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Synthesis of novel anticancer iridoid derivatives and their cell cycle arrest and caspase dependent apoptosis

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ABSTRACT

Nyctanthes arbortristis Linn (Oleaceae) is widely distributed in sub-Himalayan regions and southwards to Godavari, India commonly known as Harsingar and Night Jasmine. In continuation of our drug discovery programme on Indian medicinal plants, we isolated arbortristoside-A (**1**) and 7-O-trans-cinnamoyl 6β-hydroxyloganin (**2**) from the seeds of *N. Arbortristis*, which exhibited moderate in vitro anticancer activity. Chemical transformation of **2** led to significant improvement in the activity in derivative **8** and **15** against HepG2 (human hepatocellular carcinoma), MCF-7 (breast adenocarcinoma) cell lines. The compounds **8** and **15** were also capable of cell cycle arrest and caspase dependent apoptosis in HepG2 cell lines. These iridoid derivatives hold promise for developing safer alternatives to the marketed drugs.

Introduction

Iridoids represent the group of cyclopentan – (c) – pyran monoterpenoids. They are found as natural constituents in a large number of plant families. Iridoids were first isolated in the latter part of the nineteenth century, but it was not until 1958 that Halpern and Schmid (Halpern et al. 1958) proposed the basic skeleton of the iridoids in their investigation of the structure of plumieride, which possess various biological activities (Chopra et al. 1956). The name iridoid is a generic term derived from the names iridomyrmecin, iridolactone and iridodial compounds isolated from some species of Iridomyrmex, a genus of ants, in which they occur as defensive secretions (Roth and Eisner 1962). These compounds have been referred to as pseudoindicans. They have also been referred to as aucubin glucosides.

Arbortristoside-A (**1**) and 7-O-trans-cinnamoyl-6β-hydroxyloganin (**2**) belongs to iridoid class of compounds are the major bioactive compounds of numerous herb species such as *Nyctanthes arbortristis* Linn (Division: Magnoliophyta; Class: Magnoliopsida; Order: Lamiales; Family: Oleaceae), commonly known as Harsingar and Night Jasmine and possesses leishmanicidal (Tandon et al. 1991), antiplasmodial (Tuntiwahwuttikul et al. 2003), antispermatogenic (Gupta et al. 2006), antiallergic (Gupta et al. 1995), anti-inflammatory (Amrite et al. 2006; Patel et al. 1998; Saxena et al. 1984; Sanjita Das et al. 2008), antinociceptive (Sanjita Das et al. 2008) and analgesic activity (Saxena et al. 1987).

In continuation of our drug discovery programme on anticancer agents from Indian medicinal plants, we isolated large quantities of 7-O-trans-cinnamoyl-6β-hydroxyloganin (**2**) from the seeds of *Nyctanthes arbortristis* Linn and planned to carry out chemical transformation to improve its therapeutic application. Chemical transformation of bioactive compounds of medicinal herbs is one of the most common approaches in drug discovery to improve the therapeutic properties. For example the anticancer drugs teniposide and etoposide are derivatives of podophyllotoxin and topotecan and irinotecan are analogues of camptothecin, which have better therapeutic benefits than the parent natural products. Towards this goal, we have synthesized novel derivatives of compound **2** and evaluated their anticancer activity against HepG2 (human hepatocellular carcinoma), MCF-7 (breast adenocarcinoma), MDAMB-231 and NIH/3T3 cell lines (Table 1). Further we have studied the apoptosis inducing ability, effect on cell cycle and caspase-3 activation studies of most active compounds **8** and **15**.

Materials and methods

General chemistry

IR spectra were recorded on Perkin-Elmer RX-1 spectrometer. Using either KBr pellets (or) in neat. ^{1}H NMR, ^{13}C NMR, DEPT-90 and DEPT-135 spectra were run on Bruker Advance DPX 300 MHz and 200 MHz in $CDCl_3$. Chemical shifts are reported as values in

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Table 1
Chemical structure and in vitro anticancer activity (IC_{50} in μM) of compounds **1**, **2** and its derivatives (**3**–**15**).

