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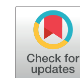
Research paper

Antiproliferative efficacy of curcumin mimics through microtubule destabilization



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ABSTRACT

Curcumin possesses an attractive chemical structure with highly conjugated diferuloylmethane core. Curcumin mimics have been designed and prepared with an additional bridged phenyl ring in conjugation. Fourteen diverse analogues were evaluated against a panel of human cancer cell lines. The best analogue of the series i.e. compound **6a** exhibited potent cytotoxicity against A431, epidermoid carcinoma cell line ($IC_{50} = 1.5$ μM) and DLD1, colorectal adenocarcinoma cell line ($IC_{50} = 6.9$ μM). In tubulin kinetics experiment, compound **6a** destabilized polymerisation process ($IC_{50} = 4.68$ μM). In cell cycle analysis, compound **6a** exerted G2/M phase arrest in A431 cells and induced apoptosis. In Ehrlich Ascites Carcinoma in Swiss-albino mice, compound **6a** showed 78.6% tumour reduction at 80 mg/kg dose and 57% solid tumour reduction at 150 mg/kg dose. Further, in acute-oral toxicity experiment in rodent model, compound **6a** was given in three different oral doses to Swiss albino mice. There were non-significant changes in various biochemical parameters and major body organs studied, including their absolute and relative weights. It was tolerable up to 300 mg/kg dose in Swiss-albino mice. The present study shows that the novel curcumin mimic **6a** is a safe and efficacious anticancer compound. However, it needs to be optimized for better efficacy.

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1. Introduction

Skin cancer is the most common form of cancer accounting approximately 40% of cases. It is an abnormal growth of skin cells occurring due to the development of abnormal cells that have the ability to spread to other parts of the body. There are three main types of skin cancer i.e. basal cell carcinoma, squamous cell carcinoma and melanoma. Among these, melanoma is the most aggressive. According to WHO, there are 2–3 million non-melanoma skin cancer and 0.23 million melanoma skin cancer cases globally at present [1,2]. More than 90% of skin cancer cases are caused by exposure to UV radiation victimizing mostly light skin people. Common treatments are surgical removal, radiation therapy and topical medication of US-FDA approved drugs like fluorouracil, imiquimod, cobimetinib etc [3] (Fig. 1). Globally, skin cancer causes around one lakh deaths annually. There is still a need for an efficient anticancer drug to tackle this disease.

Curcumin is a well known plant phenolic compound abundantly available in the rhizomes of *Curcuma longa* (Zinziberaceae). Curcumin, demethoxycurcumin and bisdemethoxycurcumin are three main components known as curcuminoids. Curcumin has been a hot molecule due to its broad range of pharmacological properties [4] like anticancer [5], anti-inflammatory [6], immunomodulatory, antibacterial [7], antimalarial activities. However, its most of the pharmacological properties are attributed to its antioxidant property. Despite of potential anticancer, antiinflammatory and

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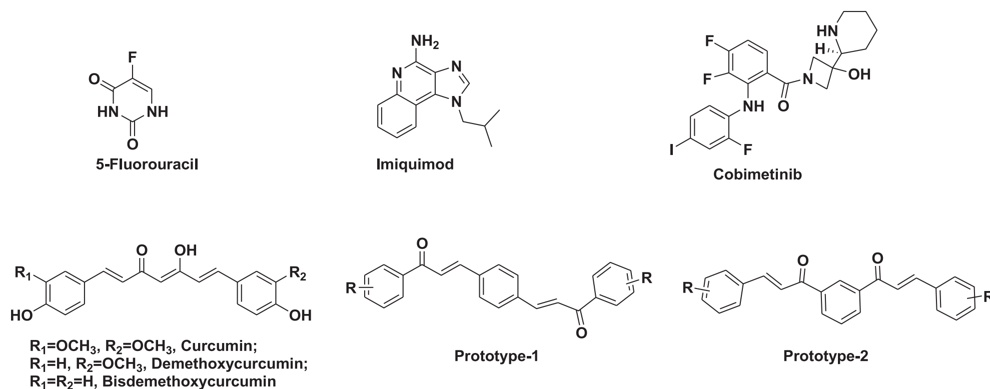


Fig. 1. Structure of three clinical drugs of skin cancer, curcuminoids and designed prototypes 1 & 2 as curcumin mimics.

immunomodulatory activities, curcumin could not be developed as a drug because of its poor bioavailability and sometimes referred as pseudo-drug [8].

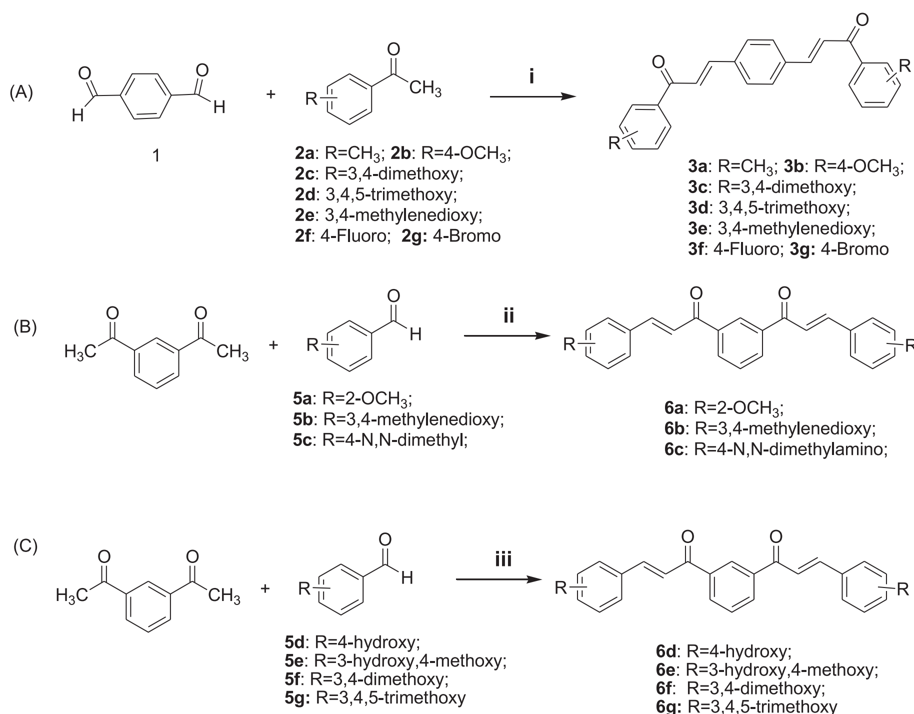
Curcumin possesses an attractive chemical structure with highly conjugated diferuloylmethane core. Two phenolic aromatic rings are linked through a conjugated aliphatic linker chain. It is in keto-enol form which is stabilized through hydrogen bonding. Researchers have utilized the structural features of curcuminoids to prepare various curcumin mimics [4]. In the present communication, we have done detailed pharmacology of potential curcumin mimics possessing an additional aromatic ring in between the aliphatic linker chain to enhance the conjugation of the system for better efficacy (Fig. 1). Analogue **6a** exhibited potential cytotoxicity against A431 skin cancer cell line, which was further evaluated for mechanistic study, and preclinical efficacy and toxicity in rodent models.

2. Results

2.1. Chemistry

2.1.1. Synthesis of curcumin mimics

The synthetic strategy was as depicted in Scheme-1. Three different approaches were adopted to prepare these curcumin mimics. For prototype-I, 1,4-terephthaldehyde was the starting substrate, which was condensed with various acetophenones in 7% methanolic alkali to afford desired compounds **3a**-**3g** in 53–96% yields. 1,3-Diacetylbenzene was the starting substrate for prototype-II compounds. The same methodology was used i.e. 7% methanolic KOH to get compounds **6a**-**6c** in moderate yields (60–78%). Some of the representative compounds of prototype-II were prepared by using 3% conc. HCl in dioxane to yield the desired curcumin mimics **6d**-**6g** in 69–96% yield. All the products



Scheme 1. i) 7% KOH in MeOH, RT, 7–8 h, 53–96%; ii) 7% KOH in MeOH, RT, 7–12 h, 60–78%; iii) 5% Con HCl in Dioxane, reflux, 7–8 h, 69–96%.

were purified through flash chromatography using silica gel (230–400 mesh) as adsorbent and ethyl acetate-hexane as eluants. Compounds were confirmed by spectroscopy.

