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Synthesis and tumor inhibitory activity of novel coumarin analogs targeting angiogenesis and apoptosis

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ABSTRACT

A sequence of coumarin analogs **5a**–**j** was obtained by multi step synthesis from hydroxy benzophenones (**1a**–**j**). The *in vitro* antiproliferative effect of the title compounds was tested against Ehrlich ascites carcinoma (EAC) and Daltons lymphoma ascites (DLA) cell lines. Among the series, compound **5c** with bromo group in the benzophenone moiety was endowed with excellent antiproliferative potency with significant IC_{50} value. Further, *in vivo* antitumor effect of compound **5c** against murine EAC and solid DL tumor model system was evident by the extended survivality. The tumor inhibitory mechanism of compound **5c** was due to the antiangiogenesis and promotion of apoptosis. These results suggest possible applications of compound **5c** which could be developed as a potent anticancer drug in the near future.

1. Introduction

Most, if not all, human cancers share six acquired capabilities that enable malignant growth as proposed by Hanahan and Weinberg. Promotion of angiogenesis and resistant to apoptosis are the two important hallmarks of cancer [1]. Tumor growth and expansion requires an ability not only to proliferate, but also to down-modulate cell death and activate angiogenesis to produce a tumor neovasculature. Thus, the promotion of apoptosis and anti-angiogenesis targeting strategies is one of the important focus in current cancer therapy [2]. The development of such novel, effective and less or no toxic compounds with multiple mode of action for targeted cancer therapy has become an innovative approach and efforts have been directed toward discovering such anticancer agents endowed with cytotoxic action [3,4].

Coumarins are an old class of compounds obtained from both natural products and synthetic methods. The pharmacological and biochemical properties and therapeutic applications of coumarins depend upon the pattern of substitution and have attracted intense interest in recent years because of their diverse pharmacological properties [5]. Among these properties, their cytotoxic effects were the most extensively examined, this reflects in anticancer activity. Studies have revealed the mechanism behind the anticancer effect of coumarin analogs which include antiangiogenesis and induction of apoptosis independently [6–12]. The current strategies in cancer drug development shifted toward the multiple mechanistic approach and several drugs have been validated and developed. Such validation of structure–system–activity–relationship of coumarins with special respect to angiogenesis and apoptosis leads to cancer-preventing activities should be continued [13]. The vast majority of coumarins, completely innocuous, may be beneficial in a variety of human cancer, in spite of some ongoing controversy [14]. Hence it is very essential to synthesize and develop novel coumarin analogs with multiple targets. In the present study efforts have been made to synthesize novel derivatives of coumarin analogs with antiangiogenic and proapoptotic activity leading to inhibition of tumor growth in mouse model systems.

2. Results and discussion

2.1. Chemistry

The synthesis of the title compounds **5a**–**j** is as outlined in Scheme 1. A series of *N*-[2-(2-aroyl-4-methylphenoxy)-acetyl]-hydrazide methanone coumarins **5a**–**j** were obtained starting from

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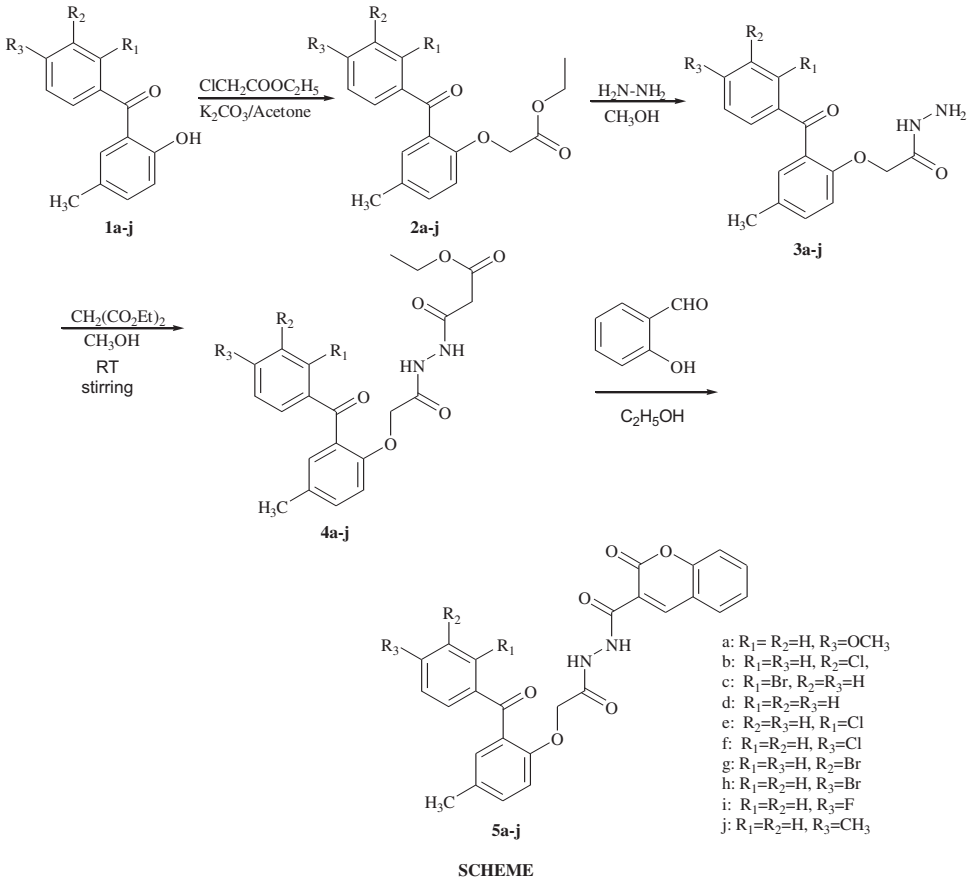

SCHEME

Scheme 1. Synthesis of coumarin conjugated benzophenone analogs.

hydroxyl benzophenones **1a**–**j**. Compounds **1a**–**j** on reaction with ethyl chloroacetate afford ethyl 2-aroyl-4-methylphenoxy acetates **2a**–**j** [19], which on treatment with hydrazine hydrate in the presence of ethanol yields 2-aroyl-4-methylphenoxy acetohydrazides **3a**–**j** [22]. Condensation of **3a**–**j** with diethyl malonate in the presence of methanol at room temperature affords {*N*-[2-(2-aroyl-4-methyl-phenoxy)-acetyl]-hydrazinocarbonyl}-acetic acid ethyl ester **4a**–**j**. Finally the title compounds **5a**–**j** were achieved by intramolecular cyclization of **4a**–**j** with *o*-hydroxy benzaldehyde in the presence of alcohol. The structures of the compounds were confirmed by IR, NMR and mass spectroscopy. In IR spectra the disappearance of O–C stretching band of ester group and appearance of amide C=O and ring C=O stretching bands were observed. Besides, the compounds were confirmed by disappearance of $COCH_2$, CH_2 and CH_3 protons and enhancement in the number of aromatic protons in ^1H NMR spectra and also by mass spectra and CHN analysis.

