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Identification of Sakurasosaponin as a Cytotoxic Principle from *Jacquinia flammea*

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The crude ethanolic extract of leaves, stem-bark and roots of *J. flammea* were tested for their cytotoxic effect against two mammalian cell lines (HeLa and RAW 264.7) and four bacterial species (*Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa*). When tested at the concentration of 100 µg/mL, the root extract showed the highest cytotoxic activity against mammalian cells followed by the stem-bark extract while the leaves extract did not show significant activity. No antibacterial activity was detected for all extracts when tested up to 500 µg/disc in the disc diffusion assay. The cytotoxic root extract was subjected to fractionation using solvents of ascending polarity: petroleum ether, chloroform, ethylacetate, butanol and water. The water fraction which showed cytotoxic activity was further subjected to routine bioassay-guided fraction to lead to the isolation of sakurasosaponin as the active principle. The recorded IC_{50} value for sakurasosaponin was 11.3 ± 1.52 and 3.8 ± 0.25 µM (n=3) against HeLa and RAW 264.7 respectively. The identification of sakurasosaponin was based on analysis of spectroscopic data.

Keywords: *Jacquinia flammea*, *Bonellia*, Theophrastaceae, sakurososaponin, cytotoxicity, saponins.

Jacquinia flammea Millsp. ex Mez (syn *Bonellia flammea* (Millsp. ex Mez) Ståhl & Källersjö; Theophrastaceae) is a shrub or small tree endemic to the Yucatan peninsula, Mexico. In the Mayan tradition where the plant is known by its common names "Chak sik"and "ink'axx" [1a, 1b], the infusion of the leaves often combined with other plants is taken orally to treat colds and fever. Previous biological activity studies on the methanolic extracts of the leaves, stem-bark and roots of *J. flammea* showed that the root extract possess activity against 10 fungal strains [1c] and cytostatic activity in plants when tested using the cucumber seeds assay model [1d]. Despite the fact that *J. flammea* is generally considered poisonous [1c] and several species of the genus (e.g. *J. barbasco*, *J. macrocarpa,* and *J. seleriana* in Mexico; *J. armillaris* in the Antilles and Venezuela; and *J. sprucei* (syn. *J. pubescens*) in Ecuador and Peru) are widely used as fish poisons [1e], the chemistry and cytotoxicity profile of *J. flammea* have not yet been studied.

Preliminary cytotoxicity studies on the EtOH extracts of leaves, stem-bark and roots of *J. flammea* were carried out using representative mammalian cell lines and bacterial test species [2a,2b]. As shown in Table 1, the root extract showed the highest toxicity in mammalian cells followed by the stem-bark extract, while the leaves extract displayed no activity up to the highest concentration tested (100 µg/mL). When the extracts were tested for antibacterial activity using representative gram-positive and gram-negative test species, no activity was observed up to the concentration of 500 µg/disc. The root extract of *J. flammea* which showed the most cytotoxic activity against mammalian cells was taken for liquid-liquid partitioning to yield fractions of low, medium-low, medium-high and high-polarity including the residual aqueous fraction. Evaluation of fractions for mammalian cell cytotoxicity activity revealed that only the aqueous fraction displayed significant activity in the 24 h assay. The IC_{50} values obtained in this study were

Table 1: Cytotoxic activity of crude extracts of *J. flammea* on HeLa cells at 24 and 48 h. Each experiment was carried out in quadruplicate determinations. The mean percent cell death and SEM from three separate experiments are shown.

Plant part	% cell death - 24h		% cell death - 48h	
	33 µg/mL	100 µg/mL	33 µg/mL	100 µg/mL
Leaves	Not active	Not active	Not active	Not active
Stem/bark	48.7 ± 1.3	80.4 ± 3.1	64.9 ± 4.5	93.7 ± 1.7
Root	88.0 ± 1.0	87.0 ± 2.2	94.1 ± 0.4	93.2 ± 1.1

28.61 ± 2.27 and 10.60 ± 1.83 µg/mL (n=3) for HeLa and Raw 264.7 cells respectively. Further bioassay-guided purification of the aqueous fraction using repetitive silica gel column chromatography led to the isolation of the active principle **1** (Figure 1).

Sakurasosaponin was isolated as a white amorphous powder. Its $[M-H]^-$ ion in the ESI-MS at *m/z* 1249 was consistent with a molecular formula of $C_{60}H_{98}O_{27}$. The COSY, DEPT, HSQC, and HMBC experiments were further used for the assignment of ^1H and ^{13}C resonances. The data proved to be identical to the ^1H and ^{13}C NMR data reported for sakurasosaponin (Figure 1), a saponin previously isolated from the leaves of *Rapanea melanphloeos,* stem-bark of *Tapeinosperma clethroides* and roots of as yet unidentified species of *Monoporus species* [3a-3c].

Sakurasosaponin (**1**) displayed a moderate cytotoxic activity against HeLa (IC_{50} = 11.3 ± 1.52 µM) and RAW 264.7 (IC_{50} = 3.8 ± 0.25 µM) cells. Under similar experimental conditions, the positive control, podophyllotoxin, gave an IC_{50} value of 32 ± 2 and 68 ± 0.9 nM against HeLa and RAW 264.7 cells respectively. Sakurasosaponin was previously reported to possess antifungal effects suggesting that it might also be responsible for the reported antifungal activity of *J. flammea* extracts [1c]. Sakurasosaponin isolated from as yet unidentified species of *Monoporus* (family Myrsaceae) has also been reported to display cytotoxicity in A2780 cancer cells (3c). Although phytochemical and cytotoxicity studies on the genus *Jacquinia* are very limited [4a, 4b], the identification of saponins as cytotoxic agents from the various species of the family Theophrastacea has been well documented [4a, 4b]. Piscicidal, spermicidal, antifungal, haemolytic, cytotoxic and molluscicidal activities by saponin-containing plants has largely been shown to be mediated through non-specific interaction with cell membrane cholesterol and/or sterols [4c]. Such interactions are now believed to lead to the formation of membrane pores and eventual cell death [4c]. The fact that prokaryotic bacterial cells do not have significant amount of sterols in their membrane and/or cell wall [4d] might thus explains why *J. flammea* extracts and the isolated active principle, sakurasosaponin (**1**), lack antibacterial activities.

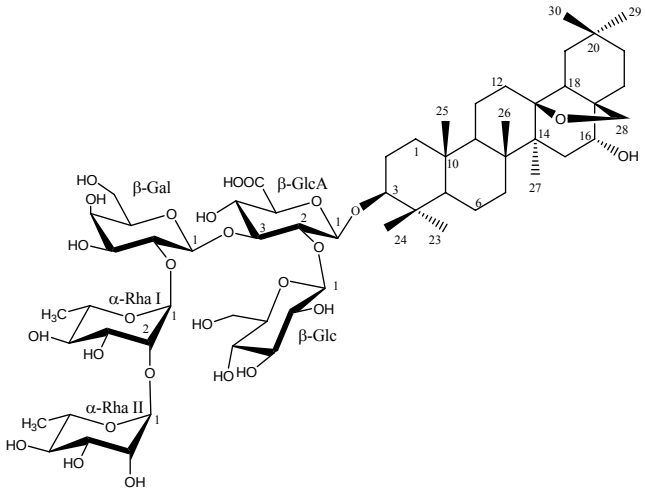

Figure 1: Structure of sakurasosaponin (**1**)

The identification of sakurasosaponin (**1**) as the cytotoxic principle of *J. flammea* is also in good agreement with the previously established structural-activity relationship of saponins. The number of sugars and glycosylation sites of saponins have been shown to play an important role for their cytotoxic effects [5a-5c]. Furthermore, the 13β,28-oxo-oleanane skeleton of saikosaponins has been shown to be essential for biological activities including antiviral, hepatoprotective, haemolytic, anti-inflammatory and antitumour effects [5c,5d]. Accordingly, sakurasosaponin derivatives which lack the 13β,28-oxo bridge moiety have been shown to be weak antifungal and molluscicidal agents [3b]. Our finding of the triterpene saponin, sakurasosaponin (**1**) which possess the 13β,28-oxo bridge in its structure as a cytotoxic agent is thus in good agreement with previous studies. For saponins which contain the 13β,28-oxo bridge structural moiety (e.g. saikosaponins and maesabalides), their haemolytic activity has been shown to be further enhanced by α-hydroxylation of the C-16 position [5c,5d]. This structural feature is also evident in sakurasosaponin (**1**) (Figure 1).

In conclusion, our bioassay-guided isolation studies on *J. flammea* resulted in the isolation of the active principle, sakurasosaponin. This appears to be the first report on the chemistry of *J. flamea* extracts. The observed biological activity may further explain some of the various traditional uses reported for the plant. In view of the cytotoxicity of the *J. flammea* extracts, care must be taken when using the plant preparations for internal use. Further research on the anticancer activity of the isolated compound is also warranted.

Experimental

General Procedures: All solvents were analytical grade (Fisher Scientific), and distilled in the laboratory prior to use. Analytical thin layer chromatography (TLC) was carried out using Silica gel 60 F_{254} (Sigma-Aldrich Company) plates. Chromatograms were sprayed with 1% vanillin (in EtOH) followed by H_2S0_4 (5% in H_2O), and then heated using hot air until colour spots are visible. Open (gravity) column chromatography separations were carried out using silica gel 70-230 mesh (Sigma-Aldrich Company). Fractions were monitored by TLC and those showing a similar composition were combined. Infrared (IR) data was collected using a Perkin-Elmer FT-IR spectrometer (Paragon1000). ESI-MS and MS/MS mass spectrometry analysis were performed using a Micromass (Manchester, UK) Quattro 11 tandem mass spectrometer with an electrospray, Z-spray ion source (+/– ve). Flow injection analysis was employed with a solvent mixture of CH_3OH and H_2O each with 0.1% HCOOH 50:50 v/v and a flow rate of 10 µl/min. The analyte concentration was 10 µg/mL. For tandem mass spectrometry, a collision cell with 2×10^{-5} torr argon, and product ion scans were employed. 1D (^{1}H and ^{13}C) spectra and 2D (COSY, HMBC, HSQC and NOESY) experiments of sakurasosaponin were recorded on a Bruker AVANCE DRX-500 spectrometer operating at 500 MHz (^{1}H) and 125 MHz (^{13}C).

Plant Collection and Extract Preparation: *Jacquinia flammea* Millsp. ex Mez was collected in July 2001 in Yucatán (aprox. 204220N and 883940W). A voucher specimen (FMay 1927) was deposited in the Herbarium of the Centro de Investigación Científica de Yucatán. Collected plants were separated into leaves, stem-bark, and roots, and left to dry for a week at room temperature followed by four days in an oven at 55°C. Plant material was ground then; portions of 250 g were extracted separately using ethanol (three times; 1200 mL, 48 h) at room temperature. The solvent was removed under reduced pressure to yield the corresponding crude extract.

Isolation of Sakurasosaponin: A portion of the crude roots extract of *J. flammea* (11 g) was suspended in 500 mL of water and subjected to successive liquid-liquid partitioning with 1 L each (three times) of petroleum ether (yield - 376.4 mg), chloroform (yield - 110.9 mg), ethyl acetate (yield - 579.2 mg), and butanol (yield - 46.7 mg) and the final residual lyophilized aqueous residue (9.45 g). The aqueous residue (9.45 g) which showed cytotoxicity against mammalian cells was subjected to Silica gel column chromatography (diameter: 3 cm, height: 25 cm) and elution done using a solvent gradient of chloroform, MeOH and water: $CHCl_3$ (200 mL); $CHCl_3$-MeOH 95:5 (200 mL), 90:10 (200 mL), 80:10 (200 mL); $CHCl_3$-MeOH-H_2O 80:10:1 (200 mL), 80:20:1 (200 mL), 80:30:1 (200 mL), 70:30:1 (200 mL), 60:30:1 (200 mL), 50:30:1 (200 mL, 40:30:1 (200 mL), 30:30:1 (200 mL), 30:20:1 (200 mL), 20:30:1(200 mL), 20:30:2 (200 mL). Fractions were collected in portions of 50 mL and combined on the basis of their TLC profile to yield 18 major fractions (A1-A18). Fraction A18 (147.2 mg and obtained from $CHCl_3$-MeOH-H_2O 20:30:1 and 20:30:2 elution) was further purified by column chromatography (diameter: 3 cm, height: 6 cm), eluting with $CHCl_3$-MeOH-H_2O 70:30:2 (300 mL) and $CHCl_3$-MeOH-H_2O 65:35:3 (200 mL). Fractions of 25 mL were collected and their composition monitored by TLC. Further column chromatography (diameter: 2.5 cm, height: 22 cm) purification of the above mentioned $CHCl_3$-MeOH-H_2O 70:30:2 elution (78.5 mg), eluting again with mixtures of $CHCl_3$-MeOH-H_2O 80:30:2 (200 mL) and $CHCl_3$-MeOH-H_2O 50:35:3 (400 mL), yielded 22.5 mg of sakurasosaponin in pure form. Additional amounts of sakurasosaponin were obtained similarly from the purification of fraction A13 ($CHCl_3$-MeOH-H_2O 50:30:1, 142 mg) to yielded 47.7 mg of pure sakurasosaponin.