Comp. No.	HepG2	MDAMB-231	MCF-7	NIH/3T3
1	89	80	87	78
2	78	76	79	84
3	469	270	310	280
4	134	125	147	140
5	113	97	98	120
6	42	49	45	55
7	56	45	44	59
8	12	10	14	19
9	145	156	151	167
10	79	83	87	92
11	74	82	76	89
12	23	16.8	32	34
13	78	82.6	76	93
14	29	32	37	45
15	14	18	16	31
Epirubicin	4.6	7.7	3.7	3.2
Paclitaxel (PTX)	2.1	1.24	4.6	3.6
Doxorubicin	1.24	3.26	3.84	4.57

ppm relative to $CHCl_3$/DMSO with TMS as internal standard. ESI mass spectra were recorded on JEOL SX 102/DA-6000. Plates for thin layer chromatography (TLC) were prepared from silica gel 60 GF254 (Merck) and activated by drying at 100 °C for 2 h. Chromatography was executed with silica gel (60–120 mesh) using mixtures of chloroform, methanol and hexane as eluants. Visualization was obtained under UV light and spraying with 10% sulphuric acid in methanol.

Background of plant

Nyctanthes arbortristis Linn (Oleaceae), commonly known as Harsingar and Night Jasmine. It is a shrub growing to 10 m tall, with flaky grey bark. The fruit is a flat brown heart-shaped to round capsule 2 cm diameter, with two sections each containing a single seed. Which have been claimed to possess multiple pharmacological activities like antibacterial, antifungal, anti-influenza, anti-inflammatory, analgesic, antipyretic, antihistaminic, antiulcer, hypnotic, tranquilizing, hepatoprotective, antidiabetic, antianemic, immunobioactivities, antioxidant, antispermatogenic, etc. (Rachna and Mridula 2011).

Collection of medicinal plant

N. arbortristis Linn seeds were purchased from the local market of Lucknow, India and the authentification was done by Botany Division of Central Drug Research Institute, Lucknow and is kept in the herbarium for future reference.

Extraction

Powdered *N. arbortristis* Linn seeds (4 kg) were placed in glass percolator with 95% ethanol (10 l) and allowed to stand for 24 h at room temperature. The percolate was collected and these processes were repeated for four times. The combined percolate was evaporated under reduced pressure at 50 °C to afford ethanol extract. The weight of extract was found to be 300 g.

Fractionation

The ethanol extract was macerated with hexane. The hexane soluble fraction was separated and evaporated under reduced pressure to afford hexane fraction (F001, 80 g). Chloroform was added to hexane insoluble portion, and the resultant solution was evaporated under reduced pressure to afford chloroform fraction (F002, 120 g). *n*-Butanol was added to chloroform insoluble portion, the *n*-butanol soluble fraction was evaporated under reduced pressure at 60 °C afford *n*-butanol fraction (F003, 80 g).

Isolation and purification of Iridoids

n-Butanol fraction (80 g) was chromatographed on a column of silica gel (60–120 mesh) and eluted with chloroform and methanol in increasing polarity. Fractions were collected and then combined on the basis of TLC pattern to get two subfractions (A and B). Fraction A was rechromatographed on silica gel, eluting with chloroform–methanol (96:4); recrystallization from methanol afforded compound **1** (80 mg). Fraction B was rechromatographed on silicagel (60–120 mesh), eluting with chloroform–methanol (90:10); recrystallization from methanol afforded compound **2** (1.5 g). The compounds visualization was done under UV light, also shown brown spot by spraying with 10% sulphuric acid in methanol.

Preparation of iridoid derivatives

General procedure for the dihydro compound

To a magnetically stirred solution of compound **2** (100 mg, 0.00018 mol) in methanol (10 ml) was added gradually $NiCl_2 \times 6H_2O$ (0.00009 mol) at rt. When the clear solution acquired a greenish colour, the whole reaction mixture was brought to 0 °C and $NaBH_4$ (0.00036 mol) was added portion wise. After addition of $NaBH_4$, the whole solution was stirred for 30 min at 0 °C to rt. Methanol was removed by vacuum, and then extracted with ethyl acetate (3 × 25 ml), the organic layer was washed with water, dried over anhyd Na_2SO_4 and evaporated under reduced pressure. Then the crude product was chromatographed on silica gel to afford the desired compound (Fig. 1).

