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Cytotoxic Agents from *Terminalia arjuna*

Original Paper

Abstract

Although a number of chemicals have been isolated from *Terminalia arjuna,* only a few have been evaluated for their biological significance. As a part of our drug discovery programme for cytotoxic agents from Indian medicinal plants, four novel cytotoxic agents arjunic acid (1), arjungenin (2), arjunetin (3) and arjunoglucoside I (4) were isolated from the bark of *T. arjuna.* Out of the four compounds, arjunic acid (1) was significantly active against the human oral (KB), ovarian (PA 1) and liver (HepG-2 & WRL-68) cancer cell lines. Further, the most active compound arjunic acid was converted into seven semi-synthetic ester derivatives 5 – 11. 2-O-Palmitoyl arjunic acid (6) showed two times more activity, while 2, 3-di-O-acetyl-, 2-O-p-anisoyl-, 2, 3-di-O-benzoyl- and 2, 3-di-O-p-nitrobenzoyl arjunic acid (7 – 10) showed 1.7 – 2.3 times less activity than the cytotoxic drug vinblastine against the liver cancer cell lines HepG-2 and WRL-68 respectively.

Key words
Terminalia arjuna · Combretaceae · arjunic acid · cytotoxic · cytotoxicity assay · human oral · colon · liver cancer cell lines

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Introduction

Over the past few years triterpenoids from higher plants have shown a wide range of biological activities [1], [2] such as cytotoxic [3], [4], antitumour [5], antiviral [6], anti-inflamatory [7], anti-HIV [8] and anti-HSV [9], [10]. The Arjun tree (*Terminalia arjuna*; Combretaceae) is a well known medicinal plant whose bark is extensively used in Ayurvedic medicine [11], particularly as cardiac tonic [12], [13], [14]. Considerable work has been carried out on the chemical investigation of different parts of *Terminalia arjuna,* which revealed the presence of a number of tannins, triterpenoid acids and their glycosides among others. Tannins and oleane triterpene derivatives are the major constituents of *T. arjuna* [15], [16], [17], [18]. In a bioassay-guided separation of cytotoxic constituents from the bark, stem and leaves of *T. arjuna*, gallic acid, ethyl gallate flavone, and leutonolin were found, in part, to be responsible for the rational underling the use of *T. arjuna* in traditional cancer treatment [19]. Further investigation of *T. arjuna* resulted in the isolation and identification of the antimutagenic agent ellagic acid [20] and the moderately cytotoxic agent ellagitanin [21]. As a part of our drug discovery programme for cytotoxic agents from Indian medicinal plants [22], [23], [24], the bark of *T. arjuna* was taken for detailed chemical investigation.

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Materials and Methods

Cell lines, chemicals and biochemicals

KB (human oral), PA 1 (human ovary), HepG-2 and WRL-68 (human liver) cancer cell lines were procured from the cell repository of the National Center for Cell Sciences (NCCS) at Pune, Maharastra, India. Purity of each compound was assessed by HPLC and NMR and was > 99%. Spots on TLC plates were visualized with a spray reagent [vanillin-ethanol-sulphuric acid (1 g: 95 mL: 5 mL)] followed by heating for 15 min at 110 °C. Bioassays were carried out as per known protocols. MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was purchased from Sigma Aldrich (Bangalore, India). The Spectra Max 190 Micro plate Elisa reader was purchased from Molecular Devices Inc. (Sunnyvale, CA, USA). Unless stated otherwise, all other reagents were purchased from Sigma Aldrich.

Plant material

The bark of *Terminalia arjuna* was collected from the medicinal plants conservatory of the Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, Uttar Pradesh, India during the month of January, 1999, identified in the Department of Botany and Pharmacognosy at CIMAP where a voucher specimen (No. 5867) is maintained.

Extraction and isolation of bioactive compounds

The air-dried *T. arjuna* bark (4.5 kg) was crushed, powdered and extracted with ethanol (3 × 5 L, 24 h each). The dried ethanolic extract (563.7 g) was dissolved in water and successively extracted with hexane (3 × 2 L), diethyl ether (3 × 2 L), ethyl acetate (3 × 2 L) and methanol (3 × 2 L) to yield hexane (10.8 g), diethyl ether (152.0 g), ethyl acetate (23.5 g) and methanol (329.2 g) extracts, respectively.