Cytotoxic assay: The murine macrophage-like cell line RAW 264.7 and the human cervical cancer cell line HeLa were purchased from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). HeLa cells were maintained in Minimum Essential Medium Eagle (Sigma-Aldrich Company, Poole, Dorset, UK), while Dulbecco's Modified Eagle Medium (Sigma-Aldrich) was used for RAW 264.7 cells. Cell viability was detected using the modified MTT (Sigma-Aldrich) assay [2a, 2b]. Cells were treated with different concentrations (prepared by 1:3 and 1:2 serial dilutions) of extracts or pure compounds. Testing of pure metabolites started at 100 µg/mL, while the evaluation of crude extracts and fractions started at 500 µg/mL. Experiments were carried out in quadruplicate and repeated three times [2a, 2b].

Antibacterial Activity: Evaluation of the antibacterial activity was carried out using the paper-disc diffusion method [2a] and *Staphylococcus aureus* (NCIMB 9518), *Bacillus subtilis* (NCIMB 10113), *Escherichia coli* (NCIMB 10219) and *Pseudomonas aeruginosa* (NCIMB) test species. Paper discs (Whatman No. 1, 5 mm diameter) impregnated with various concentrations of either crude extracts, sakurasosaponin or antibiotic positive controls were placed on the agar surface. After incubating the plates for 24 h at 37°C, the inhibitory zone was measured for each sample. Chloramphenicol, (Sigma-Aldrich) was used as a positive control. All

experiments were carried out in quadruplicate and repeated three times.

Sakurasosaponin

IR υ_{max}: 3400 (OH), 2950 (CH), 1730 (C=O, acid), 1077 (C-O) cm^{-1}.
^{1}H NMR (500 MHz, CD_3OD): δ 1.01, 1.75 (2×*m*, H-1a,b), 1.47. 2.06 (2×*br m*, H-2 a,b), 3.23 (*m*, H-3), 0.73 (*d*, *J*= 11.3 H-5), 0.98, 1.15 (*m*, H-6a,b), 1.20, 1.54 (2×*m*, H-7a,b), 1.26 (*m,* H-9), 1.22, 1.47 (2×*m*, H-11a,b), 1.22, 2.11 (2×*m*, H-12a,b), 1.17, 2.10 (2×*m*, H-15a,b), 3.89 (*br m*, H-16), 1.5 (*m,* H-18), 1.18, 2.36 (2×*m*, H-19a,b), 1.27, 2.06 (2×*m*, H-21a,b), 1.51, 1.75 (2×*m*, H-22a,b), 1.06 (s, 23-CH_3), 0.87 (s, 24-CH_3), 0.89 (s, 25-CH_3), 1.15 (s, 26-CH_3), 1.23 (s, 27-CH_3), 3.12, 3.49 (2×*d*, *J*= 7.5, 7.5 H-28a, b), 0.95 (s, 29-CH_3), 0.91 (s, 30-CH_3), 4.43 (*d*, *J*= 7.5 H-1 of GlcA), 4.86 (*d*, *J*= 7.6 H-1 of Glc), 5.20 (*d*, *J*= 7.5 H-1 of Gal), 5.40 (*br* s, H-1 of Rha I), 1.25 (*d*, *J*= 6.5 H-6 of Rha I), 4.97 (*br* s, H-1 of Rha II), 1.24 (*d*, *J*= 6.5 H-6 of Rha I)
^{13}C NMR (125 MHz CD_3OD): δ 40.4 (C-1), 27.2 (C-2), 92.1 (C-3), 40.9 (C-4), 57.0 (C-5), 18.9 (C-6), 35.4 (C-7), 43.4 (C-8), 51.4 (C-9), 38.0 (C-10), 20.0 (C-11), 33.4 (C-12), 88.5 (C-13), 45.4 (C-14), 37.5 (C-15), 78.3 (C-16), 45.5 (C-17), 52.5 (C-18), 39.9 (C-19), 32.5 (C-20), 37.2 (C-21), 32.3 (C-22), 23.4 (C-23), 16.9 (C-24), 16.9 (C-25), 18.9 (C-26), 20.1 (C-27), 78.9 (C-28), 34.0 (C-29), 25.0 (C-30), 105.9 (C-1 of GlcA), 102.7 (C-1 of Glc), 100.9 (C-1 of Gal), 101.0 (C-1 of Rha I), 18.2 (C-6 of Rha I), 104.0 (C-1 of Rha II), 18.2 (C-6 of Rha II).
ESIMS: *m/z* 1273 $[M+Na]^+$, 1249 $[M-H]^-$, and 815 $[M+Na\ -aglycone]^+$.
MS/MS daughter ion fragments of *m/z* 815: 653, 495, 477, and 361.
Daughter ion fragments of *m/z* 495: *m/z* 349 and 203.

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