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In vivo growth inhibitory and anti-angiogenic effects of synthetic novel dienone cyclopropoxy curcumin analogs on mouse Ehrlich ascites tumor

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Abstract—In the present study, four novel dienone cyclopropoxy curcumin analogs **1a**–**4a** were synthesized by nucleophillic substitution reaction with cyclopropyl bromide. The tumor inhibitory and anti-angiogenic effects of the synthetic compounds were studied on mouse Ehrlich ascites tumor (EAT) in vivo. The compounds **1a**–**4a** increased the life span (% ILS) of EAT bearing mice with corresponding significant reduction in ascites volume and cell number and induced apoptotic bodies in EAT cells. Anti-angiogenic studies of the compounds demonstrated significant reduction of microvessel density (MVD) in the peritoneum wall sections of mice and induced avascular zone in CAM model. Our findings demonstrate that the tumor growth inhibitory effects of synthetic dienone cyclopropoxy curcumin analogs **1a**–**4a** could be mediated by promoting apoptosis and inhibiting tumor angiogenesis. However, the compounds need to be explored further to assess its clinical relevance.

1. Introduction

Angiogenesis is the process of formation of new capillary blood vessels and is considered essential for tumor growth.[1] A growing tumor needs an extensive network of capillaries to provide nutrients and oxygen.[2,3] In animal models, anti-angiogenic compounds have proven very successful in inhibiting tumor development.[4,5] The inhibition of tumor angiogenesis using novel anti-angiogenic agents has gained increasing importance in cancer research and it has been demonstrated that different anti-angiogenic therapies retarded experimental tumor growth.[6–9] Several compounds with anti-angiogenic properties are currently in preclinical development for the treatment of cancer.[10,11]

Synthetic compounds are used to control the advanced stages of malignancies but most of the compounds exhibit normal tissue toxicity with undesirable side effects and development of drug resistance is the major clinical problem. Hence there is a need to discover novel compounds that selectively kill cancer cells. Recently, attention has been toward the drug derived from plant sources which are nontoxic and accessible to common man.

Indian System of Medicine has many herbal preparations with versatile medicinal properties. Hence attempts are made to synthesize compounds of natural origin. The rhizome of *Curcuma longa* L. (turmeric) has been widely used in indigenous medicine for the treatment of various ailments.[12] Curcumin a well-known cyclic diarylheptanoid has been identified as the major constituent in turmeric. Recently numerous studies have demonstrated the remarkable cancer preventive properties of curcumin.[13–22] The chemo preventive effects of curcumin have been attributed to various biological properties including neutralization of carcinogenic free radicals[13] and anti-angiogenesis action which limits the blood supply to rapidly growing malignant cells.[14,15]

Synthetic analogs of curcumin and β-diketone compounds that are structurally related to curcumin have

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been reported for their potential cytotoxic effects against a panel of human tumor cell lines.[23] In the present study we designed, synthesized, and characterized four novel dienone cyclopropoxy curcumin analogs **1a**–**4a**. To investigate the activities of these compounds, it was decided to study the tumor inhibitory and anti-angiogenic effects on Ehrlich ascites tumor in vivo. The compounds were subsequently tested for their ability to inhibit neovascularization in chorio allantoin membrane (CAM) model.

2. Results

2.1. Tumor growth inhibition of Ehrlich ascites tumor in vivo

Animal survival (Table 1): The vehicle 0.1% DMSO did not have any effect on the tumor growth. All the animals in vehicle treated controls developed tumor and died within 16–20 days. The median survival time (MST) was 18 days. All the treatments produced significant increase in MST and % ILS compared to control ($p < 0.01$). Three doses of dienone cyclopropoxy curcumin analogs treatments (**2a**, **3a**, and **4a**) on 7, 9, and 11 days after tumor transplantation showed effective antitumor response (>25% ILS) and resulted in 48%, 34%, and 28% ILS, respectively. However the compound 1a exhibited higher tumor inhibitory effect and showed 32 days of MST with 77% ILS. The weight changes were significantly higher in compound treated groups compared to control (Table 1) indicating the effect of the compounds in preventing the tumor growth. The treated groups showed reduction in body weight due to decrease in tumor burden however no toxic side effects were observed.