Further 100 g of diethyl ether extract were column chromatographed over silica gel (60 – 120 mesh, 1.5 kg; 6.5 × 1400 cm). Gradient elution of the column was carried out with hexane and ethyl acetate in the ratio of 98 : 2, 95 : 5, 90 : 10, 85 : 15, 80 : 20, 75 : 25, 70 : 30, 60 : 40, 50 : 50, 40 : 60, 30 : 70, 20 : 80, 10 : 90, and 100. Fractions of 100 mL each were collected and detected by TLC (SiO_2, chloroform:methanol 9 : 1; vanillin-sulphuric acid), which resulted in the isolation and characterization of arjunic acid, 0.004%, m. p. 280 °C (decomposed) [25], [26]; arjungenin 0.007%, m. p. 293 – 294 °C [27] and arjunetin 0.002%, m. p. 232 – 234 °C [27] (Fig. **1**).

Isolation of arjunglucoside I (4) by preparative HPLC

The column fractions eluted with hexane:ethyl acetate (5 : 95) on TLC was found to be a mixture of one major and several minor constituents. Hence, in order to purify the major compound, these fractions were subjected to preparative HPLC, which yielded a crystalline compound (0.001%, m. p. 232 – 233 °C) characterized as arjunglucoside-I (Fig. **1**) on the basis of its physical and spectroscopic data [28]. Chromatographic conditions employed for preparative HPLC were: column: Supelcosil LC-18 (25 cm × 21.2 mm, 12 μm); mobile phase: methanol : H_2O (50 : 50); flow rate: 17 mL/min; λ = 220 nm (SPD-M10Avp Shimadzu photodiode detector); column temperature: 26 °C, respectively. The HPLC used consisted of LC-8A Shimadzu semipreparative equipment.

Chemical derivatization

The potential cytotoxic activity of arjunic acid prompted us to carry out chemical derivatization of arjunic acid (Fig. **2**) and the preparation of arjunic acid ester derivatives was carried out. Arjunic acid was dissolved in dry pyridine and then the respective acid chlorides were added in 1 : 1.5 ratios. The reaction mixtures were kept overnight at room temperature (30 – 45 °C). After completion of the reaction, ice/water was added (~15 mL) and reaction solutions were extracted three times with chloroform. The combined chloroform extract was washed with water (until it was neutralized). The neutralized chloroform extracts were dried over anhydrous Na_2SO_4 and the solvent removed under vacuum. Further purification of the ester derivatives on short columns afforded the products **5** – **11** in 85 – 95% yields. All the deri-

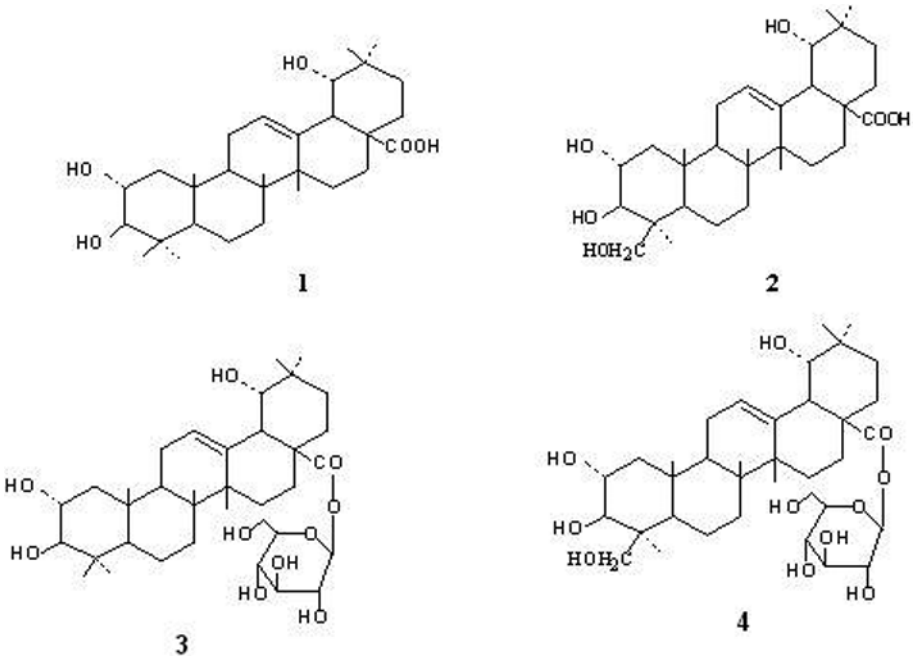

Fig. **1** Cytotoxic compounds from *Terminalia arjuna*.

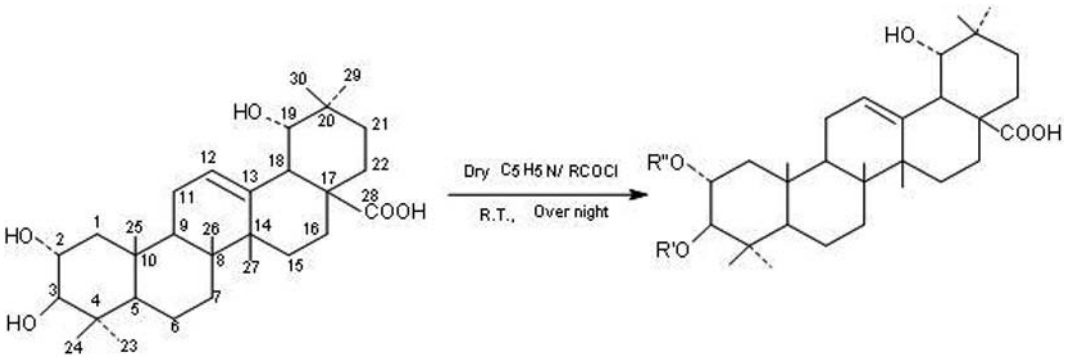

Fig. **2** General scheme for the preparation of arjunic acid ester derivatives.

vatives were characterized on the basis of their spectroscopic data (for ^{1}H, ^{13}C NMR, MS and $[\alpha]_D$ data, see Supporting Information).

Cytotoxicity assay

The *in vitro* cytotoxicity testing was carried out by the method of Woerdenbag et al. [29]. 2×10^3 cells/well were incubated in the 5% CO_2 incubator for 24 h to enable them to adhere properly to the 96-well polystyrene micro plates. Test compounds dissolved in 100% DMSO in at least 5 doses were added and left for 6 h after which the compound plus media was replaced with fresh media and the cells were incubated for another 48 h in the CO_2 incubator at 37 °C. The concentration of DMSO used in our experiments never exceeded 1.25%, which was found to be non-toxic to cells. Then, 10 μL MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide, Sigma M 2128] was added, and plates were incubated at 37 °C for 4 h. 100 μL dimethyl sulphoxide were added to all wells and mixed thoroughly to dissolve the dark blue crystals. After few minute at room temperature to ensure that all crystals were dissolved, the plates were read on a Spectra Max 190 Micro plate Elisa reader (Molecular Devices Inc.), at 570 nm. Plates were normally read within 1 h of adding the DMSO. The experiments were done in triplicate and the inhibitory concentration (IC) values were calculated as follows: % inhibition = [1 – OD (570 nm) of sample well/ OD (570 nm) of control well] × 100. IC_{90} and IC_{50} are the concentrations in μg/mL required for 90% and 50% inhibition of cell growth as compared to that of untreated control.

Supporting information

^{1}H-, ^{13}C-NMR, MS and $[\alpha]_D$ data of arjunic acid (**1**) and its derivatives (**5** – **11**) are available as Supporting Information.