2.2. Pharmacology

2.2.1. *5c is the lead compound*

The synthesized coumarin analogs were initially tested for their cytotoxic and antiproliferative activity in EAC and DLA cells *in vitro* (Table 1). Among the series of compounds **5a**–**j**, the

Table 1
IC_{50} values of compounds **5a**–**j** calculating based upon trypan blue, MTT at 48 h in EAC and DLA cells.

	EAC cells			DLA cells		
	Trypan blue assay IC_{50} values (μM)	MTT assay IC_{50} values (μM)	LDH release assay IC_{50} values (μM)	Trypan blue assay IC_{50} values (μM)	MTT assay IC_{50} values (μM)	LDH release assay IC_{50} value (μM)
Control	–	–	–	–	–	–
5a	41.2	38.4	41.2	43.5	42.0	43.4
5b	67.3	64.9	65.4	68.6	65.5	67.4
5c	**9.0**	**8.0**	**9.4**	**10.0**	**10.0**	**10.6**
5d	86.5	78.4	81.1	89.1	86.4	87.0
5e	48.4	45.2	47.6	54.8	53.8	54.4
5f	57.8	56.6	58.0	61.6	59.1	60.4
5g	68.5	66.4	68.1	71.9	68.5	69.8
5h	91.1	87.3	89.0	95.5	92.2	95.3
5i	43.4	39.7	40.8	46.1	43.4	45.7
5j	82.6	78.1	81.4	87.3	84.6	87.2
5-Fluorouracil (standard)	16.4	14.3	16.3	14.3	15.7	15.8

The bold values signify potent compound.

compound **5c** with bromo substituent at ortho position of benzoyl ring in benzophenone moiety showed promising antiproliferative potency against EAC cells with IC_{50} of 9 μM and 8 μM in Trypan blue and MTT assay, respectively. Comparable results were observed against DLA cells with IC_{50} of 10 μM in both assays (Table 1). Compound **5c** exhibits more active than other analogs and undoubtedly emerged as more active and lead compound within this subset. Effect of compound **5c** on cellular integrity as verified by LDH release assay inferred a concentration dependent increase in the LDH release with the IC_{50} of 9.4 μM and 10.6 μM in EAC and DLA cell lines, respectively (Table 1). Thus, our studies using two cell lines of different origin suggest that irrespective of the cancer type, compound **5c** could induce cytotoxicity, as shown by three independent assaying methods and further investigated for antitumor effect.

*2.2.2. Treatment of compound **5c** prevents progression of tumors in both murine ascites and solid tumor model system without any side effects*

Murine ascites and solid tumors are a suitable model system for preliminary screening and place a critical role in drug development. It is a rational and a hierarchical approach beginning with toxicology and pharmacology studies, progressing to primary tumors to identify therapeutic targets and models of metastatic disease to compare drugs using rigorous, clinically relevant outcome parameters [15]. In the present study we have chosen Murine EAC and solid DL model systems to study the preliminary antitumor property of compound **5c**. Ehrlich ascites tumor implantation induces a set of local inflammatory reaction, with increasing vascular permeability, which results in an intense edema formation, cellular migration and a progressive ascites fluid formation [16]. Ascites

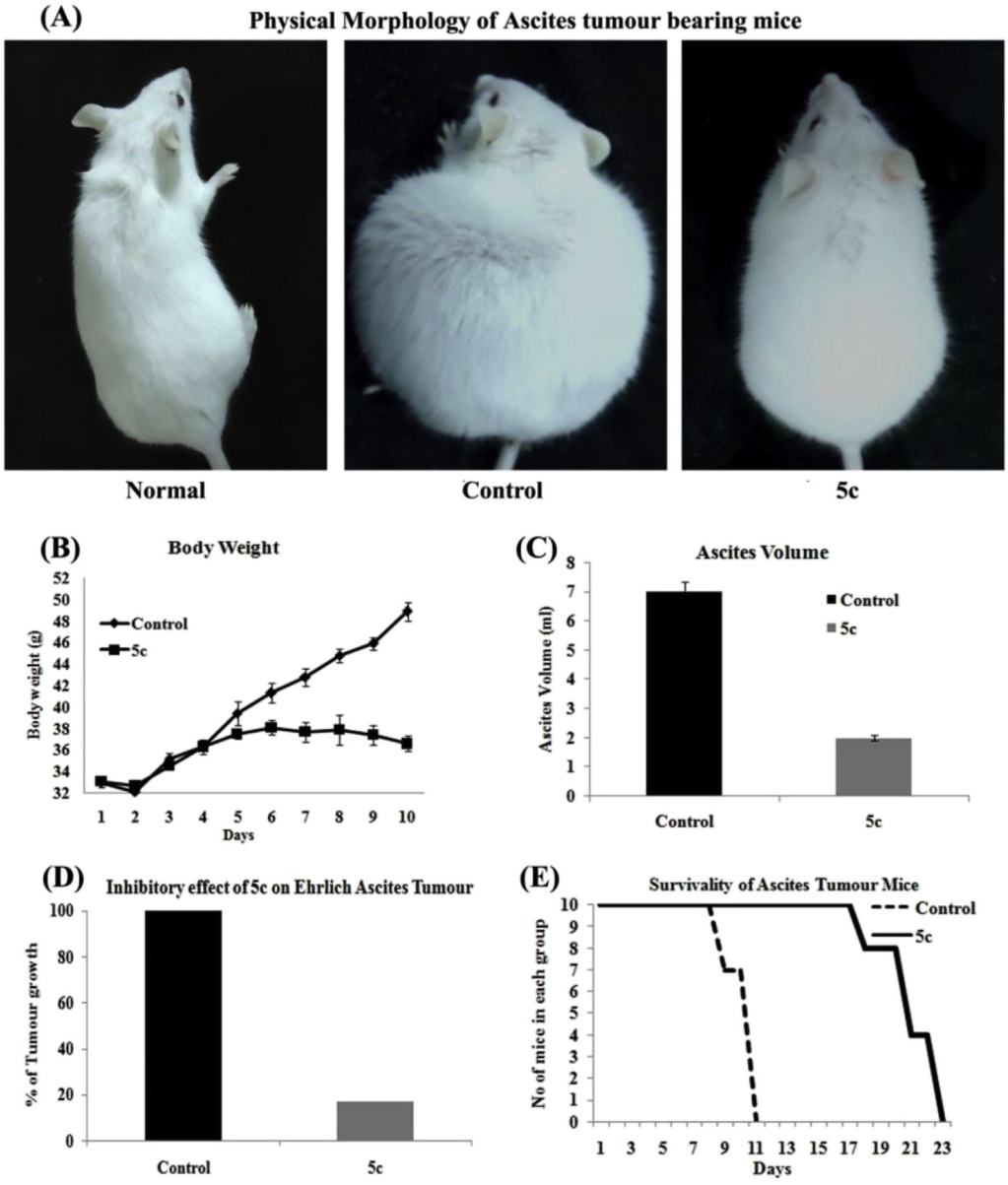

Fig. 1. Effect of compound **5c** treatment on Ehrlich Ascites Carcinoma (EAC) in mice. Ascites tumor was induced in 6–7 week old Swiss albino mice by injecting EAC cell intraperitoneally. Three doses of compound **5c** (75 mg/kg) each administered to tumor bearing mice on every alternate day after three days of tumor growth. (A) Physical morphology of normal, control and compound **5c** treated tumor mice. (B) Dose dependent decrease in body weight of mice treated with compound **5c** compared with control mice. (C) Decrease in ascites secretion. (D) Reduction in percentage of tumor growth. (E) Kaplan–Meier survival curve for. Data are reported as the mean ± S.D. of three different observations (six animals per treatment group).

fluid is the direct nutritional source for tumor cells and a rapid increase in ascites fluid with tumor growth would be a means to meet the nutritional requirement of growing tumor cells [17]. Hence decrease in ascites fluids accounts for the suppression of tumor growth.

In the present study the compound **5c** was given at the concentration of 75 mg/kg body weight i.p., as determined by LD_{50} studies on 4th, 6th and 8th day of the tumor growth. Upon treatment with compound **5c** on the murine ascites tumor, there was a dose dependent decrease in body weight (Fig. 1A and B). The ascites secretion responsible for establishment of tumor was also reduced when compared to the control (Fig. 1C and D). The mice bearing Ehrlich Ascites Carcinoma (EAC) cells survive only for 10 days after implantation of the tumor cells. No treatment continued for test animals after the 10th day and kept for survival studies. The body weight of treated animals kept for survivality analysis monitored regularly and slight increase (~ 10%) was observed. Compound **5c** significantly expanded the life span of the treated mice to 23 days (Fig. 1E). The measurement of asictes volume and tumor cells after the death of the animal indicated moderate increase inferring re-occurrence of the tumor.

The solid tumor model system is the most reliable and representative of major histological types of cancer and thereby providing the rapid action of drug delivery [15]. The important concern in the drug development process is target specific in action minimizing the side effects. Therefore the antitumor potency of compound **5c** was evaluated in solid DL tumor model system. Upon treatment of **5c** from the onset of solid DL tumor, a significant reduction in tumor volume was observed when compared with untreated mice (Fig. 2A). Most decisively, there was approximately a three-fold increase in the life span of treated animals (Fig. 2B). To study the physiological effect of compound **5c**, gross morphological and anatomical appearance of thigh tissue containing tumor of control and treated animals on the 35th day after tumor development was assessed which further confirmed the regression of tumors. The appearance of the treated animals as well as the morphology of their dissected organs like liver and spleen were comparable with those of normal animals indicating that compound **5c** treatment is not cytotoxic to organs and did not lead to visible alterations (Fig. 2C) suggesting that **5c** could be a potent target specific antitumor agent.

On the other hand, compound **5c**, despite being a more potent antitumor compound had limited or no adverse side effect as verified by hematological and serum profile parameters in non-tumor bearing mice (Table 2).

Table 2
Hematological and serum profile of non-tumor bearing mice following treatment with compound **5c** at day 10. Values are indicated in mean ± SEM.

Hematological and serum profile parameters	Normal mice	Treated mice
Alkaline Phosphatase (IU/L)	128.55 ± 2.55	132.65 ± 3.6
Creatinine (mg/dl)	0.59 ± 1.05	0.50 ± 2.35
Urea (mg/dl)	42 ± 2.78	39 ± 1.9
RBC (10^6/μl)	5.18 ± 1.45	5.98 ± 1.75
WBC (10^6/μl)	3.22 ± 1.2	3.91 ± 0.5

2.2.3. *5c as a potent inhibitor of angiogenesis*

Tumor growth and metastasis are dependent on angiogenesis as demonstrated in many *in vivo* experiments [18]. Increased neovasculature may allow not only increase in tumor growth but also

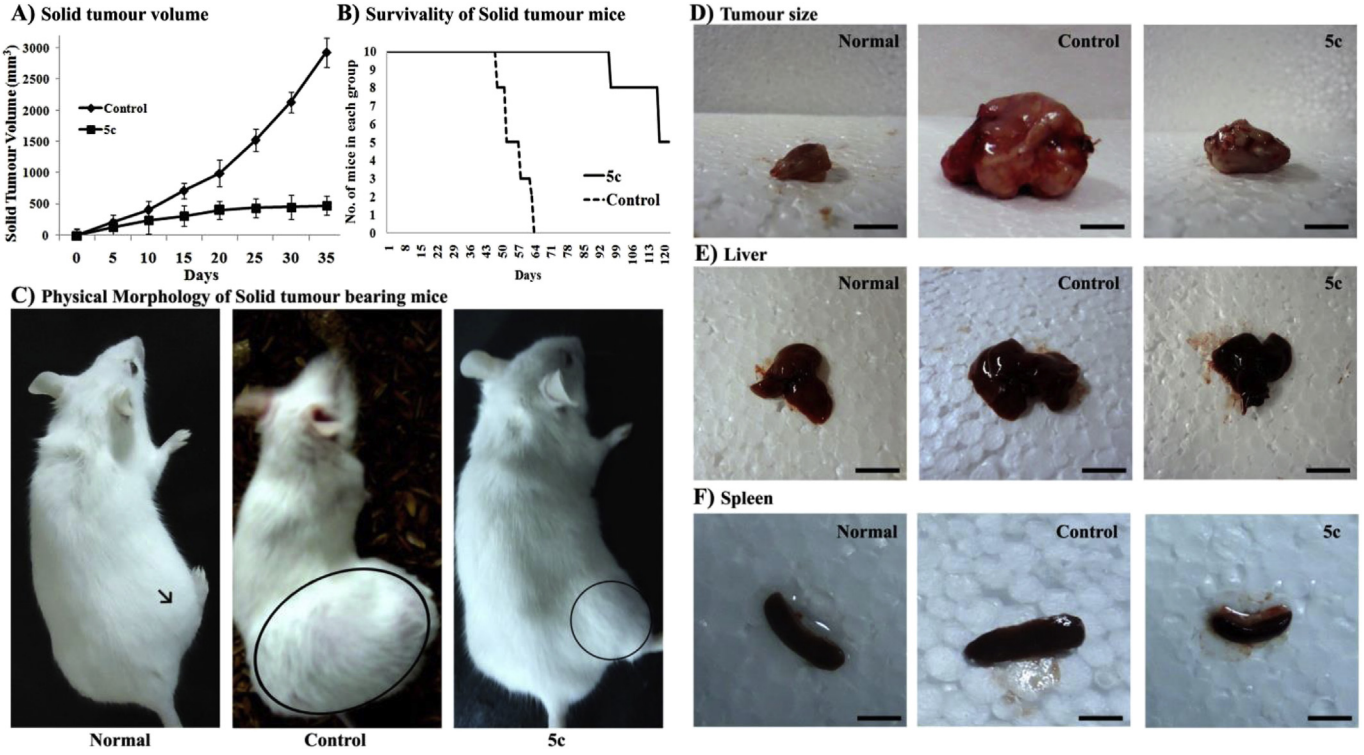

Fig. 2. Comparison of effect of compound **5c** on progression of solid tumor in mice and their selected organs at 35th day of treatment. Solid tumor was induced in 6–7 week old Swiss albino mice by injecting DLA cells subcutaneously into thigh region. Ten doses of **5c** (75 mg/kg) each administered to tumor bearing mice on every alternate day after solid tumor grew to 100 mm^3 in size (A) compound **5c** inhibited tumor growth as measured by tumor volume (B) Kaplan–Meier survival curve. (C) Physical appearance of normal, control and compound **5c** treated tumor mice. (D) Antiproliferative effect of compound **5c** on tumor size shows active tumor inhibitory properties of compound **5c**. (E) Livers and (F) spleens from normal, control and **5c** treated mice depicts that compound **5c** is not cytotoxic to organs. Data represented as the mean ± S.D. of three different observations (six animals per treatment group).

enhances hematogenous tumor rembolization. Thus inhibiting tumor angiogenesis may arrest the tumor growth and decrease the metastatic potential of tumors. Measurement of neovascularization or micro vessel density (MVD) is a widely used surrogate marker in pathological specimens and tumor models to assess the prognosis of the disease [19,20]. The current study has revealed that the compound **5c** has potent antitumor efficacy and activation of antiangiogenesis could be one of the possible underlying mechanisms of tumor inhibition. Since the angiogenesis is evident in the inner lining of the peritoneum of the EAC tumor bearing mice and it is a reliable model to study the angiogenesis dependent tumor growth [19], we verified the effect of compound **5c** for angioprevention effect. Surprisingly the compound **5c** was showed decreased angiogenesis when compared to the immense angiogenesis in the peritoneum lining of untreated tumor bearing mice (Fig. 3A). Further, the formalin fixed peritoneum sections were subjected to histopathological analysis with H&E staining to measure the Micro Vessel Density (MVD) (Fig. 3B). There was a prominent decrease in the MVD in the peritoneum sections of the treated animals with 10 ± 3 Vessels/High Power Field (V/HPF) whereas in tumor bearing mice 33 ± 2V/HPF was observed (Fig. 3C).

Angioprevention effect was further assessed by the CAM assay which is another reliable model for angiogenesis studies [21]. In the present study we have employed $rVEGF_{165}$ induced neovascularisation in both *in vivo* and *ex vivo* CAM models to study the efficacy of the compound **5c**. A clear avascular zone around the implanted disc with compound indicates the inhibition of angiogenesis in CAM (Fig. 3D and E) reconfirming the peritoneal angiogenesis results.

2.2.4. The compound **5c** induces apoptosis in Ehrlich ascites carcinoma

The resistances to apoptosis and angiogenesis are the two important characteristics which promote the establishment of the tumor and there is a direct correlation between these two characteristics [1,2]. This inhibition of angiogenesis may lead to promotion of apoptosis resulting in cell death there by tumor inhibition. Several anticancer drugs with these dual effects used in current cancer therapy and many are in clinical trials [2]. When we verified the possible proapoptotic effect of compound **5c** on EAC cells by Giemsa staining and DNA degradation assay it was found that the morphological changes in EAC cells treated with

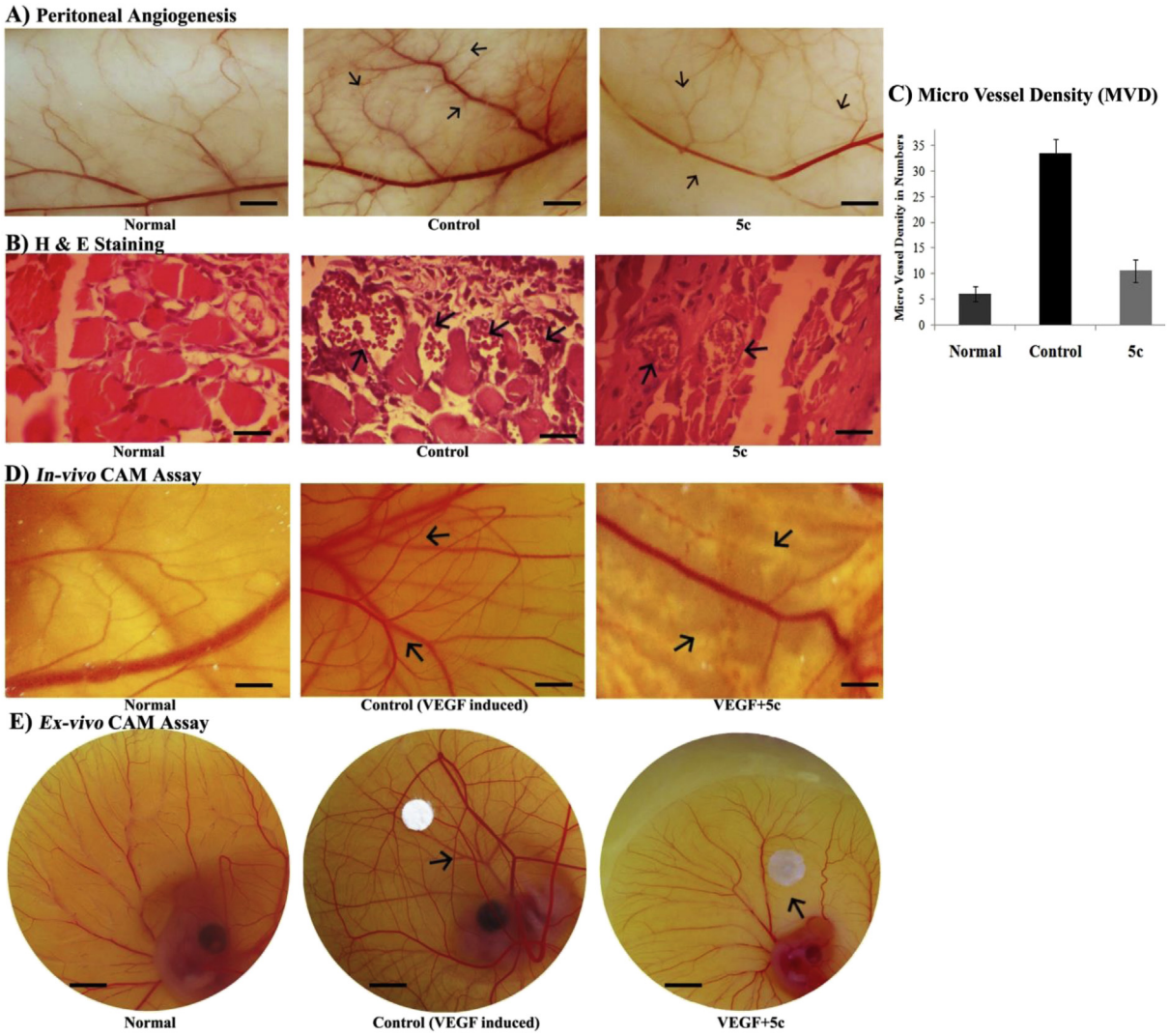

Fig. 3. Angiogenesis modulatory effect of compound **5c** reduces the neovascularization. (A) Peritoneal angiogenesis as seen by EAC induced neovascularization in compound **5c** treated compared to control and the peritoneum lining of mice was photographed. (B) Hematoxylin and Eosin stained inner peritoneum section showing varying number of micro vessel density (MVD) in normal, control and compound **5c** treated mice. (C) Depiction of decrease in Micro Vessel Density in compound 5c treated mice compared to control mice. (D) *In vivo* and (E) *ex vivo* CAM photos exhibits the angiopreventive effect of compound **5c**.

compound **5c** indicated typical apoptotic structures including cell shrinkage, condensed nucleus and formation of apoptotic bodies (Fig. 4A and B). DNA fragmentation was also apparent and provides an additional proof for the induction of apoptosis caused due to cytotoxic effect of **5c** (Fig 4C).

3. Conclusion

The complex molecular pathways that govern angiogenesis and apoptosis are two logical targets for pharmacological manipulations given the important role they play in the tumor growth and development of cancers. So targeting the tumor neovasculature and inducing apoptosis is an attractive strategy for effective cancer therapy. As an approach we synthesized a series of coumarin analogs and screened for cytotoxic and antitumor potency against two different cell lines both *in vitro* and *in vivo*. The compound **5c** emerged as a lead compound with potent cytotoxic and antitumor efficacy by inhibiting the neovascularisation and promoting apoptosis. This study highlights that the compound **5c** *N*-{2-[2-(2-bromo-aroyl)-4-methylphenoxy]-acetyl}-hydrazide methanone coumarins was identified as a promising anticancer molecule with multiple mode of actions such as antiproliferative, angiopreventive effect together with its remarkable apoptosis inducing action, making it as great interest for further studies.

4. Materials and methods

4.1. Experimental section

Chemicals were procured from Sigma Aldrich Chemical Co. TLC was performed on aluminum-backed silica plates and visualized by UV-light. Melting points were determined on a Thomas Hoover capillary melting point apparatus with a digital thermometer. IR spectra were recorded in Nujol on FT-IR Shimadzu 8300 spectrophotometer, ^{1}H NMR spectra were recorded on a Bruker 400 MHz NMR spectrophotometer in $CDCl_3$ and chemical shift were recorded in parts per million down field from tetramethylsilane. Mass spectra were obtained with a VG70-70H spectrophotometer and important fragments are given with the relative intensities in the brackets. Elemental analysis results are within 0.5% of the calculated value.

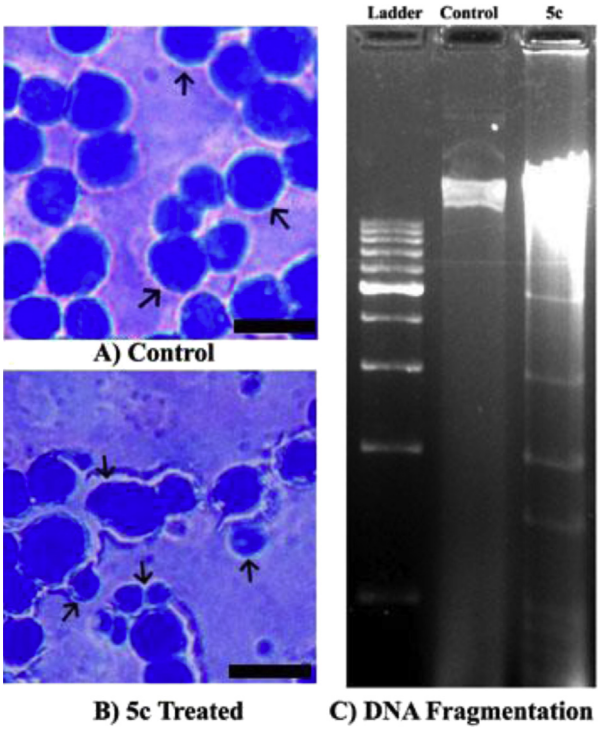

Fig. 4. Proapoptotic effect of compound **5c** on EAC bearing Mice. (A) EAC cells from control stained with 0.1% of Giemsa photograph depicting that regular shape of the cells and none of apoptotic bodies (B) EAC cells from 5c treated stained with 0.1% of Giemsa photograph showing that irregular shape and membrane blebbing of the cells and formation apoptotic bodies. (C) Observation of DNA fragmentation in control versus compound **5c** treated showing that fragmented DNA pattern in compound **5c** treated compared to those in control.

4.2. Chemistry

4.2.1. General procedure for synthesis of ethyl (2-benzoyl-4-methylphenoxy) acetates (**2a**–**j**)

A mixture of **1a**–**j** (0.028 mol) and ethyl chloroacetate (0.028 mol) in dry acetone (70 ml) and anhydrous potassium carbonate (0.056 mol) was refluxed for 7–8 h then cooled and the solvent removed under reduced pressure. The residual mass was triturated with ice water to remove potassium carbonate and extracted with ether (3 × 60 ml) and the ether layer was washed with 10% sodium hydroxide solution (3 × 40 ml) followed by distilled water (3 × 40 ml) and then dried over anhydrous sodium sulfate and evaporated to dryness to get crude solid, which on recrystallization with alcohol gave pure compounds **2a**–**j** [19].

4.2.1.1. Ethyl [2-(4-methoxybenzoyl)-4-methylphenoxy] acetate ***2a***. Yield 88%; M.p. 58–60 °C; IR (Nujol): 1660 (C═O), 1730 cm^{-1} (ester, C═O); ^{1}H NMR ($CDCl_3$): δ 1.2 (t, J = 7 Hz, 3H, CH_3 of ester), 2.25 (s, 3H, CH_3), 3.8 (s, 3H, OCH_3), 4.2 (q, J = 6 Hz, 2H, CH_2 of ester), 4.42 (s, 2H, OCH_2), 7.0 (d, J = 8.3 Hz, 2H, Ar–H), 7.15–7.41 (m, 3H, Ar–H), 7.49 (d, J = 8.5 Hz, 2H, Ar–H); EI-MS: m/z 328 (M^+, 59). Anal. Calcd. for $C_{19}H_{20}O_5$ (328): C, 69.51; H, 6.09. Found: C, 69.49; H, 6.05%.

4.2.1.2. Ethyl [2-(3-chloro-benzoyl)-4-methylphenoxy]acetate ***2b***. Yield 85%; M.p. 60–62 °C; IR (Nujol): 1670 (C═O), 1735 cm^{-1} (ester, C═O); ^{1}H NMR ($CDCl_3$): δ 1.2 (t, J = 7 Hz, 3H, CH_3 of ester), 2.3 (s, 3H, CH_3), 4.2 (q, J = 6 Hz, 2H, CH_2 of ester), 4.45 (s, 2H, OCH_2), 7.2–7.6 (m, 7H, Ar–H); EI-MS: m/z 332 (M^+, 62). Anal. Calcd. for $C_{18}H_{17}ClO_4$ (332.5): C, 64.96; H, 5.11. Found: C, 64.94; H, 5.07%.

4.2.1.3. Ethyl [2-(2-bromo-benzoyl)-4-methylphenoxy]acetate ***2c***. Yield 81%; M.p. 65–67 °C; IR (Nujol): 1665 (C═O), 1730 cm^{-1} (ester, C═O); ^{1}H NMR ($CDCl_3$): δ 1.21 (t, J = 7 Hz, 3H, CH_3 of ester), 2.3 (s, 3H, CH_3), 4.22 (q, J = 6 Hz, 2H, CH_2 of ester), 4.46 (s, 2H, OCH_2), 7.2–7.6 (m, 7H, Ar–H); EI-MS: m/z 376 (M^+, 61). Anal. Calcd. for $C_{18}H_{17}BrO_4$ (377): C, 57.29; H, 4.50. Found: C, 57.26; H, 4.53%.

4.2.1.4. Ethyl [2-benzoyl-4-methylphenoxy]acetate ***2d***. Yield 83%; M.p. 61–63 °C; IR (Nujol): 1664 (C═O), 1760 cm^{-1} (ester, C═O); ^{1}H NMR ($CDCl_3$): δ 1.2 (t, J = 7 Hz, 3H, CH_3 of ester), 2.3 (s, 3H, CH_3), 4.1 (q, J = 6 Hz, 2H, CH_2 of ester), 4.5 (s, 2H, OCH_2), 7.1–7.7 (m, 8H, Ar–H); EI-MS: m/z 298 (M^+, 60). Anal. Calcd. for $C_{18}H_{18}O_4$ (298): C, 72.48; H, 6.04. Found: 72.46; H, 6.02%.

4.2.1.5. Ethyl [2-(2-chloro-benzoyl)-4-methylphenoxy]acetate ***2e***. Yield 84%; M.p. 52–54 °C; IR (Nujol): 1672 (C═O), 1737 cm^{-1} (ester, C═O); ^{1}H NMR ($CDCl_3$): δ 1.21 (t, J = 7 Hz, 3H, CH_3 of ester), 2.31 (s, 3H, CH_3), 4.21 (q, J = 6 Hz, 2H, CH_2 of ester), 4.46 (s, 2H, OCH_2), 7.25–7.7 (m, 7H, Ar–H); EI-MS: m/z 332.5 (M^+, 61). Anal. Calcd. for $C_{18}H_{17}ClO_4$ (332.5): C, 64.96; H, 5.11. Found: C, 64.99; H, 5.07%.

4.2.1.6. Ethyl[2-(4-chloro-benzoyl)-4-methylphenoxy] acetate ***2f***. Yield 70%; M.p. 62–65 °C; IR (Nujol): 1675 (C═O),1740 cm^{-1} (ester, C═O); ^{1}H NMR ($CDCl_3$): δ 1.22 (t, 3H, CH_3 of ester), 2.35 (s, 3H, CH_3),

4.2 (q, 2H, CH_2 of ester), 4.5 (s, 2H, OCH_2), 7.2–7.7 (m, 7H, Ar–H); EI-MS: *m/z* 332.5 (M^+, 61). Anal. Calcd. for $C_{18}H_{17}ClO_4$ (332.5): C, 64.96; H, 5.11. Found: C, 64.94; H, 5.09%.

4.2.1.7. Ethyl [2-(3-bromo-benzoyl)-4-methylphenoxy]acetate ***2g***. Yield 80%; M.p. 55–57 °C; IR (Nujol): 1620 (C=O), 1725 cm^{-1} (ester, C=O); ^1H NMR ($CDCl_3$): δ 1.2 (t, $J = 7$ Hz, 3H, CH_3 of ester), 2.2 (s, 3H, CH_3), 4.3 (q, $J = 6$ Hz, 2H, CH_2 of ester), 4.5 (s, 2H, OCH_2), 7.0–7.6 (m, 7H, Ar–H); EI-MS: *m/z* 376 (M^+, 60). Anal. Calcd. for $C_{18}H_{17}BrO_4$ (377): C, 57.29; H, 4.50. Found: C, 57.29; H, 4.59%.

4.2.1.8. Ethyl [2-(4-bromo-benzoyl)-4-methylphenoxy]acetate ***2h***. Yield 86%; M.p. 59–61 °C; IR (Nujol): 1640 (C=O), 1735 cm^{-1} (ester, C=O); ^1H NMR ($CDCl_3$): δ 1.3 (t, $J = 7$ Hz, 3H, CH_3 of ester), 2.3 (s, 3H, CH_3), 4.25 (q, $J = 6$ Hz, 2H, CH_2 of ester), 4.45 (s, 2H, OCH_2), 6.9–7.4 (m, 7H, Ar–H); EI-MS: *m/z* 376 (M^+, 62). Anal. Calcd. for $C_{18}H_{17}BrO_4$ (377): C, 57.29; H, 4.50. Found: C, 57.21; H, 4.42%.

4.2.1.9. Ethyl [2-(4-fluoro-benzoyl)-4-methylphenoxy]acetate ***2i***. Yield 79%; M.p. 50–52 °C; IR (Nujol): 1610 (C=O), 1715 cm^{-1} (ester, C=O); ^1H NMR ($CDCl_3$): δ 1.25 (t, $J = 7$ Hz, 3H, CH_3 of ester), 2.2 (s, 3H, CH_3), 4.2 (q, $J = 6$ Hz, 2H, CH_2 of ester), 4.4 (s, 2H, OCH_2), 6.8–7.3 (m, 7H, Ar–H); EI-MS: *m/z* 316 (M^+, 60). Anal. Calcd. for $C_{18}H_{17}FO_4$ (316): C, 68.35; H, 5.42. Found: C, 68.27; H, 5.34%.

4.2.1.10. Ethyl [2-(4-methyl-benzoyl)-4-methylphenoxy]acetate ***2j***. Yield 79%; M.p. 57–59 °C; IR (Nujol): 1665 (C=O),1740 cm^{-1} (ester, C=O); ^1H NMR ($CDCl_3$): δ 1.2 (t, 3H, CH_3 of ester), 2.3–2.35 (s, 6H, $2CH_3$), 4.25 (q, 2H, CH_2 of ester), 4.45 (s, 2H, OCH_2), 7.2–7.8 (m, 7H, Ar–H); EI-MS: *m/z* 312 (M^+, 64). Anal. Calcd. for $C_{19}H_{20}O_4$ (312): C, 73.07; H, 6.41. Found: C, 73.04; H, 6.38%.

*4.2.2. General procedure for synthesis of 2-(2-benzoyl-4-methylphenoxy) acetohydrazides (**3a–j**)*

Compounds **2a–j** (0.027 mol) were dissolved in alcohol (20 ml) and then 80% hydrazine hydrate (0.027 mol) was added in drops and stirred for 1–2 h at room temperature. A white solid was separated, which was filtered, washed with distilled water (3 × 15 ml) and recrystallized with alcohol. A white solid of **3a–j** was obtained [22].

4.2.2.1. 2-[2-(4-Methoxybenzoyl)-4-methylphenoxy] acetohydrazide ***3a***. Yield 70%; M.p.175–177 °C; IR (Nujol): 1610 (C=O), 1645 (amide, C=O), 3100-3205 cm^{-1} (NH–NH_2); ^1H NMR (CD_3COCD_3): δ 2.2 (s, 3H, CH_3), 3.5 (bs, 2H, NH_2), 3.9 (s, 3H, OCH_3), 4.55 (s, 2H, OCH_2), 7.0 (d, $J = 8.3$ Hz, 2H, Ar–H), 7.2–7.45 (m, 3H, Ar–H), 7.7 (d, $J = 8.5$ Hz, 2H, Ar–H), 9.4 (bs, 1H, CONH); EI-MS: *m/z* 314 (M^+, 42). Anal. Calcd. for $C_{17}H_{18}N_2O_4$ (314): C, 64.96; H, 5.73; N, 8.91. Found: C, 64.94; H, 5.70; N, 8.89%.

4.2.2.2. 2-[2-(3-Chlorobenzoyl)-4-methylphenoxy]acetohydrazide ***3b***. Yield 75%; M.p.177–179 °C; IR (Nujol): 1620 (C=O), 1655 (amide, C=O), 3110–3215 cm^{-1} (NH–NH_2); ^1H NMR (CD_3COCD_3): δ 2.3 (s, 3H, CH_3), 3.7 (bs, 2H, NH_2), 4.5 (s, 2H, OCH_2), 7.1–7.6 (m, 7H, Ar–H), 9.25 (bs, 1H, CONH); EI-MS: *m/z* 318 (M^+, 47). Anal. Calcd. for $C_{16}H_{15}ClN_2O_3$ (318.5): C, 60.28; H, 4.70; N, 8.79. Found: C, 60.24; H, 4.67; N, 8.75%.

4.2.2.3. 2-[2-(2-Bromobenzoyl)-4-methylphenoxy]acetohydrazide ***3c***. Yield 72%; M.p.185–187 °C; IR (Nujol): 1625 (C=O), 1660 (amide, C=O), 3115–3220 cm^{-1} (NH–NH_2); ^1H NMR (CD_3COCD_3): δ 2.32 (s, 3H, CH_3), 3.72 (bs, 2H, NH_2), 4.55 (s, 2H, OCH_2), 7.0–7.55 (m, 7H, Ar–H), 9.3 (bs, 1H, CONH); EI-MS: *m/z* 362 (M^+, 45), 364 (M^+, 40). Anal. Calcd. for $C_{16}H_{15}BrN_2O_3$ (363): C, 52.89; H, 4.13; N, 7.71. Found: C, 52.87; H, 4.15; N, 7.73%.

4.2.2.4. 2-[2-Benzoyl-4-methylphenoxy]acetohydrazide ***3d***. Yield 75%; M.p. 179–181 °C; IR (Nujol): 1615 (C=O), 1650 (amide, C=O), 3105-3210 cm^{-1} (NH–NH_2); ^1H NMR (CD_3COCD_3): δ 2.25 (s, 3H, CH_3), 3.55 (bs, 2H, NH_2), 4.5 (s, 2H, OCH_2), 7.2–7.7 (m, 8H, Ar–H), 9.2 (bs, 1H, CONH); EI-MS: *m/z* 284 (M^+, 44), Anal. Calcd. for $C_{16}H_{16}N_2O_3$ (284): C, 67.60; H, 5.63; N, 9.85. Found: C, 67.62; H, 5.65; N, 9.83%.

4.2.2.5. 2-[2-(2-Chlorobenzoyl)-4-methylphenoxy]acetohydrazide ***3e***. Yield 75%; M.p.167–169 °C; IR (Nujol): 1622 (C=O), 1658 (amide, C=O), 3112-3218 cm^{-1} (NH–NH_2); ^1H NMR (CD_3COCD_3): δ 2.31 (s, 3H, CH_3), 3.71 (bs, 2H, NH_2), 4.52 (s, 2H, OCH_2), 7.1–7.65 (m, 7H, Ar–H), 9.3 (bs, 1H, CONH); EI-MS: *m/z* 318. Anal. Calcd. for $C_{16}H_{15}ClN_2O_3$ (318.5): C, 60.28; H, 4.70; N, 8.79. Found: C, 60.24; H, 4.74; N, 8.76%.

4.2.2.6. 2-[2-(4-Chlorobenzoyl)-4-methylphenoxy]acetohydrazide ***3f***. Yield 68%; M.p. 182–185 °C; IR (Nujol): 1625 (C=O), 1660 (amide, C=O), 3115–3220 cm^{-1} (NH–NH_2); ^1H NMR (CD_3COCD_3): δ 2.25 (s, 3H, CH_3), 3.6 (bs, 2H, NH_2), 4.55 (s, 2H, OCH_2), 7.1–7.7 (m, 7H, Ar–H), 9.3 (bs, 1H, CONH); EI-MS: *m/z* 318. Anal. Calcd. for $C_{16}H_{15}ClN_2O_3$ (318.5): C, 60.28; H, 4.70; N, 8.79. Found: C, 60.25; H, 4.68; N, 8.77%.

4.2.2.7. 2-[2-(3-Bromobenzoyl)-4-methylphenoxy]acetohydrazide ***3g***. Yield 70%; M.p.170–172 °C; IR (Nujol): 1640 (C=O), 1670 (amide, C=O), 3135-3245 cm^{-1} (NH–NH_2); ^1H NMR (CD_3COCD_3): δ 2.3 (s, 3H, CH_3), 3.7 (bs, 2H, NH_2), 4.5 (s, 2H, OCH_2), 6.9–7.5 (m, 7H, Ar–H), 9.2 (bs, 1H, CONH); EI-MS: *m/z* 362 (M^+, 42), 364 (M^+, 39), Anal. Calcd. for $C_{16}H_{15}BrN_2O_3$ (363): C, 52.89; H, 4.13; N, 7.71. Found: C, 52.83; H, 4.11; N, 7.71%.

4.2.2.8. 2-[2-(4-Bromobenzoyl)-4-methylphenoxy]acetohydrazide ***3h***. Yield 75%; M.p.184–186 °C; IR (Nujol): 1650 (C=O), 1660 (amide, C=O), 3125-3235 cm^{-1} (NH–NH_2); ^1H NMR (CD_3COCD_3): δ 2.25 (s, 3H, CH_3), 3.75 (bs, 2H, NH_2), 4.45 (s, 2H, OCH_2), 6.95–7.6 (m, 7H, Ar–H), 9.1 (bs, 1H, CONH); EI-MS: *m/z* 362 (M^+, 40), 364 (M^+, 37). Anal. Calcd. for $C_{16}H_{15}BrN_2O_3$ (363): C, 52.89; H, 4.13; N, 7.71. Found: C, 52.80; H, 4.22; N, 7.68%.

4.2.2.9. 2-[2-(4-Fluorobenzoyl)-4-methylphenoxy]acetohydrazide ***3i***. Yield 72%; M.p.164–166 °C; IR (Nujol): 1610 (C=O), 1650 (amide, C=O), 3115-3225 cm^{-1} (NH–NH_2); ^1H NMR (CD_3COCD_3): δ 2.2 (s, 3H, CH_3), 3.7 (bs, 2H, NH_2), 4.5 (s, 2H, OCH_2), 6.9–7.5 (m, 7H, Ar–H), 9.1 (bs, 1H, CONH); EI-MS: *m/z* 302 (M^+, 40). Anal. Calcd. for $C_{16}H_{15}FN_2O_3$ (302): C, 63.57; H, 5.00; N, 9.27. Found: C, 63.47; H, 5.11; N, 9.38%.

4.2.2.10. 2-[2-(4-Methylbenzoyl)-4-methylphenoxy]acetohydrazide ***3j***. Yield 71%; M.p. 186–88 °C; IR (Nujol): 1630 (C=O), 1670 (amide, C=O), 3120-3220 cm^{-1} (NH–NH_2); ^1H NMR (CD_3COCD_3): δ 2.2–2.3 (s, 6H, $2CH_3$), 3.55 (bs, 2H, NH_2), 4.6 (s, 2H, OCH_2), 7.2–7.8 (m, 7H, Ar–H), 9.35 (bs, 1H, CONH); EI-MS: *m/z* 298 (M^+, 48). Anal. Calcd. for $C_{17}H_{18}N_2O_3$ (298): C, 68.45; H, 6.04; N, 9.39. Found: C, 68.41; H, 6.0; N, 9.35%.

*4.2.3. General procedure for synthesis of N-[2-(2-benzoyl-4-methyl-phenoxy)-acetyl hydrazinocarbonyl]-ethyl acetates (**4a–j**)*

A mixture of **3a–j** (2.2 mmol) and diethyl malonate (2.4 mmol) was refluxed for 4–5 h in methanol (20 ml), cooled and poured into ice-cold water. The solid separated was filtered, dried and recrystallized from alcohol to achieve compounds **4a–j**.

4.2.3.1. N-{2-[2-(4-methoxy-benzoyl)-4-methyl-phenoxy]-acetyl} hydrazinocarbonyl-ethyl acetate ***4a***. Yield 70%; M.p. 230–232 °C; IR

(KBr): 1640 (C=O), 1660 (amide, C=O), 1730 (ester, C=O), 3200–3300 cm^{-1} (NH–NH); ^1H NMR ($CDCl_3$): δ 1.25 (t, $J = 7$ Hz, 3H, CH_3 of ester), 2.3 (s, 3H, CH_3), 3.14 (s, 2H, CH_2), 3.82 (s, 3H, OCH_3), 4.18 (q, $J = 6$ Hz, 2H, CH_2 of ester), 4.7 (s, 2H, OCH_2), 6.8–7.7 (m, 7H, Ar–H), 9.2 (bs, 2H, NH); ^{13}C NMR ($CDCl_3$): δ 13.6, 20.9, 39.3, 56.0, 59.2, 78.0, 113.7, 113.8, 123.3, 129.7, 130.1, 131.1, 131.8, 133.9, 160.6, 165.7, 170.3, 171.0, 187.0. EI-MS: *m/z* 428 (M^+, 48). Anal. Calcd. for $C_{22}H_{24}N_2O_7$ (428): C, 61.68; H, 5.6; N, 6.54. Found: C, 61.65; H, 5.4; N, 6.57%.

4.2.3.2. (N-{2-[2-(3-chloro-benzoyl)-4-methyl-phenoxy]-acetyl} hydrazinocarbonyl)-ethyl acetate ***4b***. Yield 71%; M.p. 234–236 °C; IR (KBr): 1642 (C=O), 1658 (amide, C=O), 1732 (ester, C=O), 3210–3310 cm^{-1} (NH–NH); ^1H NMR ($CDCl_3$): δ 1.3 (t, $J = 7$ Hz, 3H, CH_3 of ester), 2.31 (s, 3H, CH_3), 3.15 (s, 2H, CH_2), 4.18 (q, $J = 6$ Hz, 2H, CH_2 of ester), 4.72 (s, 2H, OCH_2), 6.75–7.71 (m, 7H, Ar–H), 9.28 (bs, 2H, NH); ^{13}C NMR ($CDCl_3$): δ 13.61, 20.92, 39.31, 59.21, 78.01, 113.72, 123.31, 128.2, 129.6, 129.7, 130.5, 131.8, 132.6, 133.5, 133.9, 139.2, 160.6, 170.31, 171.01, 187.02; EI-MS: *m/z* 432 (M^+,48); Anal. Calcd. for $C_{21}H_{21}ClN_2O_6$ (432.5): C, 58.20; H, 4.85; N, 6.47. Found: C, 58.22; H, 4.87; N, 6.45%.

4.2.3.3. (N-{2-[2-(2-bromo-benzoyl)-4-methyl-phenoxy]-acetyl} hydrazinocarbonyl)-ethyl acetate ***4c***. Yield 69%; M.p. 225–227 °C; IR (KBr): 1644 (C=O), 1654 (amide, C=O), 1736 (ester, C=O), 3205–3308 cm^{-1} (NH–NH); ^1H NMR ($CDCl_3$): δ 1.28 (t, $J = 7$ Hz, 3H, CH_3 of ester), 2.29 (s, 3H, CH_3), 3.13 (s, 2H, CH_2), 4.17 (q, $J = 6$ Hz, 2H, CH_2 of ester), 4.74 (s, 2H, OCH_2), 6.75–7.71 (m, 7H, Ar–H), 9.28 (bs, 2H, NH); ^{13}C NMR ($CDCl_3$): δ 13.62, 20.93, 39.32, 59.22, 78.02, 113.73, 123.32, 124.7, 127.2, 129.71, 131.5, 131.81, 132.3, 133.9, 134.4, 141.1, 160.62, 170.32, 171.02, 187.03; EI-MS: *m/z* 477 (M^+, 48). Anal. Calcd. for $C_{21}H_{21}BrN_2O_6$ (477): C, 52.83; H, 4.40; N, 5.87. Found: C, 52.85; H, 4.43; N, 5.85%.

4.2.3.4. (N-{2-[2-benzoyl-4-methyl-phenoxy]-acetyl}hydrazino-carbonyl)-ethyl acetate ***4d***. