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# Apoptosis and DNA intercalating activities of novel emodin derivatives†

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**www.rsc.org/advances**

Emodin belongs to the anthraquinone class of compounds and is the major bioactive compound of several herb species. A library of novel emodin derivatives was synthesized and their antiproliferative activities were evaluated against HepG2, PC-3, DU-145, MCF-7, and HEK-293 cell lines. Among these derivatives, one showed a higher order of *in vitro* anticancer activity profile similar to the market drug epirubicin. The derivative strongly inhibited the proliferation of HepG2, PC-3, DU-145 and MCF-7 cancer cell lines with $IC_{50}$ values of 3.5, 5.6, 7.5, and 4.5 μM respectively. The derivative was also capable of inducing caspase-3-mediated apoptosis in HepG2 cells by arresting the cell cycle in the S phase. Furthermore, the derivative exhibited DNA intercalating ability comparable to doxorubicin, thus making this compound a promising potential antitumor drug.

## 1. Introduction

Anthraquinones are widely distributed secondary metabolites of plants (aloe, *cascara sagrada*, *Senna* and rhubarb), microbes, lichens and insects, which possess various biological activities.[1–3] Anthraquinones isolated from microbes such as daunorubicin (**I**), doxorubicin (**II**), epirubicin (**III**) and several synthetic analogues such as mitoxantrone (**IV**) and pixantrone (**V**) (Fig. 1) are in clinical use for the treatment of various cancers. Emodin (**1**) belongs to the anthraquinone class of compounds and is the major bioactive compound of numerous herb species such as *Rheum*, *Polygonum* (Polygonaceae), *Rhamnus* (Rhamnaceae) and Cassieae (*Senna*)[4–6] and possesses immunosuppressive, anticancer, anti-inflammatory, anti-atherosclerotic, and vasorelaxant effects.[7–10] Several reports have shown that emodin (**1**) has antiproliferative effects on many kinds of cancer cell lines such as HER-2/neu over-expressing breast cancer,[11,12] leukemia,[13,14] hepatoma,[15] and lung[16] cancer cells. Also, emodin-triggered apoptosis is mediated through the caspase and mitochondria dependent pathways in proximal tubular epithelial HK-2 cells.[17] Emodin enhanced gefitinib induced cytotoxicity *via* Rad51 down regulation and ERK1/2 inactivation in human breast cancer B Cap-37 cells.[18] Various research groups have derivatized emodin with enhanced antitumor activities and DNA binding properties using semi-synthetic approaches. For instance Tan *et al.* have reported DNA binding pyrazole emodins,[19] Eger *et al.* reported annulated tetrahydropyrazine derivatives of emodins,[20] Song *et al.* developed anthracene L-rhamnopyranosides[21] and Wang *et al.* synthesized emodin quaternary ammonium salt derivatives.[22]

As a part of our drug discovery program on anticancer agents from Indian medicinal plants, we isolated large quantities of emodin (**1**) from the roots of *Rheum emodi* and planned to carry out chemical transformations to improve its therapeutic applications. Chemical transformation of the bioactive compounds of medicinal herbs is one of the most common approaches in drug discovery to improve 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 a series of novel *O*-alkylated derivatives **2–12** (Scheme 1), ester derivatives **13–16**

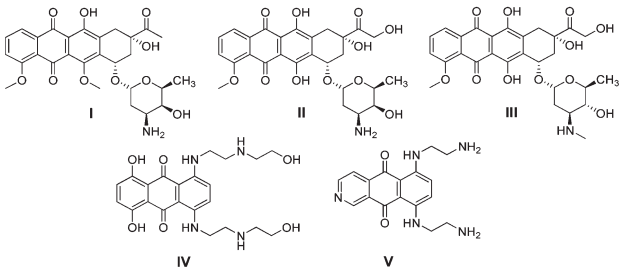


**Fig. 1** Anticancer drugs with the anthraquinone skeleton: daunorubicin (**I**), doxorubicin (**II**), epirubicin (**III**), mitoxantrone (**IV**), and pixantrone (**V**).

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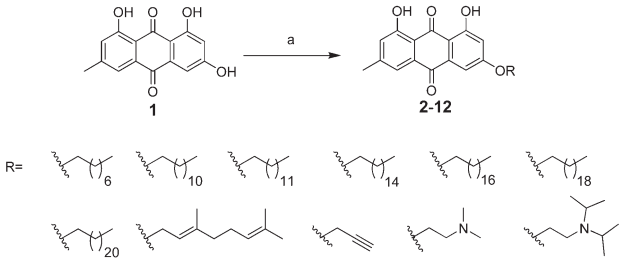
[b]*Pharmaceutics Division, CSIR-Central Drug Research Institute, Lucknow-226 001, U.P., India. E-mail: bsreddy@cdri.res.in (Biology)*

† Electronic supplementary information (ESI) available: Spectra ($^1$H NMR, $^{13}$C NMR and mass) of all compounds associated with this article. See DOI: 10.1039/c3ra23149f



**Scheme 1** Synthesis of *O*-alkylated derivatives **2–12** of emodin (**1**). Reagents and conditions: (a) RX, $K_2CO_3$, DMF, 60 °C, 4 h, yield: 62–80%.

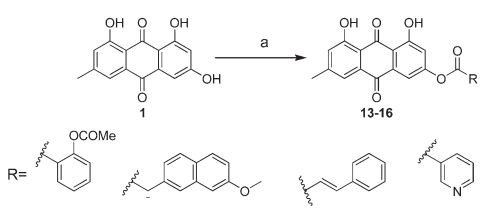
(Scheme 2) and amide derivatives **19**, **20** (Scheme 3) from **1** and evaluated their anticancer activity against HepG2 (human hepatocellular carcinoma), MCF-7 (breast adenocarcinoma), DU-145 and PC-3 (human prostate cancer cells), and HEK-293 (human embryonic kidney) cell lines (Table 1). Further, we have studied the apoptosis inducing ability, effect on cell cycle and DNA binding properties of the most active emodin derivative **11**.

## 2. Materials and methods

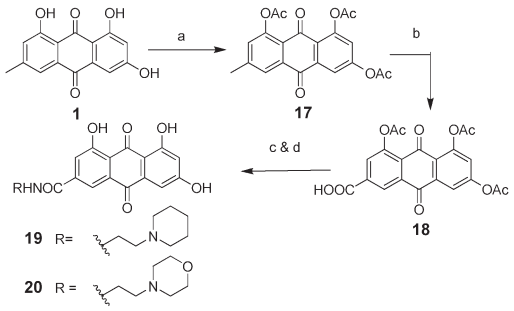
### 2.1. General chemistry

Melting points were recorded on a Buchi-530 capillary melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer RX-1 spectrometer using either KBr pellets or neat. $^1$H-NMR, $^{13}$C-NMR, DEPT-90 and DEPT-135 spectra were run on Bruker Advance DPX 300 MHz and 200 MHz spectrometers in $CDCl_3$, DMSO-$d_6$ and $CD_3OD$. Chemical shifts are reported as values in ppm relative to $CHCl_3$(7.26) in $CDCl_3$ and TMS was used as an internal standard. ESI mass spectra were recorded on a JEOL SX 102/DA-6000. Chromatography was executed with silica gel (60–120 mesh) using mixtures of chloroform, ethyl acetate and hexane as eluants. Visualization was done under UV light and spraying with 10% sulfuric acid in methanol.

**2.1.1. Plant background.** *Rheum emodi* Wall. (Family: Polygonaceae, commonly known as *Revand chini* and English name rhubarb) is a stout herb, distributed in the alpine and sub-alpine zones of the Himalayas.[23,24] The roots of this species are used widely in ayurvedic medicine. Roots of the Indian rhubarb are inferior in aroma, and are a well known stomachic, bitter and cathartic and used all over the world.



**Scheme 2** Synthesis of ester derivatives **13–16** from emodin (**1**). Reagents and conditions: (a) acid, dry DCM, DIPC, DIEA, 0 °C–rt, 6 h, yield: 60–65%.



**Scheme 3** Synthesis of amide derivatives **19** and **20** from emodin. Reagents and conditions: (a) $Ac_2O$, pyridine, 60–70 °C, 4 h, yield: 98%; (b) glacial AcOH : $Ac_2O$ (1 : 1), $CrO_3$, 70 °C, 10 h, yield: 80%; (c) benzene, $SOCl_2$, 70 °C, 4 h; (d) $RNH_2$, DCM, $Et_3N$, 0 °C, 1 h, yield: 85%.

**2.1.2. Collection of medicinal plant.** *Rheum emodi* Wall. (bark) was purchased from the local market of Lucknow, India and the authentication was carried out by the Botany Division of Central Drug Research Institute, Lucknow.

**2.1.3. Extraction.** Powdered bark of *Rheum emodi* Wall. (bark) (3 kg) was placed in a 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 four times. The combined percolate was evaporated under reduced pressure at 50 °C to afford the ethanol extract. The weight of the extract was found to be 200 g.

**2.1.4. Isolation and purification of emodin.** The alcoholic extract (200 g) was chromatographed on a column of silica gel (60–120 mesh), eluted with hexane and chloroform (70 : 30) and recrystallized from methanol to afford emodin (3 g). Compound visualization was carried out under UV light, also showing an orange spot by spraying with 10% sulfuric acid in methanol.

### 2.2. Preparation of emodin derivatives

**2.2.1. General method for *O*-alkylation (Method A).** To a magnetically stirred solution of compound **1** (100 mg, 0.37 mmol) in DMF (5 mL) at room temperature was added the respective aliphatic halide (RX) (0.33 mmol) and $K_2CO_3$ (0.92 mmol). The whole reaction mixture was stirred for 4 h at 60–70 °C. It was then extracted with ethyl acetate (3 × 25 mL). The organic layer was washed with water, dried over anhydrous $Na_2SO_4$ and evaporated under reduced pressure. Then the crude product was chromatographed on silica gel to afford the desired compound.

**2.2.2. General method for esterification (Method B).** To a magnetically stirred solution of the appropriate acid (0.44 mmol) and compound **1** (100 mg, 0.37 mmol) in dry DCM (5 mL), was added DIPC (0.55 mmol) and DIEA (0.37 mmol). The whole reaction mixture was stirred for 6 h at room temperature. It was then extracted with ethyl acetate (3 × 25 mL). The organic layer was washed with water, dried over anhydrous $Na_2SO_4$ and evaporated under reduced pressure. Then the crude product was chromatographed on silica gel to afford the desired compound.

**2.2.3. General method for preparation of amide derivatives (Method C).** Compound **1** (100 mg, 0.37 mmol) was magne-

**Table 1** Chemical structures and *in vitro* anticancer activities ($IC_{50}$ in μM) of emodin (**1**) and its derivatives (**2–20**)

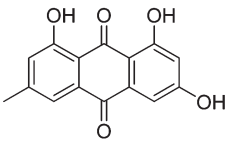
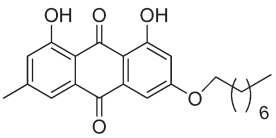
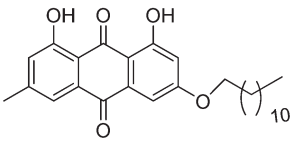
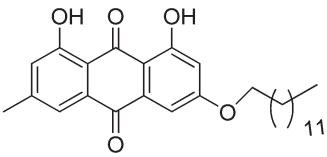
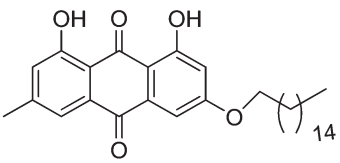
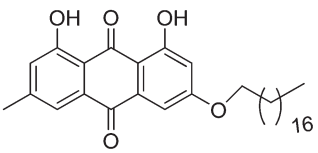
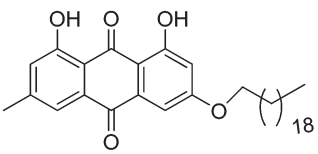
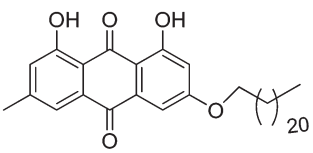
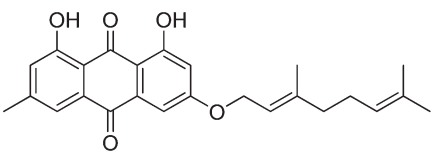
| Comp. No. | Chemical structure | $IC_{50}$ (μM) | | | | |
|---|---|---|---|---|---|---|
| | | HepG2 | PC-3 | DU-145 | MCF-7 | HEK-293 |
| **1** |  | 119 ± 0.16 | 145 ± 0.02 | 114 ± 0.06 | 162 ± 0.09 | 150 ± 0.04 |
| **2** |  | 127 ± 0.14 | 137 ± 0.01 | 124 ± 0.024 | 140 ± 0.054 | 162 ± 0.011 |
| **3** |  | 48 ± 0.12 | 76 ± 0.13 | 78 ± 0.21 | 69 ± 0.32 | 49 ± 0.024 |
| **4** |  | 127 ± 0.11 | 130 ± 0.06 | 114 ± 0.03 | 125 ± 0.87 | 122 ± 0.9 |
| **5** |  | 92 ± 0.029 | 56 ± 0.012 | 110 ± 0.9 | 87 ± 0.1 | 75 ± 0.43 |
| **6** |  | 100 ± 0.069 | 97 ± 0.024 | 82 ± 0.03 | 89 ± 0.09 | 90 ± 0.02 |
| **7** |  | 103 ± 0.022 | 120 ± 0.017 | 125 ± 0.07 | 114 ± 0.04 | 140 ± 0.09 |
| **8** |  | 36 ± 0.149 | 37 ± 0.07 | 34 ± 0.033 | 39 ± 0.012 | 42 ± 0.067 |
| **9** |  | 15 ± 0.032 | 12 ± 0.08 | 11 ± 0.099 | 10 ± 0.078 | 17 ± 0.056 |

**Table 1** (*Continued*)

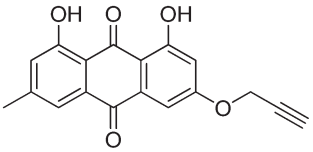
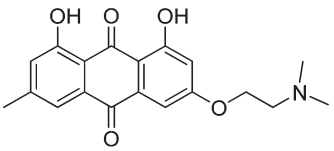
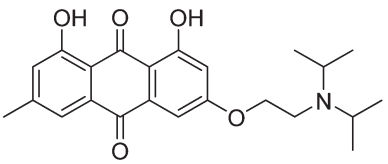
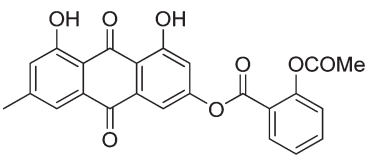
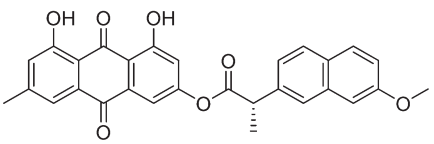
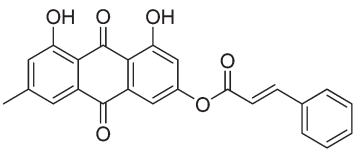
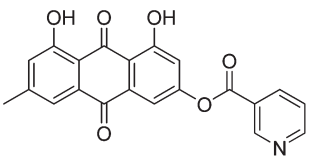
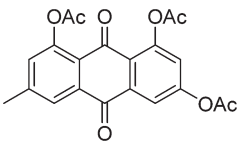
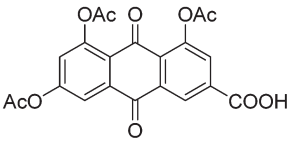
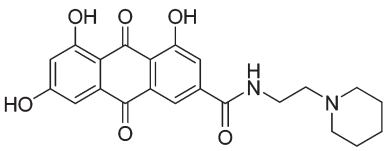
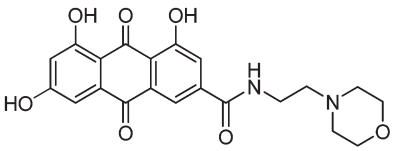
| Comp. No. | Chemical structure | $IC_{50}$ (μM) HepG2 | PC-3 | DU-145 | MCF-7 | HEK-293 |
|---|---|---|---|---|---|---|
| **10** |  | 25 ± 0.055 | 22 ± 0.02 | 29 ± 0.043 | 20 ± 0.04 | 27 ± 0.08 |
| **11** |  | 3.5 ± 0.11 | 5.6 ± 0.01 | 7.5 ± 0.013 | 4.5 ± 0.022 | 12 ± 0.9 |
| **12** |  | 98 ± 0.036 | 89 ± 0.02 | 86 ± 0.012 | 94 ± 0.011 | 84 ± 0.09 |
| **13** |  | 53 ± 0.13 | 49 ± 0.03 | 51 ± 0.045 | 45 ± 0.023 | 57 ± 0.043 |
| **14** |  | 74 ± 0.16 | 80 ± 0.011 | 86 ± 0.077 | 78 ± 0.05 | 84 ± 0.06 |
| **15** |  | 130 ± 0.01 | 112 ± 0.013 | 129 ± 0.013 | 124 ± 0.034 | 119 ± 0.07 |
| **16** |  | 25 ± 0.05 | 32 ± 0.08 | 29 ± 0.014 | 22 ± 0.09 | 34 ± 0.3 |
| **17** |  | 34 ± 0.13 | 42 ± 0.12 | 36 ± 0.02 | 24 ± 0.07 | 27 ± 0.09 |
| **18** |  | 218 ± 0.14 | 41 ± 0.019 | 40 ± 0.9 | 32 ± 0.04 | 46 ± 0.05 |

**Table 1** (*Continued*)

| Comp. No. | Chemical structure | $IC_{50}$ (μM) HepG2 | PC-3 | DU-145 | MCF-7 | HEK-293 |
|---|---|---|---|---|---|---|
| **19** |  | 19 ± 0.003 | 20 ± 0.09 | 39 ± 0.01 | 24 ± 0.07 | 42 ± 0.06 |
| **20** |  | 20 ± 0.019 | 19 ± 0.06 | 25 ± 0.014 | 21 ± 0.05 | 29 ± 0.06 |
| **III** | Epirubicin (marketed drug) | 4.6 ± 0.9 | 9.9 ± 0.03 | 5.7 ± 0.01 | 3.7 ± 0.08 | 4.2 ± 0.09 |

tically stirred in a solution of pyridine (2 mL) and acetic anhydride (1.85 mmol) at 60–70 °C for 4 h. The reaction mixture was put into cold water for crystallization, then filtered and dried. To the resultant crude acetate was gradually added 10 mL of a mixture of acetic anhydride and glacial acetic acid (1 : 1) and $CrO_3$ at 45 °C and the resulting mixture stirred for 10 h at 70 °C. The acetic anhydride and glacial acetic acid mixture was removed by vacuum. The residue was then extracted with ethyl acetate (3 × 25 mL). The organic layer was washed with water, dried over anhydrous $Na_2SO_4$ and evaporated under reduced pressure. Then the crude product was chromatographed on silica gel to afford the desired compound. The acid was magnetically stirred in a solution of benzene (2 mL) and $SOCl_2$ (5 mL) at 70 °C for 4 h. Benzene was removed by vacuum. To the residue was then added dry DCM, amine (0.25 mmol) and triethylamine (0.38 mmol) and the mixture maintained at 0 °C for 1 h. DCM was removed by vacuum. The residue was then extracted with ethyl acetate (3 × 25 mL), and the organic layer was washed with water, dried over anhydrous $Na_2SO_4$ and evaporated under reduced pressure. Then the crude product was chromatographed on silica gel to afford the desired compound.

