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Short Communications

Ichthyotoxic Properties and Essential Oils of *Syzygium malaccense* (Myrtaceae)

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ABSTRACT

The preliminary ichthyotoxic test on all parts of *Syzygium malaccense* (Myrtaceae) revealed that the leaves fraction was the most ichthyotoxic against tilapia-fish (*Tilapia oreochromis*). Three compounds, namely ursolic acid (**1**), β-sitosterol (**2**) and sitost-4-en-3-one (**3**), were isolated and their structures were elucidated with the aid of spectroscopic data and comparison with previously reported investigations. However none of these compounds gave any significant ichthyotoxicity. The volatile constituents of the leaves and fruit were determined by Gas Chromatography-Mass Spectrometer (GC-MS), with 180 and 203 compounds being identified in the aroma concentrates, respectively.

Keywords: *Syzygium malaccense*, Myrtaceae, ichthyotoxicity, volatile constituents

INTRODUCTION

Syzygium malaccense (Myrtaceae), which is widely known as the Malay apple, is a medium-sized tree which is native to India and Malaysia. Now, the tree is cultivated throughout the tropics as far as east of Hawaii, as well as Central and South America (Whistler and Craig, 2006). The fruit are eaten raw or cooked as jam or made into juice. The whole plant has a variety of medicinal uses which range from dermatological, digestive, head and throat to endocrine remedy. In Malaysia, powder from the dried leaves is applied on cracked tongue, while a preparation of the roots is used to cure itching, given to alleviate swelling, to treat dysentery and serves as an emmenagogue and abortifacient (Brown, 1935). Some volatile constituents, such as 2-phenylethanol and its derivatives, were found to be the major compounds in the fruit (Pino *et al*., 2004). This plant has also been reported as a good xanthine oxidase (Guerrero *et al*., 1998) and an aldose reductase inhibitor (Guzman *et al.,* 2005). This paper deals with ichthyotoxic properties, the characterization of the isolated components from the hexane leaves extract, and volatile constituents of the leaves and fruits of *S. malaccense*.

MATERIALS AND METHODS

General Nuclear Magnetic Resonance (NMR) spectra were recorded on a Varian VXR-500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) in $CDCl_3$. The chemical shifts are given in δ (ppm) values relative to that of the solvent [$CDCl_3$ (δ_H 7.26; δ_C 77.0)] on the tetramethylsilane scale. Infrared (IR) spectra were recorded on a Perkin-Elmer FTIR model 1725 spectrometer using KBr disc. The absorption bands were measured in cm^{-1}. The essential oils GC-MS data were determined

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using QP5050A Shimadzu GCMS with the AOC-20i Shimadzu Auto Injector (SGE 054148 non-polar column, 30 X 0.2 mm, an initial temperature of 250˚C with an interface temperature of 280˚C, column flow rate at 1.0 ml/min, dilution of sample in $CHCl_3$, mobile phase was hexane), and equipped with FID. An open column chromatography was performed with Merck Kieselgel 60 (70-230 mesh ASTM). Kieselgel $60F_{254}$ plates (0.2 mm thick, Merck) were used for TLC, and developed in the solvent system of a Hexane-Ethyl acetate (EtOAc) (9:1), (8:2) and/or b) Chloroform ($CHCl_3$)-Methanol (MeOH) (9:1), and spots were detected by either observation under UV (254 and 360 nm) and/or spraying with 10 % sulphuric acid in ethanol reagent.

Plant Material

Leaves, fruit, flower buds and barks of *S. malaccense* were collected from Kuala Selangor, in Selangor in November 2005 and identified by Mr. Shamsul Khamis, a botanist at the Institute of Bioscience.

Extraction and Isolation

The ground dried leaves (1 kg) were extracted with MeOH (3 l X 3), each time for 24 hours at the room temperature. The combined filtrates were concentrated to 900 mL, and extracted successively with hexane (3 X 500 ml) and EtOAc (3 X 500 ml). Part of the hexane soluble extract (2 g) was subjected to column chromatography on Silica gel 60 (i.d. 4 cm; length 30 cm) with $CHCl_3$-MeOH (5 to 90% MeOH), and 100% MeOH in the step-wise gradient mode. Compound **1** (7.3 mg) was afforded from the 5% MeOH fraction. Another portion (1g) of the hexane fraction was chromatographed on Silica gel 60 (i.d. 2 cm; length 30 cm) using hexane-EtOAc as a solvent, at increasing concentrations of EtOAc, i.e. from 10 to 90 %. Compounds **2** (9.1 mg) and **3** (8.4 mg) were isolated from 10 and 20% EtOAc, respectively.

Ursolic Acid (1)

White powder, mp: 282-285˚C (Lit. 283-285˚C; Gohari *et al.,* 2005) IR υ_{max} (cm^{-1}, KBr disc): 3401, 2926, 1688, 1455, 1359, 1030. ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 5.28 (1H, *t*, *J* = 17.5 Hz, H-12), 3.23 (1H, *dd*, *J* = 4.5/4.0 Hz, H-3), 2.20 (1H, *d*, *J* = 10.00 Hz, H-18), 2.01 (1H, *dd*, *J* = 4.0/3.0 Hz, Hb-22), 1.27 (3H, *s*, Me-23), 1.17 (1H, *m*, Ha-22), 1.15 (3H, *s*, Me-27), 1.09 (3H, *s*, Me-26), 1.00 (3H, *s*, Me-24), 0.92 (3H, *d*, *J* = 5.0 Hz, Me-29), 0.87 (3H, *d*, *J* = 6.5 Hz, Me-30), 0.77 (3H, *s*, Me-25). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 39.1 (C-1), 27.5 (C-2), 79.3 (C-3), 38.7 (C-4), 55.5 (C-5), 18.5 (C-6), 33.2 (C-7), 39.7 (C-8), 47.8 (C-9), 36.9 (C-10), 17.3 (C-11), 126.1 (C-12), 138.2 (C-13), 42.2 (C-14), 29.9 (C-15), 24.4 (C-16), 46.7 (C-17), 52.9 (C-18), 39.3 (C-19), 39.3 (C-20), 30.8 (C-21), 36.9 (C-22), 28.2 (C-23), 15.8 (C-24), 15.7 (C-25), 17.2 (C-26), 23.2 (C-27), 182.2 (C-28), 21.4 (C-29), 21.4 (C-29), 23.5 (C-30).

β-sitosterol (2)

Colorless needles, mp: 139-140˚C (Lit. 140-14˚C; Jiang and Wang, 2006). IR υ_{max} (cm^{-1}, KBr disc): 3402, 2936, 2869, 1640, 1466, 1382, 1049. ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 5.35 (1H, *d*, *J* = 5.0 Hz, H-6), 3.53 (1H, *m*, H-3), 1.02 (3H, *s*, Me-19), 0.92 (3H, *d*, *J* = 6.8 Hz, Me-21), 0.84 (3H, *d*, *J* = 7.5 Hz, Me-27), 0.83 (3H, *d*, *J* = 7.0 Hz, Me-26), 0.82 (3H, *t*, Me-29), 0.69 (3H, *s*, Me-18). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 37.5 (C-1), 31.9 (C-2), 72.0 (C-3), 42.5 (C-4), 141.0 (C-5), 122.0 (C-6), 32.2 (C-7), 32.2 (C-8), 50.4 (C-9), 36.7 (C-10), 21.3 (C-11), 40.0 (C-12), - (C-13), 57.0 (C-14), 24.5 (C-15), 28.5 (C-16), 56.3 (C-17), 12.1 (C-18), 19.6 (C-19), 36.4 (C-20), 19.0 (C-21), 34.2 (C-22), 26.3 (C-23), 46.1 (C-24), 29.4 (C-25), 20.1 (C-26), 19.3 (C-27), 23.3 (C-28), 12.2 (C-29).

Sitost-4-en-3-one (3)

Yellow powder, mp: 150-153 °C (Lit. 152-154°C; Mario *et al.,* 2001). IR υ_{max} (cm^{-1}, KBr disc): 2935, 2869, 1702, 1677, 1460, 1376. ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 5.73 (1H, *s*, H-4), 1.19 (3H, *s*, Me-19), 0.92 (3H, *d*, *J* = 6.5 Hz, Me-21), 0.85 (3H, *d*, *J* = 8.0 Hz, Me-26), 0.83 (3H, *d*, *J* = 9.0 Hz, Me-27), 0.81 (3H, *t*, Me-29), 0.72 (3H, *s*, Me-18). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 36.4 (C-1), 32.3 (C-2), 199.9 (C-3), 172.0 (C-4), 124.0 (C-5), 39.9 (C-6), 33.2 (C-7), 34.1 (C-8), 54.1 (C-9), 35.9 (C-10), 21.3 (C-11), 38.8 (C-12), 42.6 (C-13), 56.3 (C-14), 24.4 (C-15), 28.4 (C-16), 56.1 (C-17), 12.2 (C-18), 19.3 (C-19), 35.9 (C-20), 17.6 (C-21), 34.2 (C-22), 26.3 (C-23), 46.1 (C-24), 29.4 (C-25), 20.1 (C-26), 18.9 (C-27), 23.3 (C-28), 12.2 (C-29).

Hydro-distillation

The fresh leaves (350 g) and fruit (53 g) were separately immersed in distilled water and refluxed under Dean Stark condition for 2 hours. The obtained volatile oils for the leaves and fruit were separated from the water layer and mixed with anhydrous sodium sulphate prior to filtering to get the respective/required oils.

Ichthyotoxic Assay

A test solution prepared by adding acetone (0.5 ml) to the weighed crude extract (4.0 mg) or pure compound (1.0 mg) was dissolved in aerated water (100 ml) to give a test solution of 40 ppm or 10 ppm, respectively (Kawazu *et al.,* 1968). In the control group, only acetone (0.5 ml) was added to the 100 ml aerated water. Five tilapia fish (*Tilapia oreochromis*: 2.5 – 3.5 cm) per test solution or control were added. The results were recorded after 24 h.

RESULTS AND DISCUSSION

As part of the present study on the ichthyotoxicity of the Malaysian medicinal plants using tilapia fish (*Tilapia oreochromis*), the researchers have isolated three known compounds from the hexane fraction of the leaves of *S. malaccense*. None of them showed toxicity to tilapia, even though two sub-fractions of the hexane fraction gave a very strong ichthyotoxicity. The ichthyotoxic test was observed in 24 hours.

In the 24 hours lethality, 40 ppm has been established as the least amount of drug which can lead to or cause death in a given fish species and sizes under controlled conditions. Based on the data derived from several studies conducted previously, 50% of death in fish in the first five hours indicated that the sample contained high ichthyotoxic constituents. There are different reporting methods used for ichthyotoxicity in the literature. One common designation in reporting the average concentration required to kill at least 50% of fish in a given period of time is defined as the median tolerance limit (TLm). However, some researchers simply reported a concentration that killed all the fish in a few minutes or hours, or reported a comparison of the piscicidal activity with that of a well-known fish poison, such as rotenone (Jonathan *et al.,* 2004).

A concentrated methanol (MeOH) extract from the dried leaves of *S. malaccense* was partitioned into hexane-, ethyl acetate- (EtOAc-) and water- (H_2O-) soluble portions. The hexane-soluble portion, which showed a significant ichthyotoxic activity (lethal dose 40 ppm after 2 hours), was subjected to bioassay-guided fractionation by column chromatography on Merck Silica gel 60 to yield two active fractions at 40 ppm, namely fractions 12 and 15 (Table 1). However, the amounts of these fractions were rather small (7.9 and 11.6 mg), based on their Thin-Layer Chromatography (TLC) profiles, and they contained too many compounds in each of the fractions. Thus, no further fractionation was pursued. Fractions with reasonable quantities were subjected

TABLE 1
Ichthyotoxicity test of sub-fractions at 40 ppm (in duplicate) from hexane soluble portion

Sample	Number of dead fish at specified time interval (hours)				Total number of dead fish in 24 hours
	1	3	6	18	
Standard	-	-	-	-	0
Fraction 1	-	-	1X	-	1X
2	-	2X	-	-	2X
3	-	1X	-	-	1X
4	-	2X	-	-	2X
5	-	-	-	-	1X
6	-	-	-	-	0
7	-	-	-	-	0
8	-	-	-	-	0
9	-	-	-	-	0
10	-	-	-	-	0
11	-	1X	-	-	1X
12	-	5X	-	-	5X
13	-	1X	-	-	1X
14	-	2X	-	-	2X
15	-	3X	2X	-	5X
16	-	1X	-	-	1X
17	-	-	-	-	0

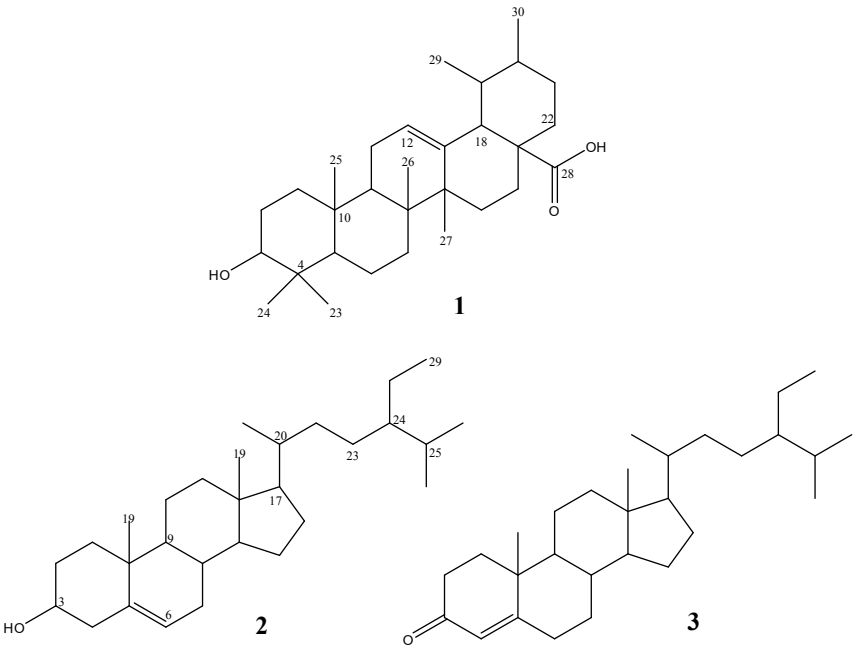


Fig. 1: Structures of 1 to 3

to another open-column chromatography to give compounds **1**, **2,** and **3**, which were identified as the ursolic acid (**1**), β-sitosterol (**2**), and sitost-4-en-3-one (**3**) respectively *(Fig. 1)*. The identity of these compounds were determined based on the spectral data and by the comparison of their physicochemical data with those in the reported literature (Gohari *et al*., 2005; Chien-Ya and Gow-Chin, 2001; Vincent *et al.,* 2001). However, the Ichthyotoxicity test on these compounds yielded no significant results.

The essential oil constituents of the leaves and fruit were also determined. In this study, the hydro-distillation-solvent extraction on the leaves and fruits of *S. malaccense* yielded 104.1 and 89.2 mg of essential oils, respectively. These concentrates were analyzed using the GC-MS, while their chemical compositions were quantified based on the GC-FID peak integration. The identification of the constituents was done by comparing them with the data available at the National Institute for Standard and Technology (NIST) and 1998 Mass Spectral Wiley library. The essential oil from the leaves contained 180 compounds, in which 15 (>1.0%) were major and identified as hexanoic acid (12.16%), methyl salicylate (8.27%), 3-hexen-1-ol (7.81%), 1-octen-3-ol (5.89%), *n*-hexadecanoic acid (5.07%), 2-hexenal (4.89%), 3-buten-2-one (3.68%), 1-hexanol (2.96%), phytol (2.95%), acetic acid (2.73%), 3-buten-2-one (2.58%), azulene (1.64%), 2-octen-1-ol (1.34%), alpha-cadinol (1.11%) and 3-hexen-1-ol (1.10%). Meanwhile, the minor compounds of the essential oil from the leaves are mostly aldehydes, ketones, sesquiterpenes, alcohols, esters and fatty acids. A wide range of different compound types were detected; however, no similarity with the components was reported from the Malay rose apple leaves (*S. malaccense*), in which (-)-*β*-caryophyllene, (+)-*α*-pinene, (-)-*β*-pinene, *p*-cymene and *α*-terpineol were found in more than 7% of the oil from Nigeria (Karioti *et al*., 2007).

Two hundred and three volatile constituents were identified from the essential oil of the fruit, with 11 major compounds such as *n*-hexadecanoic acid (18.59%), 9-octadecynoic acid (9.37%), (*Z,Z*)-9-12-octadecadien-1-ol (6.98%), (*E*)-3,7,11- trimethyl-1,6,10-dodecatrien-3-ol (6.28%), 1-octen-3-ol (2.33%), tetradecanoic acid (1.75%), phytol (1.56%), 2-ethylhexyl *p*-methoxycinnamate (1.43%), (*E*)-2-octen-1-ol (1.26%), 2-phenylethyl accetate (1.25%), and benzyl benzoate (1.00%). The minor compounds consist of a wide range of compound groups which include fatty acids, aldehydes, ketones, terpenes, alcohols, hydrocarbons, and esters. A study on the essential oil of Malay apple showed 2-phenylethyl acetate, 2-phenylethanol, 1-octen-3-ol and (*E*)-2-octen-1-ol to be the major components (Wong *et al.,* 1996). 1-octen-3-ol and (*E*)-2-octen-1-ol were also among the isolated constituents from the Cuban *S. malaccense* (Pino *et al*., 2004). Other major components were identified for the first time in the fruits.

One common major compound found in both volatile compositions of the leaves and fruit is phytol, which contributed 2.95 and 1.56% of the total oils, respectively.

CONCLUSIONS

The hexane soluble part of the leaves of *S. malaccense* was found to be most active towards tilapia fish. However, no chemical constituent was isolated from the active fractions due to their limited amounts. Three known compounds, namely ursolic acid, *β*-sitosterol and sitost-4-en-3-one, were obtained from the hexane fraction. Neverthless, none showed ichthyotoxicity. This study is the first report on these isolated compounds from *S. malaccense*. Further work with larger amount of plant is required for both isolation and characterization of the active component. Volatile compositions of the leaves and fruit were determined and different constituents were observed as compared to the previously reported investigations. These results suggested that the plants of the same species, which were collected in the different places or countries, gave large qualitative differences in the compositions of the fruit and leaves oil. The differences may be due to the different origins of the plants. Nonetheless, further systematic studies are still needed to prove this.

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