ORIGINAL ARTICLE

A terpenoid and two steroids from the flowers of *Mammea siamensis*

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Abstract

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A terpenoid and two steroids from the flowers of *Mammea siamensis*

Two steroids, β-sitosterol and stigmasterol (of their mixture in an approximate ratio 1:1) and a terpenoid, friedelin, were isolated from a chloroform extract of *Mammea siamensis* flowers by means of chromatographic techniques. Chemical structures of the isolated compounds were identified by direct comparison of their spectroscopic data with those reported in the literature and by comparing their TLC patterns with those of authentic samples. The preliminary biological acitvities, such as antimicrobial activity (disc diffusion method), DPPH-radical scavenging and brine shrimp lethality effects of various solvent extracts from *M. siamensis* flowers were examined. The chloroform extract of *M. siamensis* flowers showed antibacterial effects on *Staphylococcus aureus* and *Bacillus subtilis* with inhibition zone of 7.8 and 9.0 mm, respectively. A methanol extract exhibited inhibition zone (6.0 mm) for *B. subtilis*. However, neither extract affected the growth of either *Escherichia coli* or *Candida albicans*. Both the chloroform and the methanol extract did not show antioxidative effect in DPPH radical-scavenging assay, whereas they exhibited lethality effects on brine shrimps with LC_{50} value of 5.2 and 43.2 μg/ml, respectively. Neither the mixture of β-sitosterol and stigmasterol, or friedelin showed any effect on brine shrimps.

Key words : antioxidative effect, antimicrobial, brine shrimp lethality, β-sitosterol, stigmasterol, friedelin, *Mammea saimensis*.

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บทคัดย่อ

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สารเทอร์ปีนและสเตียรอยด์จากดอกสารภี (*Mammea siamensis*)

การสกัดแยกสารจากดอกสารภีด้วยคลอโรฟอร์มโดยเทคนิคทางโครมาโตกราฟี ได้สารผสมสเตียรอยด์ 2 ชนิด คือ สารผสมระหว่างบีตาซิโตสเตอรอลกับสติกมาสเตอรอล และสารเทอร์ปีน 1 ชนิด คือ ฟริเดลิน สูตรโครงสร้างทางเคมีของสารที่แยกได้หาโดยอาศัยข้อมูลทางด้านสเปกโตรสโคปีและเปรียบเทียบลักษณะบน TLC กับสารมาตรฐาน ได้ทำการศึกษาฤทธิ์ทางชีววิทยาเบื้องต้น เช่น ฤทธิ์ต่อการยับยั้งการเจริญเติบโตของเชื้อจุลินทรีย์เมื่อทดสอบด้วยวิธีดิสซ์ดิฟฟิวชัน ฤทธิ์ต้านอนุมูลอิสระของ DPPH และความเป็นพิษต่อเซลล์เมื่อทดสอบกับไรน้ำเค็ม (brine shrimp) ของสารสกัดจากดอกสารภี พบว่า สารสกัดด้วยคลอโรฟอร์ม มีฤทธิ์ยับยั้งการเจริญเติบโตของแบคทีเรียชนิด *Staphylococcus aureus* และ *Bacillus subtilis* โดยมีระยะการยับยั้งการเจริญเติบโตเป็น 7.8 และ 9.0 มม. ตามลำดับ สารสกัดด้วยเมทานอลมีฤทธิ์ยับยั้งการเจริญเติบโตเฉพาะต่อเชื้อ *B. subtilis* สารสกัดทั้งสองส่วนไม่มีฤทธิ์ยับยั้งการเจริญเติบโตของเชื้อ *Escherichia coli* และ *Candida albicans* สารสกัดด้วยคลอโรฟอร์มและเมทานอลไม่มีฤทธิ์ต้านอนุมูลอิสระของ DPPH แต่มีความเป็นพิษต่อเซลล์เมื่อทดสอบกับ brine shrimp โดยมีค่าความเข้มข้นที่สามารถทำให้ไรน้ำเค็มตายได้ 50% (LC_{50}) คือ 5.2 และ 43.2 ไมโครกรัม/มล. ตามลำดับ สารผสมระหว่างบีตาซิโตสเตอรอลกับสติกมาสเตอรอล และฟริเดลิน ไม่มีความเป็นพิษต่อเซลล์เมื่อทดสอบกับไรน้ำเค็ม

ภาควิชาเภสัชเวทและเภสัชพฤกษศาสตร์ คณะเภสัชศาสตร์ มหาวิทยาลัยสงขลานครินทร์ อำเภอหาดใหญ่ จังหวัดสงขลา 90110

The tree *Mammea siamensis* Kosterm (otherwise *Ochrocarpus siamensis* T. And.) (Guttiferae) is known in Thai as "Sarapee", a small, evergreen which grows up to 15 m tall and 10-30 cm in diameter; it is native to Myanmar, Thailand, Laos, Cambodia and Vietnam (Poobrasert *et al*., 1998). The flowers of this plant are used for a heart tonic, reducing of fever and enhancement of appetite in Thai traditional medicine (Wuthithammawech, 1997). It is interesting to note that several phenylcoumarins have been isolated from the flowers of this plant (Thebtaranonth *et al*., 1981). We wish to report the isolation of terpenoid and steroid compounds from the same part of the plant as well as some biological activities.

Materials and Methods

The instruments used in this study were as follows; NMR spectra were recorded at 500 MHz for ^{1}H, and 125 MHz for ^{13}C, on a Varian Unity Inova 500 MHz spectrometer; MS were recorded using a Thermo Finnigan Mat 95 XL spectrometer; IR and UV spectra were recorded using a Jasco IR-810 infrared spectrometer and a Hewlett-Packard type 8452A diode array spectrophotometer, respectively; melting points was recorded using a Buchi 520 melting point apparatus; optical rotation was measured in chloroform solution with sodium D line (590 nm) on an Jasco DIP-370 digital polarimeter. TLC was performed on Merck silica gel 60 GF_{254} plates and the detection of compounds was accomplished by exposure to UV light at 254 nm and/or by spraying with vanillin/sulfuric acid reagent. All chemicals were of analytical grade and were purchased from chemical companies.

Plant materials

The dried flowers of *M. siamensis* were purchased from a herbal drugstore in June 2001 in Hat Yai, Songkhla Province, Thailand. Voucher specimens of these plant materials have been deposited in the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand.

Preparation of extracts

Dried coarsely-powdered flowers (500 g) were macerated with chloroform (4 l) for seven days. The macerate was filtered and evaporated to give a dried mass. The marc was re-macerated with chloroform (4 l) three times, filtered and evaporated. The dried masses were combined to give the chloroform extract (21.0 g; 4.2%). The marc was dried in open air and then was macerated with methanol using the same procedure as described above to give the methanol extract (123.9 g; 24.8%).

Isolation of pure compound

A portion of chloroform extract (12.0 g) was separated using silica gel column chromatography. The column was eluted with mixtures of *n*-hexane and chloroform, starting with 40% of *n*-hexane and finishing with pure chloroform. Then, the column was eluted with chloroform and ethyl acetate, starting with 50% of chloroform and finishing with pure ethyl acetate. Fractions of 75 ml were collected and gave a total of 528 fractions. Fractions 18-31 and fractions 60-73 from the column each were pooled and then further separated by SiO_2 column chromatography to give **2** (11.6 mg) and **1** (161.4 mg), respectively.

DPPH radical-scavenging assay

Samples for testing were prepared by dissolving in absolute ethanol. The concentrations of the tested samples were 50, 100, 200 and 400 µg/ml. Each concentration was tested in triplicate.

The DPPH (1,1-diphenyl-2-picrylhydrazyl) method is one of the methods used for testing the antioxidant activity (Yamasaki *et al*., 1994). The scavenging activity of samples corresponds to the degree of quenching of the DPPH absorbance as described by Hatano *et al*. (1989). A portion of sample solution was mixed with the same volume (500 µl) of 60 µM DPPH in absolute ethanol in a vial and allowed to stand at room temperature for 30 minutes. The absorbance (A) was then measured at 520 nm. BHT (butylated hydroxy toluene), which is a well known antioxidant, was used as a comparison. The results were expressed as percentage inhibition, % inhibition = $[(A_{control} - A_{sample})/A_{control}] \times 100$, where $A_{control}$ is the final absorbance using absolute ethanol as sample. EC_{50} (effective concentration of sample at 50% inhibition) was obtained by linear regression analysis of the value of dose-response curve which was plotted between % inhibition and concentration (µg/ml).

Brine shrimp lethality test (Meyer *et al*., 1982 and Solis *et al*., 1993)

Brine shrimp eggs (*Artemia salina*) obtained locally, were hatched in a shallow rectangular dish (12 x 20 cm) filled with artificial sea water, which was prepared with a commercial salt mixture (40 g/l) and distilled water, supplemented with 6 mg/l dried yeast. A plastic divider with several 2 mm holes was clamped in the dish divide it into two unequal compartments. The eggs (ca. 300 mg) were sprinkled into the larger compartment which was darkened, while the smaller compartment was illuminated. After 48 hours the phototropic nauplii were collected by pipette from the illuminated side, having been separated by the divider from their shells.

Samples for testing were prepared by dissolving in the artificial sea water. The concentrations of the tested samples were 2, 20, 200 and 2,000 µg/ml.

An aliquot (100 µl) of each concentration was transferred into the wells of a 96-well microplate (Sero-Wel, U.K.); each concentration was done in triplicate. Control wells were set up using artificial sea water (100 µl). A suspension of nauplii containing 10-15 organisms (100 µl) was added into each well and the plate was covered and incubated at room temperature (26-30ºC) for 24 hours. The plate was then examined under a binocular microscope (x 10) and the number of dead (non-mobile) nauplii in each well was counted. An aliquot of 10% formalin solution (100 µl) was then added to each well and after 15 minutes the total number of shrimps in each well was counted. The percentage deaths at each dose and the controls were determined. In cases where deaths occurred in the controls, the data were corrected using Abbott's formula (Abbott, 1925), % deaths = [(test

- control)/control] x 100. LC_{50} (concentration at 50% lethal effect) values were then determined from the 24 hours count using the probit analysis method described by Finney (1971).

Preliminary screening for antimicrobial activity

The disc diffusion method was used to evaluate the antimicrobial activity of the plant extracts and followed a procedure as previous report (Salie *et al*., 1996). The four micro-organisms used were *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Bacillus subtilis*, and *Candida albicans*. The bacteria and the fungus (*C. albicans*) were seeded over previously sterilized Mueller-Hinton agar and Sabouraud dextrose agar media, respectively. The dried plant extracts were dissolved in dimethylsulfoxide (DMSO) to a final concentration of 1,000 mg/ml and sterilized with a 0.45 µm membrane filter. Sterile 6-mm discs were impregnated with 10 µl of extract (each disc thus contained 10 mg of plant extract) and placed

Table 1. ^{13}C NMR spectral data of stigmasterol, β-sitosterol, 1, friedelin and 2 (δ ppm) in $CDCl_3$.

Carbon	Stigmasterol[a]	β-Sitosterola	1[c]	Friedelin[b]	2[c]
1	37.06	37.36	37.23	22.3	22.27
2	31.64	31.90	31.88	41.5	41.52
3	71.77	71.77	71.81	213.2	213.31
4	41.50	42.29	42.20, 42.27	58.2	58.20
5	140.23	140.75	140.73	42.1	42.14
6	121.69	121.69	121.72	41.3	41.26
7	31.72	31.65	31.88, 31.63	18.2	18.22
8	31.95	31.93	31.88	53.1	53.08
9	50.10	50.13	50.10, 50.13	37.4	37.42
10	36.72	36.50	36.49	59.4	59.44
11	20.80	21.09	21.06	35.6	35.60
12	39.59	39.78	39.66, 39.75	30.5	30.49
13	42.50	42.32	42.30	39.7	39.67
14	56.67	56.77	56.74, 56.85	38.3	38.20
15	24.01	24.31	24.29, 24.35	32.4	32.39
16	28.55	28.26	28.91, 28.24	36.0	35.98
17	55.85	56.06	55.92, 56.03	30.0	29.97
18	11.61	11.86	11.84	42.8	42.76
19	19.23	19.40	19.39	35.3	35.32
20	40.13	36.15	40.50, 36.13	28.1	28.16
21	20.48	18.79	21.06, 18.76	32.7	32.74
22	138.04	33.95	137.31, 33.92	39.2	39.23
23	129.15	26.07	129.25, 26.03	6.8	6.82
24	51.06	45.83	51.22, 45.81	14.6	14.64
25	32.00	29.16	31.88, 29.12	17.9	17.93
26	19.00	19.83	18.96, 19.81	20.2	20.25
27	21.21	19.04	21.20, 19.01	18.6	18.66
28	25.41	23.07	25.40, 23.04	32.1	32.07
29	12.00	11.99	12.03, 11.97	35.0	35.02
30	-	-	-	31.8	31.76

[a] Alam *et al*., 1996; [b]Mahato and Kundu, 1994. [c]This work

on the surface of agar plates inoculated with the microbial culture. Each extract was tested in triplicate; the control discs contained sterile DMSO (10 µl). Norfloxacin, gentamicin and clotrimazole (10 µg/disc) in each case served as positive controls. The agar plates containing the bacteria were incubated at 37ºC for 24 hours, whereas the plates inoculated with the *C. albicans* were incubated at 30ºC for 48 hours. After incubation, the inhibition zones were recorded as the differences in diameter between that of the disc and that of the growth-free zone around the disc.

Results and Discussion

The substance **1** was obtained as a white amorphous solid. It gave positive color tests in the vanillin-sulfuric acid and Liebermann-Burchard reactions. It gave one spot on TLC chromatograms. When it was subjected to GC-MS, it showed two peaks having retention times at 17.96 and 18.60 minutes corresponding to the molecular-ion peaks at *m*/*z* 413 and 415, respectively. The IR spectrum exhibited hydroxyl (3300 cm^{-1}) absorption. The ^{1}H NMR of **1** revealed signals for two singlet methyls at δ 1.01 (H-19) and 0.68 (H-18), four doublet methyls at δ 1.02 (d, *J* = 6.63 Hz, H-21), 0.85 (d, *J* = 6.41 Hz, H-26), 0.81 (d, *J* = 7.55 Hz, H-29) and 0.80 (d, *J* = 6.41 Hz, H-27). There were three vinylic proton signals at δ 5.36 (2H, m, H-6), 5.15 (1H, dd, *J* = 15.1, 8.7 Hz, H-22) and 5.02 (1H, dd, *J* = 15.1, 8.7 Hz, H-23) and a proton signal at δ 3.53 (2H, m, H-3). The remaining proton signals were at δ 0.8-2.4. The ^{13}C NMR spectrum showed a total of 47 carbon signals, among them four olefinic carbon signals (δ 140.73, 138.31, 129.25 and 121.72) and one oxygen-attached carbon signal (δ 71.81) were observed. The remaining carbons showed signals having chemical shifts between 11 and 57 ppm. From the above spectroscopic data, seemed to be a mixture of β-sitosterol and stigmasterol. Direct comparison of the ^{13}C NMR data of **1** with those reported in a literature (Alam *et al*., 1996; Wright *et al*., 1978) (Table 1) showed they were identical. Therefore **1** was identified as a mixture of β-sitosterol and stigmasterol (Figure 1). From the ^{1}H NMR spectrum, integration of proton signals at δ 5.36 (H-6), 5.15 (H-22), 5.02 (H-23) and 3.53 (H-3) were in the ratio 2:1:1:2. Thus, the ratio of β-sitosterol and stigmasterol in **1** was approximately 1:1.

Compound **2** was obtained as a white needles, m.p. 261-262ºC (uncorr.), $[\alpha]D^{25}$ -26.5 ($CHCl_3$). The MS spectrum showed a molecular-

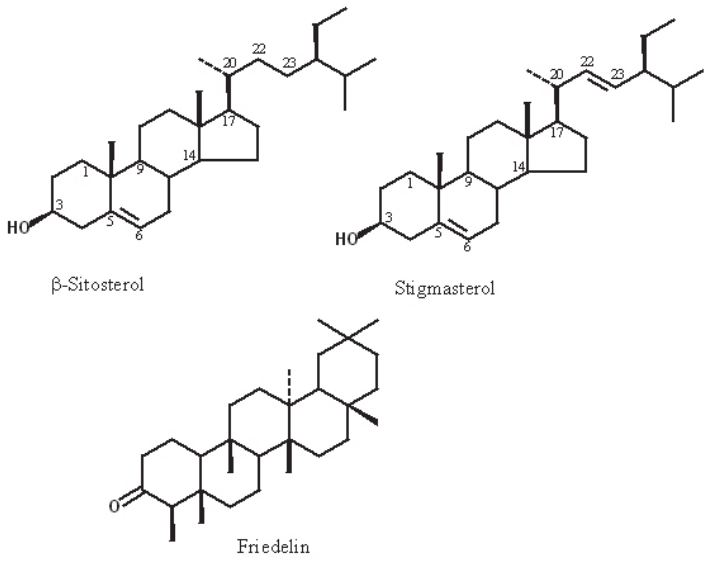

Figure 1. Chemical structures of β-sitosterol, stigmasterol and friedelin

Table 2. Preliminary screening of the activities of *M. siamensis* extracts, and isolates 1 and 2.

