

Research Article

Anti-leishmanial and Anti-cancer Activities of a Pentacyclic Triterpenoid Isolated from the Leaves of *Terminalia arjuna* Combretaceae

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Abstract

Purpose: *Terminalia arjuna* Roxb (Combretaceae) is commonly known as Arjjhan and Arjun in Bengal, India. The anti-leishmanial and anticancer activities of a pentacyclic triterpenoid isolated from its leaves were evaluated.

Methods: Dried and crushed leaves of *Terminalia arjuna* were de-fatted with petroleum ether (40 - 60 °C) and extracted with methanol. The extract was subjected to column chromatography and column fractions were monitored on TLC plates developed in a suitable solvent system. Structures of the isolated pure compounds were elucidated by infra-red spectroscopy, mass spectrometry and nuclear magnetic resonance spectroscopy. One of the compounds was screened for anti-leishmanial activity against promastigotes of *Leishmania donovani*, and anti-cancer activity on K562 leukaemic cell line.

Results: A pentacyclic triterpenoid, characterized as 3 β -hydroxyurs-12-en-28-oic acid (together with β -D-glucoside) and designated ursolic acid, was successfully isolated. The structure was determined on the basis of spectroscopic analyses. In vitro anti-leishmanial activity against promastigotes of *Leishmania donovani* (strain AG 83) and anti-cancer activity on K562 leukaemic cell line were shown by the isolated ursolic acid.

Conclusion: The methanol extract of the leaves of *Terminalia arjuna* led to the isolation of a pentacyclic triterpenoid, ursolic acid. This compound demonstrated in vitro anti-leishmanial and anti-cancer activities

Keywords: *Terminalia arjuna* Roxb, Pentacyclic triterpenoid, Ursolic acid, Spectroscopy, Anti-leishmanial, Anti-cancer

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INTRODUCTION

Terminalia arjuna Roxb (Combretaceae) is commonly known as Arjjhan and Arjun in Bengal, India. It is a large tree, often with buttressed trunk, smooth grey bark and about 20 - 25 m in height. Its leaves are usually sub-opposite, oblong or elliptic-long, pale dark green above and pale-brown beneath, 10 - 20 cm long and hard. The flowers are yellowish-white while the fruits are 2.5 - 5.0 cm ovoid or ovoid-oblong, fibrous-woody, and glabrous. It is common on the banks of rivers, streams and dry watercourses in sub-Himalayan tract, West Bengal as well as in central and south India. The bark of the plant is known to contain a crystalline compound, arjunine, a lactone, arjunetin, essential oil and reducing sugar. Besides these, it also contains 34 % calcium carbonate, 9% of other salts of calcium, 13% tannin and aluminum, magnesium, organic acids, colouring matter and other substances [1]. In Indian traditional medicine, the fruits of the plant are used as a tonic [2]. Externally, its leaves are used as a cover on sores and ulcer. The bark is anti-dysenteric, antipyretic, astringent, cardiogenic, lithotriptic and tonic [3] while the powder of the bark acts as a diuretic in cirrhosis of liver and gives relief in symptomatic hypertension. A decoction of the thick bark made with milk is given every morning on an empty stomach or its powder with milk and gur as a cardiogenic [4]. The bark powder is also given with honey in fractures and contusions with echymosis. Furthermore, the extract of the bark, as an astringent, is used for cleaning sores, ulcers and cancers, etc. An ointment made from the bark by mixing with honey is used to cure acne while the ashes of the bark are prescribed in scorpion stings [5].

The present work was undertaken to isolate and characterize a pentacyclic triterpenoid (ursolic acid) from the methanol extract of the leaves of *T. arjuna* and, and evaluate its anti-leishmanial and anti-cancer activity on K562 leukemic cell line.

EXPERIMENTAL

Plant material

The leaves of *Terminalia arjuna* were collected in January 2008 from Nadia, West Bengal, India. The plant material was taxonomically identified by Dr. Lakhmi Narashimhan, Scientist, Botanical Survey of India, Central National Herbarium, Howrah, West Bengal, India. The voucher specimen [CNH/I-I/(216)/2008/Tech.II/216] was preserved in the above herbarium for future reference.