2.1.2. Isolation of curcuminoids

Curcumin (CUR), demethoxycurcumin (DC) and bisdemethoxycurcumin (BDC) were isolated from the rhizomes of *Curcuma longa* obtained from Central Research Farm, CIMAP, Lucknow. The dried and ground rhizomes (100 g) were extracted with chloroform (1000 mL) in a soxhlet apparatus for 5 h to get curcumin rich extract (3.84 g). The crude mass was purified through Flash chromatography using glass column (230 mmX25 mm, lengthXdia) and silica gel (230–400 mesh, 118 g) and eluted successively with 20%, 40%, 60%, 80% chloroform-hexane, chloroform, 1%, 2%, 3%, 4%, 5%, 6% acetone-chloroform and 1%, 2%, 3% 4% methanol-chloroform. The similar fractions were pooled to get CUR (3% acetone-chloroform), DC (5% acetone-chloroform) and BDC (3% methanol-chloroform). The solvent was distilled off to get pure compounds in powder form. These were confirmed by spectroscopy.

2.1.3. Purity profile of compound ***6a*** by UPLC

The integration on the peaks was performed at λ_{max} i.e. 300 nm with data acquisition in PDA (190–400 nm). The normalized peak area of total impurities, the retention time of ***6a***, and the resolutions between ***6a*** and its adjacent peaks were selected as optimization criteria. Under optimized chromatographic condition, ***6a*** was eluted at 2.150 min without any interference of neighbouring peaks. As a reference method, the functions for peak purity analysis in the chromatographic data processing by Empower were applied. The purity of the synthesized ***6a*** was found to be 97.30%

2.2. Biological evaluation

2.2.1. Antiproliferative activity against human cancer cell lines

All the curcumin mimics (**3a**-**3g** and **6a**-**6g**) were evaluated for cytotoxicity against a panel of human cancer cell lines by MTT assay [9]. Human cancer cell lines, A549 (Lung), MCF-7 (Breast), WRL-68 (Liver), DLD1 (Colorectal), and A431 (Epidermoid) were originally obtained from American type of cell culture collection (ATCC), USA and stock were maintained in laboratory. Tamoxifen and podophyllotoxin were used as standard anticancer drugs (positive control) for cytotoxicity while natural CUR, DC, and BDC were used for comparison purpose. $IC_{50} > 100\ \mu M$ was considered as inactive. For structure activity relationship, we mainly took A431 cell line data into consideration. We had two structural differentiated series i.e. Series I of 4-bis (*E/E*) phenyl)-3-oxo-benzene (compounds **3a**-**3g**) and Series II of 1,3-bis (*E/E*) phenyl-1-oxo-2-enyl benzene (Compounds **6a** to **6g**). Series I compounds were not much effective as compared to Series II compounds. In Series II compounds **6a**, **6b**, **6f** and **6g** exhibited potential cytotoxicity. All the four analogues possessed methoxy or methylenedioxy substitutions in both the terminal phenyl rings. But, more the number of alkoxy substitutions less was the cytotoxicity. Compound **6a** with one alkoxy group was the best analogue ($IC_{50} = 1.5\ \mu M$) of the series. Presence of two methoxy groups in both the terminal phenyl rings decreased cytotoxicity of compounds **6f** ($IC_{50} = 6.9\ \mu M$) while introducing third methoxy group as in compound **6g** cytotoxicity was reduced drastically ($IC_{50} = 10\ \mu M$). Presence of other substitutions like hydroxyl or N,N-dimethylamino in the both terminal phenyl rings either reduced the activity or were inactive at 100 μM concentration.

Compounds **6a** and **6f** were further evaluated for interaction with tubulin polymerisation process (cytoskeleton USA) [10,11]. Podophyllotoxin (PDT), a standard microtubule destabilizer was used as control positive for antitubulin effect and curcumin was used for comparison purpose. The cytotoxicity and antitubulin data are represented in Table 1.

2.2.2. Soft agar colony assay

Soft agar assay allows for semi-quantitative evaluation by utilizing the ability of cells to grow in soft agar and form colony. It is the gold standard assay for cellular transformation *in-vitro* [12]. Hence, the cytotoxicity of compound **6a** was further confirmed by Soft agar colonisation assay [13]. It suppressed growth of A431 cells by 13–99% at 1.5–50 μg/mL concentrations ($IC_{50} = 6.2\ \mu g/mL$) (Table 2). The inhibition of A431 colony formation by **6a** clearly indicated potential cytotoxicity of it, which is quite significant and concentration dependent (Fig. 2). Soft agar colonisation assay is used to confirm cellular anchorage-independent growth *in-vitro* and it is an astringent method to detect the tumorigenic potential of transformed cells and tumour suppressive effects on transformed cells [14].

2.2.3. Cell cycle analysis

Cell division cycle is an ordered set of events culminating in cell growth and cell division into two daughter cells after DNA duplication. In cell cycle analysis compound **6a** (Fig. 3) induced G2/M phase arrest at concentration dependent manner. Flow cytogram

Table 1
In-vitro cytotoxicity of curcumin mimics against various human cancer cell lines by MTT assay (IC_{50} in μM).

S. no.		A549	A431	WRL-68	MCF-7	DLD1	Tubulin Polymerisation inhibition IC_{50} in μM
1.	**3a**	Inactive	68.7	Inactive	Inactive	>40	–
2.	**3b**	Inactive	Inactive	Inactive	64.6	>40	–
3.	**3c**	85.6	96.7	41.7	Inactive	>40	–
4.	**3d**	32	18.6	70.9	Inactive	16.1	–
5.	**3e**	Inactive	Inactive	80.1	30.6	>40	–
6.	**3f**	Inactive	Inactive	81.8	Inactive	>40	–
7.	**3g**	85.2	Inactive	72.2	**9.2**	>40	–
8.	**6a**	30.7	**1.5**	Inactive	16.1	6.9	4.68
9.	**6b**	18.7	**10.0**	Inactive	Inactive	16.5	–
10.	**6e**	20.3	16.9	Inactive	21.2	16.2	–
11.	**6f**	>40	**6.9**	56.7	10.8	**9.7**	14.39
12.	**6g**	Inactive	**10.0**	38.7	26.6	>40	–
13.	**CUR**	43.9	Inactive	27.8	22.1	>40	5.12
14.	**DC**	28.2	Inactive	26.3	92.1	>40	–
15.	**BDC**	ND	Inactive	45.4	48.9	>40	–
16.	Podophyllotoxin	5.61	Inactive	3.02	4.16	0.11	0.78
17.	Tamoxifen	17.3	Inactive		8.61	16.3	–

$IC_{50} > 100\ \mu M$ considered as inactive.

Table 2
Effect of compound **6a** on Percent colony formation in A431 cells in soft agar after 24 h incubation (no. of seeded cells = 5×10^4 cells/mL, area of 60 mm plate = 2826 mm^2).

Condition	Concentration In μg/mL	Avg. % live cells	A431 live cells (% dead cells)	A431 IC_{50} in μg/mL
Control	–	100	0	
Compd. **6a**	1.5	86.86	13.14 ± 4.9**	6.21
	5	61.46	38.54 ± 0.31**	
	10	34.73	65.25 ± 2.60**	
	20	5.59	94.41 ± 1.16**	
	50	0.75	99.25 ± 0.03**	

**p < 0.01 (Dunnett test).

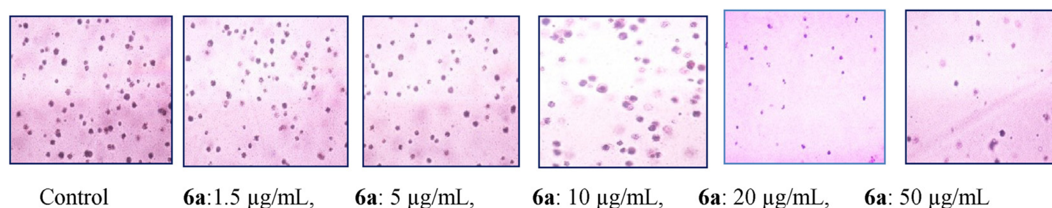


Fig. 2. Antiproliferative activity of **6a** by soft agar colony assay against A431 cells; Control **6a**: 1.5 μg/mL, **6a**: 5 μg/mL, **6a**: 10 μg/mL, **6a**: 20 μg/mL, **6a**: 50 μg/mL.

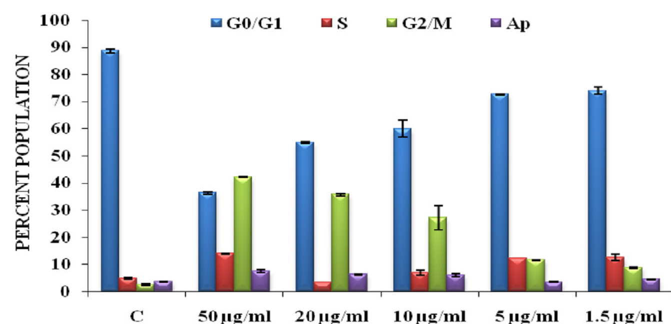


Fig. 3. Cell cycle analysis of compound **6a** in A431 cells.

clearly showed induction of apoptosis at all experimental concentrations. There was S phase arrest also which was relatively less than G2/M phase arrest.

2.2.4. Apoptosis induction by Annexin V-FITC

Compound **6a** induced early apoptosis by 1.1% and late apoptosis by 2.5% while necrosis was 21.6% at its IC_{50} (1.5 μM) while at 2XIC_{50} (3 μM) early and late apoptosis were much pronounced (6% & 9.8%) as compared to necrosis (20%) (Fig. 4).

2.2.5. Tubulin polymerisation assay

Microtubules are polymers of α/β-tubulins existing in dynamic equilibrium. This dynamic equilibrium of microtubules is very crucial for cellular functions especially cell transport and cell division which achieved with the help of several microtubule associated proteins. Any impede in tubulin-microtubule equilibrium disrupts tubulin dynamics which leads to cell cycle arrest and apoptosis. The microtubule interfering agents induce mitotic arrest which leads to increase in 4 N cells in G2/M phase in cell cycle. Compound **6a** showed inhibition (IC_{50} = 4.68 μM) of polymerisation of tubulins to microtubules (Fig. 5). Its destabilization effect was better than the curcumin (IC_{50} = 5.12 μM) but much lower than the standard depolymerisation compound podophyllotoxin (PDT, IC_{50} = 0.78 μM). Another compound **6f** showed very poor destabilization effect (IC_{50} = 14.39 μM).

2.2.6. Effect of *6a* on actin–tubulin cytoskeleton network

Further, the phenotypic effect of compound **6a** was observed on actin-tubulin cytoskeleton network. A431 cancer cells were immune-stained and analysed under Confocal microscope (Fig. 6). Compound **6a** exhibited destabilization effect on microtubule filaments on concentration dependent manner which was clearly observed at 20 μM concentration. This effect was quite similar to curcumin (20 μM). But this destabilization effect was much lower than colchicine (1 μM).

2.2.7. In-vivo anticancer activity against Ehrlich Ascites Carcinoma (EAC)

Ehrlich Ascites Carcinoma is a spontaneous murine mammary adenocarcinoma adapted to ascites form and performed by serial intraperitoneal passages in outbred mice [15]. In our first experiments of EAC with potent curcumin mimic **6a**, there was no loss of weight and mortality of animals (Tables 3 and 4). Compound **6a** reduced EAC by 47.95%, 68.53% and 77.67% at 20 mg/kg, 40 mg/kg and 60 mg/kg doses respectively (Tables 3a and 3b). Further enhancement of doses of **6a** to 80 mg/kg and 100 mg/kg showed only slight improvement in efficacy but one mortality (01/07) at 100 mg/kg dose group and loss in bodyweight were also observed (Tables 4a and 4b).

The results of second experiment of compound **6a** against EAC with solid tumour were much encouraging. Compound **6a** reduced the tumour by 43% and 56.86% at 100 mg/kg and 150 mg/kg doses respectively with no mortality (Tables 5a and 5b). In case of 5-fluorouracil 51% tumour was reduced at 22 mg/kg dose with two mortalities (02/07). The efficacy of **6a** was quite comparable to clinical drug 5-flurouracil though at relatively higher doses but lower toxicity.

2.2.8. Safety studies

In the present experiment, acute oral toxicity of the lead compound **6a** was carried out in Swiss albino mice at 5, 50 and 300 mg/kg once orally. No mortality and morbidity was observed throughout the experimental period in the animals studied. There were no changes observed in gait, posture and response of animals receiving up to 300 mg/kg dose. Blood and serum biochemistry samples upon analysis showed non-significant changes in all the blood and serum parameters studied like haemoglobin (Hb), RBC, WBC, differential leukocyte count (DLC), SGPT, ALP, creatinine,

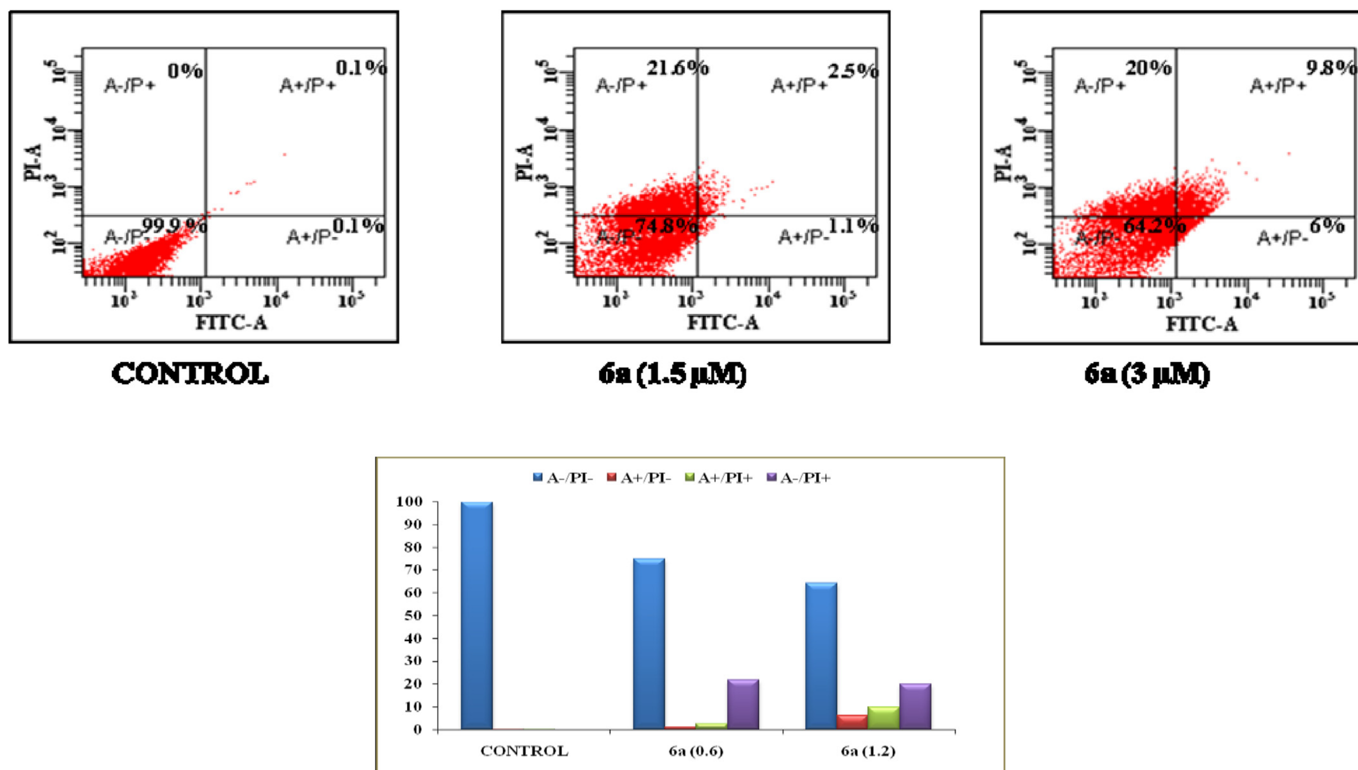


Fig. 4. Induction of apoptosis and necrosis by compound **6a** in A431 cells by Annexin V-FITC assay.

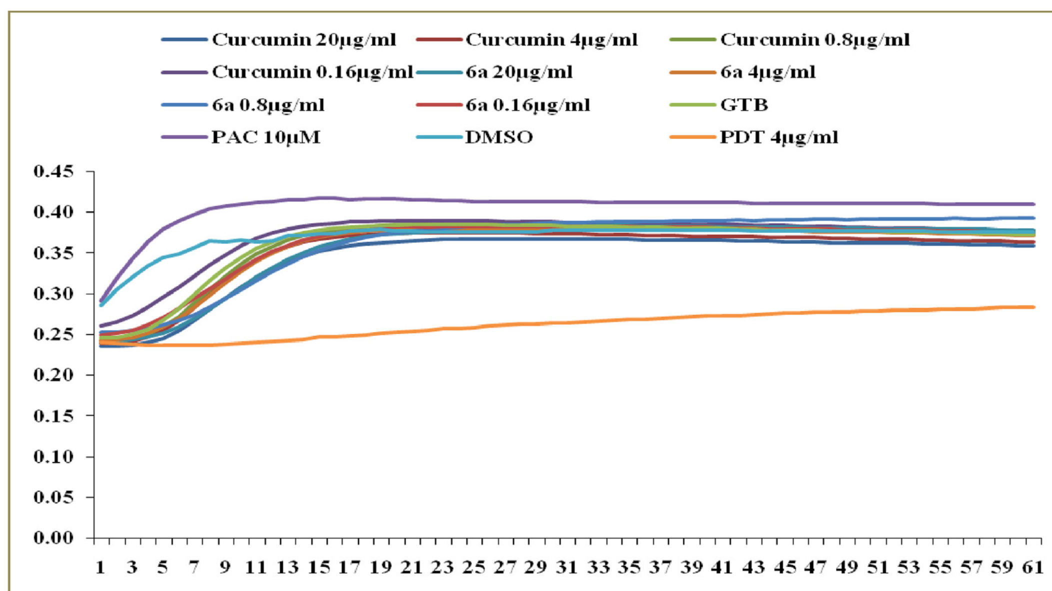


Fig. 5. Tubulin polymerisation kinetics graph of **6a** at various concentrations, curcumin was taken for comparison, Paclitaxel (PAC) as standard microtubule stabilizer and podophyllotoxin (PDT as standard microtubule inhibitor.

triglycerides, cholesterol, albumin, total bilirubin and serum protein (Table 6 & Fig. 8). Animals on gross pathological study showed no significant changes in any of the major body organs studied, including their absolute and relative weights (Fig. 7). Therefore, the experiment showed that **6a** is well tolerated by the Swiss albino mice up to the dose level of 300 mg/kg body weight as a single acute oral dose.

3. Discussion

Plants are a rich source of diverse pharmacologically active secondary metabolites. Drug development from natural resources has a major contribution and it is much more in case of anticancer drug development from plants. Turmeric, widely used in traditional Asian kitchen also exhibits diverse pharmacological activities. Curcumin, demethoxycurcumin and bisdemethoxycurcumin are

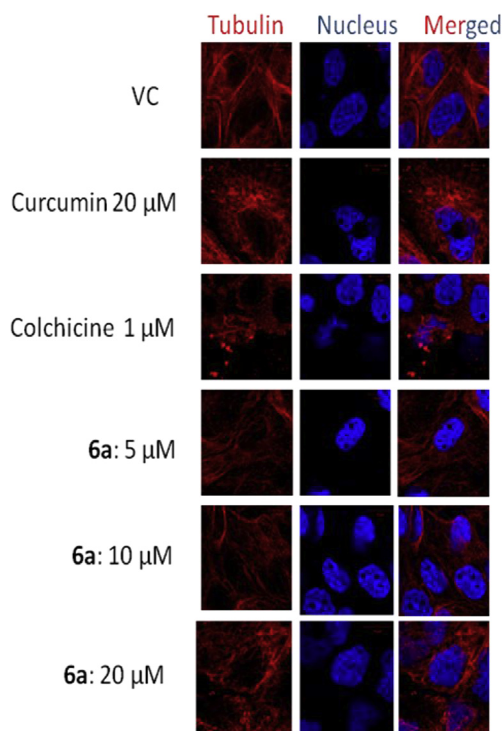


Fig. 6. Effect of compound **6a** on actinetubulin cytoskeleton structure with confocal microscopy. (Curcumin at 20 μM, colchicine at 1 μM, and **6a** at 5 μM, 10 μM and 20 μM.

Table 3a
Effect of compound **6a** on body weight of mice bearing Ehrlich Ascites Carcinoma.

Treatment	Dose	Body weight (g)			
		Day 1	Day 5	Day 9	Day 12
Control	0.2 mL N.S. i/p	22.0	23.7	28.66	32.5
Compd. **6a**	20 mg/kg i/p	23.28	24.42	30.14	32.42
Compd. **6a**	40 mg/kg i/p	23.14	25.14	30.42	32.71
Compd. **6a**	60 mg/kg i/p	22.85	25.42	30.0	35.33
5-FU	20 mg/kg i/p	22.85	22.57	21.71	21.42

the three main components of turmeric. The purpose of this study was to prepare compounds as curcumin mimics possessing an extra aromatic ring at centre with better anticancer efficacy.

The pharmacological activity of curcuminoids is mainly due to their antioxidant property [16]. Presence of phenolic groups was considered important in some studies while others pointed out the β-diketone. Recently, Cai et al. (**2017**) has reported some of these compounds as antidiabetic agents through α-glycosidase inhibition [17].

Our synthesized curcumin mimics exhibited poor antioxidant property as compared to curcuminoids [data not shown]. However,

Table 4a
Effect of compound **6a** on body weight of mice bearing Ehrlich Ascites Carcinoma at higher doses.

Treatment	Dose	Body weight (g)			
		Day 1	Day 5	Day 9	Day 12
Control	0.2 mL N.S. i/p	24.77	26.33	30.55	35.5
Compd. **6a**	80 mg/kg i/p	25.85	27.57	28.42	30.33
Compd. **6a**	100 mg/kg i/p	25.85	27.14	27.57	28.57
5-FU	20 mg/kg i/p	25.85	25.85	24.57	24.14

these compounds exhibited significant cytotoxicity as compared to curcuminoids. Compound **6a** showed potential anticancer activity against skin cancer and colorectal cancer cells. The cytotoxicity of **6a** was further confirmed by soft agar colony assay where it killed 13–99% of skin cancer cells in concentration dependent manner.

As evident from tubulin polymerisation kinetic experiment, compound **6a** exhibited potential cytotoxicity through microtubule destabilization and induction of apoptosis in A431 cells. Previously, curcumin has been reported to depolymerise interphase microtubules at 25 μM and 40 μM concentrations [18]. Later, it was found that curcumin bioactivity involves non-specific microtubule disruption, similar to that of colchicines and other antitubulin drugs used to combat uncontrolled cancerous cell growth [19]. Confocal microscopy showed degradation of microtubules clearly at 20 μM concentration of **6a**.

Cell cycle is an important phenomenon for cell growth and proliferation. Induction of cell cycle arrest in cancer cells through various mechanisms is a strategy to control cancer cell proliferation. Cell cycle check points play an important role in regulation of cell cycle [20]. These checkpoints control system by sensing defects that occur during essential processes such as DNA replication, chromosome segregation etc. and inducing cell cycle arrest in response until the defects are repaired [21]. Some compounds interfere with CDK/cyclin complexes leaving cells stuck at the G2/M phase border, while others affect CaMKII phosphorylation, inducing G1 phase arrest. Among several other mechanisms interference with RNA function and inhibition of protein synthesis are important. As a result of cell cycle interruption, many compounds ultimately induce apoptosis [22]. Compound **6a** induced mainly G2/M phase arrest in A431epidermoid carcinoma cells in concentration dependent manner. In general, interference in microtubule dynamics disturbs mitosis and such compounds cause G2/M phase arrest [23]. Induction of cell cycle arrest in cancer cells constitutes one of the most prevalent strategies to stop or limit cancer spreading [24,25].

EAC is also known as undifferentiated carcinoma which is more malignant than a differentiated carcinoma and difficult to treat. In *in-vivo* efficacy studies compound **6a** exhibited potential cytotoxicity. Its efficacy was comparable to clinical drug 5-fluorouracil though relatively at higher doses. But the toxicity of compound **6a** was relatively much less than 5-fluorouracil. Ehrlich ascites

Table 3b
In-vivo efficacy of compound **6a** against Ehrlich Ascites Carcinoma.

Treatment	Dose	Day 12					
		Av. Volume of ascitic fluid (ml)	Av. Weight of ascitic fluid (g)	Av. No. of tumor cells	% Tumor cell growth	% Tumor growth inhibition	Mortality
Control	0.2 mL N.S. i/p	13.44	13.24	415.04×10^{7}	100	–	0/10
Compd. **6a**	20 mg/kg i/p	12.85	12.21	$216.0 \times 10^{7***}$	52.05	47.95	0/7
Compd. **6a**	40 mg/kg i/p	12.05	12.08	$130.61 \times 10^{7***}$	31.47	68.53	0/7
Compd. **6a**	60 mg/kg i/p	10.08	9.75	$92.64 \times 10^{7***}$	22.33	77.67	0/7
5-FU	20 mg/kg i/p	0.47	0.42	$13.17 \times 10^{7***}$	3.18	96.82	0/7

$^{***}p < 0.001$ (Dunnett test).

Table 4b
In-vivo efficacy of compound **6a** on against Ehrlich Ascites Carcinoma at higher doses.

