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Original article

Cytotoxic tirucallane triterpenes from the stem of *Luvunga scandens*

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ABSTRACT

Two tirucallane triterpenes, namely flindissol (1) and 3-oxotirucalla-7,24-dien-21-oic-acid (2), were isolated from the dichloromethane extract of the stem of *Luvunga scandens* (Roxb.) Buch-Ham ex Wight & Arn, Rutaceae. This is the first report of their isolation from this plant. Their structures were constructed by high resolution mass and 2D NMR spectroscopic data. The cytotoxic potential of the two pure compounds 1 and 2 were determined by MTT assay against human breast adenocarcinoma cell line (MCF-7). Compounds 1 and 2 showed potent cytotoxicity against MCF-7 cell line with IC₅₀ values of 13.8 μM and 27.5 μM, respectively. This result suggested their potential activity as antitumor agents.

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Introduction

Development of new cytotoxic drugs is essential since the emerging resistance by cancer cells towards existing drugs. Medicinal plants are an ideal target for the discovery of potential bioactive compounds or lead structures for new cytotoxic drugs. Novel antitumor agents such as paclitaxel (Taxol) (Wani et al., 1971; Bills et al., 2002), vincristine and vinblastine (Hamburger et al., 1991; Tyler, 1986) were reported from plant sources.

The genus *Luvunga* (Rutaceae) is comprised by only about twelve species. *Luvunga scandens* (Roxb.) Buch-Ham ex Wight & Arn is mainly distributed in tropical and humid environments

throughout China, Cambodia, India, Laos, Indonesia, Philippines, Malaysia, Myanmar, Thailand and Vietnam. This plant is used for the treatment of several ailments by traditional folk medicine. A decoction of the stem of *L. scandens* is orally administered to treat malaria and fatigue (Eswani et al., 2010; Ong et al., 2011). A few reports of the phytochemical (Chopra, 2006), antifungal and insecticidal activity of *L. scandens* have been documented (Singh and Maurya, 2005; Garg and Jain, 1999).

The present study was aimed to isolate and elucidate the structure of two compounds from the dichloromethane extract of the stem of *Luvunga scandens* by spectroscopic techniques, and the evaluation of their cytotoxicity.

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Materials and methods

General experimental procedures

The NMR spectra were recorded on a Bruker 400 MHz NMR Spectrometer using CDCl₃. The HRMS spectra were recorded using a Thermo LTQ-FT mass spectrometer (RMS 2 ppm with external calibration) in the Mark Wainwright Analytical Centre at The University of New South Wales, Sydney, Australia. IR spectra were measured on a Perkin Elmer infrared spectrophotometer. The UV spectra were obtained on a Shimadzu UV-1800 UV/VIS spectrophotometer. Column chromatography was performed on silica gel (70-230 and 230-400 mesh, Merck, Germany) and Sephadex LH-20 (40-70µm, GE healthcare). Organic solvents and TLC plates were purchased from Merck, Germany.

Dulbecco's modified Eagle medium (DMEM), Fetal Bovine Serum (FBS), Penicillin-Streptomycin and Triple Express were purchased from GIBCO. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Invitrogen (USA). Doxorubicin hydrochloride was obtained from Sigma Aldrich (USA).

Plant material

The stems of *Luvunga scandens* (Roxb) Buch-Ham ex Wight & Arn, Rutaceae, were collected from Bukit Pelindung, Kuantan, Pahang, Malaysia, in September 2012. Samples were further identified by Dr. Shamsul Khamis at Institute of Bioscience, Universiti Putra Malaysia. A voucher specimen (No. PIU 0204) was deposited in the Herbarium of the Faculty of Pharmacy, International Islamic University Malaysia.

Extraction and isolation

The dried and powdered stem (730 g) of *L. scandens* was extracted by maceration using *n*-hexane, dichloromethane (DCM) and methanol (MeOH) as solvents. The concentrated DCM extract (133 g) was successively partitioned by *n*-hexane (1.8 l), *n*-hexane - DCM (1:1, 1.2 l), DCM (1.2 l), DCM-MeOH (1:1, 1.5 l), and MeOH (600 ml). The DCM-MeOH (1:1) fraction (67 g) was subjected to column chromatography (CC) for fractionation over silica gel (70-230 mesh) with a mobile phase of DCM-EtOAc (100 ml of each) of 100:0, 90:10, 80:20, 70:30, 60:40, 50:50 and 0:100 (v/v); and finally by EtOAc-MeOH (50:50). The fractions with similar TLC profile were combined to yield four fractions. Fraction 2 (62.2 g) was further extracted using the above-mentioned method to obtain compound 1 (32 mg) and 2 (17 mg).

Flindissol (1)

White amorphous powder; m.p. 184-187°C; UV (MeOH) λ_{max}: 211 nm; IR (KBr) ν_{max}: 3395, 2950, 2372, 1717, 1446, 979 cm⁻¹; ESI-MS: [M + Na]⁺ m/z 479.3482 (calculated for C₃₀H₄₈O₃Na, 479.3496); ¹H NMR (400 MHz, CDCl₃): δ 0.80 (3H, s, CH₃-18), 0.93 (3H, s, CH₃-19), 0.93 (3H, s, CH₃-29), 0.95 (3H, s, CH₃-28), 0.99 (3H, s, CH₃-30), 1.71 (3H, s, CH₃-27), 1.73 (3H, s, CH₃-26), 3.49 (1H, bs, H-3), 4.71 (1H, m, H-23), 5.17 (1H, bs, OH-21), 5.19 (1H, d, J = 8.0 Hz, H-24), 5.27 (1H, bs, H-7). ¹³C NMR (100 MHz, CDCl₃): δ 13.3 (C-19), 17.4 (C-11), 18.4

(C-27), 21.6 (C-29), 22.6 (C-18), 23.8 (C-6), 26.0 (C-26), 26.8 (C-28), 28.4 (C-30), 28.7 (C-2), 28.7 (C-16), 31.9 (C-12), 34.1 (C-15), 34.1 (C-22), 34.5 (C-10), 37.0 (C-4), 40.3 (C-1), 44.2 (C-13), 45.2 (C-17), 50.0 (C-20), 50.9 (C-9), 51.3 (C-14), 52.0 (C-5), 73.1 (C-23), 75.8 (C-3), 99.7 (C-21), 118.4 (C-7), 124.5 (C-24), 137.6 (C-25), 146.2 (C-8).

3-Oxotirucalla-7,24-dien-21-oic acid (2)

White amorphous powder; m.p. 238-241°C; UV (MeOH) λ_{max}: 204 nm; IR (KBr) ν_{max}: 3754, 3396, 2960, 1710, 1202 cm⁻¹; ESI-MS: [M + H]⁺ m/z 455.3510 (calculated for C₃₀H₄₇O₃, 455.3520); ¹H NMR (400 MHz, CDCl₃): δ 0.92 (3H, s, CH₃-18), 1.00 (3H, s, CH₃-19), 1.03 (3H, s, CH₃-30), 1.07 (3H, s, CH₃-28), 1.13 (3H, s, CH₃-29), 1.50 (1H, m, H-1), 1.50 (2H, m, H-11), 1.61 (3H, s, CH₃-26), 1.70 (3H, s, CH₃-27), 1.77 (1H, m, H-5), 1.99 (1H, m, H-1), 1.99 (2H, m, H-23), 2.11 (2H, m, H-6), 2.11 (1H, m, H-17), 2.29 (1H, m, Heq-2), 2.29 (1H, m, H-9), 2.29 (1H, m, H-20), 2.75 (1H, ddd, J = 8.4, 8.4, 4.8 Hz, Hax-2), 5.11 (1H, bt, H-24), 5.34 (1H, bs, H-7). ¹³C NMR (100 MHz, CDCl₃): δ 12.7 (C-19), 17.7 (C-26), 17.9 (C-11), 21.6 (C-18), 21.7 (C-29), 24.4 (C-6), 24.5 (C-28), 25.7 (C-27), 26.0 (C-23), 27.2 (C-16), 27.4 (C-30), 30.0 (C-12), 32.3 (C-22), 33.5 (C-15), 34.9 (C-2), 35.1 (C-10), 38.6 (C-1), 43.3 (C-13), 47.3 (C-20), 47.9 (C-4), 48.2 (C-9), 49.8 (C-17), 51.0 (C-14), 52.4 (C-5), 118.2 (C-7), 123.5 (C-24), 132.4 (C-25), 145.5 (C-8), 181.9 (C-21), 217.1 (C-3).

In vitro cytotoxicity assay

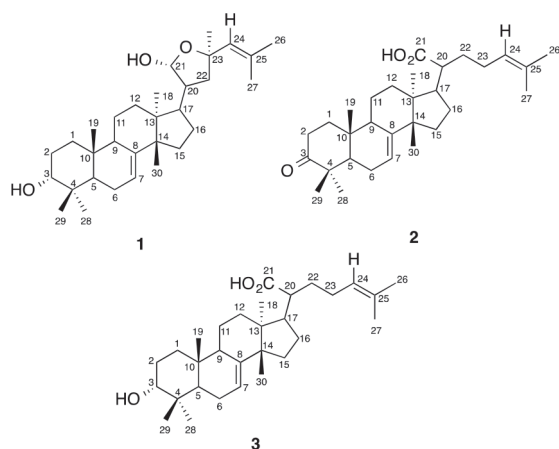
The cytotoxic activity of the isolated compounds against MCF-7 cells was measured by MTT assay according to Mosmann (1983) with some modifications. A total of 2 × 10⁵ cells/ml were plated in a 96-well plate overnight; then, the cells were treated in triplicate with various concentrations of the compounds. After 24 h of incubation, the cells were washed with phosphate buffer saline (PBS) and assayed by addition of 30 µl of MTT solution (5 mg/ml MTT in PBS). After 4 h of incubation, the formazan crystals were dissolved in 120 µl DMSO and left in the dark at room temperature for an additional 1 h. The absorbance measured at 570 nm and 630 nm used as reference wavelength was performed using a microplate reader (TECAN infinite M200). The wells with untreated cells were taken as the control, wells with medium with 0.5% (v/v) of EtOH were used as solvent control and wells with Doxorubicin were used as positive control. Results were expressed as a percentage of the average absorbance of sample treated cells with respect to untreated ones. The half maximal inhibitory concentration (IC₅₀) was defined as the concentration of compound that caused a 50% reduction in cell viability against MCF-7 cell line. Viability of cells (%) was calculated using the following equation:

$$\text{Viability of cells (\%)} = [(A_{\text{Sample}} - A_{\text{Blank}}) / (A_{\text{Untreated}} - A_{\text{Blank}})] \times 100$$

Results and discussion

Compound 1 was obtained as a white amorphous powder. The ¹H-NMR and ¹³C-NMR data together with the DEPT 135 spectrum proved the presence of 30 C-atom signals corresponding to seven Me, eight sp³ CH₂, seven sp³ CH, two sp² CH groups, and four sp³ and two sp² quaternary C-atoms. Accurate mass measurements of compound 1 obtained by

ESI-MS yielded a parent mass at m/z 479.3482 in positive ionization mode which, in conjunction with the data of other spectra, suggested the sodium adduct $[M + Na]^+$ of a compound with a molecular formula of $C_{30}H_{48}O_3$ (calculated mass 479.3496, $[C_{30}H_{48}O_3 + Na]^+$), accounting for 7 degrees of unsaturation. The 1H -NMR and ^{13}C -NMR data of compound 1 were identical to those of the tirucallane triterpene flindissol, which has the unusual 3- α configuration of the A-ring hydroxyl group (Birch et al., 1963) and has been previously isolated from several species of the Rutaceae family (Guang-Yi et al., 1988; Bevan et al., 1967; Waterman, 1973). This is the first report of flindissol found in *L. scandens*.



The NMR spectra of compound 2 were similar to those of 1, with exception to the additional one sp^3 and two sp^2 quaternary C-atoms and lack of three sp^3 CH. The three sp^3 CH signals (δ_C 75.8, 99.7 and 73.1) of compound 1 were bound to oxygen at C-3, C-21 and C-23, and their absence in compound 2 indicated a change in the 3-hydroxy substituent and a modification of the side-chain lactol. The appearance of two additional sp^2 quaternary C-atoms in 2 suggests the presence of COOH (δ_C 181.9) and C=O (δ_C 217.1) moieties, which affirm the suggested structural modifications. In the 1H NMR spectrum, the presence of a 3-oxo moiety was confirmed by the coupling pattern ddd at δ_H 2.75 for the H-2 axial proton. This was further confirmed by tracing of cross peaks between the neighboring coupled protons at δ 2.75 (H_{ax} -2) and 1.99 and 1.50 (H-1, both m) in the COSY spectrum. Two olefinic proton signals, appeared at δ 5.34 (H-7, bs) and 5.11 (H-24, bt), correlated in the HSQC spectrum (showing one-bond C-H correlations) to carbons at δ 118.2 (C-7) and 123.5 (C-24), respectively.

Comparison of ^{13}C -NMR data of 2 with 3 α -hydroxy-tirucalla-7,24-dien-21-oic acid (α -elemolic acid) (3) (Guang-Yi et al., 1988; Tessier et al., 1982; Cotterrell et al., 1970) showed good agreement except for a keto group at C-3 (δ_C 217.1) in 2, instead of a secondary alcohol group (δ_C 74.2) in 3. These observations suggest that 2 differed from 3 only in oxidation of the 3 α -hydroxyl function to the corresponding 3-oxo. A comparison of ^{13}C -NMR data with those reported for 3-oxotirucalla-7,24-dien-21-oic acid at 75 MHz (Ma, et al., 2000) showed identical signals. This was further evidenced

by the ESI-MS mass spectrum with $[M + H]^+$ at m/z 455.3510 for 2, which suggested the molecular formula $C_{30}H_{46}O_3$ (calcd mass 455.3520, $[C_{30}H_{46}O_3 + H]^+$), accounting for 8 degrees of unsaturation. Accordingly, compound 2 was established as 3-oxotirucalla-7,24-dien-21-oic acid, a tirucallane triterpene first isolated from oleoresin of *Aucoumea klaineana* (Tessier et al., 1982) and later identified in *Xanthoceras sorbifolia* (Ma et al., 2000), *Rhus javanica* L. (Lee et al., 2005), *Pancovia pedicellaris* (Soh et al., 2009), and *Feroniella lucida* (Sriyatep et al., 2014), which displays inhibitory activity against HIV-1 protease (Ma et al., 2000; Nimmanpipug et al., 2009) and cytotoxicity (Lee et al., 2005).

Compounds 1 and 2 were assayed for their dose-response effect on the viability of human breast adenocarcinoma cell line (MCF-7) by MTT assay. Doxorubicin was used as positive control to compare the cytotoxicity of the pure 1 and 2. In order to determine the growth inhibition, the MCF-7 cells were incubated with various concentrations of the compounds for 24 h. The inhibition concentration required reduction of 50% of cell viability (IC_{50}) was determined by comparison to the untreated control. Based on the results of the MTT assay (Table 1), both compounds isolated from stems of *L. scandens* inhibited the growth of human breast adenocarcinoma cells. A bar chart of the percentage of viability of cells was plotted against sample concentrations (Fig. 1). Compound 1 was comparatively more cytotoxic at a lower IC_{50} value (13.8 μM) than that of compound 2 (27.5 μM), and less than that of doxorubicin.

Table 1

Cytotoxicity data (IC_{50} value in μM) of compounds 1 and 2 against MCF-7 cell line.

Components	KI
1	13.8
2	27.5
Doxorubicin	6.21

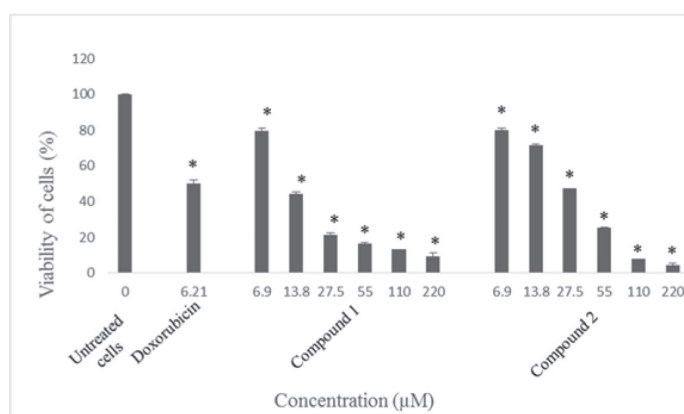


Figure 1 – Cytotoxic activity of compounds 1-2 against MCF-7 cell line. Untreated cells and Doxorubicin were used as negative and positive control respectively. The error bars indicate mean standard error of triplicate values. Asterisks represent statistically significant ($p < 0.05$) data compared to controls (untreated cells).

Conclusions

It is concluded from spectroscopic evidence that two tirucallane triterpenes were isolated from the dichloromethane extract of the stems of *Luvunga scandens*. The tirucallane triterpenes flindissol (1) and 3-oxotirucalla-7,24-dien-21-oic acid (2) were isolated for the first time from this plant. The cytotoxicity of compounds 1 and 2, as determined by MTT assay against MCF-7 cell line, are consistent with the findings that medicinal plants are an ideal source of cytotoxic agents, thus, subsequent research towards the discovery of more cytotoxic agents should continue.

Authors' contributions

PNHA and MT contributed in collecting plant material, extraction, purification, biological testing and writing of the manuscript. MT and DS designed the study and supervised the laboratory work. MFR contributed in running and advising NMR results. RWR contributed in MS analysis. RWR and FA gave critical reading of the manuscript. MT, RWR, MHS and CMH contributed in interpretation of NMR and MS data.

Conflicts of interest

The authors declare no conflicts of interest.

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