General procedure for the amino hydroxylation

$K_2[OsO_2(OH)_2]$ (1.5 mol%) was dissolved with stirring in 10 ml of aqueous solution of $LiOH{\cdot}H_2O$. After the addition of *t*-BuOH, $(DHQ)_2PHAL$ was added and the mixture immersed in a cooling bath set 0 °C. After the addition of compound **2**, N-bromo acetamide was added in one portion, which resulted in an immediate colour change to green and the mixture vigorously stirred at the same temperature. The reaction was monitored by TLC, and pH (full conversion is indicated when the reaction mixture attains pH 7). After 4 h, the reaction mixture was treated with Na_2SO_3 and stirred at rt

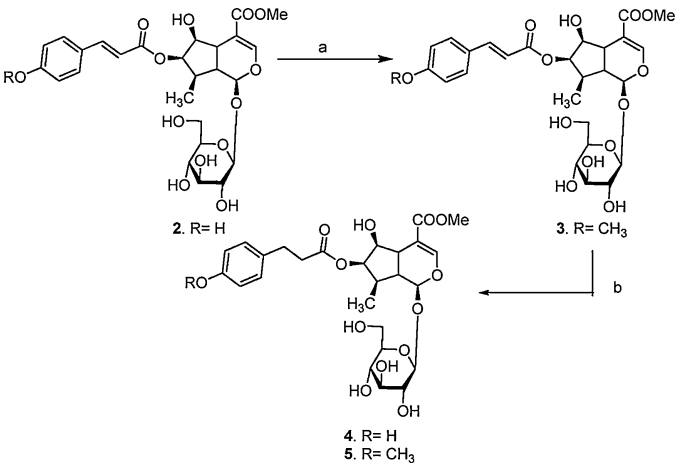

Fig. 1. Regioselective hydrogenation of compounds **2** and **3** by using $NaBH_4/NiCl_2{\cdot}6H_2O$.

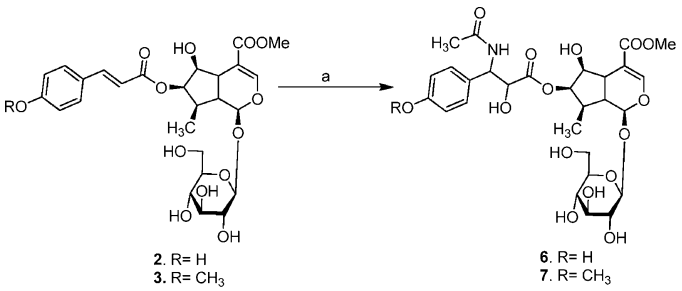

Fig. 2. Amino hydroxylation of compounds **2** and **3** by using $K_2[OsO_2(OH)_2]$.

for 30 min. It was then extracted with ethyl acetate (3 × 25 ml), the organic layer was washed with water, dried over anhyd Na_2SO_4 and evaporated under reduced pressure. Then the crude product was chromatographed on silica gel to afford the desired compound (Fig. 2).

General procedure for the decinnamylation

To a magnetically stirred solution of compound **2** (100 mg, 0.00018 mol) in a mixture of solvents (H_2O:THF:EtOH (1:1:1), 10 ml) was added hydroxylamine. Sulphuric acid salt (0.0009 mol) and triethylamine (0.012 mol) at rt. The whole solution was stirred for 6 h at 100–110 °C. Solvents were removed by vacuum, and then extracted with ethyl acetate (3 × 25 ml), the organic layer was washed with water, dried over anhyd Na_2SO_4 and evaporated under reduced pressure. Then the crude product was chromatographed on silica gel to afford the desired compound **8** (Fig. 3).

General procedure for the O-methylation of aromatic hyroxy group

To a magnetically stirred solution of compound **2** (100 mg, 0.00018 mol) in DMF (5 ml) at room temperature was treated with the dimethyl sulphate (0.00027 mol), TBAB (0.00018 mol) and K_2CO_3 (0.00027 mol). The whole reaction mixture was stirred for 4 h at 100 °C. It was then extracted with ethyl acetate (3 × 25 ml), the organic layer was washed with water, dried over anhyd Na_2SO_4 and evaporated under reduced pressure. Then the crude product was chromatographed on silica gel to afford the desired compound **3** (Fig. 1).

General procedure for the O-alkylation

To a magnetically stirred solution of compound **2** (100 mg, 0.00018 mol) in DMF (5 ml) at room temperature was treated with the ethyl α-bromo isobutyrate (0.00021 mol) and K_2CO_3 (0.00027 mol).The whole reaction mixture was stirred for 4 h at 70 °C. It was then extracted with ethyl acetate (3 × 25 ml), the organic layer was washed with water, dried over anhyd Na_2SO_4 and evaporated under reduced pressure. Then the crude product was chromatographed on silica gel to afford the desired compound (Fig. 4).

General procedure for the esterification

To a magnetically stirred solution compound **2** (100 mg, 0.00018 mol) in dry DCM (5 ml) maintained 0 °C after it added Z-2-methyl but-2-enoyl chloride (0.00018 mol) and triethylamine (0.00027 mol) at 0 °C for 1 h. DCM was removed by vacuum. It was then extracted with ethyl acetate (3 × 25 ml), the organic layer was washed with water, dried over anhyd Na_2SO_4 and evaporated under reduced pressure. Then the crude product was chromatographed on silica gel to afford the desired compound (Fig. 4).

General procedure for the acetylation

Compound **2** (100 mg, 0.00018 mol) magnetically stirred in a solution of pyridine (2 ml) and acetic anhydride (0.00163 mol) at 60–70 °C for 4 h. The reaction mixture was put into cold water for crystallization, then filtered and dried to get desired compound (Fig. 4).

General procedure for the oxidation

To a magnetically stirred solution of compound **8** (100 mg, 0.00018 mol) in dry acetone (3 ml) maintained 0 °C after it added Jones reagent at 0 °C for 1 h. Acetone was removed by vacuum. It was then extracted with ethyl acetate (3 × 25 ml), the organic layer was washed with water, dried over anhyd Na_2SO_4 and evaporated under reduced pressure. Then the crude product was chromatographed on silica gel to afford the desired compound **10** (Fig. 3).

Biological study

Materials

HepG2, MDA-MB-231, MCF-7, and NIH/3T3 cell lines used in the present study was obtained from ATCC (American Type Culture Collection, USA). Annexin V FITC Apoptosis Detection Kit was purchased from Calbiochem. MTT dye and Caspase-3 Activation Kit was procured from Sigma–Aldrich. Absorbance was recorded using Eliza Plate Reader. Flow cytometry was performed using a FACScan (Becton Dickinson, Mountain View, CA) flowcytometer.

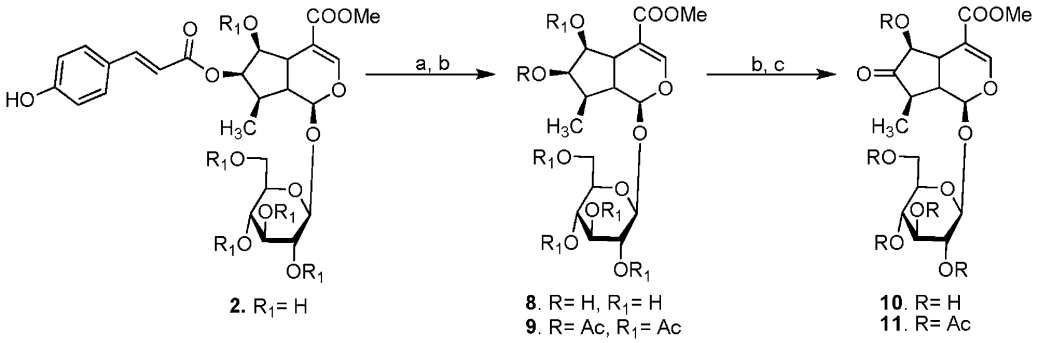

Fig. 3. Decinnamoylation of compound **2** by $NH_2OH{\cdot}H_2SO_4$ followed by using oxidation and acetylation.

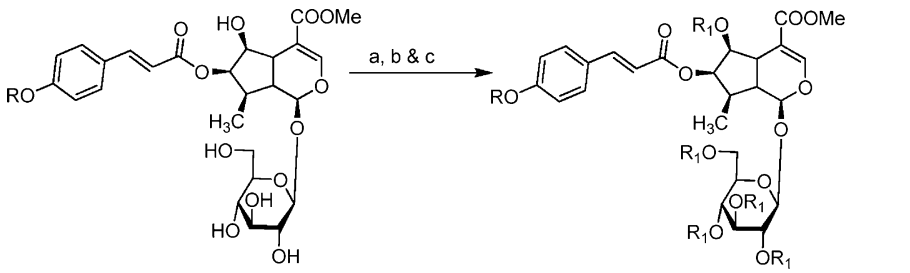

Fig. 4. Synthesis of ester analogues of compound **2**.