Treatment	Dose	Day 12					
		Av. Volume of ascitic fluid (ml)	Av. Weight of ascitic fluid (g)	Av. No. of tumor cells	% Tumor cell growth	% Tumor growth inhibition	Mortality
Control	0.2 mL N.S. i/p	12.62	12.03	340.31×10^{7}	100	–	0/10
Compd. **6a**	80 mg/kg i/p	6.91	6.91	$72.85 \times 10^{7***}$	21.41	78.59	0/7
Compd. **6a**	100 mg/kg i/p	6.28	6.22	$62.44 \times 10^{7***}$	18.35	81.65	1/7
5-FU	20 mg/kg i/p	0.58	0.69	$12.36 \times 10^{7***}$	3.64	96.36	0/7

***$p < 0.001$ (Dunnett test).

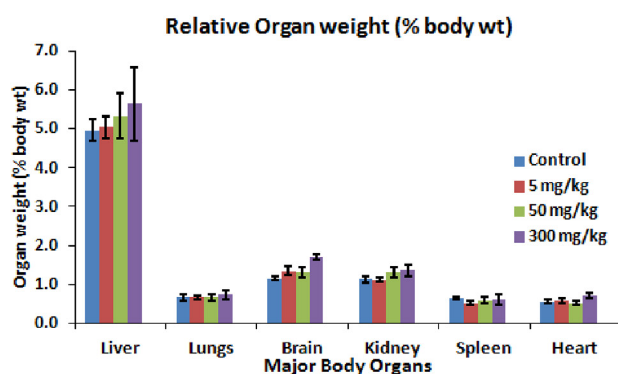
Table 5
Effect on body weight of mice and *In-vivo* efficacy of compound **6a** on against Ehrlich Ascites Carcinoma (solid tumour).

Treatment Groups	Av. Body weights (g) of animals on days			Day 13		%Tumor Growth Inhibition	Mortality
	1	5	9	Av. Body weights (g)	Av. Tumor weights (mg)		
Compd. **6a** (100 mg/kg i/p)	27.0	27.14	26.71	27.4	592.35**	43.03	0/7
Compd. **6a** (150 mg/kg i/p)	26.57	26.00	26.14	27.0	448.5***	56.86	0/7
5-FU(22 mg/kg i/p)	26.42	25.14	24.71	24.85	509.58***	50.99	2/7
Normal Control NS (0.2 mL i/p)	25.0	26.7	26.7	26.9	1039.8	–	0/10

**$p < 0.01$ (Dunnett test); ***$p < 0.001$ (Dunnett test).

Table 6
Effect of **6a** as a single acute oral dose at 5, 50 and 300 mg/kg body weight on hematological and serum biochemical parameters in swiss albino mice (Mean ± SE; n = 6).

Parameters		Effect of 6a in various parameters as a single oral dose in Swiss albino mice			
		Control	5 mg/kg	50 mg/kg	300 mg/kg
Body weight (gm)	24.87 ± 0.49	24.51 ± 0.72	24.08 ± 0.63	20.80 ± 1.37	
Haematological parameters	Haemoglobin (gm/dL)	11.50 ± 0.41	11.85 ± 0.46	11.67 ± 0.42	11.91 ± 0.46
	RBC (million/mm^3)	7.01 ± 0.48	8.08 ± 0.45	7.40 ± 0.35	6.78 ± 0.27
	WBC (1000*/mm^3)	11.80 ± 0.31	12.59 ± 0.77	12.38 ± 0.53	11.42 ± 0.94
Liver Function test	ALP (U/L)	336.0 ± 23.94	348.23 ± 25.62	268.57 ± 17.12	321.95 ± 23.11
	SGOT (U/L)	25.79 ± 2.39	29.62 ± 1.26	29.68 ± 2.42	31.87 ± 2.06
	SGPT (U/L)	24.08 ± 3.16	20.91 ± 1.04	27.37 ± 1.82	32.83 ± 1.89
	Albumin (g/dL)	2.23 ± 0.18	2.21 ± 0.08	2.20 ± 0.10	1.97 ± 0.10
	Serum Protein (mg/ml)	1.69 ± 0.28	1.68 ± 0.23	1.39 ± 0.12	1.57 ± 0.15
	Total Bilirubin (mg/dL)	0.19 ± 0.01	0.20 ± 0.04	0.21 ± 0.02	0.27 ± 0.02
Kidney Function test	Creatinine (mg/dL)	0.42 ± 0.03	0.45 ± 0.04	0.32 ± 0.02	0.39 ± 0.03
Cholesterol profile	Triglycerides (mg/dL)	156.79 ± 15.13	145.70 ± 11.82	152.84 ± 10.57	146.29 ± 14.76
	Cholesterol (mg/dL)	80.12 ± 6.06	82.26 ± 6.54	87.25 ± 5.10	79.67 ± 7.27



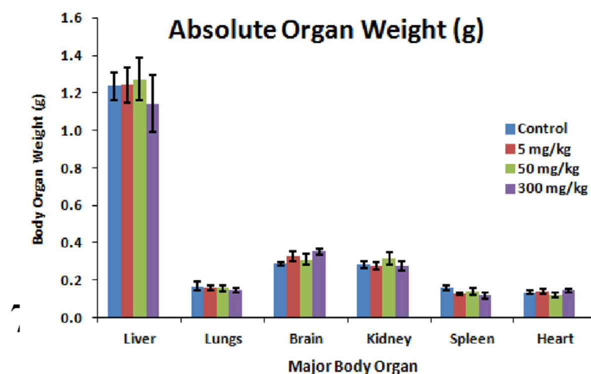


Fig. 7. Effect of **6a** as a single acute oral dose at 5, 50 and 300 mg/kg on absolute and relative organ weight in Swiss albino mice (Mean ± SE; n = 6).

tumor cells are much more difficult to break than most nonmalignant somatic cells [26].

Drug safety is an important parameter while assessing efficacy of an anticancer agent. Acute oral toxicity is used to assess the ability of a substance to cause adverse effects within a short period of time following dosing or exposure. When *in-vivo* testing is done, estimation of rodent acute toxicity is an important task in safety assessment of drug candidates. Compound **6a** was well tolerable up to 300 mg/kg dose in Swiss-albino mice. However, sub-acute, chronic or sub-chronic experiments with compound **6a are** needed to be carried out for any adverse effect on prolonged exposure [27].

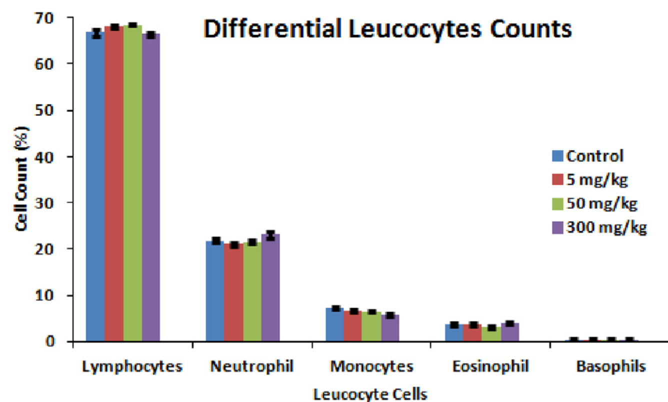


Fig. 8. Effect of **6a** as a single acute oral dose at 5, 50 and 300 mg/kg body weight on differential leukocyte counts in Swiss albino mice (Mean ± SE; n = 6).

4. Conclusion

The present study showed that introduction of bridged phenyl ring enhances anticancer efficacy of curcumin mimics. Compound **6a**, the best representative of the series exhibits potential cytotoxicity against human epidermoid carcinoma cells. Its cytotoxic efficacy was through microtubule destabilization. Its *in-vivo* efficacy was quite comparable to clinical drug 5-fluorouracil at relatively higher doses. *In-vivo* toxicity studies showed it quite safe up to 300 mg/kg dose in mice model. All these results clearly suggest that compound **6a** has significant potential for further translation into clinical application. Also it may further be optimized for better efficacy and lower toxicity in future.