Extraction and isolation

The shade-dried and powdered leaves of *Terminalia arjuna* (2.4 kg) were de-fatted with petroleum ether (40 - 60 °C) and extracted with methanol (3×8 L) at 40 - 45 °C. The combined methanol extract was concentrated and 29 g of the extract was applied to a column of silica gel 60 (400 g) and washed with 100 % petroleum ether. Gradient elution was carried out with a mixture of chloroform:petroleum ether (1:9, 1:4, 3:7, 2:3 and 1:1) and of chloroform:methanol (99:1, 49:1, 97:3, 24:1, 19:1, 9:1). A total of 72 fractions (50ml) were collected and fractions giving similar spots on TLC were combined. Fractions eluted with chloroform-methanol (99:1) were combined and subjected to re-chromatography over silica gel (20 g). Fractions (collected 15 ml lots) eluted with chloroform:methanol mixture (99:1) furnished a pure compound (1.8 g).

Thin layer chromatography (TLC)

TLC of the isolate was carried out on silica gel 60F₂₅₄ and spots were visualized by spraying with Libermann-Buchard's reagent followed by heating. Silica gel (silica gel 60, Merck) was used for column chromatography.

Determination of melting point

The melting point of the isolate was measured on a Yanagimoto micromelting apparatus and the data are uncorrected.

Infra-red spectroscopy (IR)

The IR spectra of the isolate was determined with Jasco 7300FTIR spectrometer using potassium bromide pellets, the sample, being in a crystalline state.

Nuclear magnetic resonance (NMR)

¹H and ¹³C NMR spectra of the isolate were recorded at 500 and 125 MHz, respectively using a Jeol ECP-500 spectrometer in C₅D₅N with TMS as internal standard. .

Mass spectroscopy

Mass spectroscopy was performed on the isolate using a Jeol MS-700 mass spectrometer to determine the molecular weight of the compound.

Evaluation of anti-leishmanial activity

Leishmania donovani strain AG 83 was originally obtained from an Indian kala-azar patient [7] and maintained in golden hamsters by serial passage. Two months later, the hamster was sacrificed and its spleen isolated and macronized. The splenic culture was made in Medium-199 (L-glutamine with Hepes buffer without NaHCO₃) supplemented with 10 % foetal bovine serum (pH 7.2). The logarithm phases of promastigotes (2×10⁶ cells/ml) were incubated with or without the isolates along with Medium-199 at 22 °C. The isolated compound (ursolic acid) was dissolved in 0.2 % DMSO and then added to the culture in graded doses of 3µg/ml, 5µg/ml, 10µg/ml, 15µg/ml and 30µg/ml. After 2 h of treatment, all tubes were centrifuged at 8000 g for 10 min, the supernatant was decanted and the pellets washed with 20 mM phosphate buffer saline (PBS). Each pellet was dissolved in

100 µl (2 mg/ml) of MTT in phosphate buffer saline [8].

All the tubes were incubated at 22 °C for 4 h and then centrifuged at 8000 g for 10 min. All the pellets were dissolved in 500 µl DMSO and assessed by UV spectrophotometry at 570 nm. Percent of lysis of promastigotes by the compounds was calculated using the standard formula of Tim Mosmann [9]. IC₅₀ (50% inhibitory concentration of the isolated compound or IC₅₀) dose was evaluated by linear regression analysis using Prism 3 software. Chloroform treatment served as control.

Assessment of anti-cancer activity

The human chronic myelogenous leukaemia cell line K562 obtained from a patient in blast crisis of chronic myeloid leukemia was used for this test. The cells were grown in RPMI-1640 medium supplemented with 10% faetal calf serum (Gibco, USA), air and 5 % CO₂. The isolate was added to a medium containing 1×10⁶ cells/ml, 2 mM L-glutamine and 50 µg/ml gentamycin, and kept at 37 °C in a fully humidified atmosphere. After 18 h of incubation at 37 °C in 5 % CO₂ incubator, the tubes were centrifuged at 8000 g for 10 min. The supernatant was decanted, and the pellets taken and washed with 20 mM of phosphate buffered saline solution. Each pellet was dissolved in 100 µl (2mg/ml) MTT solution in a tube, as previously described [9], incubated at 22 °C for 4 h and centrifuged at 8000 g for 10 min. All the pellets were dissolved in 500 µl DMSO and read spectrophotometrically at 500nm. Lysis (%) of K562 cells by the isolate was calculated using the standard formula of Tim Mosmann shown in Eq 1 [9]. Chloroform treatment served as control.

$$\text{Lysis (\%)} = 100 - (T - PC) / (C - PC) \times 100 \dots (1)$$

where T is the test isolate, PC the positive control, and C the control. IC₅₀ dose was evaluated by linear regression analysis using Graph Pad Prism 3 software.