### 2.3. Biological studies

**2.3.1. Cell lines and culture.** The human cancer cell lines *e.g.* HepG2 (hepatocellular carcinoma), DU-145 and PC-3 (human prostate cancer cells), MCF-7 (breast adenocarcinoma), and normal non-transformed cell type, human embryonic kidney (HEK-293), used in the present study were obtained from the ATCC (American Type Culture Collection, USA). Mouse skin fibroblasts were isolated from mice maintained in RPMI 1640 (Roswell Park Memorial Institute, Merck) with 10% FBS (Merck), supplemented with 1% antibiotic and antimycotic solution (Gibco, USA) at 37 °C in a humidified incubator with 5% $CO_2$. All stock solutions of the compounds were prepared in cell culture grade DMSO (dimethyl sulfoxide) and stored at −20 °C. Compounds were diluted in culture media prior to use in experiments. Annexin V FITC apoptosis detection kits were purchased from Calbiochem. MTT dye was procured from Sigma-Aldrich. All the flow cytometry experiments were performed using a FACScan (Becton Dickinson, Mountain View, CA) flow cytometer, equipped with a single 488 nm argon laser. All the experiments with live animals were done under the animal protocol approved by the CDRI Institutional Animal Ethics Committee.

**2.3.2. MTT assay for cell viability.** Cell viability was assessed by MTT assay, which is based on the reduction of MTT by mitochondrial dehydrogenases of viable cells to form a purple formazan product. Briefly, cells ($5 \times 10^3$/well) were plated in 96 well plates. After incubating overnight, the cells were treated with six different concentrations (100, 50, 20, 10, 5, 2.5 μM) as triplicates of emodin derivatives for 48 h. Subsequently, 10 μL of MTT (10 mg $mL^{-1}$) was added to each well and incubated for 3 h. The MTT formazan formed by viable cells was dissolved in 100 μL of DMSO and shaken for 10 min. The absorbance was measured on an ELISA reader. Each test was repeated at least three times. The concentration of the compound which gives the 50% growth inhibition value corresponds to $IC_{50}$, calculated using GraphPad prism 5.

**2.3.3. Apoptosis studies.** Quantitation of apoptotic cells by annexin V staining was carried out according to the manufacturer's instructions. Briefly, cells ($5 \times 10^5$ cells/well) were seeded in 6 well plates and treated with 5, 10 and 20 μM of derivative **11** for 24 h. After incubation, cells were washed with PBS stained with 1.25 μL of annexin V-FITC and 10 μL of media binding reagent and incubated for 20 min. After that 10 μL of propidium iodide (PI) was added and samples were analysed using a flow cytometer. Annexin V-FITC was 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. Each experiment was repeated two times independently.

**2.3.4. Cell cycle analysis.** For cell cycle distribution studies post-treatment, cells were fixed in 70% ethanol overnight, rehydrated in PBS with ribonuclease A (100 μg $mL^{-1}$) and

Triton-X (1%) for 30 min at room temperature then PI (50 μg $ml^{-1}$) added before incubation in the dark for 30 min and analysis with a flow cytometer. Experiments were repeated two times independently.

**2.3.5. Caspase-3 activation assay.** The activity of caspase-3 was determined using a caspase-3 colorimetric assay kit (Sigma Aldrich) according to the manufacturer's protocol. Briefly, HepG2 cells ($1 \times 10^6$ cells/well) were treated with 5, 10 and 20 μM of derivative **11** for 24 h. The cells were harvested and lysed by addition of lysis buffer. 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.

**2.3.6. DNA intercalation assay.** Calf thymus DNA (8.8 μM) was loaded with Tris buffer containing ethidium bromide (4.4 μM). To each well were added compounds **9**, **11**, **19**, etoposide and DOX at 5, 10 and 20 μM. After incubation for 30 min at 25 °C fluorescence was measured by using a fluorimeter in duplicate experiments with two control wells (no compound = 100% fluorescence and no DNA = 0% fluorescence). Doxorubicin was used as a positive control for the DNA intercalation assay while etoposide was used as a negative control. Fluorescence readings are reported as % fluorescence readings compared to controls.

### 2.4. Statistical analysis

All data are expressed as mean ± standard deviation for three experiments. A probability value of $p < 0.05$ was considered statistically significant. All the statistical analysis was performed using Graph Pad Prism version 5.00 for Windows (Graph Pad Software, USA).

## 3. Results and discussion

### 3.1. Chemistry

**3.1.1. *O*-Alkylation.** In order to synthesize *O*-alkylated derivatives emodin (**1**) was reacted with various long chain alkyl halides, geranyl bromide, 3-bromopropyne, 2-bromo-*N*,*N*-dimethylethanamine and *N*-(2-bromoethyl)-*N*-isopropylpropan-2-amine under basic conditions using $K_2CO_3$ and DMF as a solvent (Scheme 1). Out of three hydroxyls of emodin (**1**), two hydroxyls are involved in chelation with a carbonyl group, therefore only *meta*-substituted derivatives **2–12** were obtained. The presence of chelated hydroxyls was confirmed from $^1$H-NMR spectral data (please see ESI†).

**3.1.2. Esterification.** Since emodin (**1**) contains free hydroxyl groups we planned to prepare several esters to study their anticancer activities. Emodin (**1**) was reacted with the respective aromatic acids in the presence of DIPC and DIEA in dry DCM at 0 °C to rt (Scheme 2), which provided the *meta*-substituted ester derivatives **13–16**.[25,26]

**3.1.3. Amide formation.** To transform the methyl group into an amide functionality, emodin (**1**) was converted to its triacetate **17** with acetic anhydride in the presence of pyridine and subsequently oxidized with chromium trioxide in the presence of glacial acetic acid and acetic anhydride to provide acid derivative **18** (Scheme 3).[27] The resultant acid **18** was converted to its acid chloride by using $SOCl_2$ in benzene at 70 °C, then further reacted with alkylamine in the presence of triethylamine *in situ* at 0 °C to obtain amides **19** and **20** (Scheme 3).

### 3.2. Biology

**3.2.1. Anti-proliferative activity.** All the emodin derivatives (**2–20**) were evaluated for *in vitro* anticancer activity using an MTT assay across different molar concentrations (2.5, 5, 10, 20, 50, 100 μM) as triplicates. The growth-inhibitory effects were studied in four human cancer cell lines, HepG2 (hepatocellular carcinoma), DU-145 and PC-3 (human prostate cancer cells), MCF-7 (breast adenocarcinoma) and one non-cancer cell line, HEK-293 (human embryonic kidney). The parent compound, emodin (**1**) has an $IC_{50}$ of 119, 145, 114, 162 and 150 μM against HepG2, PC-3, DU-145, MCF-7 and HEK-293 respectively (Table 1). All the derivatives **2–20** prepared from emodin (**1**) exhibited improved *in vitro* anticancer activity against HepG2, PC-3, and MCF-7 cell lines compared to parent compound **1** (Table 1). Except **2**, **7**, **19**, all other derivatives also exhibited improved activity against the DU-145 cell line. Among all these derivatives (**2–20**), the *O*-alkylated derivative with a basic amine group (**11**) turned out to be the most potent antiproliferative agent with $IC_{50}$ values of 3.5, 5.6, 7.5 and 4.5 μM against HepG2, PC-3, DU-145 and MCF-7 cancer cell lines respectively, which are comparable to those of the marketed anthraquinone class of anticancer drug (epirubicin $IC_{50}$ values of 4.6, 9.9, 5.7 and 3.7 μM). The *O*-geranylated derivative (**9**) also showed significant antiproliferative activity against all the cancer cell lines ($IC_{50}$ values of 15, 12, 11 and 10 μM) followed by *O*-propyne derivative **10** ($IC_{50}$ values of 25, 22, 29 and 20 μM). Among other *O*-alkylated derivatives (**2–8**), compound **8** with a C-22 alkyl chain showed good antiproliferative activity ($IC_{50}$ values of 36, 37, 34, 39 and 42 μM) followed by derivative **3** with a C-12 alkyl chain ($IC_{50}$ values of 48, 76, 78, 69 and 49 μM). Esters **13–17** prepared from **1** also exhibited improved activity over the parent compound. The nicotinic acid derivative (**16**) and triacetate (**17**) have better activity profiles than other ester derivatives **13–15** with $IC_{50}$ values of 25, 32, 29, 22 μM and 34, 42, 36, 24 μM against HepG2, PC-3, DU-145 and MCF-7 cancer cell lines respectively. The amides **19** and **20** prepared from acid derivative **18** exhibited good antiproliferative activities with $IC_{50}$ values of 19, 20, 39 and 24 μM and 20, 19, 25 and 21 μM against HepG2, PC-3, DU-145 and MCF-7 cancer cell lines respectively. The most active compounds **9**, **11** and**19** were also tested against mouse skin fibroblasts (Fig. S1, ESI†). These compounds did not show any significant cytotoxicity against them.

**3.2.2. Apoptosis studies.** Most of the currently used cytotoxic drugs induce cell killing by the use of a process known as apoptosis.[28] In this study we measured the apoptosis inducing ability of derivative **11** in HepG2 cells by flow cytometry. Annexin V specially binds to phosphatidylserine and has been employed in the determination of apoptotic cells. Annexin V/PI staining was performed to determine early (annexin V positive, PI negative), late apoptotic (annexin V positive, PI positive) and necrotic (annexin V negative, PI positive) cells followed by 24 h

treatment with 5, 10 and 20 μM concentration of derivative **11** in HepG2 cells.

In Fig. 2, compared to the control, compound treatment increased the number of both annexin V and PI positive cells for all tested concentrations. It is evident from the above data that derivative **11** induces apoptosis in HepG2 cells.

**3.2.3. Cell cycle analysis.** In this study we also examined the effect of derivative **11** on the cell cycle of HepG2 cells by flow cytometry in PI (propidium iodide) stained cells after treatment with **11** for 24 h. Fig. 3 shows the DNA distribution histograms of HepG2 cells in the absence (control) and presence (5, 10, and 20 μM) of derivative **11**. The treatment of HepG2 cells with **11** led to an obvious increase in the percentage of cells in the S phase, accompanied by a corresponding reduction in the percentage of cells in the G0/G1 phase in a concentration dependent manner. Treatments with 5, 10 and 20 μM of derivative **11** caused an increase of the S-phase population to 28.05%, 27.45%, and 66.59% respectively, compared with the S-phase population of 15.80% of untreated HepG2 cells, indicating the induction of S-phase arrest by derivative **11**. The above results suggest that the antiproliferative mechanism of derivative **11** on HepG2 cells is due to the S-phase arrest of the cell cycle.

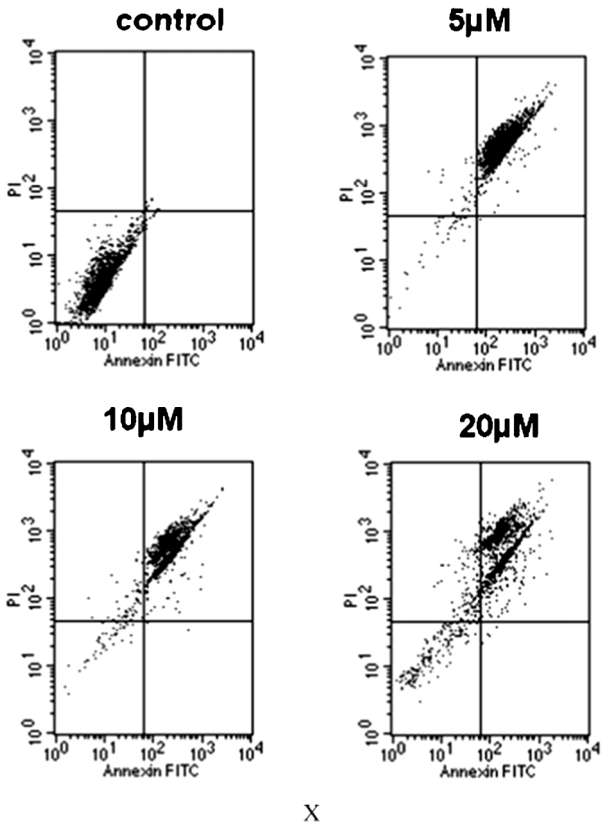



**Fig. 2** Analysis of apoptosis induction by compound **11** in the HepG2 cell line. Cells were treated with different concentrations of **11** for 24 h and apoptosis was analyzed using annexin V and propidium iodide double staining by flow cytometry.

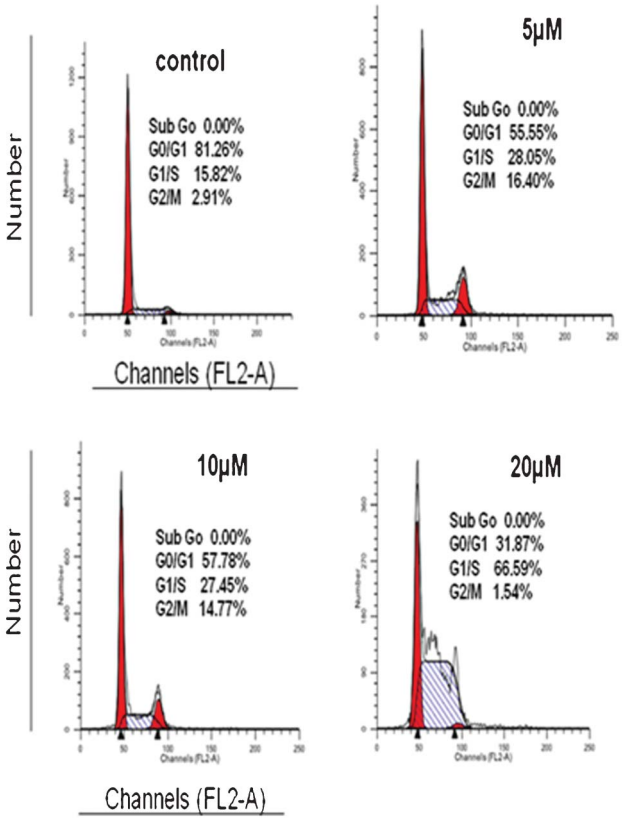



**Fig. 3** Effect of emodin derivative **11** on cell cycle progression of HepG2 cells. Cells were treated with different concentrations of derivative **11** starting from 5, 10 and 20 μM for 24 h, washed with PBS, fixed in 70% ethanol and stained with PBS containing PI (50 μg $mL^{-1}$), as described in the materials and methods section. The percentages of cells at each cell cycle phase are shown, respectively.