Cell culture

MDA-MB-231 and MCF-7 cells were routinely maintained in Dulbecco's Modified Eagle Medium DMEM (Sigma–Aldrich) while HepG2, NIH/3T3 cells were maintained in Roswell Park Memorial Institute (RPMI 1640, Merck) supplemented with 10% FBS (Merck) and 1% Antibiotic–antimycotic solution (Merck) at 37 °C in a humidified incubator with 5% CO_2. All stock solutions of compounds were prepared in cell culture grade DMSO and stored in −20 °C. Compounds were diluted in culture media prior to use in experiments.

Cell viability assay

Antiproliferative activity of the compounds were tested by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, cells (4×10^3 cells/well) were cultured in 96 well tissue culture plates and treated with different concentrations of compounds for 48 h. At the end of incubations, 10 μl of MTT (10 mg/ml) was added to the wells and incubated for 3 h. Absorbance was recorded at 540 nm using Eliza Plate Reader. All the experiments were repeated at least thrice independently. Values are expressed as mean + SD.

Apoptosis studies

Quantitation of apoptotic cells by Annexin V staining was carried out according to the manufacturer's instructions (Calbiochem). Briefly, HepG2 cells (5 × 105 cells/well) were seeded in 6 well plates and treated with 5, 10 and 20 μM of compounds **8** and **15** for 24 h. After the incubations, cells prepared as a suspension in 500 μl of cold PBS, centrifuged for 5 min at 1000 × *g* then resuspended in 500 μl cold 1× binding buffer and added 1.25 μl of Annexin V FITC and incubated for 15 min at RT in dark, centrifuge at 1000 × *g* for 5 min at rt, remove supernatant, gently resuspend in 500 μl cold 1× binding buffer and added 10 μl PI. Flow cytometry was performed using a FACScan (Becton Dickinson, Mountain View, CA) flowcytometer, equipped with a single 488-nm argon laser. Annexin V-FITC were analyzed using excitation and emission settings of 488 nm and 535 nm (FL-1 channel); PI, 488 nm and 610 nm (FL-2 channel). Debris and clumps were gated out using forward and orthogonal light scatter. The experiment was repeated three times independently.

Cell cycle analysis

For cell cycle distribution studies post-treatment, cells were fixed in 70% ethanol, rehydrated in PBS with ribonuclease A (100 μg/ml) and Triton-X (10 μg/ml) for 30 min at room temperature then added PI (50 μg/ml) and incubated in dark for 30 min and analyzed with FACScan flow cytometer (Becton Dickinson, Mountain View, CA) equipped with an argon laser to give 488 nm light.

Caspase-3 activation assay

The activity of caspase-3 was determined using caspase-3 colorimetric assay kit (Sigma–Aldrich) according to the manufacturer's protocol. Briefly, HepG2 cells (1×10^6 cells/well) were treated with 0 μM, 10 μM and 20 μM compounds **8** and **15** for 24 h. The cells were harvested and lysed by addition of lysis buffer. Samples of the cell lysates were mixed with colorimetric substrate (Ac-DEVD-pNA) and incubated at 37 °C in the dark for 4 h. The absorbance was measured at 405 nm in an ELISA reader. Caspase-3 activity was expressed as the change of the activity compared to the control.

Statistical analysis

Each experiment was observed in triplicate. The data are presented as mean ± SD and compared using Student's *t*-test. $p < 0.05$ or less was considered to be statistically significant.

Results and discussion

Chemistry

Chemical transformation of the active compound was carried out to study the structure–activity relationship. Initially the selective hydrogenation of olefinic bond (Khurana and Sharma 2004) of cinnamoyl group was carried out on compound **2** using $NaBH_4$, $NiCl_2{\cdot}6H_2O$ to obtain the dihydro derivatives **4** and **5** (Fig. 1).

To increase the hydrophilic character of the compound asymmetric amino hydroxylation was carried out on **2** and **3** using $K_2[OsO_2(OH)_2]$, which provided aminohydroxylated (Subba Rao et al. 2005) derivatives **6** and **7** (Fig. 2).

