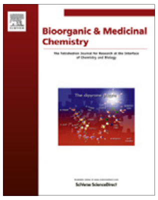
Bioorganic & Medicinal Chemistry 20 (2012) 3049–3057

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc



Synthesis and anticancer activity of 2-benzylidene indanones through inhibiting tubulin polymerization

A.P. Prakasham [a], A.K. Saxena [b], Suaib Luqman [a], Debabrata Chanda [a], Tandeep Kaur [b], Atul Gupta [a], D.K. Yadav [a], C.S. Chanotiya [a], Karuna Shanker [a], F. Khan [a], Arvind S. Negi [a,*]

[a] *CSIR, Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), Kukrail Picnic Spot Road, Lucknow 226 015, UP, India*
[b] *CSIR, Indian Institute of Integrative Medicine (CSIR-IIIM), Canal Road, Jammu 180 001, India*

ARTICLE INFO

Article history:
Received 30 December 2011
Revised 24 February 2012
Accepted 25 February 2012
Available online 14 March 2012

Keywords:
Anticancer, Indanones
Gallic acid
Tubulin polymerization inhibition
Acute oral toxicity

ABSTRACT

In an attempt to discover a potent and selective anticancer agent, gallic acid has been modified to benzylidene indanones as tubulin polymerization inhibitors. These compounds were evaluated against several human cancer cell lines and also evaluated for inhibition of tubulin polymerase in in vitro assays. Three of the analogues exhibited strong cytotoxicity against human cancer cell lines $IC_{50} = 10–880$ nM and also showed tubulin polymerization inhibition ($IC_{50} = 0.62–2.04$ μM). Compound **9j**, the best candidate of the series was found to be non-toxic in acute oral toxicity in Swiss-albino mice up to 1000 mg/kg dose.

1. Introduction

Cancer, the uncontrolled growth of cells is a leading cause of death worldwide and accounted for 7.6 million deaths in 2008 which is around 13% of total deaths.[1,2] While great efforts have been made to tackle the disease over past few decades, it continues to be a major health menace. Medicinal Chemists are tirelessly exploring for a better and suitable cancer therapeutic. Combretastatins are naturally occurring anticancer agent isolated from African willow tree *Combretum caffrum*.[3–5] Combretastatin A4 (**1**, CA4) is the most active compound of the series, its water soluble prodrugs are in the final stage of clinical trials[6,7] and more likely, at least one of the analogues will succeed finally.[5] CA4 binds to the β-subunit of tubulin at the same site of colchicine and thus strongly inhibits tubulin polymerization.[8,9] Combretastatin A4 phosphate inhibits inherent angiogenesis in tumor cells, thus acts as vascular disrupting agent.[10] According to the structure–activity relationship of this class of compounds, a diaryl system should be separated through a double bond or a cyclic ring system and one of the rings must have a 3,4,5-trimethoxy substitutions.[4] A 3,4,5-trimethoxyphenyl unit (**2**, Chart 1) is considered to interact with tubulin to induce tubulin polymerization inhibition, and occurs some of the antitubulins like combretastatins, colchicine (**3**), podophyllotoxin (**4**) etc.[11]

In continuation of our work on modification of gallic acid (**5**) to anticancer agents,[12–15] we designed and synthesized some indanone moieties previously.[12] The best analogue of the series (Compound **7**) exhibited $IC_{50} = 2.2$ μM against breast cancer (MCF-7).[12] To optimize the previous lead we planned to introduce an α,β-unsaturated ketone very similar to chalcones for better activity. The prototype (benzylidene ring) thus synthesized on to indanone **7**, also have a triarylethylene (TAE) type arrangement known as estrogen receptor ligand. Hence, our pharmacophore (Chart 2) should have the affinity to estrogen receptors which are over-expressed in hormone dependent breast cancer.[16] Hence, we synthesized 2-benzylidene indanones starting with gallic acid (**5**).

2. Results and discussion

2.1. Chemistry

The 2-benzylidene indanone series of compounds **9a–p** were synthesized as outlined in Scheme 1. First, gallic acid was modified to two of the intermediates, that is, 3,4,5-trimethoxybenzaldehyde and 3,4,5-trimethoxy acetophenone as described in literature.[17] The chalcone (**6f**) and subsequently 3-(3′,4′,5′-trimethoxyphenyl)-4,5,6-trimethoxy indanone-1 (**7**) were synthesized as per our reported methods.[12] Briefly, gallic acid (**5**) was modified to 3,4,5-trimethoxybenzoic acid methyl ester (**5a**) by refluxing with dimethyl sulfate in 30% aqueous alkali. The ester group of **6a** was reduced with lithium aluminium hydride to get corresponding

---

* Corresponding author. Tel.: +91 522 2717529x327; fax: +91 522 2342666.
E-mail address: arvindcimap@rediffmail.com (A.S. Negi).

http://dx.doi.org/10.1016/j.bmc.2012.02.057

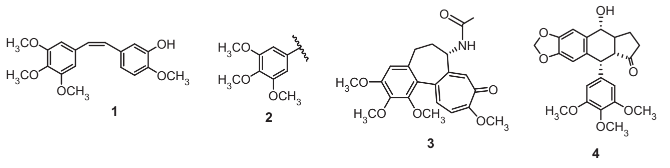


Chart 1. 3,4,5-Trimethoxyphenyl unit and natural antitubulins.

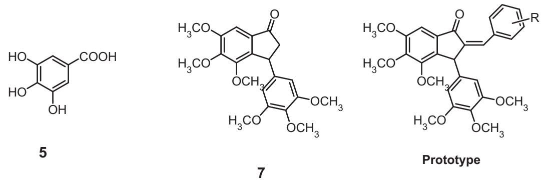
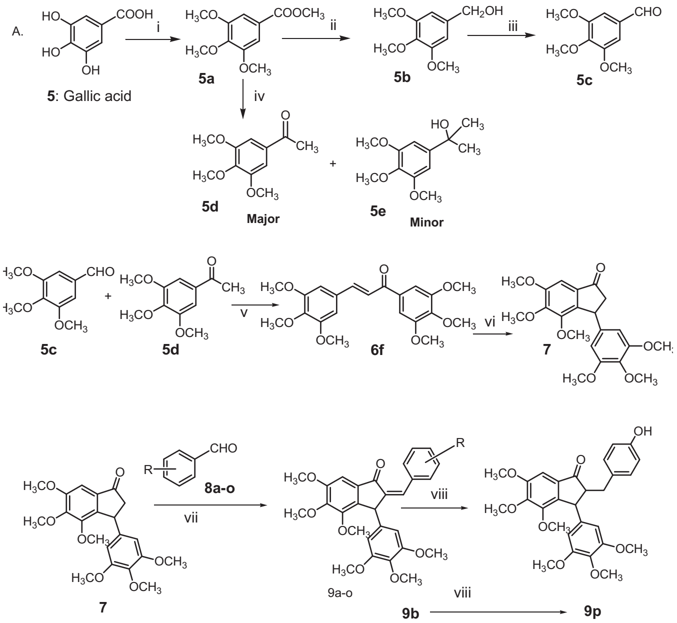


Chart 2. Gallic acid, indanone and proposed 2-benzylidene indanone prototype.

benzyl alcohol (**5b**) in 64% yield. The alcoholic group of **5b** was oxidized using pyridinium chlorochromate (PCC) to yield 3,4,5-trimethoxybenzaldehyde (**5c**). Gallic acid was converted to 3,4,5-trimethoxyacetophenone (**5d**) using Grignard reaction. Aldehyde **5c** and acetophenone **5d** on treatment with 7% aqueous-methanolic alkali yielded hexamethoxychalcone (**6f**). Chalcone **6f** was subsequently transformed to indanone **7** by treating it with trifluoroacetic acid. Finally, 2-benzylidene indanones (**9a–p**) were prepared on treating indanone **7** with various aromatic aldehydes (**8a–p**) in 2.5% methanolic KOH at room temperature for 1–3 h in good yields after recrystallization from chloroform–hexane. (See Table 1 for % yields). The benzylated benzylidene indanones (**9b**) on hydrogenolysis by Pd–C in THF yielded a saturated



Scheme 1. (a): R = H; (b): R = 4-benzyloxy; (c): R = 4-OCH_3; (d): R = 2-OCH_3; (e): R = 3,4-dimethoxy; (f): R = 2,4-dimethoxy; (g): R = 3,4,5-trimethoxy; (h): R = 2,3,4-trimethoxy; (i): R = 2,4,5-trimethoxy; (j): R = 3,4-methylenedioxy; (k): R = 2,3-methylenedioxy; (l): R = 4-nitro; (m): R = 3-nitro; (n): R = 4-fluoro; (o): R = 4-trifluoromethyl. Reagents and conditions: (i) Me_2SO_4, 30% aqueous KOH, reflux, 4 h, 60%; (ii) $LiAlH_4$, THF, reflux, 1 h, 64%; (iii) PCC, DCM, RT, 1 h, 86%; (iv) MeI, Mg turnings, THF, 20 min at rt then reflux 1 h, 46%; (v) 7% KOH in methanol–H_2O (2:1), rt, 1 h, 94%; (vi) TFA, sealed tube, 120 °C, 4 h, 30%; (vii) various aromatic aldehydes (**8a–o**), 2.5% methanolic KOH, rt, 1–3 h, 70–95%; (viii) **9b**, THF, 10% Pd–C, H_2-balloon, rt, 6 h, 40%.