**3.2.4. Capase-3 activation.** To find out whether the induction of apoptosis by **11** is caspase mediated or not, we examined the caspase-3 activation in HepG2 cells. Caspases are cysteinyl aspartate peptidases and cleave substrate proteins at aspartate residues upon receiving an apoptotic signal. Among these, caspase-3 is a key protein in the apoptosis mechanism since it executes both extrinsic and intrinsic pathways.[29,30] Our studies indicated a concentration dependent increase in caspase-3 activity with derivative **11** treatment as compared with the untreated control. Caspase-3 activation was enhanced 2.8, 7.01 and 13.64 fold by **11** in cells treated with 5, 10 and 20 μM respectively, when compared with the control group (Fig. 4). These results confirmed that the apoptosis induction is due to caspase-3 activation by derivative **11** in HepG2 cells.

**3.2.5. DNA interaction.** In this study we evaluated the DNA interacting abilities of antiproliferative emodin derivatives **9**, **11**, and **19** using an ethidium bromide displacement assay. The fluorescence intensity of ethidium bromide increases upon the binding of DNA and subsequent displacement of ethidium is apparent by a decrease in emission intensity upon addition of a competitive inhibitor.[31] We have included DOX, a known DNA interacting anticancer drug for comparison. Calf thymus DNA was preincubated with ethidium bromide for 30 min in buffer before newly synthesized compound addition.

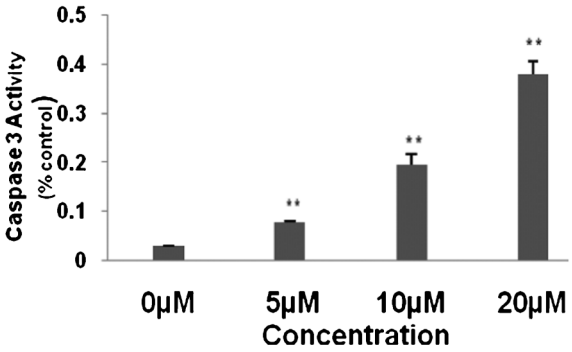



**Fig. 4** Measurement of caspase-3 activities. Dose-dependent induction of caspase-3 in HepG2 cell lines. Colorimetric determination of general caspase was done as described in the materials and methods section. Each data point is the representation of triplicate treatments. (**$P < 0.01$ *vs.* negative control).

Derivatives **9**, **11** and **19** and etoposide (negative control) were incubated with ethidium bromide DNA complex for 30 min and the fluorescence measured. As shown in Fig. 5, DOX has a very strong effect in this assay, while derivatives **9**, **11** and **19** have comparable effect. Considering the nearly equipotent DNA binding affinity of the derivative **11** with DOX, we can conclude that the antiproliferative effect of the derivative **11** is due to DNA intercalation.

## 4. Conclusions

In conclusion we have isolated large quantities of emodin (**1**) from the roots of *R. emodi* and prepared novel derivatives **2–20** and evaluated their *in vitro* anticancer activities in HepG2, PC-3, DU-145 and MCF-7 cancer cell lines and a normal cell line HEK-293. All the derivatives showed better therapeutic effect than the parent compound. Among these derivatives **11** showed comparable *in vitro* anticancer activity to that of the marketed drug epirubicin (**III**). Compound **11** was capable of inducing cell cycle arrest and caspase dependent apoptosis in HepG2 cell lines. Along with those derivatives **9**, **11** and **19** have shown comparable DNA intercalation with DOX. These synthetic emodin derivatives may hold promise for developing an alternative to the existing anthraquinone class of anticancer agents. Further work is in progress in our laboratory to evaluate their *in vivo* anticancer potentials and in-depth mechanistic aspects.

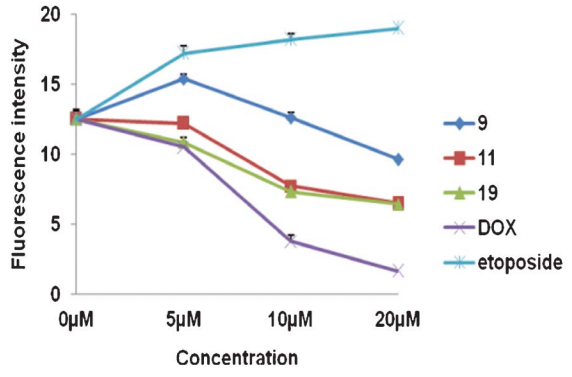



**Fig. 5** DNA intercalating activity of emodin derivatives **9**, **11** and **19**, doxorubicin and etoposide. Ethidium bromide was preincubated with calf thymus DNA prior to compound addition. Fluorescence was measured 30 min after compound addition. Doxorubicin was used as a positive control for the DNA intercalation assay while etoposide was used as a negative control. Error bars represent the standard deviation of the mean, $n = 3$.

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