SUPPLEMENTARY MATERIAL

***Ailanthus altissima* (Miller) Swingle fruit - new acyl β -sitosteryl glucoside and *in vitro* pharmacological evaluation**

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β -Sitosterol-3-*O*-(6'-*O*-13"-octadecenoyl)- β -D-glucoside (**1**), a new acyl β -sitosteryl glucoside, along with three known compounds β -sitosterol-3-*O*- β -D-glucoside (**2**), β -sitosterol (**3**) and methyl gallate (**4**) have been isolated from the ethyl acetate soluble fraction of methanolic extract of *Ailanthus altissima* fruits. Their structures were elucidated through spectroscopic data including 2D NMR, ESI-MS, methanolysis and oxidative cleavage of double bond. Antibacterial, antifungal, cytotoxic, phytotoxic and insecticidal activities were evaluated of compound **1**, crude extract and its fractions so far for the first time. Pharmacological activities results showed that *n*-butanol fraction was good-active against *Pseudomonas aeruginosa* and *Salmonella typhi* bacteria, and moderate-active against *Microsporum canis* fungus. Crude extract, and *n*-butanol and aqueous fractions showed good cytotoxicity. Moreover, compound **1**, extract and all fractions and showed notable phytotoxicity at higher concentrations, whereas all inactive against assayed insects.

Keywords: Simaroubaceae; *Ailanthus altissima*; β -Sitosterol-3-*O*-(6'-*O*-13"-octadecenoyl)- β -D-glucoside; antimicrobial; cytotoxicity; phytotoxicity

Table S1: ^1H - (CD_6CO , 500 MHz) and ^{13}C - (CDCl_3 , 125 MHz) NMR spectral data of compound **1**

C No.	Multiplic	δ_{C}	δ_{H}
1	CH_2	37.3	1.83 (1H, m), 1.03 (1H, m)
2	CH_2	27.2	1.88 (1H, m), 1.55 (1H, m)
3	CH	79.7	3.49 (1H, t, $J = 7.5$ Hz)
4	CH_2	38.9	2.31 (1H, m), 2.26 (1H, m)
5	C	140.3	---
6	CH	122.1	5.36-5.34 (1H, m)
7	CH_2	32.0	1.92 (2H, m)
8	CH	31.9	1.45 (1H, m)
9	CH	50.2	0.88 (1H, m)
10	C	36.7	---
11	CH_2	21.1	1.45 (2H, m)
12	CH_2	39.8	1.98 (1H, m), 1.12 (1H, m)
13	C	42.3	---
14	CH	56.8	0.93 (1H, m)
15	CH_2	24.3	1.56 (1H, m), 1.10 (1H, m)
16	CH_2	28.3	1.88 (1H, m), 1.28 (1H, m)
17	CH	56.1	1.08 (1H, m)
18	CH_3	11.9	0.67 (3H, s)
19	CH_3	19.4	0.96 (3H, m)
20	CH	36.2	1.32 (1H, m)
21	CH_3	18.8	0.89 (3H, d, $J = 6.4$ Hz)
22	CH_2	34.0	1.31 (1H, m), 0.95 (1H, m)
23	CH_2	26.2	1.30 (1H, m), 1.15 (1H, m)
24	CH	45.8	0.90 (1H, m)
25	CH	29.2	1.83 (1H, m)
26	CH_3	19.8	0.79 (3H, m)
27	CH_3	19.0	0.82 (3H, m)
28	CH_2	22.7	1.41 (2H, m)
29	CH_3	12.0	0.81 (3H, t, $J = 7.4$ Hz)
1'	CH	101.2	4.38 (1H, d, $J = 8.0$ Hz)
2'	CH	73.9	3.39 (1H, t, $J = 9.0$ Hz)
3'	CH	73.5	3.26 (1H, t, $J = 9.0$ Hz)
4'	CH	70.3	3.15 (1H, t, $J = 8.5$ Hz)
5'	CH	76.1	3.55 (1H, m)
6'	CH_2	63.4	4.37 (1H, dd, $J = 11.5, 1.5$ Hz), 4.18 (1H, dd, $J = 11.5, 7.5$ Hz)
1''	C	174.4	---
2''	CH_2	34.2	2.32 (2H, t, $J = 7.5$ Hz)
3''	CH_2	25.0	1.60 (2H, m)
4''-11''	$\text{CH}_2 \times 8$	29.1-29.8	1.24-1.26 (16H, br s)
12''	CH_2	25.6	2.79 (2H, m)
13''	CH	128.1	5.32-5.30 (1H, m)
14''	CH	130.0	5.32-5.30 (1H, m)
15''	CH_2	27.2	2.00 (2H, m)
16''	CH_2	31.9	1.26 (2H, m)
17''	CH_2	23.1	1.26 (2H, m)
18''	CH_3	14.1	0.85 (3H, t, $J = 7.5$)

Figure S1: ^{13}C NMR of Compound 1

Figure S2: ^1H NMR of Compound 1

Figure S3: Important HMBC (—→) and COSY (—) correlations of **1**

3.4. Pharmacological evaluation assays

3.4.1. Antibacterial bioassay

The antibacterial potential of methanolic extract and its fractions and compound **1** of *A. altissima* fruits was determined against Gram-positive *Staphylococcus aureus*, *Bacillus subtilis*, and Gram-negative *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella flexneri* bacteria by agar well diffusion method using Mueller-Hinton agar medium (Alves et al. 2000). Test samples were prepared by dissolving 3 mg in 1 mL DMSO. The strains were grown in Mueller-Hilton agar and broth. The strains were incubated at 37 °C for 24 hrs and diluted to approximately 10⁶ CFU/mL. 100 µL of 10⁶ CFU/mL containing bacterial suspension was spread over Mueller-Hilton agar and homogenized. After solidification, wells of 6 mm-diameters were made using sterile cork-borer. 100 µL test samples, imipenem 10 µg (positive control) and DMSO (negative control) were inoculated in respective wells and all wells were incubated at 37 °C for 24 hrs. Antibacterial activity was determined by measuring the diameter of zone of inhibition and results were expressed as: < 9 mm inactive; 9-12 mm partially active; 13-15 mm low active, 16-18 mm good active; >18 mm very active/significant. All reactions were performed in triplicate. Minimal inhibitory concentration (MIC) was measured by two fold dilution at various concentrations.