Table 1
Cytotoxicity and tubulin polymerization inhibition by 2-benzylidene indanones

S.No.	Compd	Yield (%)[a]	Mp (°C)	IC_{50} (μM)[b] human cancer cell lines				IC_{50} (μM)[c] tubulin polymerization inhibition
				MCF-7 (breast)	HCT (Colon)	THP-1 (leukemia)	A549 (lung)	
1	**7**	30	106-07	2.20	75.9	–	55.4	1.88
2	**9a**	89	139-41	42.5	14.9	32.7	47.5	In[e]
3	**9b**	80	188-90	In	In	In	In	In
4	**9c**	81	188-90	3.18	18.6	0.88	13.4	4.67
5	**9d**	78	191-92	In	In	29.3	62.6	In
6	**9e**	85	179-80	80.8	In	62.0	In	0.62
7	**9f**	87	191-92	1.0	11.1	In	71.6	3.04
8	**9g**	78	118-20	38.9	39.7	64.9	67.6	4.62
9	**9h**	86	163-65	8.88	79.6	In	93.0	In
10	**9i**	95	203-05	56.5	In	81.5	87.2	4.21
11	**9j**	77	199-201	0.01	0.10	4.9	11.0	0.63
12	**9k**	77	180-82	57.2	45.9	In	82.2	In
13	**9l**	79	190-92	88.1	97.3	89.5	In	In
14	**9m**	77	215-17	In	In	In	In	In
15	**9n**	76	156-58	0.68	In	2.88	26.9	0.73
16	**9o**	70	188-90	68.9	92.0	8.67	61.5	0.60
17	**9p**	40	63-65	33.4	97.1	26.7	19	In
18	PDT[d]	–	–	3.5	–	–	–	0.74
19	Paclitaxel	–	–	0.006	–	–	–	–
20	Tamoxifen	–	–	0.027	–	–	–	–

Positive controls	Conc (μM)	% Inhibition			
		MCF-7	HCT	THP-1	A549
5-Fluorouracil	20	–	65	74	–
Paclitaxel	1	–	–	–	69
Adriamycin	1	65	–	–	–

[a] Isolated yield.
[b] Inactive, if IC_{50} > 100 μM.
[c] Inactive, if IC_{50} > 10 μM.
[d] Podophyllotoxin.
[e] Inactive.

2-benzylindanone (**9p**). All the compounds were characterized by spectroscopic means (See Supplementary data).

2.2. Biological evaluation

2.2.1. Anticancer activity

All these compounds were evaluated against four human cancer cell lines, that is, MCF-7 (breast), HCT (colon) and THP-1 (Leukemia) and A549 (Lung) by Sulphorhodamine B assay.[18] Cytotoxicity data for compounds **9a–p** are depicted in Table 1. Compounds exhibiting IC_{50} >100 μM were considered inactive. Our main interest was to optimize indanones **7** for better anticancer activity against MCF-7 breast cancer. Most of the analogues exhibited activity, and analogues **9g**, **k** and **o** exhibited much higher activity than the parent indanone **7** (2.20 μM). Overall, the anticancer activity of 2-benzylidene indanones was better than the previously synthesized indanone series.[12]

2.2.2. Tubulin polymerization inhibition

The results of tubulin polymerization inhibition assay[19,20] are represented in Table 1. Four of the analogues (**9f**, **k**, **o** and **p**) exhibited strong activity comparable to podophyllotoxin, the standard antitubulin agent. Analogue **9k** exhibiting highest anticancer activity also showed strong inhibition of tubulin. Thus, these 2-benzylidene indanones possess anticancer cancer activity through inhibition of tubulin polymerization. Rest of the analogues were found inactive ($IC_{50} \geqslant 10$ μM).

There are several natural antitubulins like colchicine, combretastatins and podophyllotoxin interacting with the tubulin. A 3,4,5-trimethoxyphenyl moiety has been found to exert tubulin polymerization inhibition in these compounds.[11] On the other hand taxol and taxotere are also considered tubulin interacting agents but these stabilize the polymerization process.[11] Microtubule polymerization and depolymerisation are tightly controlled during mitosis. Any disruption of this process causes cell cycle arrest and subsequently cell death.[21] The anticancer effect of these benzylidene indanones having this type arrangement is expected due to cytotoxicity of cancer cells through disturbance of mitotic spindle function which leads to cell apoptosis.[22]

2.2.3. In vivo acute oral toxicity

The most active analogue of the series, that is, **9j** was further evaluated for in vivo acute oral toxicity[23,24] in Swiss-albino mice at 5, 50, 300 and 1000 mg/kg as single oral dose. No observational changes, morbidity and mortality was observed throughout the experimental period except in case of 1000 mg/kg dose group where two of the animals showed sign of excitement, hyperactivity for initial 10 min. Blood and serum sample upon analysis showed non-significant changes in all the parameters studied like RBC, WBC count, different leukocyte count, hemoglobin, serum total cholesterol, triglycerides, creatinine level, SGPT and SGOT activity (Table 2, Fig. 2). However, SGPT and ALKP activity showed significant increase in group of animals treated at 1000 mg/kg. Similarly, animals on gross pathological study showed no changes in any of the organs studied including their absolute and relative weights (Fig. 1). Overall, the experiment showed that compound **9j** was well tolerated by the Swiss-albino mice up to the dose level of 1000 mg/kg body weight as a single acute oral dose. However, sub-acute and chronic experiments need to be carried out to look for any adverse effect on repeated exposure to the compound **9j** for its future development.

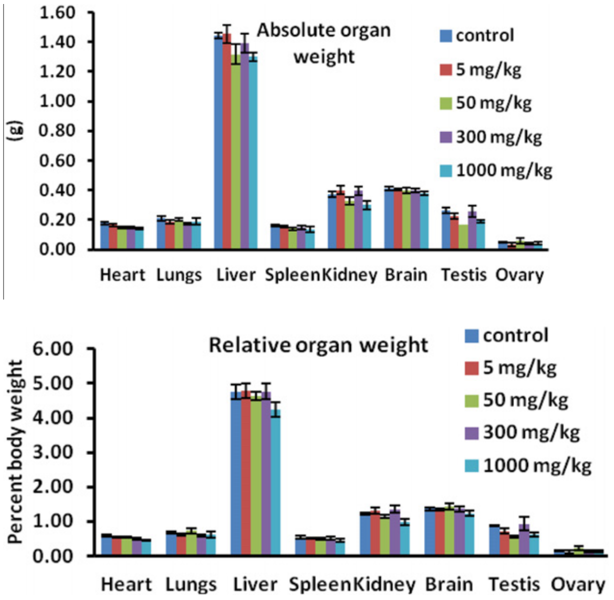


Figure 1. Effect of **9j** as a single acute oral dose at 5, 50, 300 and 1000 mg/kg on absolute and relative organ weight in Swiss-albino mice ($n = 6$).

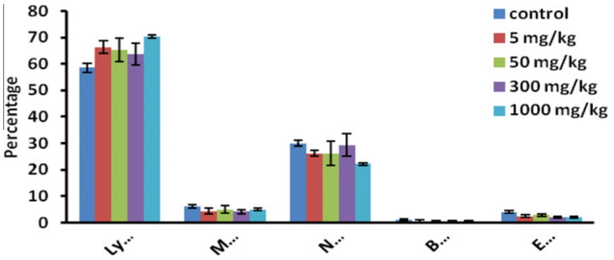


Figure 2. Effect of **9j** as a single acute oral dose at 5, 50, 300 and 1000 mg/kg body weight on differential leucocytes counts in Swiss-albino mice ($n = 6$).

