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Original Article

Cytotoxic Compounds from *Calotropis gigantea* (Linn.) and *Amoora rohituka* (Roxb.)

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Calotropis gigantea (Linn) and *Amoora rohituka* (Roxb.) have long been used in folkloric medicine for the treatment of various ailments in Bangladesh. The present study was designed to investigate the cytotoxicity of isolated compounds from these two plants. Two compounds, named β-boswellic acid (compound-1) and 2-methoxy-14-calamenenone (compound-2) were isolated from the flowers of *Calotropis gigantea* and stem bark of *Amoora rohituka*, respectively. The structures of these compounds were confirmed on the basis of IR, Mass and NMR spectroscopy, and their cytotoxic activity against A431 (human vulval-derived epidermoid carcinoma) cell line was examined. When compared with standard drug doxorubicin (IC_{50}: 0.31 ± 0.02 µg/mL), compound-1 exhibited significant cytotoxic activity against A431 cell line with IC_{50} value of 0.36 ± 0.07 µg/mL whereas the IC_{50} of compound-1 was 0.56 ± 0.05 (µg/mL). On comparison with ampicillin trihydrate (IC_{50}: 7.21 ± 0.47 µg/mL), compound-1 also showed moderate cytotoxicity against brine shrimp nauplii with IC_{50} value of 15.26 ± 0.57 µg/mL.

Keywords: *Calotropis gigantea*, *Amoora rohituka*, cytotoxicity

Resistance development by tumor cells to chemotherapeutic agents is a major problem in cancer treatment. One way to encounter this, is to search for compounds with cytotoxic properties. The biological and chemical diversities in medicinal plants provide opportunities to find out compounds with cytotoxic properties (1). Sixty percent of currently used anticancer agents are derived in one way or another from medicinal plants (2). Medicinal Plants have provided an extraordinary source of new compounds with therapeutic activity and the secondary metabolites represents an efficient alternative for obtaining new drugs (1, 3). So, the systematic screening of plant species with the purpose of discovering new bioactive compounds is a valuable approach in research laboratories. Having concern on this fact, this study was designed to determine *in vitro* the cytotoxic activity of isolated

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compounds from *Calotropis gigantea* (Linn) and *Amoora rohituka* (Roxb.) using A431 (human vulval-derived epidermoid carcinoma) cells line. *Calotropis gigantea* (Linn.) (Family: Asclepiadaceae) and *Amoora rohituka* (Roxb) (Family: Meliaceae) are two important plants that grow in forests and roadsides of many districts of Bangladesh and have wide folk medicinal use (4, 5). Several researchers also reported the isolation of different types of compounds from different parts of these *Calotropis gigantea* (6-16) and *Amoora rohituka* (17-26). But a few of isolated compounds from *Calotropis gigantea* and *Amoora rohituka* have potent antineoplastic, antimicrobial and insecticidal activities. Therefore, these plants have merits for further screening on bioactive compounds.

Materials and methods

Plant materials

Flowers of *Calotropis gigantea* (Linn.) (Family: Asclepiadaceae) and stem bark of *Amoora rohituka* (Roxb.) (Family: Meliaceae) were selected for the chemical investigation. Both plant materials were collected in the month of September, 2015 from Rajshahi district of Bangladesh. The plant materials were taxonomically identified by Professor A.T.M Naderuzzaman, Department of Botany, University of Rajshahi. A voucher specimen for *Calotropis gigantea* (No. 1A. Alam, collection date 15.08.2004) was kept in the Department of Botany, University of Rajshahi and for *Amoora rohituka,* voucher specimen was deposited under the accession number DACB-28927 at the Bangladesh National Herbarium.

Extraction and isolation

The shed-dried powdered flower (1.0 kg) of *Calotropis gigantea* was extracted with ethyl acetate (1.5 L) at room temperature. The solvent was completely removed by rotary vacuum evaporator from the crude extract to yield a residue of 38 g. Then, crude ethyl acetate extract (10 g) was applied on silica gel (60-120 mesh) chromatography using n-hexane with a gradient of ethyl acetate up to 100% and followed by chloroform. Sixty four (64) fractions were collected. Based on TLC profile, 7~13 fractions were combined (1.8 g) and re-chromatographed on silica gel column eluting with n-hexane and ethyl acetate (19:1) with increasing portions of ethyl acetate and finally with methanol. Fractions 20~23 afforded compound-1 as colourless crystals (37 mg).

The stem bark powder (650 g) of *Amoora rohituka* was successively extracted with ethyl acetate and dichloromethane at room temperature. The extracts were filtered through a filter paper and concentrated with a rotary evaporator under reduced pressure at 60 ^{o}C to obtain 7.8 g ethyl acetate extract and 4.3 g dichloromethane extract. Then, preparative thin layer chromatography (PTLC) was applied to the separation and final purification of compound-2 as orange oil from dichloromethane extract (27-28).

Analytical methods

Infrared (IR) spectra were taken on FTIR-8900 spectrophotometer (Shimadzu Kyoto, Japan) and High Resolution TOF Mass Spectra were obtained using a Waters LCT Premier mass spectrometer (UK) coupled with a Waters AQUITY HPLC system, with data acquisition achieved using MassLynx software, version 4.0. GCMS-QP2010S (Shimadzu Kyoto, Japan) spectrometer was used for taking GC-MS. NMR spectra were recorded on Bruker 400 MHz FT spectrometer (DPX-400, Switzarland). All the spectra were taken in Analytical Research Division, Bangladesh Council of Scientific and Industrial Research (BCSIR) laboratories, Dhaka-1205, Bangladesh.

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Cytotoxicity assay

To examine the cytotoxicity of isolated compounds, A431 (human vulval-derived epidermoid carcinoma) cell line was used in this study. This study was conducted in Graduate School of Bioagricultural Sciences, Nagoya University, Japan. The cytotoxicity of each compound was evaluated by previously reported procedures (29, 30). The A431 cell line was cultured in a serum-free RD medium [RPMI 1640 medium (Kyokuto, Tokyo Japan)-DMEM (Kyokuto) 1:1, vol/vol] containing five factors (10 µg/ml of insulin, 5 µg/ml of transferrin, 10 µM 2-mercaptoethanol, 10 µM 2-aminoethanol and 10 nM Selenite) (31, 32). In the proliferation assays, the cells were plated at 1.0 x 10^4 per well into 24-well tissue culture plates coated with type-I collagen and cultured in the same medium. The cells were allowed to attach and spread for 12 h prior to their incubation with each test compound and doxorubicin (used as standard drug) at various concentrations (none, 0.02, 0.2 and 20 µg/ml). On day 4, the cells were harvested by 0.05% trypsin/0.01% EDTA and the cell numbers were counted with a Zf counter to determine IC_{50} values.

Brine shrimp lethality bioassay

The experiment was carried out using the method described by Meyer (33). In brief, *Artemia salina* Leach (brine shrimp eggs) was allowed to hatch and mature as nauplii (Larvae) in seawater for 48 h at 25 °C. Serially diluted test solutions were added to the seawater (5 mL), containing 10 nauplii. After 24 h of incubation, the numbers of survivors in each vial were counted and noted. The percentage of mortality of the nauplii was calculated for each concentration and The LC_{50} (50% lethal concentration, µg/mL) values were determined using probit analysis from triplicate experiments. Ampicillin trihydrate was used as positive control.

Results

In this study, isolated and purified compound 1 and 2 were characterized by IR, Mass and NMR spectral data. The IR spectrum of compound-1 exhibited the absorptions for a hydroxyl group (3448.5 cm^{-1}), a carbonyl carbon of a carboxylic acid group (1705.8 cm^{-1}) and an olefinic structure (1627.8 cm^{-1}). The mass spectrum of compound-1 showed a molecular ion peak at m/z 456.3603 suggesting a molecular formula of $C_{30}H_{48}O_3$. In ^{1}H-NMR spectrum of compound-1, the appearance of a olefinic proton at δ 5.14 (1H, t, J = 3.5 Hz, H-12), a methine proton at δ 1.30 (1H, d, J = 8.9 Hz, H-18) and two methyl group signals at δ 0.78 (3H, d, J = 5.6 Hz, H-29) and δ 0.79 (3H, d, J = 3.2 Hz, H-30) unambiguously confirmed the presence of Δ^{12}-ursane skeleton. In addition, the ^{1}H-NMR spectrum of compound-1 also showed a hydroxylated methine proton signal at δ 4.08 (1H, t, J = 3.5, H-3) and five methyl group signals, each as singlet at δ 1.32 (C-23), δ 0.89 (C-25), δ 0.96 (C-26), δ 0.92 (C-27) and δ 0.99 (C-28). ^{13}C-NMR spectrum of compound-1 showed the presence of 30 signals, one of which was assigned to a carbonyl carbon (δ 183.7) of a carboxylic acid group. In ^{13}C-NMR spectrum of compound-1, two signals at δ 124.8 (C-12) and δ 139.6 (C-13) were assigned to a double bond that was consistent with that of Δ^{12}-ursane skeleton. Finally, the structure of compound-1 was confirmed as β-boswellic acid (3β-hydroxy-urs-12-ene-24β-oic acid) (Fig. 1) by a comparison with the ^{1}H-, ^{13}C-NMR and mass spectral data reported in the literature (34).

The ^{1}H-NMR spectrum of compound-2 indicated two aromatic proton signals at δ 6.72 (1H, s, H-1) and δ 6.98 (1H, s, H-4). One methoxyl signal at δ 3.78 (3H, s, CH_3-O-) and two methyl signals at δ 0.99 and δ 0.76 (each 3H, d, J = 6.5, H-12 &13) were observed in its ^{1}H-NMR. In addition, ^{1}H-NMR spectrum of compound-2 also exhibited one downfield methyl proton signal at δ 2.19 (3H, s, H-

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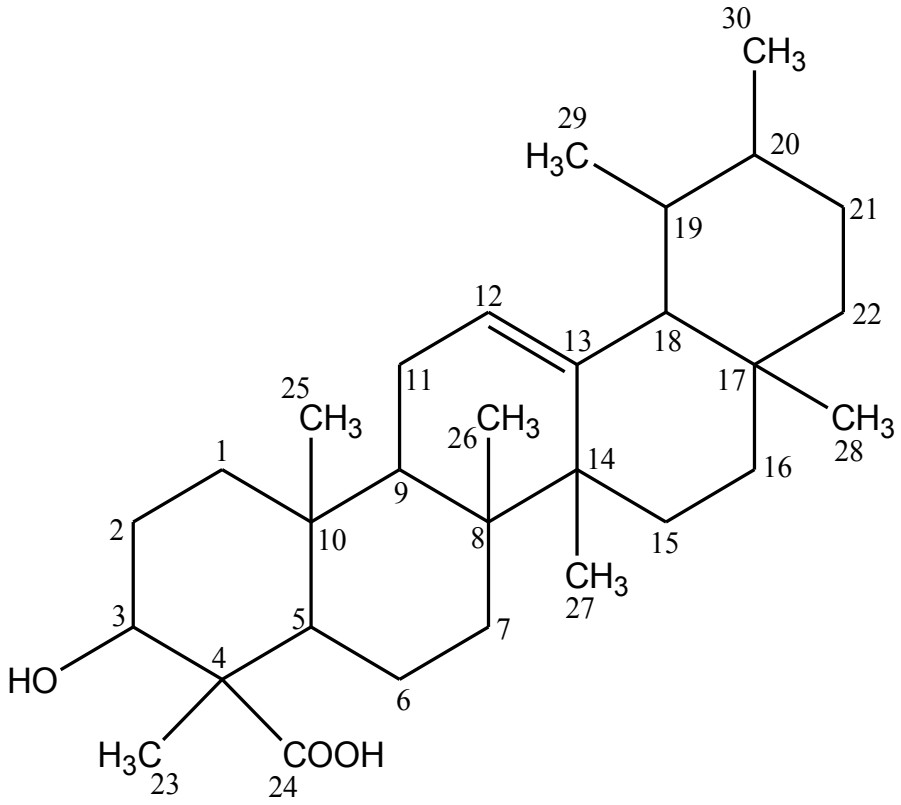

Figure 1. Chemical structure of compound-1: 3β-hydroxy-urs-12-ene-24β-oic acid.

15) which is conjugated with benzene ring. The ^{13}C-NMR spectrum of compound-2 displayed 14 signals while the DEPT experiment indicated the presence of four methyls, two methylenes, four methines and five quarternary carbons. The ^{13}C-NMR spectrum of compound-2 included six olefinic carbon signals (δ 108.9, δ 156.7, δ 133.2, δ 131.2, δ 131.5 and δ 140.3) indicating the presence of a one benzene ring in its structure. In addition, its ^{13}C-NMR spectrum also showed one carbonyl carbon signal (δ_C 198.7) with four methyl carbon signals (δ 20.7, δ 20.2, δ 14.5 & δ 61.8). Finally, based on spectroscopic analysis and a comparison of the NMR data with literature (35), compound-2 has been identified as 2-Methoxy-14-calamenenone (Fig. 2).

Properties of compound-1 (i.e., β-boswellic acid)

Compound -1 was a white amorphous powder; with melting point 228 ºC; IR bands (neat): 3448.5, 2956.7, 1705.8 (s), 1627.8, 1456.2, 1244.0, 1022.2 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$, δ, ppm, *J*/Hz): 1.91 (2H, dd, *J* = 10.5 & 4.0 Hz, H-1), 2.21 (2H, dd, *J* = 14.0 & 4.0 Hz, H-2), 4.08 (1H, t, *J* = 3.5 Hz, H-3), 1.46 (1H, dd, *J* = 11.8 & 3.1 Hz, H-5), 1.67 (2H, dd, *J* = 14.1 & 3.0 Hz, H-6), 1.50 (2H, dd, *J* = 14.0 & 3.2 Hz, H-7), 1.60 (1H, t, *J* = 3.5 Hz, H-9), 1.98 (2H, t, *J* = 3.5, H-11), 5.14 (1H, t, *J* = 3.5 Hz, H-12),

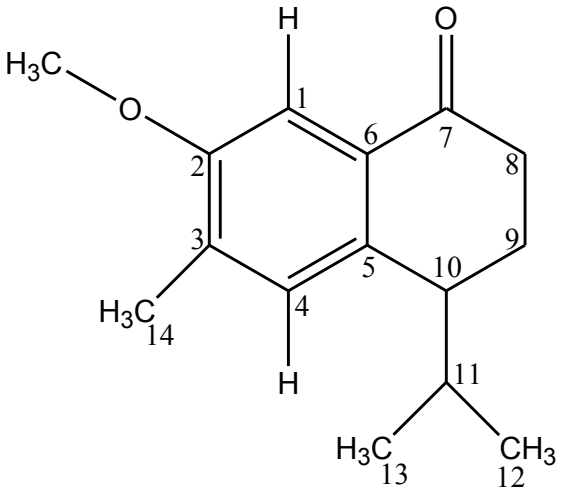

Figure 2. Chemical structure of compound-2: 2-Methoxy-14-calamenenone.

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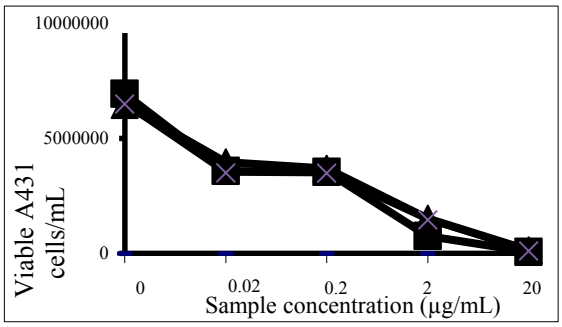

Figure 3. Cytotoxicity assay. Concentration-cell viability curves of compound-1, compound-2 and doxorubicin was determined following 12 h exposure on A431 cell line.

0.84 (2H, d, *J* = 12.3 Hz, H-15), 1.03 (2H, dd, *J* = 16.0 & 2.0 Hz, H-16), 1.30 (1H, d, *J* = 8.9 Hz, H-18), 1.24 (1H, t, *J* = 3.5 Hz, H-19), 1.29 (1H, m, H-20), 1.63 (2H, m, H-21), 1.20 (2H, t, *J* = 4.0 Hz, H-22), 1.32 (3H, s, H-23), 0.89 (3H, s, H-25), 0.96 (3H, s, H-26), 0.92 (3H, s, H-27), 0.99 (3H, s, H-28), 0.78 (3H, d, *J* = 5.6 Hz, H-29), 0.79 (3H, d, *J* = 3.2 Hz, H-30); ^{13}C-NMR (150 MHz, $CDCl_3$, δ, ppm): 34.2 (C-1), 26.4 (C-2), 71.9 (C-3), 47.7 (C-4), 55.3 (C-5), 21.3 (C-6), 33.7 (C-7), 39.6 (C-8), 47.2 (C-9), 37.7 (C-10), 23.6 (C-11), 124.8 (C-12), 139.6 (C-13), 39.7 (C-14), 28.1 (C-15), 26.3 (C-16), 33.2 (C-17), 59.1 (C-18), 40.2 (C-19), 40.1 (C-20), 31.2 (C-21), 41.5 (C-22), 23.4 (C-23), 183.7 (C-24), 15.7 (C-25), 16.9 (C-26), 21.3 (C-27), 23.2 (C-28), 17.5 (C-29), 28.7 (C-30); EI-MS (m/z): 456.3603 (M^+), 443.3375 (54), 425.3866 (28), 407.3864 (29), 238.0080 (21), 218.2954 (100), 203.3854 (26), 159.4275 (9), 133.5075 (15).