2.2. Ascites volume and cell number (Fig. 1)

The inhibitory effect of dienone cyclopropoxy curcumin analogs **1a**–**4a** on EAT cells in vivo was further examined in terms of total number of cells and volume of ascites in mice treated with vehicle or compounds. The mean value of cell number and ascites volume in control animals was found to be $1730.40 \pm 0.65 \times 10^6$ cells/mouse (Fig. 1a) and 7.16 ± 0.30 ml, respectively (Fig. 1b). All the compound treatments showed significant decrease in ascites volume and cell number compared to control ($p < 0.01$). The compound **1a** treated group which demonstrated highest % ILS (Table 1) showed maximum reduction in cell number and ascites volume compared to other compounds treated groups.

Table 1. Effect of synthetic novel dienone cyclopropoxy curcumin analogs **1a**–**4a** on survival of mice bearing Ehrlich ascites tumor

Treatment groups	MST (days)[a]	ILS (%)	Av. wt[b] changes
1. Control (0.1% DMSO)	18	—	+10.44
2. **1a**-100	32**	77.7	−4.44**
3. **2a**-100	26.8**	48.8	−3.55**
4. **3a**-100	24.2**	34.4	−3.00**
5. **4a**-100	23.2**	28.8	−2.04**

1a–**4a**-100 indicates dose in mg/kg body wt. Vehicle or compounds were administered on day 7, 9, and 11 after tumor cell inoculation to different treatment groups of 10 animals each.

[a] Median survival time (MST) and ILS % was calculated from the mortality data within the observation period.

[b] Determined on 12th day of treatment.

** Significant from control ($p < 0.01$).

2.3. Changes in the morphology of EAT cells (Fig. 2)

The inhibitory effect of synthetic dienone cyclopropoxy curcumin analogs on EAT cell growth may be due to induction of apoptosis. The EAT cells were stained with Geimsa or with nuclear stain (acridine orange: ethidium bromide) and the slides were observed under microscope and photographed. The apoptotic bodies and nuclear condensation are evident in compound treated groups.

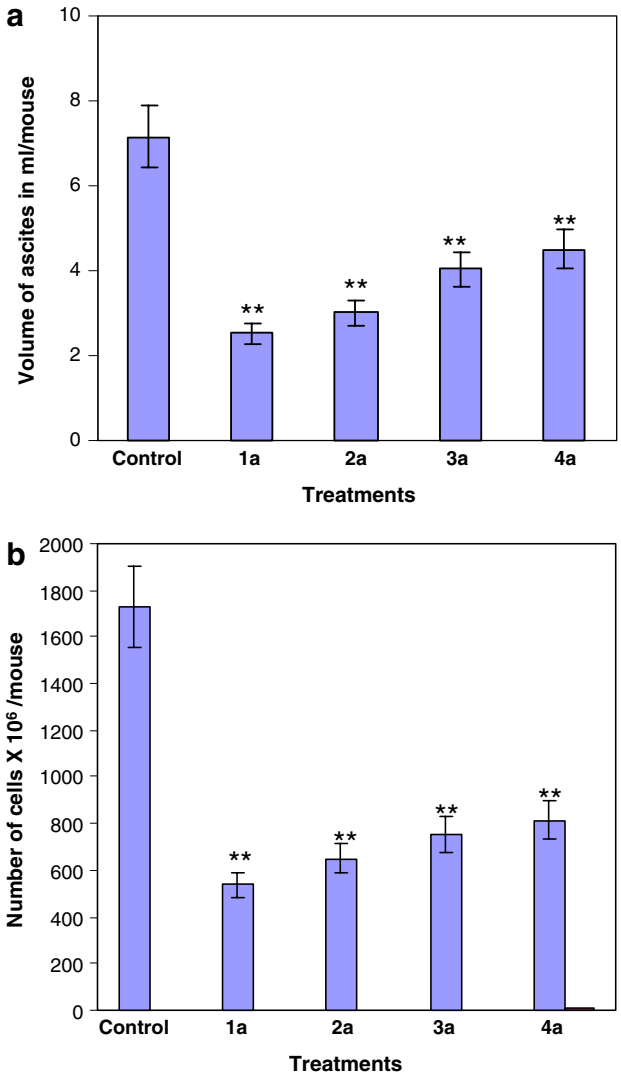


Figure 1. Effects of synthetic dienone cyclopropoxy curcumin analogs **1a**–**4a** on ascites volume and cell number of EAT bearing mice. The bar graph represents the effect of the compounds on ascites volume (a) and cell number (b). All the treatments showed a significant difference in ascites volume and cell number from the control ($p < 0.01$). The error bars represent standard deviation of the mean.

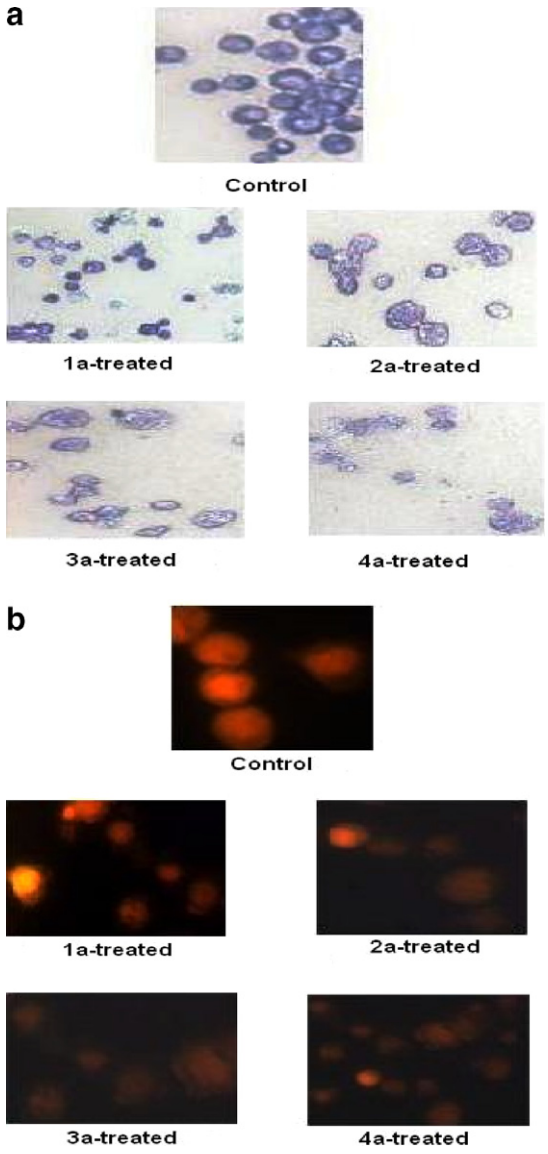


Figure 2. Changes in the morphology of EAT cells after treatment with synthetic dienone cyclopropoxy curcumin analogs **1a**–**4a**. EAT cells with or without compounds **1a**–**4a** treatment in vivo were washed with PBS, fixed, and stained with Giemsa stain (a) or with mixture of acridine orange: ethidium bromide (b). The cells were viewed under compound or fluorescent microscope and photographed. The apoptotic bodies and condensed nuclei are evident in the compound treated groups.

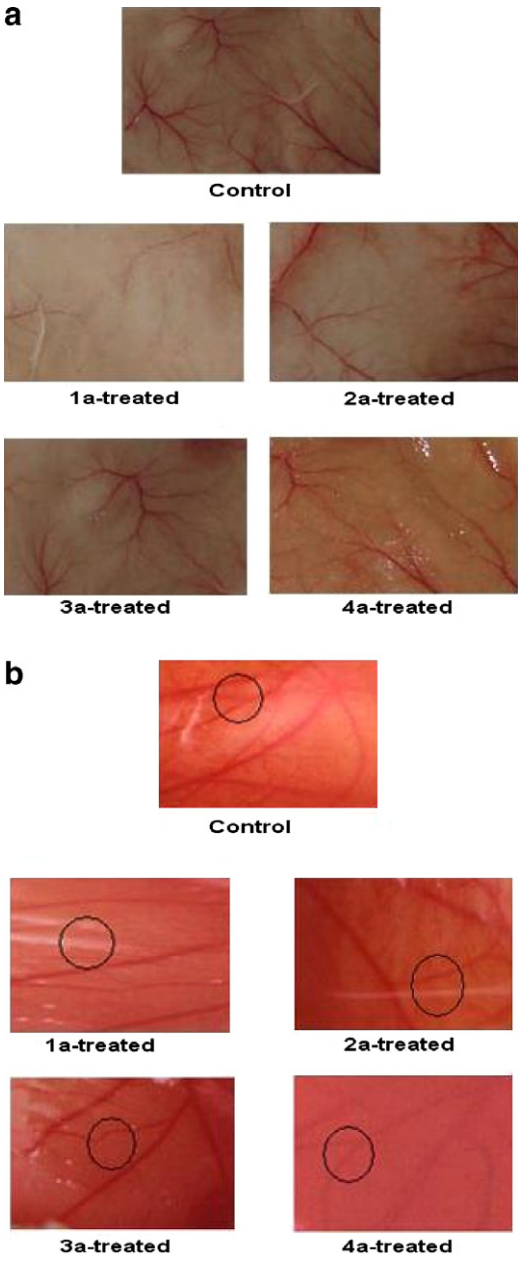


Figure 3. Suppression of angiogenesis in vivo by synthetic novel dienone cyclopropoxy curcumin analogs **1a**–**4a**. Peritoneal lining of tumor bearing mice treated with vehicle (0.1% DMSO) and curcumin analogs were inspected for anti-angiogenesis effects (a). Inhibitions of angiogenesis were prominent in compound treated mice compared to control. CAM assay model (b)—curcumin analogs **1a**–**4a** or the vehicle was applied on the CAM of 11 days old chick embryo. Decreased vasculature was observed in treated groups compared to control. Dotted circles indicate the area covered by the cover slip.

2.4. Inhibition of tumor induced neovascularization by synthetic dienone cyclopropoxy curcumin analogs (Figs. 3 and 4)

Significant inhibitions of blood vessel formation were observed in the peritoneal wall of compound treated mice compared to control (Fig. 3). The MVD (Fig. 4) studies using hematoxylin and eosin stained peritoneal wall sections demonstrated significant decrease in MVD count in all the compounds **1a**–**4a** treated groups compared to control ($p < 0.01$). The peritoneal sections from EAT bearing control mice showed MVD count 5.9 ± 0.94/HPF, whereas in compound treated mice **1a**–**4a** the MVD count was decreased to 2.8 ± 0.44, 3.0 ± 0.63, 4.2 ± 1.24, and 4.4 ± 1.49, respectively. The strong angioinhibitory effect of the compounds was evident in this study.

2.5. Angioinhibitory effects of the synthetic dienone cyclopropoxy curcumin analogs on CAM

The anti-angiogenic activity was evaluated by observing the formation of avascular zone under the cover slip (Fig 3), control CAM treated with 0.1% DMSO showed

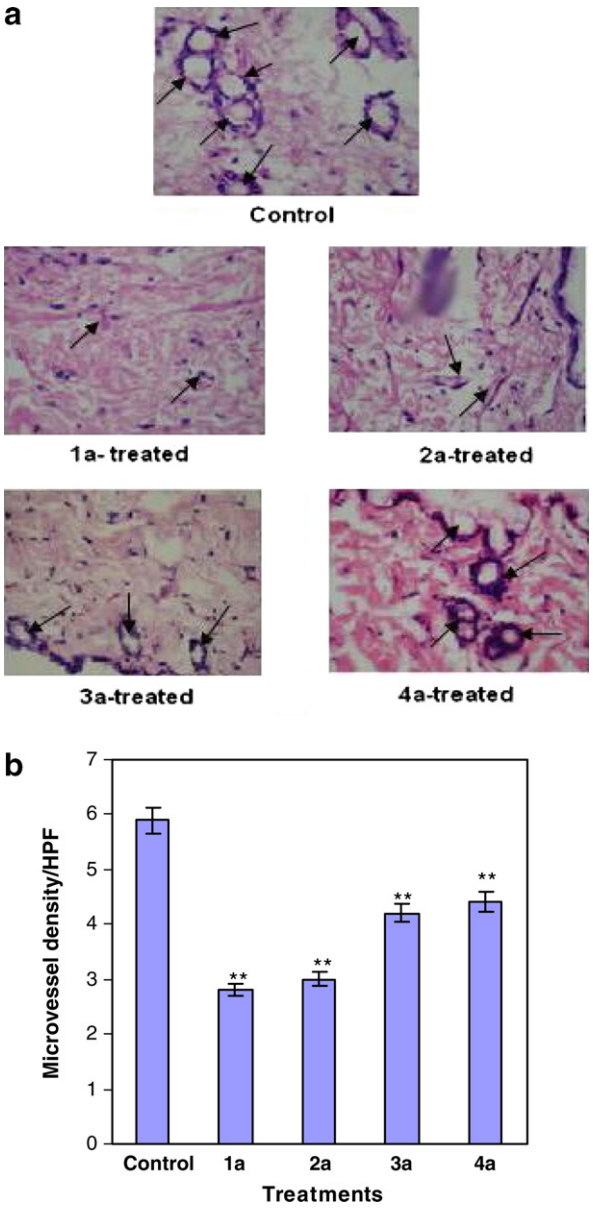


Figure 4. Histopathology and MVD count of the peritoneal wall sections of EAT bearing mice with or without synthetic novel dienone cyclopropoxy curcumin analogs **1a**–**4a** treatment in vivo. The arrow indicates the blood vessel present in section (a). Hematoxylin and eosin staining of peritoneal wall sections were observed to study MVD. The bar graph shows the effect of compounds on MVD count (b) of the peritoneal section of tumor bearing mice. All the compound treatments resulted in a significant decrease in MVD count compared to control ($p < 0.01$). The error bars represent standard deviation of the mean.

no changes in vasculature. The microvessel density decreased in all the compounds **1a**–**4a** treated CAM model.

3. Discussion

The basic principle of cancer chemotherapy is to use drugs that have targets and preferably nonoverlapping toxicity. Thus a logical chemotherapeutic strategy is the combined use of apoptosis inducing compounds and cytotoxic agents.[24–26] Analogs of curcumin are attractive targets for anti-angiogenesis. Examination of curcumin suggests that the two aromatic regions might be critical for potential ligand-receptor binding. A reasonable approach following standard medicinal chemistry design concepts was to explore compounds with systematic differences in the carbon chain connecting the two aromatic regions and substitution of cyclopropyl ring to the hydroxy phenyl group. The substitutions of cyclopropyl to hydroxyl group in aromatic ring led to the synthesis of four novel dienone cyclopropoxy curcumin analogs **1a**–**4a.**

The results on Ehrlich ascites tumor showed that the administration of curcumin analogs **1a**–**4a** on day 7, 9, and 11 after tumor cell inoculation produced effective antitumor response (>25% ILS). The compounds also exhibited corresponding reduction in mean ascites volume and cell number. Our findings demonstrated the potent anti-cancer activity of the dienone cyclopropoxy curcumin analogs against EAT in vivo. The compounds effectively reduced the ascites tumor burden and produced no side effects. Although the antitumor property of curcumin and its synthetic derivatives on in vivo and in vitro studies has been reported earlier,[27,28] this is for the first time the tumor inhibiting effects of synthetic novel dienone cyclopropoxy curcumin analogs have been demonstrated. Morphology of EAT cells after compound treatments exhibited apoptotic bodies, nuclear condensation, and intra nucleosomal fragmentation. These results indicate that the compounds **1a**–**4a** induced inhibition of EAT cell growth was due to the induction of apoptosis in EAT cells. As the compounds are injected intraperitoneally (ip) directly into the peritoneal cavity where the tumor is growing, the effect would be immediate and direct on the tumor cells. Induction of apoptosis by curcumin analogs both in vitro and in vivo studies has been reported[29–31] which may explain the tumor growth inhibitory and apoptotic effects of the compounds **1a**–**4a**. Apoptosis has been reported to be the cause of tumor cell death by chemotherapy.[32]

In the control EAT bearing mice, extensive peritoneal angiogenesis was observed which may be due to the secretion of the angiogenesis inducing factors in the ascites fluid. Involvement of VEGF in the formation of malignant ascites has been well documented.[33,34] Treatment of synthetic dienone cyclopropoxy curcumin analogs to EAT bearing mice significantly decreased peritoneal angiogenesis suggesting the inhibition of the secretion of such factors and thereby preventing the formation of new blood vessels. In recent report the natural curcumin has mediated its anti-angiogenic activity by inhibiting FGF-induced neovascularization.[35]

Microvessel density (MVD) counts have become the morphological gold standard to assess the neovasculature in tumors. MVD counts are reflective of the angioarchitectural properties of the tumor in that they are a representative of the average intercapillary distance. This is in fact an important parameter as it is the goal of an anti-angiogenic tumor therapy to reduce the intercapillary distance to a degree that it becomes rate-limiting for the growth of the tumor.[36] Hematoxylin and eosin staining of peritoneal wall tissue sections of EAT

bearing mice treated or untreated with the compounds **1a**–**4a** were examined for the microvessel density count. Control EAT bearing peritoneum section showed MVD of 5.9 ± 0.94/HPF, whereas in dienone cyclopropoxy curcumin analogs treated groups the MVD was drastically decreased. Thus our results demonstrate that dienone cyclopropoxy curcumin analogs inhibited the blood vessel formation and microvessel density which support the earlier findings that natural curcumin inhibits endothelial proliferation.[37,38] Our study agrees with the previous report on aromatic enone and dienone analogs of curcumin which showed anti-angiogenic inhibition pattern equivalent or better than the parent natural product.[39]