2.2.4. Molecular docking experiments

The binding affinity obtained in the docking experiment allowed the activity of the best analogue **9j** to be compared to that of the

Table 2
Various hematological and serum biochemical parameters after ingestion of **9j** in Swiss-albino mice ($n = 6$; $P < 0.001$)

Parameters	Dose of **9j** at mg/kg body weight as a single oral dose				
	Control	5 mg/kg	50 mg/kg	300 mg/kg	1000 mg/kg
Body weight (g)	30.42 ± 1.25	30.38 ± 0.77	28.37 ± 1.42	29.35 ± 1.63	30.69 ± 0.83
SGPT (U/L)	9.30 ± 0.89	6.90 ± 0.17	9.77 ± 0.77	9.16 ± 1.14	19.68 ± 1.78
SGOT (U/L)	20.47 ± 2.07	18.74 ± 1.54	20.66 ± 1.15	26.55 ± 3.23	19.38 ± 3.49
ALKP (U/L)	83.29 ± 9.77	82.51 ± 3.04	94.24 ± 8.83	90.11 ± 5.42	171.12 ± 15.91
Hemoglobin (g/dL)	13.38 ± 1.00	11.31 ± 1.14	12.93 ± 0.98	11.46 ± 0.76	12.21 ± 1.46
Serum total cholesterol (mg/dL)	116.07 ± 8.86	109.42 ± 5.46	125.69 ± 5.90	117.57 ± 3.45	118.60 ± 6.07
Serum triglycerides (mg/dL)	101.29 ± 5.18	112.69 ± 3.75	90.62 ± 8.41	113.15 ± 3.65	97.44 ± 6.96
Serum creatinine (mg/dL)	0.47 ± 0.06	0.53 ± 0.04	0.45 ± 0.05	0.56 ± 0.07	0.42 ± 0.09

Table 3
Comparison of binding affinity of Podophyllotoxin and **9j** against tubulin polymerase

S.No.	Compd	Docking energy (Kcal/mol)	Binding pocket residues (5 Å)	Atoms of ligand involved in docking	Amino acid residue involved in docking interaction	Length of hydrogen bond (Å)	No of H-bonds
1	**9j**	−27.811	GLY237, VAL238, THR239, THR240, CYS241	C3-H14284	LYS352	2.371	5
			LEU242, LEU248, ALA250, ASP251, LEU252	H39-H14284		1.693	
			LYS254, LEU255, ASN258, MET259, THR314	H56-N11726			
			VAL315, ALA316, ALA317, VAL318, ASN349	H56-H14213		2.080	
			ASN350, VAL351, LYS352, THR353, ALA354	H56-H14214	ASN-258	2.070	
			ILE378			1.768	
2	Podophyllotoxin	−22.078	GLY237, VAL238, THR239, THR240, CYS241	C10-H14213		2.537	3
			LEU242, LEU248, ALA250, ASP251, LEU252	H36-H14213	THR240	2.227	
			LYS254, LEU255, ASN258, MET259, THR314			1.664	
			VAL315, ALA316, ALA317, VAL318, ASN349	H36-N11726			
			ASN350, VAL351, LYS352, THR353, ALA354				
			ILE378				

standard antitubulin podophyllotoxin (Table 3). Compound **9j** showed high binding affinity (high negative docking energy) against well known human anticancer target tubulin polymerase (PDB ID: 1SA1). Compound **9j** showed significant binding affinity at the binding site pocket amino acid residues better than podophyllotoxin (Fig. 3).

When we compared interactions of binding pocket residues of tubulin polymerase, we found that compound **9j** (Fig. 3) and podophyllotoxin form hydrophobic bonds (H-bonds), leading to more stability and potency in these cases (Table 3). The docking results showed that compound **9j** docked onto tubulin polymerase with a low interaction energy (−27.811 kcal mol^{-1}) and formed two H-bonds of length 2.371 and 1.693 Å to the basic amino acid residue Lys-352 and formed three H-bonds of length 2.08, 2.07 and 1.768 Å to the polar amide amino acid residue Asn-258 (Fig. 4), leading to more stability and potency in this compound. Other residues of the binding site pocket (within a radius of 5 Å) are summarized in Table 3. While, the docking results for podophyllotoxin with tubulin polymerase showed a docking energy of −22.078 kcal mol^{-1} and the

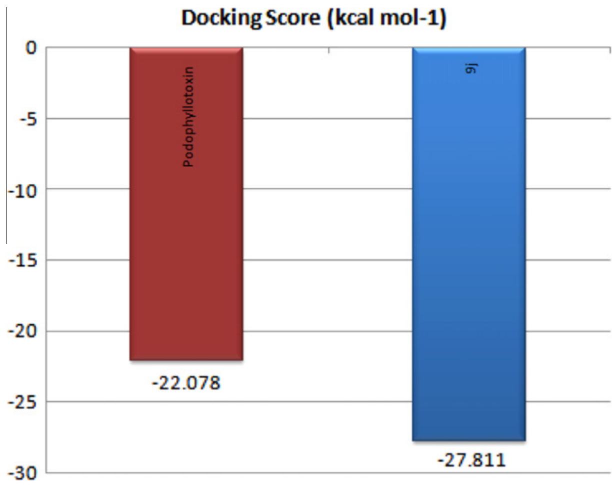


Figure 3. Binding affinities of podophyllotoxin and **9j** against tubulin polymerase. Negative docking scores (kcal mol^{-1}) indicate higher binding affinity.

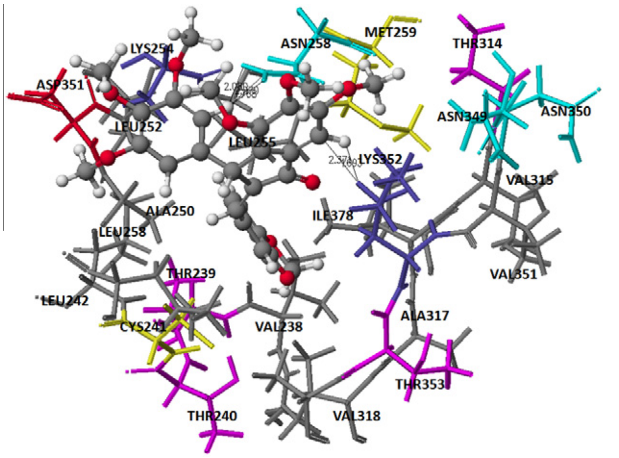


Figure 4. Compound **9j** docked onto target tubulin polymerase (Docking energy = −27.811 kcal mol^{-1}).

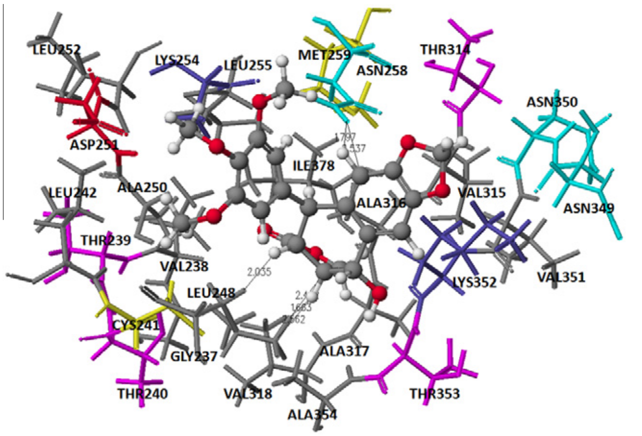


Figure 5. Podophyllotoxin docked on to tubulin polymerase (Docking energy = −22.078 kcal mol^{-1}).

formation of three H-bonds of length 2.537, 2.227 and 1.664 Å to the polar nucleophilic residue Thr240 (Fig. 5), leading to more stability and potency in this case. Other residues of the binding site pocket (within a radius of 5 Å) are summarized in Table 3.

3. Conclusions

In summary, 16 analogues of 2-benzylidene indanones were synthesized and evaluated for in vitro anticancer activity. It was found that some analogues achieved promising cytotoxicity with IC$_{50}$ values in nM against some human cancer cell lines. Of particular note is that three analogues showed improved cytotoxicity against MCF-7 breast cancer cell line. The analogues of this series also showed strong inhibition of tubulin polymerase. The best compound of the series, that is, **9j** showed 220 times higher activity than the parent indanone **7** and was found to be safe up to the 1000 mg/kg dose in Swiss-albino mice. In docking experiments **9j** showed better binding affinity with tubulin polymerase than the podophyllotoxin. In conclusion, the optimization of indanone **7** at 2-position yielded some better analogues as benzylidene indanones. The present study gives an insight to future optimization of the series.

4. Experimental

4.1. General experimental procedures

The starting substrate gallic acid was procured from S.d. Fine chemicals, India. Melting points (mp) were determined on E-Z Melt automated MP apparatus, Stanford Research System, USA and were uncorrected. Reactions were monitored on Merck precoated TLC plates, visualizing in UV (254 and 365 nm) and also by spraying with 2% ceric sulfate solution in 10% aqueous sulfuric acid followed by charring at 80–100 °C. Column chromatography was carried out on silica gel (100–200 mesh, Thomas Baker, India). FT-NMR spectra were obtained on Bruker–Avance 300 MHz using tetramethylsilane as internal standard and chemical shifts expressed in δ ppm. All the ^{1}H and ^{13}C NMR are reported. The abbreviations used in ^{1}H NMR are as; s, singlet; d, doublet; t, triplet; dd, double doublet, bs, broad singlet, bd, broad doublet, bt, broad triplet, m, multiplet. Electrospray mass were recorded on Shimadzu LC–MS after dissolving compounds in methanol. FT-IR spectra were recorded on Perkin–Elmer SpectrumBX.

4.2. Syntheses

4.2.1. Synthesis of 1-(3,4,5-trimethoxyphenyl)- 3-[3′,4′,5′-trimethoxyphenyl]-2-propen-1-one (6f)

To a stirred solution of 3,4,5-trimethoxyacetophenone (**5d**, 2.1 g, 10.0 mmol) in 7% aqueous-methanolic KOH (60 mL), 3,4,5-trimethoxybenzaldehyde (**5c**, 2.06 g, 10.5 mmol) was added after 10 min. The reaction mixture was stirred for 2 h at room temperature. After completion, the solvent was evaporated and the residue was acidified with dil. HCl (10%, 10 mL). It was extracted with ethyl acetate (3 × 20 mL), washed with water, dried over anhydrous sodium sulfate and evaporated in vacuo. The residuethus obtained was recrystallised from chloroform–hexane (10%) to get the desired chalcone (3.65 g, 94%).

mp = 128–130 °C; ^{1}H NMR (CDCl$_3$, 300 MHz): δ 3.89(s, 12H, 4×OCH$_3$), 3.94(s, 6H, 2×OCH$_3$), 6.85 (s, 2H, 2′ and 6′-CH), 7.29 (s, 2H, 2″ and 6″-CH), 7.29–735 (d, 1H, 2CH=, J = 15.6 Hz), 7.68–7.73 (d, 1H, 3-CH=, J = 15.6 Hz). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 56.69, 56.69, 56.90, 56.90, 61.40, 61.40, 106.16, 106.16, 106.66, 106.66, 121.73, 130.78, 134.02, 145.39, 153.56, 153.56, 153.56, 153.90, 153.90, 153.90, 189.86. ESIMS (MeOH): 389 [M+H]$^+$, 411 [M+Na]$^+$; IR (KBr): 2935, 1689, 1597, 1509, 1469, 1124.

4.2.2. Synthesis of 3-(3′,4′,5′-trimethoxyphenyl)-4,5,6-trimethoxy-indan-1-one (7)

1,3-Bis-(3,4,5-trimethoxyphenyl)-propenone (**6f**, 1 g, 2.58 mmol) was taken in trifluoroacetic acid (1 mL) in a sealed glass-tube and heated at 120 °C for 4 h. On completion, it was poured into cold water and extracted with ethyl acetate. The organic layer was washed with water, dried over anhydrous sodium sulfate and evaporated to get a residue. The residue was purified through silica gel column to get 3-(3′,4′,5′-trimethoxyphenyl)-4,5,6-trimethoxy-indan-1-one (**7**, 300 mg, 30%).

mp = 106–107 °C ; ^{1}H NMR (CDCl$_3$, 300 MHz): δ 2.58–2.65 (br d, 1H, 2-CH, J = 9.6 Hz), 3.13–3.22 (dd, 1H, 2-CH, J = 8.1 and 9.6 Hz) , 3.42 (s, 3H, OCH$_3$), 3.78 (s, 6H, 2×OCH$_3$), 3.81 (s, 3H, OCH$_3$), 3.93 (s, 6H, 2×OCH$_3$), 4.50–4.53 (br s, 1H, 3-CH), 6.31 (s, 2H, 2′ and 6′-CH, aromatic), 7,09 (s, 1H, 7-CH, indanone ring). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 42.30, 47.48, 56.58, 56.68, 56.68, 60.44, 61.14, 61.14, 100.81, 105.03, 132.65, 137.75, 140.42, 140.42, 144.38, 149.21, 150.89, 153.84, 153.84, 155.45, 205.08 . ESIMS (MeOH): 389 [M+H]$^+$, 411 [M+Na]$^+$; IR (KBr): 2999, 2937, 1706, 1590, 1466, 1129, 1101.

4.2.3. General procedure for the synthesis of 2-benzylidene 1-indanones (9a–o)

To a solution of Indanone **7** (250 mg, 0.65 mmol) in 2.5% methanolic potassium hydroxide (10 mL) aromatic aldehyde (**8a**–**o**, 0.72 mmol) was added. The reaction mixture was stirred at room temperature for 1–3 h. On completion, the organic solvent was evaporated and the crude mass was acidified with dil. HCl (5%) and extracted with ethyl acetate (30 mL×3), washed with water, dried over anhydrous sodium sulfate and evaporated in vacuo. The residue thus obtained was purified through column chromatography over silica gel by eluting with ethyl acetate–hexane. The purified compounds (**9a**–**o**) were recystallised from chloroform–hexane (1:4).

4.2.3.1. 3-(3′,4′,5′-Trimethoxyphenyl)-4,5,6-trimethoxy, 2-benzylidene-indan-1-one (9a). ^{1}H NMR (CDCl$_3$, 300 MHz): δ 3.43 (s, 3H, OCH$_3$), 3.69 (s, 9H, 3×OCH$_3$), 3.88 (s, 6H, 2×OCH$_3$), 5.26 (s, 1H, 3-CH), 6.40 (s, 2H, 2′ and 6′-CH, aromatic), 7.15 (s, 1H, 7-CH, indanone ring), 7.21 (br s, 3H, phenyl ring of benzylidene), 7.48 (br s, 2H, phenyl ring of benzylidene), 7.67 (s, 1H, benzylidene-CH). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 46.53, 56.57, 56.67, 56.67, 60.66, 61.25, 61.30, 101.83, 106.39, 106.39, 106.39, 128.70, 128.70, 131.43, 129.83, 131.43, 132.46, 134.79, 135.00, 137.15, 137.33, 140.31, 141.44, 149.17, 150.36, 153.34, 153.34, 155.32, 194.17. ESIMS (MeOH): 477 [M+H]$^+$, 499 [M+Na]$^+$, 515 [M+K]$^+$; IR (KBr): 3003, 2825, 1696, 1625, 1590, 1471, 1126.

4.2.3.2. 3-(3′,4′,5′-Trimethoxyphenyl)-4,5,6-trimethoxy, 2-(4″-benzyloxybenzylidene)-indan-1-one (9b). ^{1}H NMR (CDCl$_3$, 300 MHz): δ 3.48 (s, 3H, OCH$_3$), 3.76 (s, 9H, 3×OCH$_3$), 3.92 (s, 3H, OCH$_3$), 3.96 (s, 3H, OCH$_3$), 5.06 (s, 2H, OCH$_2$, benzyl), 5.29 (s, 1H, 3-CH), 6.50 (s, 2H, 2′ and 6′-CH of 3-phenyl), 6.87–6.90 (d, 2H, 2″ and 6″-CH of benzylidene ring, J = 8.4 Hz), 7.24 (s, 1H, 7-CH of indanone ring), 7.39–7.41 (br s, 5H, phenyl ring of benzylidene), 7.50–7.53 (d, 2H, 3″ and 5″-CH, J = 8.1 Hz), 7.69 (s, 1H, benzylidene-CH). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 46.75, 56.60, 56.60, 56.69, 60.68, 61.30, 61.30, 70.45, 101.80, 106.36, 106.36, 115.16, 115.16, 127.60, 127.88, 127.88, 128.58, 129.07, 129.07, 132.62, 133.67, 133.67, 134.82, 136.77, 137.12, 137.31, 137.78, 141.22, 148.88, 150.37, 153.39, 153.39, 155.24, 160.41, 194.26. ESIMS (MeOH): 583 [M+H]$^+$; IR (KBr): 3083, 2934, 1688, 1596, 1508, 1124, 1103.