5. Materials and methods

5.1. General

Curcumin, demethoxycurcumin and bisdemethoxycurcumin were isolated from the rhizomes of *Curcuma longa*. For synthesis, substrates, reagents and other chemicals were procured from Avra Chemicals India and used without purification. Melting points were determined in open glass capillaries on E-Z Melt automated melting point apparatus, Stanford Research System USA and were uncorrected. Reactions were monitored on pre-coated silica gel TLC-GF$_{254}$ aluminium sheets (Merck) and visualized done under UV light (254 nm and 365 nm) and subsequent charring with 2% ceric sulphate in 10% sulphuric acid (aqueous). Compounds were purified through column chromatography and Flash chromatography system (CombiFlash R$_f$200i, Teledyne-ISCO, USA) using glass columns (13 cm lengthx2 cm i.d.) and silica gel (230–400 mesh, Avra Chemicals) using UV detector (230 nm and 254 nm). The NMR spectra were obtained on Bruker Avance-300 MHz & 500 MHz instruments with tetramethylsilane (TMS) as an internal standard. Chemical shifts are given in δ ppm values. ^{1}H–^{1}H coupling constant (J) values are given in Hz. ESI mass spectra were recorded on APC3000 LC-MS-MS (Applied Biosystem) and High Resolution Mass (HRMS) on Agilent 6520Q-TOF after dissolving the compounds in methanol. The purity profile was determined on Ultra Performance Liquid Chromatography (UPLC) ACQUITY UPLC H-Class Bio System, Waters USA. DMEM (Dulbecco's Modified Essential Eagle Medium) and FBS (Fetal Bovine Serum) were purchased from Gibco, India. RNaseA, Crystal Violet Dye, Trypsin-EDTA, HEPES, Antibiotic-Antimycotic (Ab-Am) Solution, Phosphate Buffer Saline (PBS), Citric Acid and Propidium Iodide (PI) were acquired from Sigma-Aldrich, USA. Sodium bicarbonate ($NaHCO_3$), Agar, Sodium Citrate and Di-sodium Hydrogen Phosphate were obtained from Himedia Laboratories, India. Solvents including DMSO, ethanol and iso-propanol were procured from Merck, India Ltd.

5.2. Chemical synthesis

5.2.1. General procedure for the synthesis of curcumin mimics **3a-3g** by alkaline method

1,4-Terephthaldehyde (300 mg, 1.12 mmol) in 7% methanolic KOH (10 mL) was stirred at room temperature. To this acetophenone derivative (2.4 mmol) was added and reaction mixture was further stirred for 7–8 h. On completion methanol was evaporated and water was added to it, acidified with 5% HCl (10 mL) and extracted with ethyl acetate (3 × 20 mL). Organic layer was washed with water, dried over anhydrous sodium sulphate and dried *in-vacuo*. The residue was purified through column chromatography using ethyl acetate-hexane as eluents to get pure compound.

1,4-Bis-(E/E)-[1-(4-methylphenyl)-prop-3-oxo-1-enyl]benzene **(3a):** Yield = 64%; m.p. = 234–235 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ2.43 (s, 6H, 2XCH_3), 7.24–7.31 (m, 6H, CH, aromatic), 7.53–7.58 (d, 2H, =CH, J = 15.9 Hz), 7.66 (s, 2H, CH, aromatic), 7.75–7.781 (d, 2H, CH = , J = 15.9 Hz), 7.94 (m, 4H, CH, aromatic); ESI-MS (MeOH): For $C_{26}H_{22}O_2$, 367 $[M+H]^+$, 405$[M+K]^+$; HRMS (ESI-TOF) *m/z* [M+H] calcd for $C_{26}H_{22}O_2$ 367.1698, found 367.1696.

1,4-Bis-(E/E)-[1-(4-methoxylphenyl)-prop-3-oxo-1-enyl]benzene **(3b):** Yield = 92%; m.p. = 239–242 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ3.90 (s, 6H, 2XOCH_3), 7.01 (d, 4H, CH, aromatic), 7.51–7.56 (d, 2H, =CH, J = 14.7 Hz), 7.61 (s, 4H, CH, aromatic), 7.77–7.83 (d, 2H, CH = , J = 15.9 Hz), 8.10 (d, 4H, CH, aromatic); ESI-MS (MeOH): For $C_{26}H_{22}O_4$, 399 $[M+H]^+$

1,4-Bis-(E/E)-[1-(3,4-dimethoxylphenyl)-prop-3-oxo-1-enyl]benzene **(3c):** Yield = 61%; m.p. = 218–220 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ4.02 (s, 12H, 4XOCH_3), 6.93–6.96 (d, 2H, CH, aromatic, J = 8.7 Hz), 7.47–7.52 (d, 2H, =CH, J = 15 Hz), 7.73–7.78 (d, 2H, CH = , J = 15 Hz), 7.52–8.11 (m, 8H, CH, aromatic), ; ESI-MS (MeOH): For $C_{28}H_{26}O_6$, 481$[M+Na]^+$; HRMS (ESI-TOF) *m/z* [M+H] calcd for $C_{28}H_{26}O_6$ 459.1807, found 459.1801.

1,4-Bis-(E/E)-[1-(3,4,5-trimethoxylphenyl)-prop-3-oxo-1-enyl] benzene **(3d):** Yield = 96%; m.p. = 216–218 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ3.93 (s, 18H, 6XCH_3), 7.27 (s, 4H, CH, aromatic), 7.48–7.53 (d, 2H, =CH, J = 15.6 Hz), 7.76 (s, 4H, CH, aromatic), 7.76–7.81 (d, 2H, CH = , J = 15.6 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ56.8, 61.3, 106.6, 123.1, 129.3, 133.7, 137.3, 143.2, 143.8, 153.6, 189.2; ESI-MS (MeOH): For $C_{30}H_{30}O_8$, 519 $[M+H]^+$, 541$[M+Na]^+$.

1,4-Bis-(E/E)-[1-(3,4-methylenedioxyphenyl)-prop-3-oxo-1-enyl] benzene **(3e):** Yield = 53%; m.p. = 242–245 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ6.10 (s, 4H, 2xO-CH_2-O), 6.92–6.95 (d, 2H, CH, aromatic, J = 8.1 Hz), 7.53–7.58 (d, 2H, =CH, J = 15.6 Hz), 7.57 (d, 4H, CH, aromatic) 7.68–7.70 (d, 4H, CH, aromatic, J = 6.9 Hz), 7.79–7.84 (d, 2H, CH = , J = 15.6 Hz); ESI-MS (MeOH): For $C_{26}H_{18}O_6$, 465$[M+K]^+$.

1,4-Bis-(E/E)-[1-(4-fluorophenyl)-prop-3-oxo-1-enyl]benzene **(3f):** Yield = 94%; m.p. = 242–245 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ7.27–7.33 (m, 6H, CH, aromatic), 7.60–7.65 (d, 2H, =CH, J = 15.6 Hz), 7.77 (bs, 4H, CH, aromatic), 7.86–7.92 (d, 2H, CH = , J = 15.6 Hz), 8.12–8.15 (m, 2H, CH, aromatic); ESI-MS (MeOH): For $C_{24}H_{16}F_2O_2$, 397 $[M+Na]^+$; Negative mode: 373 $[M-H]^-$; HRMS (ESI-TOF) *m/z* [M+H] calcd for $C_{24}H_{16}O_2$ 375.1196, found 375.1194.

1,4-Bis-(E/E)-[1-(4-bromophenyl)-prop-3-oxo-1-enyl]benzene **(3g):** Yield = 63%; m.p. = 262–264 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ7.48–7.53 (d, 2H, =CH, J = 15.6 Hz), 7.63–7.68 (m, 6H, CH, aromatic), 7.71–7.82 (m, 4H, CH, 2XCH = and 2xaromatic), 7.94 (m, 4H, CH, aromatic); ESI-MS (MeOH): For $C_{24}H_{16}Br_2O_2$, 535$[M+K]^+$; negative mode: 495 $[M-H]^-$.

5.2.2. General procedure for the synthesis of curcumin mimics **6a**-**6c** by alkaline method

1,3-Dicetylbenzene (400 mg, 2.47 mmol) was taken in 7% methanolic KOH (10 mL). To this stirred solution benzaldehyde derivative (5 mmol) was added and reaction mixture was further stirred at room temperature for 7–8 h. The work up and purification procedure was followed as in section 2.2.1 to get the pure product.

1,3-Bis-(E/E)-[3-(3,4-methylenedioxyphenyl)-1-oxo-2-enyl]-benzene (**6b**): Yield=60%; m.p.=205-208°C; ^{1}H NMR (300MHz, $CDCl_3$): δ6.16 (s, 4H, 2XO-CH_2-O), 6.83 (m, 2H, CH, aromatic), 7.12 (s, 2H, CH, aromatic, J=8.1Hz), 7.43 (d, 2H, CH, aromatic), 7.76-7.81 (d, 2H, =CH, J=16.5Hz), 7.90-7.95 (d, 2H, CH=, J=15.5Hz), 8.26 (d, 2H, CH, aromatic), 8.46 (d, 2H, CH, aromatic); ^{13}C NMR (75MHz, $CDCl_3$): δ101.5, 102.5, 107.9, 108.1, 108.6, 209.4, 120.4, 127.1, 128.7, 129.9, 130.1, 133.4, 137.4, 139.2, 145.6, 148.9, 150.6, 189.6; ESI-MS (MeOH): For $C_{26}H_{18}O_6$, 449 [M+Na]$^+$, 465[M+K]$^+$.