The compounds **1a**–**4a** with minor structural differences has exhibited varying degree of tumor growth inhibition and anti-angiogenic activities against EAT in vivo. In the present findings, the compound **1a** with the bioactive cyclopropoxy group at para-position and methoxy groups at ortho- and meta-positions in rings A and B showed potent in vivo antitumor and anti-angiogenic activities against mouse tumor, whereas in the absence of methoxy group at meta-position decreased the tumor inhibitory effect of **2a** compared to the compound **1a**. However, the compounds **3a** and **4a** with no methoxy groups in the structure have exhibited almost comparable tumor response. Thus the structural modifications have profound influence on antitumor and angioinhibitory activities of curcumin analogs. From our findings we can conclude that all the four novel synthetic dienone cyclopropoxy curcumin analogs **1a**–**4a** can be considered as promising antitumor and anti-angiogenic compounds. However, further investigations are needed to understand the mechanism of action of the compounds and to examine the possible utility of the compounds in cancer therapy.

4. Experimental

4.1. Chemistry

Chemicals were purchased from Sigma–Aldrich. Melting points were determined using SELACO-650 hot stage melting point apparatus and were uncorrected. Nuclear magnetic resonance (^{1}H NMR) spectra were recorded on Shimadzu AMX 400-Bruker, 400 MHz spectrometer using DMSO as a solvent and TMS as internal standard (chemical shift in δ ppm). Spin multiplets are given as s (singlet), d (doublet), t (triplet), and m (multiplet). Mass and purity were recorded on a LC-MSD-Trap-XCT. Elemental (CHNS) analyses were obtained on Vario EL III Elementar. Silica gel column chromatography was performed using Merck 7734 silica gel (60–120 mesh) and Merck made TLC plates.

Substituted curcumin analogs **1a**–**4a** were synthesized by the method summarized in Scheme 1. The target key intermediates **1**, **2**, **3**, and **4** were synthesized by using reported procedure.[40]

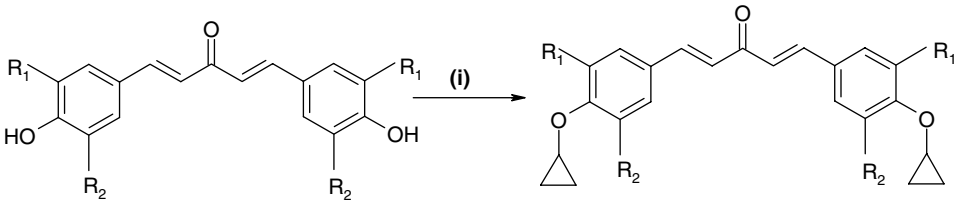
4.1.1. Synthesis of 1,5-bis-(4-cyclopropoxy-3,5-dimethoxy-phenyl)-penta-1,4-dien-3-one (1a). A solution of 1,5-bis-(4-hydroxy-3,5-dimethoxy-phenyl)-penta-1,4-dien-3-one **(1)** (0.386 g, 1 mmol) in dimethylformamide (5 ml) was taken, anhydrous potassium carbonate (0.557 g, 4 mmol) was added and stirred for 10 min, then cyclopropyl bromide diluted with 2 ml of DMF (0.244 g, 2 mmol) was added. The reaction mixture was heated to 60 °C for 6 h, and monitored by TLC. Upon completion, the solvent was removed under reduced pressure, residue was taken in water, and extracted with ethyl acetate. Finally water wash was given to the organic layer and dried with anhydrous sodium sulfate. The solvent was evaporated to get crude product which was purified by column chromatography over silica gel (60–120 mesh) using chloroform/methanol (9:1) as an eluent.

Yield: 70%. Mp: 159–162 °C.

^{1}H NMR (DMSO, 400 MHz): δ 0.48–0.73 (m, 8H), 2.42 (m, 2H), 3.73 (m, 12H), 6.26 (m, 4H), 7.03 (s, 2H), 7.66 (s, 2H).

MS: 466.20.

Anal. Calcd for $C_{27}H_{30}O_7$ (in %): C, 69.45; H, 6.43; O, 24.02. Found: C, 69.51; H, 6.48; O, 24.01.



1. $R_1 = OCH_3$, $R_2 = OCH_3$

2. $R_1 = OCH_3$, $R_2 = H$

3. $R_1 = H$, $R_2 = H$

4. $R_1 = CH_3$, $R_2 = CH_3$

1a. $R_1 = OCH_3$, $R_2 = OCH_3$

2a. $R_1 = OCH_3$, $R_2 = H$

3a. $R_1 = H$, $R_2 = H$

4a. $R_1 = CH_3$, $R_2 = CH_3$

Scheme 1. Reagents and condition: (i) DMF, K_2CO_3, cyclopropyl bromide, 60 °C, 6 h.