4.2.3.3. 3-(3′,4′,5′-Trimethoxyphenyl)-4,5,6-trimethoxy,2-(4″-methoxybenzylidene)-indan-1-one (9c). ^{1}H NMR (CDCl$_3$, 300 MHz): δ 3.49 (s, 3H, OCH$_3$), 3.76 (s, 9H, 3×OCH$_3$), 3.82 (s, 3H, OCH$_3$), 3.91 (s, 3H, OCH$_3$), 3.94 (s, 3H, OCH$_3$), 5.29 (s, 1H, 3-CH), 6.51 (s, 2H, 2′ and 6′-CH of 3-phenyl), 6.79–6.82 (d, 2H, 2″ and 6″-CH of benzylidene ring, J = 8.7 Hz), 7.24 (s, 1H, 7-CH of indanone ring), 7.49–7.52 (d, 2H, 3″ and 5″-CH of benzylidene ring), 7.68 (s, 1H, benzylidene-CH). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 46.20, 55.25, 56.14, 56.14, 56.21, 56.21, 60.20, 60.83,101.35, 105.96, 105.96, 113.86, 113.86, 126.91, 132.16, 133.17, 133.17, 134.44, 136.72, 136.84, 137.21, 140.74, 148.41, 149.91, 152.93, 152.93, 154.78, 160.76, 193.79. ESIMS (MeOH): 507 [M+H]$^+$, 529 [M+Na]$^+$, 545 [M+K]$^+$; IR (KBr): 3080, 2937, 2836, 1691, 1602, 1470, 1129, 1104.

4.2.3.4. 3-(3′,4′,5′-Trimethoxyphenyl)-4,5,6-trimethoxy, 2-(2″-methoxybenzylidene)-indan-1-one (9d). ^{1}H NMR (CDCl$_3$, 300 MHz): δ 3.35 (s, 3H, OCH$_3$), 3.67 (s, 6H, 2×OCH$_3$), 3.69 (s, 3H, OCH$_3$), 3.74 (s, 3H, OCH$_3$), 3.89 (s, 3H, OCH$_3$), 3.93 (s, 3H, OCH$_3$), 5.23 (s, 1H, 3-CH), 6.28 (s, 2H, 2′ and 6′-CH of 3-phenyl), 6.74–6.82 (b m, 2H, 4″ and 5″-CH of benzylidene ring), 7.22–7.26 (b m, 2H, 3″-CH of benzylidene ring and 7-CH of indanone ring), 7.38 (bd, 1H, 6″-CH of benzylidene ring), 7.99 (s, 1H, benzylidene-CH). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 45.98, 55.34, 56.00, 56.00, 56.19, 60.10, 60.73, 60.81, 101.32, 105.78, 105.78, 110.46, 119.70, 123.76, 130.28, 130.56, 132.51, 136.40, 137.33, 140.53, 140.84, 148.58, 149.93, 152.62, 152.62, 154.75, 157.96, 158.33, 193.45. ESIMS (MeOH): 508 [M+2H]$^+$, 529 [M+Na]$^+$, 545 [M+K]$^+$; IR (KBr): 3083, 2941, 1695, 1621, 1469, 1335, 1125, 1099.

4.2.3.5. 3-(3′,4′,5′-Trimethoxyphenyl)-4,5,6-trimethoxy, 2-(3″,4″-dimethoxybenzylidene)-indan-1-one (9e). ^{1}H NMR (CDCl$_3$, 300 MHz): δ 3.56 (s, 3H, OCH$_3$), 3.73 (s, 6H, 2×OCH$_3$), 3.75 (s, 9H, 3×OCH$_3$), 3.86 (s, 3H, OCH$_3$), 3.90 (s, 3H, OCH$_3$), 5.28 (s, 1H, 3-CH), 6.39 (s, 2H, 2′ and 6′-CH of 3-phenyl), 6.79–6.81 (d, 1H, 5-CH of benzylidene ring, J = 8.1 Hz), 6.97 (s, 1H, 2-CH of benzylidene ring), 7.15–7.18 (d, 1H, 6-CH of benzylidene ring, J = 8.4 Hz), 7.22 (s, 1H, 7-CH of indanone ring), 7.68 (s, 1H, benzylidene-CH). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 46.83, 56.31, 56.52, 56.56, 56.56, 56.68, 60.75, 61.26, 61.30, 101.85, 106.22, 106.22, 111.22, 113.88, 126.94, 127.68, 132.47, 135.44, 137.17, 137.28, 140.91, 140.91, 148.78, 149.22, 150.40, 151.11, 153.49, 153.49, 155.27, 194.20. ESIMS (MeOH): 537 [M+H]$^+$, 559 [M+Na]$^+$, 575 [M+K]$^+$; IR (KBr): 3016, 2992, 2938, 1685, 1597, 1511, 1132, 1106.

4.2.3.6. 3-(3′,4′,5′-Trimethoxyphenyl)-4,5,6-trimethoxy, 2-(2″,4″-dimethoxybenzylidene)-indan-1-one (9f). ^{1}H NMR (CDCl$_3$, 300 MHz): δ 3.42 (s, 3H, OCH$_3$), 3.72 (s, 9H, 3×OCH$_3$), 3.77 (s, 3H, OCH$_3$), 3.80 (s, 3H, OCH$_3$), 3.89 (s, 3H, OCH$_3$), 3.93 (s, 3H, OCH$_3$), 5.22 (s, 1H, 3-CH), 6.31–6.34 (d, 1H, 6″-CH of benzylidene ring, J = 7.5 Hz), 6.34 (s,1H, 3″-CH of benzylidene ring), 6.41 (s, 2H, 2′ and 6′-CH of 3-phenyl ring), 7.23 (s, 1H, 7-CH of indanone ring), 7.39–7.42 (d, 1H, 5″-CH of benzylidene ring), 8.05 (s, 1H, benzylidene-CH). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 46.81, 55.79, 55.93, 56.54, 56.54, 56.54, 56.68, 60.62, 61.27, 98.29, 101.79, 104.91, 106.27, 106.27, 116.92, 129.89, 132.48, 133.04, 136.91, 137.77, 138.00, 141.11, 148.70, 150.36, 153.22, 153.22, 155.13, 160.81, 162.82, 194.15. ESIMS (MeOH): 537 [M+H]$^+$, 559 [M+Na]$^+$, 575 [M+K]$^+$; IR (KBr): 3079, 2938, 2835, 1682, 1600, 1469, 1503, 1125, 1100.

4.2.3.7. 3-(3′,4′,5′-Trimethoxyphenyl)-4,5,6-trimethoxy, 2-(3″,4″,5″-trimethoxybenzylidene)-indan-1-one (9g). ^{1}H NMR (CDCl$_3$, 300 MHz): δ 3.51 (s, 3H, OCH$_3$), 3.66 (s, 3H, OCH$_3$), 3.72 (s, 6H, 2×OCH$_3$), 3.74 (s, 6H, 2×OCH$_3$), 3.79 (s, 3H, OCH$_3$), 3.82 (s, 3H, OCH$_3$), 3.83 (s, 3H, OCH$_3$), 5.19 (s, 1H, 3-CH indanone ring), 6.49 (s, 2H, 2′ and 6′-CH of 3-phenyl of indanone ring), 6.61 (s, 2H, 2″ and 6″-CH of benzylidene ring), 7.13 (s, 1H, 7-CH of indanone ring), 7.55 (s, 1H, benzylidene-CH). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 46.70, 60.42, 60.42, 60.42, 60.66, 60.66, 60.66, 61.15, 61.18, 61.18, 101.78, 105.84, 106.55, 106.55, 109.30, 109.30, 130.11, 132.13, 135.30, 136.83, 136.83, 137.26, 140.11, 140.34, 150.32, 153.39, 153.46, 153.46, 153.46, 155.27, 193.93. ESIMS (MeOH): 567 [M+H]$^+$, 589 [M+Na]$^+$, 605 [M+K]$^+$; IR (KBr): 3083, 2940, 2837, 1689, 1590, 1505, 1128.

4.2.3.8. 3-(3′,4′,5′-Trimethoxyphenyl)-4,5,6-trimethoxy, 2-(2″,3″,4″-trimethoxybenzylidene)-indan-1-one (9h). ^{1}H NMR (CDCl$_3$, 300 MHz): δ 3.39 (s, 3H, OCH$_3$), 3.67 (s, 9H, 3×OCH$_3$), 3.72 (s, 3H, OCH$_3$), 3.75 (s, 3H,OCH$_3$), 3.80 (s, 3H, OCH$_3$), 3.87 (s, 3H, OCH$_3$), 3.90 (s, 3H, OCH$_3$), 5.21(s, 1H, 3-CH), 6.32 (s, 2H, 2′ and 6′-CH, aromatic),6.48–6.51 (d, 1H, 5″-CH of benzylidene ring), 7.10–7.14 (d, 1H, 6″-CH of benzylidene ring), 7.21 (s, 1H, 7-CH, aromatic), 7.88 (s, 1H, benzylidene-CH). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 46.04, 55.93, 56.05, 56.05, 56.05, 56.19, 60.14, 60.78, 60.78, 61.53, 101.32, 105.79, 105.79, 106.65, 121.72, 125.76, 129.71, 132.49, 136.53, 137.30, 139.43, 140.67, 141.98, 148.45, 149.89, 152.73, 152.73, 153.72, 154.75, 154.97, 193.39. ESIMS (MeOH): 568 [M+2H]$^+$, 589 [M+Na]$^+$, 605 [M+K]$^+$; IR (KBr): 3071, 2937, 1686, 1592, 1496, 1304, 1126, 1094.