To find out the role of the cinnamoyl moiety in compound **2** we attempted to remove the cinnamoyl group from compound **2**. Hydrolysis of compound **2** under basic conditions such as NaOH or LiOH or KOH desired compound was not formed. Therefore $NH_2OH{\cdot}H_2SO_4$ (Fenoglio et al. 1996; Sun et al. 2001) was used to selectively provide decinnamoylated derivative **8** (Fig. 3). The resultant compound was acetylated to give compound **9**. Compound **8** was also subjected to oxidation reaction, which led to generate a keto compound **10** and subsequent acetylation gave **11**.

Since the compound **2** contain free aromatic hydroxyl group we prepared few ester derivatives (**12**–**15**) to study their anticancer activity (Fig. 4).

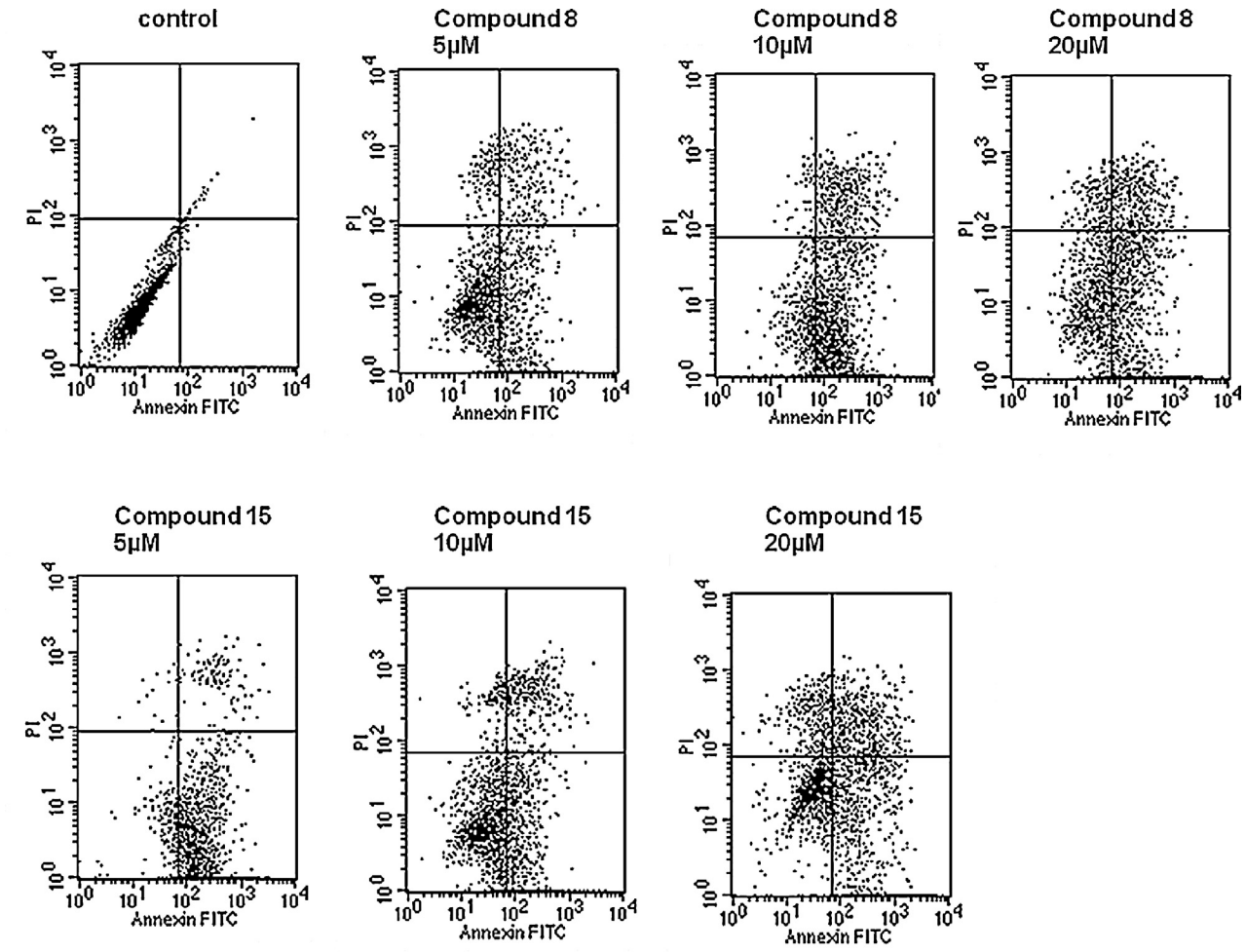

Fig. 5. Determination of apoptosis inducing ability of **8** and **15** by flow cytometry: HepG2 cells (5 × 105 cells) with 70–80% confluence were incubated in RPMI supplemented with 10% FBS without (control) with 5, 10 and 20 μM of derivatives **8** and **15**. After 24 h, the cells were harvested, stained with Annexin-VFITC and PI, and analyzed by flow cytometry.

Biological evaluation

Antiproliferative activity

All the compounds were screened against three cancer cell lines (HepG2, MCF-7, MDA-MB-231) and a non-cancer cell line (NIH/3T3). Except compounds **3**, **4**, **5** and **9** most of the derivatives prepared from iridoid exhibited improved in vitro anticancer activity against three cancer cell lines with better therapeutic index (Table 1). Out of these active derivatives, **8** and **15** exhibited improved activity against all cancer cell lines. Derivatives **8** showed IC_{50} of 12, 10, 14 μM and derivative **15** showed IC_{50} of 14, 16, 18 μM against HepG2, MCF-7, MDA-MB-231 cell lines respectively.

Apoptosis inducing ability studies by flow cytometry