Statistical analysis

Experimental results were expressed as mean \pm SEM of three parallel measurements. Statistical analysis was carried out using Student's t-test followed by ANOVA. Differences were considered statistically significant values at $p < 0.05$.

RESULTS

Isolation of ursolic acid

The compound obtained from the methanol extract of the leaves of *Terminalia arjuna* was a pale yellow powder having a melting point of 271 – 274 °C. The compound was positive to Libermann-Burchard test giving brownish violet colour, indicating the presence of triterpene skeleton. The IR spectrum exhibited strong absorptions at 3400 cm^{-1} (OH) and 1692 cm^{-1} (C=O). The mass spectrum showed a strong molecular ion peak at m/z 456 which corresponds to the molecular formula, $\text{C}_{30}\text{H}_{48}\text{O}_3$.

^1H NMR data

^1H NMR δ (500 MHz, CD_3OD): 5.21 (m, 1H, H-12), 3.14 (m, 1H, H-3), 2.20 (d, 1H, J 18,19 = 11.3 Hz, H-18), 2.02-1.15 (m, 22H), 1.10 (s, 3H, C-23 Me), 0.96 (s, 3H, c-27 Me), 0.95 (s, 3H, c-26 Me), 0.93 (s, 3H, c-24 Me), 0.87 (d, 3H, c-29), 0.83 (d, 3H, c-30), 0.76 (s, 3H, c-25); A multiplet at 5.21 ppm which integrated for one proton is assigned to the olefinic proton of C-12 which is coupled to protons at C-11.

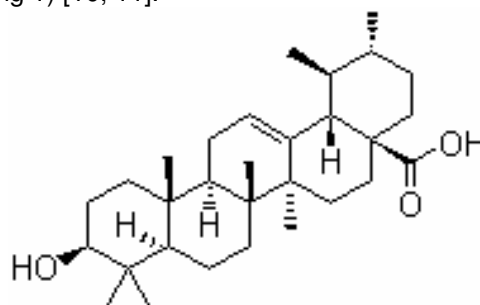
^{13}C -NMR data

^{13}C -NMR δ (125 MHz, CD_3OD , DEPT experiment): 181.6 (C-28), 139.6 (C-13), 126.8 (C-12). 79.6 (C-3), 56.7 (C-5), 54.3 (C-18), 47.6 (C-17), 47.6 (C-9), 42.8 (C-14), 40.7 (C-8), 40.4 (C-20), 40.4 (C-19), 39.9 (C-4), 39.8 (C-1), 38.1 (C-22), 38.1 (C-10), 34.3 (C-7), 31.7 (C-21), 29.2 (C-15), 28.7 (C-23), 27.8 (C-2), 25.3 (C-16), 24.3 (C-11), 24.0 (C-27),

21.5 (C-30), 19.4 (C-6), 17.8 (C-29), 17.6 (C-26), 16.3 (C-25), 16.0 (C-24).

MS m/z (%): 456 (M⁺), 248 (100), 219 (&.7), 207 (27), 203 (42.6), 189 (10.1), 133 (32.2), 119 (11.3), 69 (13.3).

In order to distinguish between the two triterpenes, ^{13}C values of the compounds were examined. The fundamental difference between the two triterpenes is at C-29 and C-30 of the ring E. In the α type triterpene, both methyl groups at ring E are secondary while in the β type, the two methyl groups are tertiary. By comparing the carbon-13 values of the compound against that of α and β type triterpenes, the compound was found to show a closer resemblance to the α type. From the foregoing evidence, it was inferred that the triterpene core of the compound is 3β -hydroxyurs-12-en-28-oic acid or ursolic acid (Fig 1) [10, 11].