Properties of compound-2 (i.e., 2-Methoxy-14-calamenenone).

Compound-2 was an orange oil; with ^{1}H-NMR (400 MHz, $CDCl_3$, δ, ppm, *J*/Hz): 6.72 (1H, s, H-1), 6.98 (1H, s, H-4), 1.88 ~ 1.98 (2H, m, H-8), 2.55 ~ 2.59 (2H, m, H-9), 2.30 ~ 2.39 (2H, m, H-10), 2.30 ~ 2.39 (2H, m, H-11), 0.99 (3H, d, J = 6.5 Hz, H-12), 0.76 (3H, d, J = 6.5 Hz, H-13), 2.19 (3H, s, H-14), 3.78 (3H, s, CH_3-O-); ^{13}C-NMR (150 MHz, $CDCl_3$, δ, ppm): 108.9 (C-1), 156.7 (C-2), 133.2 (C-3), 131.2 (C-4), 131.5 (C-5), 140.3 (C-6), 198.7 (C-7), 38.4 (C-8), 24.5 (C-9), 44.2 (C-10), 31.7 (C-11), 20.7 (C-12), 20.2 (C-13), 14.5 (C-14), 61.8 ($\underline{C}H_3$-O-).

Table 1. *In vitro* cytotoxicity of isolated compounds against A431 cell line

Sample	IC_{50} (µg/mL)
Compound-1	0.36± 0.07
Compound-2	0.56± 0.05
Doxorubicin	0.31± 0.02

IC_{50} values are expressed as mean ± S.E.M (Standard error of mean)

Cytotoxicity properties

In cytotoxicity assay, relationships between concentration (µg/mL) of each test sample and number of viable A431 cells are presented in Fig 3. Cell viability was reduced in a dose-dependent manner after exposure of A431 cells to test samples. The IC_{50} values of test compounds on the A431 cells are summarized in Table 1.

In this assay, compound-1 showed strong cytotoxic effect on A431 cells than compound-2 and IC_{50} values were found to be 0.36 ± 0.07 µg/mL for compound-1 and 0.56 ± 0.05 µg/mL for compound-2. Compound-2 exhibited moderate cytotoxic effect in comparison with doxorubicin (IC_{50}: 0.31 ± 0.02 µg/mL).

Toxicity on brine shrimp

In brine shrimp lethality bioassay, compound-1 and compound-2 showed positive results indicating that these were biologically active. The LC_{50} results of the two compounds evaluated in this screening are listed in Table 2. In comparison to

Table 2. Toxicity of crude extracts and isolated compounds of *Calotropisgigantea* and *Amoorarohituka* against brine shrimp nauplii.

Test sample	LC_{50} (µg/mL)
Amphicillintrihydrate	7.21 ± 0.47
Compound-1	15.26 ± 0.57
Compound-2	27.67 ± 0.40

LC_{50} values are expressed as mean ± S.E.M (Standard error of mean)

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ampicillin trihydrate (LC_{50}: 7.21 ± 0.47 µg/mL) used as positive control, the cytotoxicity exhibited by compound-1 was promising with the LC_{50} values of 15.26 ± 0.57 µg/mL whereas the LC_{50} of compound-2 was 27.67 ± 0.40 µg/mL.

Discussion

Here compound-1 and compound-2 were identified as β-boswellic acid and 2-methoxy-14-calamene-none, respectively. This is the first time report on cytotoxicity of β-boswellic acid and 2-methoxy-14-calamenenone against A431 cell line.

The results provide evidence on the merit of possible using of *Calotropis gigantea* and *Amoora rohituka* and also chemical constituents derived from them for the treatment of cancer. To evaluate the mechanism by which these purified compounds showed biological activities, further studies should be carried out.

Conflict of interest

The authors declared no conflict of interest.

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