4.2.3.9. 3-(3′,4′,5′-Trimethoxyphenyl)-4,5,6-trimethoxy, 2-(2″,4″,5″-trimethoxybenzylidene)-indan-1-one (9i). ^{1}H NMR (CDCl$_3$, 300 MHz): δ 3.55 (s, 3H, OCH$_3$), 3.63 (s, 3H, OCH$_3$), 3.72 (s, 3H, OCH$_3$), 3.75 (s, 9H, 3×OCH$_3$),3.88 (s, 3H, OCH$_3$), 3.91 (s, 3H, OCH$_3$), 3.92 (s, 3H, OCH$_3$), 5.26 (s, 1H, 3-CH), 6.44 (s, 1H, 3″-CH of benzylidene ring), 6.54 (s, 2H, 2′ and 6′-CH of 3-phenyl ring), 6.93 (s, 1H, 6″-CH of benzylidene ring), 7.23 (s, 1H, 7-CH of indanone ring), 8.79 (s, 1H, benzylidene-CH). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 46.39, 55.87, 55.87, 55.87, 55.98, 56.20, 56.37, 60.24, 60.73, 60.80, 96.44, 101.37, 105.70, 105.70, 114.28, 115.12, 129.27, 132.32, 136.57, 136.57, 137.13, 140.29, 142.62, 148.10, 149.90, 151.95, 152.90, 152.90, 154.69, 155.14, 193.65. ESIMS (MeOH): 567 [M+H]$^+$, 589 [M+Na]$^+$, 605 [M+K]$^+$; IR (KBr):3080, 2937, 1678, 1601, 1507, 1472, 1309, 1207, 1126.

4.2.3.10. 3-(3′,4′,5′-Trimethoxyphenyl)-4,5,6-trimethoxy,2-(3″,4″-methylenedioxybenzylidene)-indan-1-one (9j). ^{1}H NMR (CDCl$_3$, 300 MHz): δ 3.43 (s, 3H, OCH$_3$), 3.76 (s, 9H, 3×OCH$_3$), 3.91 (s, 3H, OCH$_3$), 3.94 (s, 3H, OCH$_3$), 5.26 (s, 1H, 3-CH), 5.94–5.96 (br s, 2H, O-CH$_2$-O), 6.49 (br s, 2H, 2′ and 6′-CH of 3-phenyl), 6.73–6.74 (d, 1H, 5″-CH of benzylidene ring), 7.05–7.10 (m, 2H, 2″-CH and 6″-CH of benzylidene ring), 7.28 (s, 1H, 7-CH of indanone ring), 7.61 (s, 1H, benzylidene-CH). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 46.71, 56.58, 56.58, 56.58, 60.61, 61.26, 61.26, 101.78, 101.90, 106.49, 106.49, 108.70, 110.69, 127.99, 128.95, 132.53, 134.87, 137.20, 137.20, 138.17, 141.22, 148.23, 148.96, 149.37, 150.34, 153.43, 153.43, 155.28, 194.09. ESIMS (MeOH): 521 [M+H]$^+$, 543 [M+Na]$^+$, 559 [M+K]$^+$; IR (KBr): 3081, 2940, 1699, 1599, 1491, 1469, 1334, 1270, 1106.

4.2.3.11. 3-(3′,4′,5′-Trimethoxyphenyl)-4,5,6-trimethoxy,2-(2″,3″-methylenedioxybenzylidene)-indan-1-one (9k). ^{1}H NMR (CDCl$_3$, 300 MHz): δ 3.40 (s, 3H, OCH$_3$), 3.70 (s, 3H, OCH$_3$), 3.71 (s, 6H, 2×OCH$_3$), 3.91 (s, 3H, OCH$_3$), 3.94 (s, 3H, OCH$_3$), 5.31 (s, 1H, 3-CH), 5.95 and 6.05 (two distinct s, 2H, –O–CH$_2$–O), 6.39 (s, 2H, 2′ and 6′-CH, aromatic), 6.65–6.70 (m, 2H, 4″and 6″-CH of benzylidene ring), 6.81–6.90 (dd, 1H, 5″-CH of benzylidene ring), 7.23 (s, 1H, 7-CH of indanone), 7.71 (s, 1H, benzylidne-CH). ^{13}C NMR (CDCl$_3$, 75 MHz): δ 45.99, 56.03, 56.03, 56.19, 60.13, 60.74, 60.83, 100.98, 101.33, 105.73, 105.73, 109.17, 117.15, 121.07, 122.54, 127.32, 132.22, 136.55, 137.04, 140.79, 141.37, 146.80, 147.26, 148.79, 149.90, 152.70, 152.70, 154.80, 193.15. ESIMS (MeOH): 521[M+H]$^+$, 543 [M+Na]$^+$; IR (KBr): 3084, 2937, 1701, 1591, 1507, 1458, 1251, 1125, 1110.

4.2.3.12. 3-(3′,4′,5′-Trimethoxyphenyl)-4,5,6-trimethoxy,2-(4″-nitrobenzylidene)-indan-1-one (9l). ^1H NMR ($CDCl_3$, 300 MHz): δ 3.44 (s, 3H, OCH_3), 3.71 (s, 9H, $3\times OCH_3$), 3.92 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 5.27 (s, 1H, 3-CH), 6.37 (s, 2H, 2′ and 6′-CH of 3-Phenyl), 7.22 (s, 1H, 7-CH of indanone ring), 7.57–7.60 (d, 2H, 2″ and 6″-CH of benzylidne ring), 7.67 (s, 1H, benzylidene-CH), 8.08–8.11 (d,2H, 3″ and 5″-CH of benzylidene ring). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 46.50, 56.63, 56.63, 56.71, 60.69, 61.29, 61.36, 101.89, 106.35, 106.35, 123.79, 123.79, 131.65, 131.65, 131.78, 131.98, 136.80, 137.47, 141.26, 141.35, 143.96, 147.94, 149.71, 150.35, 153.57, 153.57, 155.61, 193.41 . ESIMS (MeOH): 544 $[M+Na]^+$, 560 $[M+K]^+$ negative mode: 520 $[M-H]^-$; IR (KBr): 3079, 2939, 1693, 1592, 1507, 1469, 1343, 1124, 1109.

4.2.3.13. 3-(3′,4′,5′-Trimethoxyphenyl)-4,5,6-trimethoxy,2-(3″-nitrobenzylidene)-indan-1-one (9m). ^1H NMR ($CDCl_3$, 300 MHz): δ 3.43 (s, 3H, OCH_3), 3.71 (s, 3H, OCH_3), 3.76 (s, 6H, $2\times OCH_3$), 3.93 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 5.38 (s, 1H, 3-CH), 6.48 (s, 2H, 2′ and 6′-CH of 3-phenyl), 7.10–7.18 (m, 1H, 5″-CH of benzylidene ring), 7.26 (s, 1H, 7-CH of indanone ring), 7.45–7.50 (d, 1H, 6″-CH of benzylidene ring), 7.69 (s, 1H, 2″-CH of benzylidene ring), 8.10–8.13 (d, 1H, 4″-CH of benzylidene ring), 8.46 (s, 1H, benzylidene-CH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 46.24, 56.05, 56.05, 56.29, 60.24, 60.79, 60.92, 101.44, 105.89, 105.89, 123.75, 124.51, 127.62, 128.08, 129.36, 131.17, 131.52, 135.80, 135.80, 136.88, 137.43, 141.16, 142.51, 148.14, 149.27, 153.09, 155.15, 193.31. ESIMS (MeOH): 521 $[M]^+$, 544 $[M+Na]^+$, Negative mode: 520 $[M-H]^-$; IR (KBr): 3079, 2924, 1696, 1627, 1590, 1525, 1466, 1351, 1124, 1107.

4.2.3.14. 3-(3′,4′,5′-Trimethoxyphenyl)-4,5,6-trimethoxy,2-(4″-fluorobenzylidene)-indan-1-one (9n). ^1H NMR ($CDCl_3$, 300 MHz): δ 3.47 (s, 3H, OCH_3), 3.73 (s, 9H, $3\times OCH_3$), 3.93 (s, 6H, $2\times OCH_3$), 5.25 (s, 1H, 3-CH), 6.43 (s, 2H, 2′ and 6′-CH of 3-phenyl), 6.93–6.99 (t, 2H, 2″ and 6″-CH of benzylidene ring), 7.27 (s, 1H, 7-CH of indanone ring), 7.46–7.51 (t, 2H, 3″ and 5″-CH of benzylidene ring), 7.65 (s, 1H, benzylidene-CH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 46.53, 56.59, 56.59, 56.69, 60.67, 61.29, 61.29, 101.81, 106.30, 106.30, 115.76, 116.05, 130.95, 132.35, 133.39, 133.39, 133.50, 133.75, 137.09, 137.21, 139.83, 141.31, 149.17, 150.37, 153.43, 153.43, 155.36, 194.03. ESIMS (MeOH): 495 $[M+H]^+$, 517 $[M+Na]^+$; IR (KBr): 3075, 2937, 1692, 1599, 1508, 1314, 1126, 1112.

4.2.3.15. 3-(3′,4′,5′-Trimethoxyphenyl)-4,5,6-trimethoxy,2-(4″-trifluoromethylbenzylidene)-indan-1-one (9o). ^1H NMR ($CDCl_3$, 300 MHz): δ 3.45 (s, 3H, OCH_3), 3.73 (s, 9H, $3\times OCH_3$), 3.92 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 5.29 (s, 1H, 3-CH), 6.36 (s, 2H, 2′ and 6′-CH of 3-phenyl), 7.24 (s, 1H, 7-CH of indanone ring), 7.51–7.57 (br s, 4H, 2″, 3″, 5″ and 6″-CH of benzylidene ring), 7.69 (s, 1H, benzylidene-CH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 46.40, 56.57, 56.57, 56.70, 60.68, 61.27, 61.35, 101.84, 106.22, 106.22, 125.51, 125.56, 125.56, 131.15, 132.22, 132.22, 132.22, 132.98, 137.07, 137.29, 138.45, 141.38, 142.68, 149.51, 150.37, 153.46, 153.46, 155.50, 193.72. ESIMS (MeOH): 545 $[M+H]^+$, 567 $[M+Na]^+$; IR (KBr): 3078, 2935, 2831, 1695, 1627, 1591, 1473, 1426, 1321, 1290, 1139, 1171, 1102.

4.2.3.16. 3-(3′,4′,5′-Trimethoxyphenyl)-4,5,6-trimethoxy, 2-(4″-hydroxybenzyl)-indan-1-one (9p). To a solution of benzylidene indanone **9b** (200 mg, 0.34 mmol) in dry THF (10 mL), 10% Pd–C (500 mg) was added and stirred for 6 h at room temperature. On completion, the reaction mixture was filtered through celite, washed with methanol. The filterate was evaporated and the residue thus obtained was purified through silica gel column by eluting with ethyl acetate-hexane to get desired benzyl-indanone **9p**. ^1H NMR ($CDCl_3$, 300 MHz): δ 2.58–2.66 (bt, 1H, benzylic, *J* = 3.6 Hz), 2.82 (br s, 1H, benzylic), 3.31 (distorted doublet, 1H, 2-CH), 3.39 (s, 3H, OCH_3), 3.61 (s, 9H, $3\times OCH_3$), 3.92 (s, 6H, $2\times OCH_3$), 4.57–4.59 (br d, 1H, 3-CH, *J* = 6.6 Hz), 5.93 (br s, 2H, 2′ and 6’-CH of 3-phenyl), 6.13 (s, 1H, exchangeable, OH, Phenolic), 6.69–6.78 (m, 2H, 3″ and 5″-CH of benzyl ring),7.07–7.27 (m, 3H, 2″and 6″-CH of benzyl ring and 7-CH of indanone ring). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 47.92, 56.35, 56.35, 56.63, 60.56, 61.25, 61.37, 100.83, 101.03, 104.39, 104.39, 115.30, 115.79, 130.01, 130.96, 130.96, 131.22, 131.22, 136.49, 140.39, 143.38, 150.84, 153.10, 153.37, 154.56, 155.18, 155.50, 207.41. ESIMS (MeOH): 495 $[M+H]^+$, 517 $[M+Na]^+$, Negative mode: 493 $[M-H]^-$; IR (KBr): 3423, 2938, 1702, 1595, 1508, 1238, 1126.

4.3. Biological evaluation

4.3.1. Cell culture

Human cancer cell lines MCF-7 (breast), HCT (colon) and THP-1 (Leukemia) and A549 (Lung) were American type of cell culture collection (ATCC) obtained from National Cancer Institute (NCI), Frederick, USA. Cells were grown in tissue culture flasks in complete growth medium (RPMI-1640 medium) with 2 mM glutamine, pH 7.4 supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin and 100 units/mL penicillin in a CO_2 incubator (37 °C, 5% CO_2, 90% RH). The cells at subconfluent stage were harvested from the flask by treatment with trypsin (0.05% in PBS, pH 7.4, containing 0.02% EDTA). Cells with viability of more than 98% were used for the determination of cytotoxicity. The required concentration of cells in cell suspension was prepared in complete growth medium.

4.3.2. Cytotoxicity evaluation by sulphorhodamine B assay

Stock solutions of 2×10^{-2} M of compounds were prepared in DMSO and further dilution was carried out with complete growth medium containing 50 μg/mL of gentamycin to obtain working test solutions of required concentration. In vitro cytotoxicity against various human cancer cell lines was determined using 96 well cell culture plates. The 100 μM of cell suspension was added to each well of the 96 well cell culture plates. The cells were allowed to grow in CO_2 incubator (37 °C, 5% CO_2, 90% RH) for 24 h. The test material in complete growth medium (100 μL) was added after 24 h incubation to the wells containing cell suspension. The plates were further incubated for 48 h (37 °C in an atmosphere of CO_2 and 90% relative humidity) in a CO_2 incubator and then the cell growth was stopped by gently layering trichloroacetic acid (50% TCA, 50 μL) on top of the medium in all the wells. The plates were incubated at 4 °C for 1 h to fix the cells attached to the bottom of the wells. The liquid portion of all the wells were gently pipetted out and discarded. The plates were washed five times with distilled water to remove TCA, growth medium, low molecular weight metabolites, serum proteins etc. and air dried. Cell growth was measured by staining with Sulphorhodamine B dye. The adsorbed dye was dissolved in Tris–HCl buffer (100 μM/well, 0.01 M, pH 10.4) and the plates were gently stirred for 10 min on a mechanical stirrer. The optical density (OD) was recorded on ELISA reader at 540 nm. paclitaxel, 5-fluorouracil, adriamycin and tamoxifen were used as standard anticancer drugs.

4.3.3. In vitro tubulin polymerization inhibition assay

The tubulin polymerization experiment was performed using ‘assay kit’ (Cytoskeleton USA) by following manufacturer’s protocol. Briefly, tubulin protein (3 mg/mL) in tubulin polymerization buffer (80 mM PIPES, pH 6.9, 2 mM $MgCl_2$, 0.5 mM EGTA, 1 mM GTP and 15% glycerol) was placed in pre-warmed 96-well microtiter plates at 37 °C in the presence of test compounds with variable concentrations. All samples were mixed well and polymerization was monitored kinetically at 340 nm every min for 1 h using Spectramax plate reader. Podophyllotoxin was used as standard inhibitor of tubulin polymerase and DMSO as negative control. The IC_{50} value was determined from dose-dependent analysis and is defined as the concentration that inhibits the rate of polymerization by 50%.

4.3.4. In vivo acute oral toxicity in Swiss-albino mice

In view of potent anti-cancer activity of **9j**, acute oral toxicity of the same was carried out in Swiss-albino mice for its further development into drug product. Experiment was conducted in accordance with the Organization for Economic Co-operation and Development (OECD) test guideline No 423 (1987).

For the study, 30 mice (15 male and 15 female) were taken and divided into five groups comprising 3 male and 3 female mice in each group weighing between 20–25 g. The animals were maintained at 22 ± 5 °C with humidity control and also on an automatic dark and light cycle of 12 h. The animals were fed with the standard rat feed and provided ad libitum drinking water. Mice of group 1 were kept as control and animals of groups 2, 3, 4 and 5 were kept as experimental. The animals were acclimatized for 7 days in the experimental environment prior to the actual experimentation. The compound **9j** was suspended by ultrasonication in cremophore EL (20% in distilled water) using traces of ethanol as a co-solvent and was given at 5, 50, 300 and 1000 mg/kg body weight to animals of groups 2, 3, 4 and 5, respectively. Control animals received only vehicle.