Sample	Antimicrobial activity (Inhibition zone, mm)				DPPH radical scavenging (EC_{50}, μg/ml)	Brine shrimp lethality (LC_{50}, μg/ml)
	S. aureus	*B. subtilis*	*E. coli*	*C. albicans*		
Chloroform extract	7.8	9.0	-ve	-ve	>200	5.2
Methanol extract	-ve	6.0	-ve	-ve	>200	43.2
1	n	n	n	n	>200	>1000
2	n	n	n	n	>200	>1000
Gentamicin	25.5	25.0	n	n	n	n
Norfloxacin	n	n	39.0	n	n	n
Clotrimazole	n	n	n	32.9	n	n
BHT	n	n	n	n	16.5	n

-ve = no inhibition zone; n = not tested

ion peak at *m*/*z* 426. The ^{1}H NMR of **2** revealed signals for seven singlet methyls at δ 1.18 (H-28), 1.05 (H-27), 1.01 (H-26), 1.00 (H-30), 0.96 (H-29), 0.87 (H-25) and 0.73 (H-24), a doublet methyl at δ 0.88 (d, *J* = 6.64 Hz, H-23), a methine proton at δ 2.25 (q, *J* = 6.64 Hz, H-4), and methylene protons at δ 2.40 (ddd, *J* = 13.96, 5.26, 2.06 Hz, H-2) and δ 2.31 (ddd, *J* = 13.96, 7.09, 1.15 Hz, H-2), respectively. No vinylic proton signal was observed. The remaining proton signals were at δ 1.2-2.00. These ^{1}H NMR data showed that **2** seemed to be friedelin (Akihisa *et al*., 1992). The ^{13}C NMR spectrum showed a total of 30 carbons, among them a ketone carbon at δ 213.31 was observed. The remaining 29 carbons showed signals having chemical shifts between 6 and 59 ppm. The ^{13}C NMR data of **2** were identical with those reported in a literature for friedelin (Mahato and Kundu, 1994; Akihisa *et al*., 1992) (Table 1) and the optical rotation was according to that of previous report (Buckingham, 1998). Therefore **2** was identified as friedelin (Figure 1).

From Table 2, the chloroform extract of *M. siamensis* showed antibacterial effects on *S. aureus* and *B. subtilis*, with inhibition zone of 7.8 and 9.0 mm, respectively. It showed a smaller inhibition zone when compared with that of a standard antibiotic, gentamicin. The methanol extract exhibited positive test only for *B. subtilis*. However, neither extract had any effect on the growth of gram-negative bacteria or the fungus. Neither the chloroform or the methanol extracts showed antioxidant effect in the DPPH radical-scavenging assay, whereas they exhibited lethality effect on brine shrimp with LC_{50} values of 5.2 and 43.2 μg/ml, respectively. However, the isolated compounds from chloroform extract, **1** and **2** showed no any lethality effect on brine shrimp. It could be concluded that the lethality effect of chloroform extract would be affected by other constituent(s) of the extract.

The compound β-sitosterol is a very common chemical constituent of medicinal plants, which possesses valuable biological activity, such as antihypercholesterolaemic and estrogenic effect (Buckingham, 1998). Recently, it was reported to show an important gastroprotective activity in several experimental ulcer models in rats (Navarrete *et al*., 2002). β-Sitosterol and fatty acids from *Mallotus peltatus* leaf extract were also reported to show antibacterial and anti-inflammatory activities (Chattopadhyay *et al*., 2002). The finding that β-sitosterol from the chloroform extract of *M. siamensis*, and the extract itself, possess antibacterial activity to gram-positive bacteria provide additional support for the use of the flowers of this plant as a fever remedy and enhance appetite in the Thai traditional medicine.

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