3 β -hydroxyurs-12-en-28-oic acid

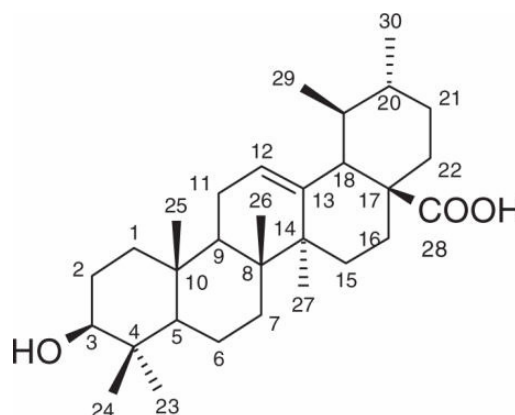


Fig 1: Structure of ursolic acid

Anti-leishmanial activity

The number of live promastigotes, which was counted by MTT reduction, was inhibited by the isolated compound in a dose-dependent manner as shown in Table 2.

Table 2: Anti-leishmanial activity of isolate (ursolic acid) Anti-leishmanial activity of isolated compound Ursolic acid with respect to Control which is 0.1 % CHCl₃

Drug dose (µg/ml)	Lysis (%) with respect to control (0.1 % CHCl ₃)	IC ₅₀ value for ursolic acid
3	51.54	3.51
5	67.27	
10	76.33	
15	82.79	
30	90.59	

Anti-cancer activity

The results of the anti-cancer test on the isolated compound using Cancer Cell Line, K562 are given in Table 3.

Table 3: Anticancer activity of isolated compound (ursolic acid)

Drug dose (µg/ml)	Lysis (%) with respect to control (0.1% CHCl ₃)	IC ₅₀ value for ursolic acid
5	38.4	7.40
10	61.3	
15	87.4	

DISCUSSION

Chromatographic separation of the defatted methanol extract of the leaves of *T. arjuna* led to the isolation of a pentacyclic triterpenoid, ursolic acid, characterized as 3β-Hydroxyurs-12-en-28-oic acid, together with β-D-glycoside. The structure was determined on the basis of IR, MASS and NMR spectroscopy. In vitro anti-leishmanial activity against promastigotes of *Leishmania*

donovani (strain AG 83) and anti-cancer activity on K562 leukaemic cell line were shown by the isolated Ursolic acid.

In the past, medicinal plants used in the preparation of folk remedies have provided modern medicines for the treatment of leishmaniasis caused by protozoan parasites. For example, the compound, diospyrin1 (a bis-naphthoquinone derivative) obtained from the bark of *Diospyros montana*, binds with the parasite's topoisomerase I, thus inhibiting the catalytic activity of the enzyme [12]. On the other hand, the compounds, meglumine antimoniate (glucantim) and sodium stibogluconate (pentostam) causes exerts its effect by disrupting the energy production of the parasite and thus inhibiting trypanothione metabolism [13].

Medicinal plants used in folk medicine have also been the source of modern medicines used in cancer therapy. Such agents include vincristin and vinblastin, both of which are vinca alkaloids from *Vinca roseus*. Their mechanism of action involves blocking the metaphase in the cell cycle while calcitonin causes significant suppression of cellular proliferation of K562 cells *in vitro* [14]. The mechanisms of anti-leishmanial and anti-cancer activities of *T. arjuna* have not yet been determined but may be similar to those of the aforementioned compounds, respectively.

However, this work buttresses the need to continue to investigate traditional remedies with a view to isolating their active constituents since an estimated 60 % of people living in developing countries depend on traditional medicine for their primary health care [15]. Modern therapy derived from medicinal herbs promises a practical approach to the development of effective and affordable drugs. However, further work (including *in vivo* studies) will still be required to establish the effectiveness and full potentials of ursolic acid isolated from *T. arjuna*.

CONCLUSION

The polar extract of the leaves of *Terminalia arjuna* contains a pentacyclic compound, ursolic acid, and a glycoside, β -D-glycoside. The former showed both anti-leishmanial and anti-cancer activities.

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