3.4.2. Antifungal bioassay

The *in vitro* antifungal activity of methanolic extract and its fractions and compound **1** of *A. altissima* fruits was evaluated by agar tube dilution method (Choudhary et al. 1995) against *Candida albicans*, *Candida glabrata*, *Fusarium solani*, *Microsporum canis* and *Aspergillus flavus* fungi. Each sample (20 mg) was dissolved in 1 mL sterile DMSO. The strains were cultured in sabouraud dextrose agar (SDA), the acidic (pH 5.5-5.6) media was prepared by mixing 32.5 g/500 ml distilled water containing glucose or maltose 2%. The media (4 mL) was transferred to screw caps tube and autoclaved at 121 °C for 15 min. Cooled the tubes at 50 °C and 66.6 µL test samples were employed. Solidify the tubes and each tube was inoculated with 4 mm diameter fungus plates. The plates were incubated for 3-7 hours at 27-29 °C. For positive control, amphotericin B used for *A. flavus* and miconozale used for others. DMSO was used as negative control. The test solution diffused with the growth of inoculated microorganisms. The percentage inhibition of fungal growth was measured. The results were expressed in percentage inhibition as: 0-39 low active; 40-59 moderate active; 60-69 good active; >70 significant active. All reactions were performed in triplicate.

3.4.3. Cytotoxicity/Brine shrimp lethality bioassay

To check *in vitro* cytotoxicity/brine shrimp activity, reported protocol was employed (Alves et al. 2000). The *Artemia salina* (shrimps) eggs were stored at 4 °C. Artificial seawater was prepared by dissolving 38 g sea salt in 1 liter double distilled water (pH 7.4), filtered it and was placed in small partition of hatching tray. 1 Milligram of shrimp eggs (*A. salina*) was added to the large partition of the tray, which was darkened with aluminum foil and incubated at 37 °C. The shrimp's larvae were attracted by illuminated partition through perforation of the wall. It was permitted to stand for 48 hours at 25 °C to hatch and mature the shrimps. Each sample (20 mg) was dissolved in 2 mL respective solvent. From these solution, 5, 50 and 500 µL were transferred to vials in triplicate and adjusted at 10, 100, 1000 µg/mL concentrations and the solvents were evaporated. After 2 days, the shrimp larvae were matured. In each veil, added 10 shrimp's larvae using Pasteur pipette and 5 mL seawater, and then incubated at 25-27 °C 24 hours under illumination. After 24 hours, the surviving larvae were counted and determine the LD₅₀ using Probit method by Finney computer program.

3.4.4. Phytotoxic bioassay

Phytotoxic activity of methanolic extract and its fractions of *A. altissima* fruits was evaluated against the *Lemna minor* using standard procedure (McLaughlin et al. 1991). The growth medium for *Lemna* was prepared by dissolving various constituents in distilled water (1L) and its pH was adjusted at 5.5-5.6 by adding KOH pellets. The medium was then autoclaved at 121 °C for 15 min. The each sample (30 mg) was dissolved in methanol/ethanol (10 ml) to prepare a stock solution. Three concentrations were prepared of 10, 100 and 1000 µg/ml by taking 10, 100 and 1000 µl of the stock in petri dish as three petri plates of each concentration. The solvent was allowed to evaporate overnight under sterile conditions. Each plate was inoculated with growth medium (20 mL) and *Lemna minor* plants (10), each containing a rosette of three fronds. Three other plates each were supplemented with growth media and reference growth inhibitors, served as negative control. All plates were kept in the growth cabinet for seven days. Total number of fronds per petri plate was counted and recorded on seventh day. Percent growth inhibition was calculated as % regulation as follows.

$$\% \text{ Regulation} = 100 - \frac{\text{No. of fronds in test sample}}{\text{No. of frond in -ve control}} \times 100$$

3.4.5. Insecticidal activity

Crude methanolic extract and its fractions and compound **1** of *A. altissima* fruits were evaluated against *Tribolium castaneum*, *Rhyzopertha dominica*, and *Callosobruchus analis* insects by impregnated filter paper test (Tabassum et al. 1997). Each sample was prepared by dissolving 20 mg in 2 mL volatile solvent (methanol/acetone). A filter paper placed in the petri plate (5 cm or 50 mm) and the whole sample of different concentrations was loaded over the filter paper and plates left for 24 hours to evaporate the solvent. After 24 hours, 10 healthy and active insects of same size and age of each species were placed in each petri plate and the plates were incubated at 27 °C for 24 hours with 50% relative humidity in growth chamber. Permethrin was used as positive control and volatile solvent was used as negative control. Total number of survivors per petri plate was counted and recorded on third day. The % mortality was calculated by the following formula:

$$\% \text{ Mortality} = 100 - \frac{\text{No. of insects alive in test sample}}{\text{No. of insects alive in control}} \times 100$$

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