative RP-C_{18} HPLC column and a mixture of water and methanol (35:65 v/v) as eluent, with a flow rate of 3.0 mL/min. The retention times of compounds **1** and **2** were 40 and 42 min, respectively.

Fraction 5 was purified by a reversed-phase ODS column eluted with mixtures of water and methanol, using a step-gradient elution starting from 10% methanol to 100% methanol, to give thirteen pooled fractions (fractions A–M). Compound **3** (17 mg) was obtained from fraction D after being purified using a semi-preparative RP-C_{18} HPLC column and a mixture of water and acetone (62:38 v/v) as eluent with a flow rate of 3.0 mL/min. The retention time of compound **3** was 30 min.

Compounds **4** (22 mg), **5** (17 mg) and **6** (14 mg) were obtained from fraction E after being purified using a semi-preparative RP-C_{18} HPLC column and a mixture of water and acetone (60:40 v/v) as eluent with a flow rate of 3.0 mL/min. The retention times of compounds **4**, **5** and **6** were 30, 35, and 40 min, respectively.

2.6. Identification of compounds *1* and *2*

Compound **1**; Yellow powder; UV λ_{max}(EtOAc) nm: 251 and 324; IR υ_{max}(KBr) cm^{-1}: 3351 (br) (OH), 2928 (CH_2=CH_2), 1699 (C=O), 1608 (C=C, Ar), 1499–1367 (C=C), 1120 (C–O); HRESI-MS *m*/*z*: 347.1494 $[M+H]^+$ (calc. 347. 1489 for $C_{19}H_{23}O_6$), ^{13}C and ^1H NMR: Table 1.

Compound **2**; Yellow powder; UV λ_{max}(EtOAc) nm: 251 and 324; IR υ_{max}(KBr) cm^{-1}: 3370 (br) (OH), 2927 (CH_2=CH_2), 1694