1,3-Bis-(E/E)-[3-(3,4-methylenedioxyphenyl)-1-oxo-2-enyl]-benzene (**6b**): Yield = 60%; m.p. = 205–208 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ6.16 (s, 4H, 2XO-CH_2-O), 6.83 (m, 2H, CH, aromatic), 7.12 (s, 2H, CH, aromatic, J = 8.1 Hz), 7.43 (d, 2H, CH, aromatic), 7.76–7.81 (d, 2H, =CH, J = 16.5 Hz), 7.90–7.95 (d, 2H, CH = , J = 15.5 Hz), 8.26 (d, 2H, CH, aromatic), 8.46 (d, 2H, CH, aromatic); ^{13}C NMR (75 MHz, $CDCl_3$): δ101.5, 102.5, 107.9, 108.1, 108.6, 209.4, 120.4, 127.1, 128.7, 129.9, 130.1, 133.4, 137.4, 139.2, 145.6, 148.9, 150.6, 189.6; ESI-MS (MeOH): For $C_{26}H_{18}O_6$, 449 [M+Na]$^+$, 465[M+K]$^+$.

1,3-Bis-(E/E)-[3-(4-N,N-dimethylphenyl)-1-oxo-2-enyl]-benzene (**6c**): Yield = 71%; m.p. = 149–152 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ3.04 (s, 12H, 4XN-CH_3), 6.68 (d, 4H, CH, aromatic, J = 8.7 Hz), 7.35–7.40 (d, 2H, =CH, J = 15.6 Hz), 7.56–7.61 (d, 5H, CH, aromatic), 7.80–7.85 (d, 2H, CH = , J = 15.6 Hz), 8.15–8.18 (d, 2H, CH, aromatic), 8.60 (s, 1H, CH, aromatic); ^{13}C NMR (75 MHz, $CDCl_3$): δ40.4, 111.1, 112.2, 113.3, 116.8, 122.88, 128.3, 129.1, 131.1, 132.1, 139.7, 146.9, 152.6, 190.3; ESI-MS (MeOH): For $C_{28}H_{28}N_2O_2$, 425 [M+H]$^+$, 447 [M+Na]$^+$, 463[M+K]$^+$.

5.2.3. General procedure for the synthesis of curcumin mimics **6d**-**6g** by acidic method

1,3-Dicetylbenzene (500 mg, 3.08 mmol) and particular benzaldehyde derivative (6.2 mmol) was taken in dioxane (10 mL). To this conc. hydrochloric acid (0.5 mL) was added and reaction mixture was refluxed for 4–5 h. On completion, water was added to reaction mixture, extracted with ethyl acetate, washed with water and dried over anhydrous sodium sulphate.

1,3-Bis-(E/E)-[3-(4-hydroxyphenyl)-1-oxo-2-enyl]-benzene (**6d**): Yield = 69%; m.p. = 235–238 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ6.92–7.08 (d, 4H, CH, aromatic), 7.69–7.76 (bd, 8H, CH, aromatic & 2X = CH), 8.20 (bd, 2H, CH, 2XCH =), 8.62 (bs, 1H, CH, aromatic); ^{13}C NMR (75 MHz, $CDCl_3$): δ116.3, 119.1, 127.0, 128.2, 129.4, 131.2, 132.4, 139.4, 145.3, 189.1; ESI-MS (MeOH): For $C_{26}H_{22}O_2$, 371 [M+H]$^+$, 393 [M+Na]$^+$, 409[M+K]$^+$.

1,3-Bis-(E/E)-[3-(4-hydroxyphenyl)-1-oxo-2-enyl]-benzene (**6d**): Yield = 69%; m.p. = 235–238 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ6.92–7.08 (d, 4H, CH, aromatic), 7.69–7.76 (bd, 8H, CH, aromatic & 2X = CH), 8.20 (bd, 2H, CH, 2XCH =), 8.62 (bs, 1H, CH, aromatic); ^{13}C NMR (75 MHz, $CDCl_3$): δ116.3, 119.1, 127.0, 128.2, 129.4, 131.2, 132.4, 139.4, 145.3, 189.1; ESI-MS (MeOH): For $C_{26}H_{22}O_2$, 371 [M+H]$^+$, 393 [M+Na]$^+$, 409[M+K]$^+$.

1,3-Bis-(E/E)-[3-(3,4-dimethoxyphenyl)-1-oxo-2-enyl]-benzene (**6f**): Yield = 72%; oil; ^{1}H NMR (300 MHz, $CDCl_3$): δ3.93 (s, 6H, 2XOCH_3), 3.96 (s, 6H, 2XOCH_3), 6.91 (bd, 2H, CH, aromatic), 7.08–7.34 (d, 4H, CH, aromatic), 7.41–7.46 (d, 2H, =CH, J = 15.6 Hz), 7.61 (t, 1H, CH, aromatic), 7.78–7.84 (d, 2H, CH = , J = 15.6 Hz), 8.19 (bd, 2H, CH, aromatic), 8.62 (s, 1H, CH, aromatic); ^{13}C NMR (75 MHz, $CDCl_3$): δ56.4, 110.6, 111.6, 119.9, 123.9, 128.1, 129.3, 132.6, 139.3, 146.3, 149.7, 152.1, 190.3; ESI-MS (MeOH): For $C_{28}H_{26}O_6$, 459 [M+H]$^+$, 481 [M+Na]$^+$, 497[M+K]$^+$; HRMS (ESI-TOF) *m/z* [M+H] calcd for $C_{28}H_{26}O_6$ 459.1807, found 459.1802.

1,3-Bis-(E/E)-[3-(3,4,5-trimethoxyphenyl)-1-oxo-2-enyl]-benzene (**6g**): Yield = 96%; oil; ^{1}H NMR (300 MHz, $CDCl_3$): δ4.02 (s, 18H, 6XOCH_3), 6.98 (s, 4H, CH, aromatic), 7.36–7.41 (d, 2H, CH = ,J = 15 Hz), 7.68 (bs, 1H, CH, aromatic), 7.76–7.81 (d, 2H, =CH, J = 15.6 Hz), 8.22 (bs, 2H, CH, aromatic), 8.64 (s, 1H, CH, aromatic); ^{13}C NMR (75 MHz, $CDCl_3$): δ56.3, 61.0, 105.9, 120.9, 128.3, 129.1, 130.1, 132.4, 138.8, 146.0, 153.5, 189.9; ESI-MS (MeOH): For $C_{30}H_{30}O_8$, 519 [M+H]$^+$, 541 [M+Na]$^+$, 557[M+K]$^+$.

5.2.4. Purity profile of **6a** by UPLC

The purity profile of most potent compound **6a** was checked by reverse phase Ultra Performance Liquid Chromatography (UPLC, Waters USA) using C-18 column (BEH130 Å, 1.7 × 50 mm, 1.7 μm, Waters, USA, thermostated at 35 ± 0.1 °C). Analysis was performed using isocratic elution of acetonitrile and water (70:30, v/v) at flow rate of 0.20 mL/min. The injection volume was 2 μL. The purity of the compounds was calculated by peak integration.

5.3. Biological evaluation

5.3.1. Antiproliferative activity by MTT assay

The assay was performed as per reported method [9]. Human cancer cell lines, A549 (Lung), MCF-7 (Breast), DLD1 (Colorectal), A431 (epidermoid) and WRL-68 (Liver) were originally obtained from American type of cell culture collection (ATCC), USA and grown at 37 °C in DMEM supplemented with 10% FBS and Ab-Am (antibiotic-antimitotic) solution in a CO_2 incubator (New Brunswick/Eppendorff, Germany) under 5%CO_2 and 95% relative humidity. Briefly, 2×10^3 cells/well were incubated in the 5% CO_2 incubator for 24 h to enable them to adhere properly to the 96 well polystyrene microplate (Grenier, Germany). Test compounds **3a**-**3g** and **6a**-**6g** were dissolved in dimethylsulphoxide (DMSO, Merck, Germany), in at least five concentrations, were added into the wells and left for 4 h. After the incubation, the compound and media were replaced with fresh media and the cells were incubated for another 24 h in the CO_2 incubator at 37 °C. The concentration of DMSO was always kept below 1.25%, which was found to be non-toxic to cells. Then, from 5 mg/mL 10 μL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to each well and plates were incubated at 37 °C for 4 h. 100 μL DMSO was added to all wells and mixed thoroughly to dissolve the formazan crystals. The plates were read on SpectraMax 190 Microplate reader (Molecular Devices Inc. USA) at 570 nm within 1 h of DMSO addition.

The cytotoxic effect of compound was calculated as % inhibition in cell growth as per formula: [1- (Absorbance of drug treated cells/ Absorbance of untreated cells) X100]. Dose dependent curves were used to determine 50% inhibitory concentration (IC_{50}).