In order to characterize the cellular basis of antiproliferative effects of selected derivatives **8** and **15**, we initially investigated the apoptosis inducing ability by flow cytometry in HepG2 cells. Annexin-V specially binds to phosphatidylserine and has been employed for determination of apoptotic cells. Annexin-V/PI staining was performed to determine early, late apoptotic and necrotic cells followed by treatment with 5, 10 and 20 μM concentration of derivatives **8** and **15** HepG2 cells (Muktapuram et al. 2012). When HepG2 cells were stained with Annexin-V/PI and analyzed with flow cytometer, early and late apoptosis (Annexin V-stained) cells were found to be increased in a dose-dependent manner (Fig. 5), which indicates the induction of apoptosis by **8** and **15**.

Cell cycle arrest studies

To determine the possible effect of these compounds on cell cycle progression, HepG2 cells were treated with 5, 10 and 20 μM of **8** and **15** then stained with propidium iodide (Fig. 6) (Narender et al. 2013). To evaluate the distribution of actively dividing cells in the cell cycle before the induction of extensive apoptosis, cells were treated for 24 h, and the percentage of apoptotic and nonapoptotic cells in each phase of the cell cycle was determined by DNA flow cytometry (Sinha et al. 2011).

Caspase-3 activity in HepG2 cells

To find out whether the induction of apoptosis by **8** and **15** via caspase mediated or not, we examined the caspase-3 activation in HepG2 cells. Caspases belongs to cysteine proteases class, are the important proteins that regulate the apoptotic response (Fig. 7). Among these, caspase-3 is a key protein in the apoptosis mechanism since it executes both extrinsic and intrinsic pathways (Kamal et al. 2011; Wang et al. 2007). Our studies indicated that a concentration dependent increase in caspase-3 activity with derivatives **8** and **15** treatment as compared with the untreated control. Caspase-3 activation was enhanced 7.66 and 11.83 folds by **8** and 1.86, 4.66 folds by **15** in cells treated with 10 and 20 μM respectively, when