Result and Discussion

Triterpenoids are an integral part of the human diet and in the last decade many triterpenoids have been reported to possess a wide range of cytotoxic activity. Out of many triterpenoids, ursolic and oleanolic acids have been studied in detail for their cytotoxic activities such as inhibition of tumor genesis, tumor promotion, angiogenesis [30], ultraviolet-B (UVB) radiation photocarcinogenesis [31], induction of tumor cell differentiation, invasion of tumor cells, metastasis [30] and effect on acute myeloid leukemia [32]. The recent isolation of cytotoxic triterpenoids from *Akebla trifoliate* and *Clematis lingustifolia* [33] and cytotoxic triterpenoid saponins from *Quillaja saponaria* Molina (soap tree) [34] prompted us to undertake a detailed chemical investigation of *T. arjuna* bark, which resulted in the isolation and characterization of two triterpenic acids arjunic acid (**1**) and arjungenin (**2**) and two saponins, arjunetin (**3**) and arjunglucoside (**4**), respectively.

Compounds **1** – **4** were tested against the human oral (KB), liver (WRL-68 and HepG-2) and ovarian (PA-1) cancer cell lines, respectively, and the results are presented in Table **1**. All the compounds had cytotoxic activity against the various cancer cell lines. Although arjunic acid (**1**) was 5 times less active against liver (HepG-2) cancer cell line with respect to the cytotoxic drug vinblastine, it was the most active cytotoxic constituent of *T. arjuna* against all tested cancer cell lines. This encouraged us to prepare derivatives of arjunic acid, and a total of seven derivatives (**5** – **11**) of arjunic acid (**1**) was prepared and their cytotoxic activities were evaluated (Table **1** and Fig. **3**). All compounds (**1** and **5** – **11**) were active against the oral, ovarian and liver cancer cell lines, but among the seven derivatives (**5** – **11**) of arjunic acid, compound **6** and **7** – **10** were highly active (7 – 9 times) against the liver cancer cell lines (HepG-2) and (WRL-68), respectively, in comparison to the starting material arjunic acid (**1**). On the other hand compounds **5** and **6** were significantly (4 to 5 times) active against the oral cancer (KB) cell line with respect to arjunic acid (**1**). When the activity results, IC_{90}, of derivatives were compared with the reference cytotoxic drug, vinblastine, compound **6** showed two times more activity than vinblastine against the liver cancer HepG-2, while compounds **7** – **10** showed 1.7 to 2.3 times less activity against the liver cancer cell line WRL-68. But it was interesting to note that with respect to the IC_{50} values, compounds **7** – **10** showed 1.5 – 2.4 times more activity than vinblastine against the liver cancer cell line WRL-68.

While studying the structure-activity relationship (SAR) of arjunic acid and its derivatives it was observed that acetylation of arjunic acid, such as to give 2,3-di-*O*-acetylarjunic acid (**7**), resulted in a ~7-fold increase (IC_{90}) in activity against the liver cancer (WRL-68) cell line. Further increasing the chain length from the 2-carbon diacetate (**7**) to the 12-carbon 2-*O*-laurylarjunic acid (**11**) and the 15-carbon compounds, **5** and **6**, decreased the activity 7- to 10-fold against the liver cancer cell line WRL-68 with respect to compound **7**. But it was interesting to note that replacement of the 2,3-diacetyl function with mono- or diaryl groups as in the case of compounds **8** – **10** did not show any effect on activity against the liver cancer cell line WRL-68.

Table **1** IC_{50} and IC_{90} (μg/mL) values for four isolated compounds **1** – **4** and ester derivatives **5** – **11** of arjunic acid (**1**) against human cancers cell lines*

Compounds	*KB*		*PA-1*		*HepG-2*		*WRL-68*	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
Arjunic acid (**1**)	5.00 ± 0.05	35.00 ± 0.06	7.50 ± 0.09	10.00 ± 0.09	0.70 ± 0.05	9.00 ± 0.04	7.50 ± 0.13	40.00 ± 0.24
Arjungenin (**2**)	–	–	40.00 ± 0.26	60.00 ± 0.13	–	–	–	–
Arjunetin (**3**)	–	–	–	–	15.00 ± 0.03	20.00 ± .020	–	–
Arjunglucoside I (**4**)	–	–	30.00 ± 0.16	70.00 ± 0.24	–	–	–	–
2,3-*di*-*O*-palmitoylarjunic acid (**5**)	0.70 ± 0.07	7.00 ± 0.08	30.00 ± 0.03	40.00 ± 0.03	0.04 ± 0.03	6.00 ± 0.05	8.50 ± 0.09	45.00 ± 0.11
2-*O*-palmitoylarjunic acid (**6**)	0.95 ± 0.08	8.50 ± 0.05	9.00 ± 0.03	20.00 ± 0.02	0.45 ± 0.03	1.00 ± 0.03	10.00 ± 0.12	50.00 ± 0.98
2,3-*di*-*O*-acetylarjunic acid (**7**)	45.00 ± 0.09	75.00 ± 0.08	9.00 ± 0.10	20.00 ± 0.10	1.00 ± 0.01	45.00 ± 0.01	1.00 ± 0.12	6.00 ± 0.87
2-*O*-*p*-anisoylarjunic acid (**8**)	4.50 ± 0.07	55.00 ± 0.09	6.50 ± 0.01	9.00 ± 0.09	9.00 ± 0.22	60.00 ± 0.37	1.00 ± 0.38	6.00 ± 0.42
2,3-*di*-*O*-benzoylarjunic acid (**9**)	4.00 ± 0.08	50.00 ± 0.03	2.50 ± 0.02	5.00 ± 0.02	6.00 ± 0.16	65.00 ± 0.64	0.60 ± 0.43	4.50 ± 0.28
2,3-*di*-*O*-*p*-nitrobenzoyl arjunic acid (**10**)	3.00 ± 0.04	15.00 ± 0.01	45.00 ± 0.06	60.00 ± 0.05	1.00 ± 0.84	45.00 ± 0.39	1.00 ± 0.77	6.00 ± 0.82
2-*O*-lauroylarjunic acid (**11**)	3.50 ± 0.01	40.00 ± 0.11	9.00 ± 0.06	20.00 ± 0.05	5.00 ± 0.25	55.00 ± 0.19	12.00 ± 0.01	60.00 ± 0.07
vinblastine	0.05 ± 0.02	0.82 ± 0.09	0.03 ± 0.06	0.45 ± 0.07	0.07 ± 0.03	2.00 ± 0.03	1.45 ± 0.03	2.61 ± 0.03

* Cancer cell lines with their ATCC No. and source organ in parenthesis: KB; CCL 17 (oral cancer), PA-1, CRL 1572 (ovary cancer), HepG-2; HB-8065 (liver cancer), WRL 68; CL 48 (liver cancer). (–) = inactive (IC_{50}/IC_{90} > 100 μg/mL).

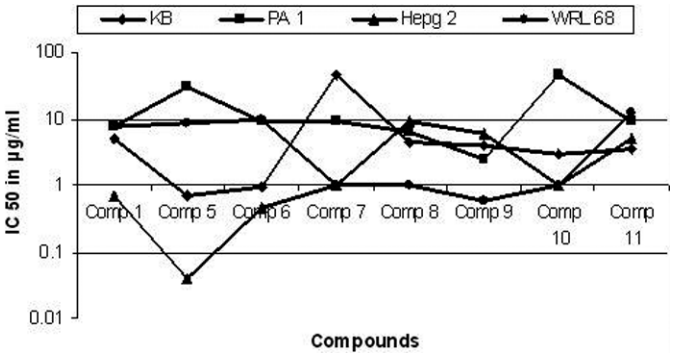

Fig. **3** IC_{50} (μg/mL) values of arjunic acid (**1**) and its ester derivatives (**5** – **11**) against four human cancer cell lines.

From the above results, it may be concluded that arjunic acid ester derivatives **6** and **7** – **10** possess potential cytotoxic activity against the liver cancer cell lines HepG 2 and WRL 68, respectively. These results may be of great help in cytotoxic drug development from the very common and widely distributed tree *T. arjuna*.

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