4.3.5. Observational, hematological, biochemical and gross pathological study

The animals were checked for mortality and any signs of ill health at hourly interval on the day of administration of drug and there after a daily general case side clinical examination was carried out including changes in skin, mucous membrane, eyes, occurrence of secretion and excretion and also responses like lachrymation, pilo-erection respiratory patterns etc. Also changes in gait, posture and response to handling were recorded. In addition to observational study, body weights were recorded, blood and serum samples were collected from all the animals on 7th day of the experiment and were analysed for total RBC, WBC, differential leukocyte count, hemoglobin percentage and biochemical parameters like total cholesterol, triglycerides, creatinine, SGPT and SGOT activity. The animals were then sacrificed and were necropsed for any gross pathological changes. Weights of vital organs like liver, heart, kidney etc. were recorded.

4.3.6. Molecular docking experiments

The chemical structures of the derivatives were constructed using the Scigress Explorer v.7.7.0.47 (formerly CaChe) software package (Fujitsu Ltd., Tokyo, Japan). Energy minimization of the compounds with cleaned geometries was achieved through the MO-G application in Scigress, which computes and minimizes an energy related to the heat of formation. MO-G solves the Schrödinger equation for the best molecular orbital and geometry of the ligand molecule. The augmented molecular mechanics (MM2/MM3) parameter was used to optimize the energy of each molecule up to its lowest stable energy state. This energy minimization process was performed until the energy change was less than 0.001 kcal mol^{-1} or the molecules had been updated almost 300 times.[25]

3D chemical structure of podophyllotoxin was retrieved from the PubChem compound database at NCBI (http://www.pubchem.ncbi.nlm.nih.gov). Crystallographic 3D structures of target proteins were retrieved from the Brookhaven Protein Databank (http://www.pdb.org). The valency and hydrogen bonding of each ligand as well as each target protein were subsequently checked using the Workspace module of the Scigress Explorer software. Hydrogen atoms were added to the protein targets to achieve the correct ionization and tautomeric states of amino acid residues such as His, Asp, Ser, and Glu.

Molecular docking of the compounds against selected target was achieved using the FastDock Manager and FastDock Compute engines that are available with the Scigress Explorer software. To perform the automated docking of ligands into the active sites, we used a genetic algorithm with a fast and simplified potential of mean force (PMF) scoring scheme. Other parameters were left as their default values. Structure-based screening involves docking candidate ligands into protein targets and then applying a PMF scoring function to estimate the likelihood that the ligand will bind to the protein with high affinity.[26]

The spectra of the compounds are available with this article in the online version.

Acknowledgements

The authors are very much thankful to the Director, CSIR–CIMAP for constant encouragement and support. The CSIR Diamond Jubilee Research Internship to one of the authors (APP) is duly acknowledged. This research was supported by Council of Scientific and Industrial Research (CSIR), India. The most of the work is covered in Indian patent (No. 133NF2011) and US patent Provisional application no. 2769DEL2011, dt. 22-09-2011.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.02.057.

References and notes

1. WHO Factsheet No. 297, Feb. 2011.
2. Cancer facts and figures, 2009.
3. Pettit, G. R.; Singh, S. B.; Boyd, M. R.; Hamel, E.; Pettit, R. K.; Schmidt, J. M.; Hogan, F. *J. Med. Chem.* **1995**, *38*, 1666.
4. Tron, G. C.; Pirali, T.; Sorba, G.; Pagliai, F.; Busacca, S.; Genazzani, A. A. *J. Med. Chem.* **2006**, *49*, 3033.
5. Kingston, D. G. I. *J. Nat. Prod.* **2009**, *72*, 507.
6. (a) Zweifel, M.; Jayson, G. C.; Reed, N. S.; Osborne, R.; Hassan, B.; Ledermann, J.; Shreeves, G.; Poupard, L.; Lu, S. P.; Balkissoon, J.; Chaplin, D. J.; Rustin, G. J. *Ann. Oncol.* **2011**, *22*, 2036; (b) Ng, Q. S.; Mandeville, H.; Goh, V.; Alonzi, R.; Milner, J.; Camell, D.; Meer, K.; Padhani, A. R.; Aunders, M. I.; Hoskin, P. J. *Ann. Oncol.* **2011**. http://dx.doi.org/10,1093/annonc/mdr332.
7. (a) He, X.; Li, S.; Huang, H.; Zhiming, L.; Chen, L.; Ye, S.; Huang, J.; Zhang, J.; Lin, T. *Brit. J. Clin. Pharmacol.* **2011**, *71*, 860; (b) Kanthou, C.; Tozer, G. M. *Expert Opin. Ther. Targets* **2007**, *11*, 1443; (c) Banerjee, S.; Wang, Z.; Mohammad, M.; Sarkar, F. H.; Mohammad, R. M. *J. Nat. Prod.* **2008**, *71*, 492.
8. (a) Ding, X.; Zhang, Z.; Li, S.; Wang, A. *Oncol. Res.* **2011**, *19*, 303; (b) Lin, C. M.; Ho, H. H.; Pettit, G. R.; Hamel, E. *Biochemistry* **1989**, *28*, 6984.
9. Ducki, S.; Mackenzie, G.; Lawrence, N. J.; Snyder, J. P. *J. Med. Chem.* **2005**, *48*, 457.
10. (a) Nagaiah, G.; Remick, S. C. *Future Oncol.* **2010**, *6*, 1219; (b) Ahmed, B.; Van, E. L. I.; Bouma-Ter, S. J. C.; Van Der, S. D. W.; Van, E. A. M.; Joosten-Achjanie, S. R.; Lambin, P.; Landuvt, W.; Griffioen, A. W. *Int. J. Cancer* **2003**, *105*, 20.
11. Srivastava, V.; Negi, A. S.; Kumar, J. K.; Gupta, M. M.; Khanuja, S. P. S. *Bioorg. Med. Chem.* **2005**, *13*, 5892.
12. Saxena, H. O.; Faridi, U.; Srivastava, S.; Kumar, J. K.; Darokar, M. P.; Luqman, S.; Chanotiya, C. S.; Krishna, V.; Negi, A. S.; Khanuja, S. P. S. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 3914.
13. Saxena, H. O.; Faridi, U.; Kumar, J. K.; Luqman, S.; Darokar, M. P.; Shanker, K.; Chanotiya, C. S.; Gupta, M. M.; Negi, A. S. *Steroids* **2007**, *72*, 892.
14. Srivastava, V.; Saxena, H. O.; Shanker, K. S.; Kumar, J. K.; Luqman, S.; Gupta, M. M.; Khanuja, S. P. S.; Negi, A. S. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 4603.
15. Srivastava, V.; Negi, A. S.; Kumar, J. K.; Faridi, U.; Sisodia, B. S.; Darokar, M. P.; Luqman, S.; Khanuja, S. P. S. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 911.
16. Labrie, F. *Nat. Clin. Pract. Endocrinol. Metabol.* **2007**, *3*, 584.
17. *Book: Vogel's Text Book of Practical Organic Chemistry*; Furniss, B. S., Hannaford, A. J., Smith, P. W. G., Tatchell, A. R., Eds., 5th ed.; Longman: England, 1998; p 395.
18. Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMohan, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst.* **1990**, *82*, 1107.
19. Shelanski, M. L.; Gaskin, F.; Cantor, C. R. *Proc. Natl. Acad. Sci.* **1973**, *70*, 765.

20. Lee, J. C.; Timasheff, S. N. *Biochemistry* **1977**, *16*, 1754.
21. Wang, L. G.; Liu, X. M.; Kreis, W.; Budman, D. R. *Cancer Chemother. Pharmacol.* **1999**, *44*, 355.
22. Jordan, M. A.; Wilson, L. *Nat. Rev. Cancer* **2004**, *4*, 253.
23. Allan, J. J.; Damodaran, A.; Deshmukh, N. S.; Goudar, K. S.; Amit, A. *Food Chem. Toxicol.* **2007**, *45*, 1928.
24. *Fundamentals of Experimental Pharmacology*; Ghosh, M. N., Ed.1st ed.; Scientific Book Agency: Kolkata, 1984; p 156.
25. Yadav, D. K.; Khan, F.; Negi, A. S. *J. Mol. Model.* **2011**. http://dx.doi.org/10.1007/s00894-011-1265-3.
26. Meena, A.; Yadav, D. K.; Srivastava, A.; Khan, F.; Chanda, D. *Chem. Biol. Drug Dis.* **2011**, *78*, 567.