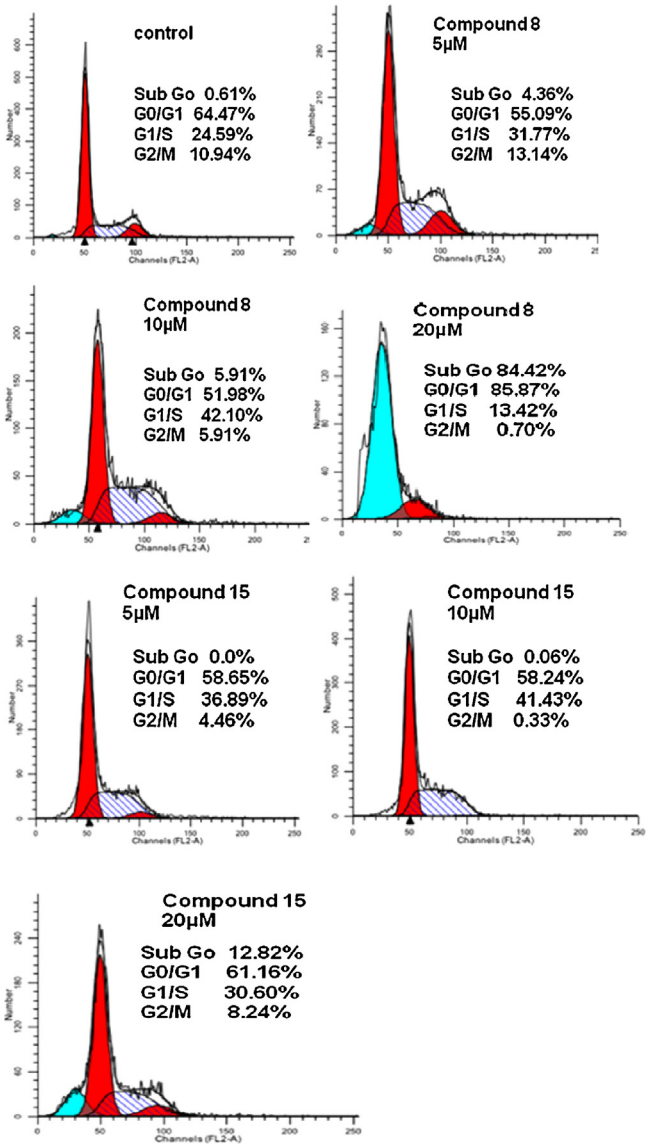

Fig. 6. Effect of derivatives **8** and **15** treatment to HepG2 cells on cell cycle distribution. The cells treated with derivatives **8** and **15** for 24 h were collected and stained with PI and analyzed by flow cytometry. Following FACS analysis, cellular DNA histograms were further analyzed by Modfit LT V3.2.1. The data are representative examples for duplicate tests.

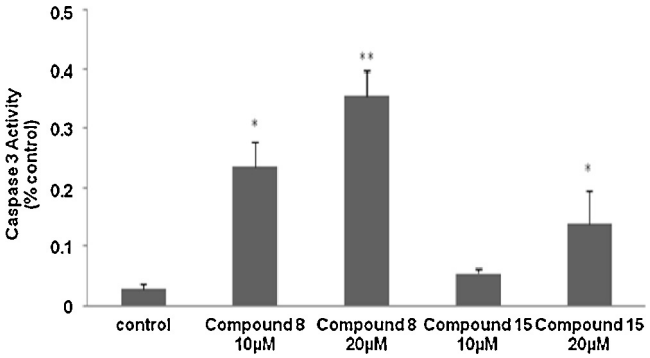

Fig. 7. Effect of **8** and **15** on caspase-3 activity in HepG2 cells. After 24 h treatment with or without derivatives **8** and **15**, caspase-3 activities were determined by mixing the cell lysates with colorimetric substrate (Ac-DEVD-pNA). The absorbance was measured at 405 nm in an ELISA reader. Caspase-3 activity was expressed as the change of the activity compared to the control. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs control.

compared with control group (Fig. 7). These results confirmed that the apoptosis induction is due to caspase-3 activation by **8** and **15** in HepG2 cells.

Conclusions

In conclusion we have discovered in vitro anticancer activity in arbortristoside-A (**1**) and 7-O-trans-cinnamoyl-6β-hydroxyloganin (**2**) for the first time. Thirteen derivatives of compound **2** prepared and evaluated their anticancer activity. Derivative **8** and **15** turned out to be potent anticancer compounds against three cancer cell lines. Our mechanistic studies indicated that both derivatives **8** and **15** induce apoptosis and arrests cell cycle. The induction of apoptosis appears to be due to caspase-3 activation. Further work is in progress in our laboratory develop potent anticancer agent of iridoid class.

Supporting information

Spectral data of compounds **1–15** are available at http://www.sciencedirect.com.

Conflict of interest

The authors have no conflict of interest.

Disclosure statement

The authors have nothing to disclose.

Acknowledgments

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