4.1.2. Synthesis of 1,5-bis-(4-cyclopropoxy-3-methoxy-phenyl)-penta-1,4-dien-3-one (2a). This compound was prepared according to the procedure described for **1a** from 1,5-bis-(4-hydroxy-3-methoxy-phenyl)-penta-1,4-dien-3-one (**2**) (0.326 g, 1 mmol), K_2CO_3 (0.552 g, 4 mmol), and cyclopropyl bromide (0.242 g, 2 mmol).

Yield: 79%. Mp: 94–96 °C.

^{1}H NMR (DMSO, 400 MHz): δ 0.48–0.73 (m, 8H), 2.42 (m, 2H), 3.71 (s, 6H), 6.61 (d, 2H), 6.7 (s, 2H), 6.75 (d, 2H), 7.03 (s, 2H), 7.66 (s, 2H).

MS: 406.18.

Anal. Calcd for $C_{25}H_{26}O_5$ (in %): C, 73.85; H, 6.40; O, 19.69. Found: C, 73.87; H, 6.45; O, 19.68.

4.1.3. Synthesis of 1,5-bis-(4-cyclopropoxy-phenyl)-penta-1,4-dien-3-one (3a). This compound was prepared according to the procedure described for **1a** from 1,5-bis-(4-hydroxy-phenyl)-penta-1,4-dien-3-one (**3**) (0.266 g, 1 mmol), K_2CO_3 (0.552 g, 4 mmol), and cyclopropyl bromide (0.241 g, 2 mmol).

Yield: 80%. Mp: 234–236 °C.

^{1}H NMR (DMSO, 400 MHz): δ 0.48–0.73 (m, 8H), 2.42 (m, 2H), 6.73 (m, 4H), 7.20 (s, 2H), 7.19 (m, 4H), 7.66 (s, 2H).

MS: 346.16.

Anal. Calcd for $C_{23}H_{22}O_3$ (in %): C, 79.73; H, 6.40; O, 13.86. Found: C, 79.74; H, 6.35; O, 13.85.

4.1.4. Synthesis of 1,5-bis-(4-cyclopropoxy-3,5-dimethyl-phenyl)-penta-1,4-dien-3-one (4a). This compound was prepared according to the procedure described for **1a** from 1,5-bis-(4-hydroxy-3,5-dimethyl-phenyl)-penta-1,4-dien-3-one (**4**) (0.322 g, 1 mmol), K_2CO_3 (0.552 g, 4 mmol), and cyclopropyl bromide (0.242 g, 2 mmol).

Yield: 75%. Mp: 232–234 °C.

^{1}H NMR (DMSO, 400 MHz): δ 0.48–0.73 (m, 8H), 2.42 (m, 2H), 2.35 (m, 12H), 6.80 (m, 4H), 7.03 (s, 2H), 7.66 (s, 2H).

MS: 402.42.

Anal. Calcd for $C_{27}H_{30}O_3$ (in %): C, 80.56; H, 7.51; O, 11.92. Found: C, 80.50; H, 7.47; O, 11.85.

4.2. Biology: in vivo anti-cancer and angioinhibitory effects of synthetic novel dienone cyclopropoxy curcumin analogs

4.2.1. Animals and tumor model. Inbred Swiss albino mice, 6–8 weeks old, weighing 25 ± 5 g of either sex, were used for the experiments. They were bred and maintained in the animal house, Department of Zoology, Manasagangotri, Mysore, India. Ehrlich ascites tumor was grown in adult Swiss albino mice intraperitoneally. Cell viability was tested by trypan blue exclusion assay. Experimental animals were prepared by injecting 5×10^6 viable tumor cells into intraperitoneal cavity of Swiss mice. Tumor growth was followed by recording the animal weights. EAT cells begin their exponential growth phase from the 7th day after tumor cell injection and the animal succumbs to the ascites tumor burden on day 16–20 after injection.

Compounds: Synthetic dienone cyclopropoxy curcumin analogs **1a**–**4a** were used as compounds for the experiments. The compounds were weighed and dissolved in 0.1% DMSO to get required concentrations. The compound treatments were initiated on the day 7 of tumor transplantation on the advanced stage of tumor when the cells enter into exponential growth period.