5.3.2. Soft agar colony assay

Briefly, human epidermoid cancer A431 cells (5×10^4 per mL) were seeded separately in 24 well plates with or without compound **6a** treatment for 24 h [13]. A bottom jelly layer was made with 0.72% agar and 4 mL media in a 90 mm Petri dish followed by the formation of top layer with cells and 3 mL media in 0.36% agar. Petri dishes were incubated in a CO_2 incubator for 15–20 days till the appearance of the colonies followed by staining with Crystal Violet (0.04% in 2% ethanol) and incubation for 1 h at room temperature. After the incubation period, colony was counted using inverted microscope and pictures were captured accordingly.

5.3.3. Cell cycle analysis

The effect of compound **6a** on cell division cycle of A431 cells was assessed by flow cytometry with PI-stained cellular DNA [28]. In brief, cells (2×10^6 per mL) were grown in a 6 well plate at 37 °C in 5%CO_2 with or without compound treatment for 24 h. After incubation, cells were collected and washed with PBS followed by fixing in 70% ethanol (add drop wise through vortexing) for overnight at 4 °C. The fixed cells were centrifuged at high rpm to remove ethanol followed by washing with PBS and addition of DNA extraction buffer. The samples were incubated for 5 min at room temperature and centrifuged again. The obtained pellet was dissolved in PI staining solution followed by RNaseA (200 μg/mL) treatment and incubation for 30 min at room temperature. Finally, cell cycle analysis was done using Flow Cytometric approach on a BD Biosciences LSR II (San Jose, CA, USA) and the analysis was performed through Facs Diva Software, version 6.1.3. Both the soft agar colony formation and cell cycle experiment was performed in duplicates and results are expressed as Mean ± SD.

*5.3.4. Apoptosis vs necrosis induction by **6a** by Annexin V-FITC assay*

For Annexin V-FITC apoptosis assay by Flow cytometry [29], 1×10^6 cells/mL cells of A431 were seeded in 6-well plate and left overnight before treatment with compound **6a** at various concentrations (IC_{50} value and $2XIC_{50}$) for 24 h. All cells apoptotic and adherent were collected and centrifuged at 5000 rpm for 5 min at 4 °C and washed twice with cold PBS. Pellets were dissolved in 100 μL 1X binding buffer and incubated with 5 μL FITC-annexin V and 5 μL propidium iodide (PI) (protocol followed as described in BD Bioscience Kit) for 15 min. Samples were maintain with 500 μL 1x binding buffer and stained cells were analysed by FACS Diva software of flow cytometer within 1 h. The annexin V-FITC binds to phosphatidiylserine present on membrane surface in case of apoptotic cells whereas PI labelled the cellular DNA in necrotic cell. This combination allows the differentiation among viable cells (annexin V negative, PI negative), early apoptotic cells (annexin V positive, PI negative), late apoptotic cells (annexin V positive, PI positive), and necrotic cells (annexin V positive, PI positive).

5.3.5. Tubulin polymerisation assay

Tubulin kinetics (polymerization) experiment was performed using 'assay kit' from Cytoskeleton, USA, as per reported protocol [10,11]. In brief, 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 (PDT) was used as standard destabilizer and paclitaxel as standard stabilizer 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%.

*5.3.6. In vivo anticancer activity of **6a** against Ehrlich Ascites Carcinoma (EAC)*

The *in-vivo* anticancer assay was performed through Ehrlich Ascites Carcinoma (EAC) model [30]. EAC cells were collected from the peritoneal cavity of the female Swiss mice (each weighing 20–28 g) harbouring 8–10 days old ascitic tumour. 1×10^7 EAC cells were injected intraperitoneally in each of the Swiss mice selected for the experiment on day zero. The next day, animals were randomized and divided into different groups. The treatment groups contained 7 animals each and control group contained 10 animals. Three treatment groups were treated with 20 mg/kg, 40 mg/kg and 60 mg/kg of potent compound **6a** intra-peritoneally from day 1–9. One more of the treatment group received anticancer drug 5-fluorouracil (20 mg/kg, i.p) and it served as positive control. The tumour bearing control group was similarly administered normal saline (0.2 mL, i.p.). On day 12, animals were sacrificed and ascitic fluid was collected from peritoneal cavity of each mouse for the evaluation of tumour growth. Percent tumour growth inhibition was calculated based on the total number of tumour cells present in the peritoneal cavity as on day 12 of the experiment using the following formula.

$$\%\ \text{tumour growth inhibition} = \frac{\text{Avg. no. of cells in control gp} - \text{Avg. no. of cells in treated gp}}{\text{Avg. no. of cells in control group}} \times 100$$

The experiment was further done at higher doses of 80 mg/kg and 100 mg/kg of **6a** with some marginal improvement in the activity.

*5.3.7. In vivo anticancer activity of curcumin mimic **6a** against Ehrlich tumour (solid)*

Ehrlich ascites carcinoma (EAC) cells were collected from the peritoneal cavity of the Swiss mice harbouring 8–10 days old ascitic tumour. 1×10^7 EAC cells were injected intramuscularly in right thigh of 24 Swiss male mice (20–28 g each) selected for the experiment on day 0. The next day, animals were randomized and divided into three groups. Two treatment groups contained 7 animals each and one control group contained 10 animals. One of the treatment groups was treated with potent compound **6a** (100 mg/kg, and 150 mg/kg, i/p) from day 1–9. Another treatment group was treated with 5-fluorouracil (22 mg/kg, i.p) from day 1–9 and it served as positive control. The control group was similarly administered normal saline (0.2 mL, i.p.) from day 1–9. On day 9 and 13, tumour bearing thigh of each animal was shaved and longest and shortest diameters of the tumour were measured with the help of Vernier callipers. Tumour weight of each animal was calculated using the following formula.

$$\text{Tumour weight (mg)} = \frac{\text{Length (mm)} \times [\text{width(mm)}]^2}{2}$$

The percent tumour growth inhibition was calculated on day 13 by comparing the average values of treated groups with that of control group. Tumour growth in saline treated control animals was taken to be 100%.

5.3.8. Acute oral toxicity

Compound **6a**, the most potent anticancer compound of this series was further evaluated for safety studies through acute oral toxicity in Swiss albino mice. The Experiment was conducted in accordance with the Organization for Economic Co-operation and Development (OECD) test guideline No 423 (1987). For the acute

oral toxicity study, 24 mice (12 male and 12 female) were taken and divided into four groups comprising 3 male and 3 female mice in each group weighing between 20 and 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 mice feed and provided *ad libitum* drinking water. Mice of group 1 (Group I) were kept as controls and animals in groups 2, 3 and 4 (Groups II, III & IV) were kept as experimental. The animals were acclimatized for 7 days prior for the experimental environment adaptation. Compound **6a** was dissolved in few drop of DMSO then solubilised in 0.7% CMC and was given at 5, 50 and 300 mg/kg body weight of animals of groups 2, 3 and 4 (Groups II, III & IV) respectively once orally. Control animals received only vehicle.

The animals were checked for mortality and any signs of ill-health at hourly interval on the day of administration of the drug and thereafter a daily general case side clinical examination was carried out including changes in the skin, mucous membrane, eyes, occurrence of secretion and excretion and also responses like lachrymation, piloerection, respiratory patterns etc. Also, changes in gait, posture and response to handling were also recorded [31]. In addition to the observational study, body weights were recorded, blood and serum samples were collected from all the animals on the 7 t h day of the experiment. The samples were analyzed for total RBC, WBC, differential leukocyte count, haemoglobin percentage and biochemical parameters like ALP, SGPT, SGOT, total cholesterol, triglycerides, creatinine, bilirubin and serum protein. The animals were then sacrificed and were necropsied for any gross pathological changes. Weights of vital organs like liver, heart, kidney etc. were recorded [32].

5.3.9. Statistical analysis

Data expressed as mean or representative of one of three similar experiments unless otherwise indicated. The MTT, Soft agar colony formation, Annexin V FITC, and cell cycle experiments were performed in duplicates and results are expressed as Mean ± SD. The MTT absorbance was taken from the SoftMax Pro Microplate data acquisition and analysis software version 5.3 (Moleular Devices Corporation., Sunnyvale CA, USA). The IC_{50} value was calculated from Table curve 2D windows version 4.07 (SPSS Inc., Chicago, IL, USA). Cell cycle was performed on a BD Bioscience LSR II (San Jose, CA, USA) and analysis was done through Facs Diva Software, version 6.1.3. For multiple comparisons each value was compared by one way ANOVA following Dunnett test, Tukey test and Student t-test in GraphPad Instat version 3.06.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ejmech.2018.03.063.

Supplementary information

The spectra of compounds are being submitted as Supplementary information..

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