4.2.2. Animal survival. After 7 days of tumor cell injection, the animals were divided into groups of 10 each and were treated as follows: control: 0.2 ml of 0.1% DMSO was given on day 7, 9, and 11 of tumor transplantation. Compound treated groups—the compounds **1a, 2a, 3a, and 4a**—were given to four different groups of tumor bearing mice. The compound 100 mg/kg body wt was injected ip into the mice using 26 gauge needle on day 7, 9, and 11 of tumor transplantation. All the mice were weighed on the day of tumor inoculation and at weekly intervals. Animal survival was recorded up to 40 days. The tumor response was assessed on the basis of MST and increase in life span (% ILS). Median survival time (MST) and % ILS were calculated from the mortality data within the observation period. Increase in life span was calculated by the formula.

$$\%\,\mathrm{ILS} = \frac{\mathrm{MST\ of\ treated\ group} - \mathrm{MST\ of\ control\ group}}{\mathrm{MST\ of\ control\ group}} \times 100$$

Enhancements of life span by 25% are more over that of the control was considered as effective antitumor response.[41]

4.2.3. Tumor growth inhibition and anti-angiogenesis. After 7 days of tumor cell injection, the animals were divided into five groups of 10 each and the control group received 0.2 ml of 0.1% DMSO on day 7, 9, and 11 of tumor transplantation. The compounds (**1a**, **2a**, **3a**, and **4a**) were given to four different groups of tumor bearing mice. The tumor inhibitory effects of the compounds on EAT cell growth were assessed by measuring cell number and ascites volume. On day 12 the control and compounds **1a**–**4a** treated tumor bearing mice were sacrificed, an incision was made in the abdominal region and EAT cells along with the ascites fluid were harvested into a beaker containing 2 ml saline and centrifuged at 3000 rpm for 10 min at 4 °C. Subtracting the volume of saline added previously from the volume of the supernatant gave the volume of ascites fluid. After harvesting the EAT cells, the cells were resuspended in 0.9% saline and counted using a haemocytometer.

4.2.4. Changes in the morphology of EAT cells. *Giemsa staining:* EAT cells from the control and treated groups **1a**–**4a** were smeared on clean glass slides, air-dried, and fixed in a solution of methanol/acetic acid (3:1). The slides were hydrated with PBS, then stained with 0.1% Giemsa solution, and observed under compound microscope.

4.2.5. Acridine orange/ethidium bromide staining. Nuclear staining was performed according to the method of Srinivas et al.[42] The EAT cells collected from both control and compounds **1a**–**4a** treated groups were smeared on clean glass slides, air-dried, and fixed in a solution of methanol/acetic acid (3:1). The slides were hydrated with PBS, then stained with mixture of acridine orange/ethidium bromide (1:1). The cells were immediately washed with PBS and viewed under fluorescent microscope and photographed.

4.2.6. Anti-angiogenic effects of the compounds. *Peritoneal angiogenesis:* The peritoneum of the mice was cut open and the inner lining of the peritoneal cavity were examined for angiogenesis in both control and compounds **1a**–**4a** treated tumor bearing mice and photographed.

4.2.7. Histopathology of mice peritoneum tissue. Peritoneal tissues from tumor bearing control mice and mice treated with compounds **1a**–**4a** were fixed in 10% formalin, embedded in paraffin, and 5-μm sections were routinely stained with hematoxylin and eosin. The sections were observed under low power (10×) of light microscope to identify the highly vascularized areas. The microvessel density (MVD) was counted in 10 fields of these vascularized areas under high power (40×) and the average MVD/HPF was noted.

4.2.8. Angioinhibitory effects of the compounds on in vivo chorio allantoin membrane. Chorio allantoin membrane assay was performed according to the method of Chandru and Sharada.[43] The fertilized eggs were divided into different treatment groups. Control, the saline treated group, and compound treated groups with minimum of six eggs in each group were maintained separately and observed. The fertilized eggs were incubated for 5 days at 37 °C in a humidified and sterile atmosphere. A window was made under aseptic conditions on the egg shell to check for the proper development of the embryo. The windows were resealed and incubation was continued. On day 11 the windows were opened and the compounds **1a**–**4a** (0.1 mM) or vehicle was loaded on the cover slips separately, air-dried, and inverted over the CAM and the windows were closed. The windows were resealed and the embryo was allowed to develop further.

The windows were opened and observed on day 13 and inspected for changes for the microvessel density in the area under the cover slip and examined under a microscope for avascular zone and photographed.

4.2.9. Statistical analysis. All data were analyzed by one-way ANOVA. Values of $p < 0.05$ were considered significant.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.08.051.

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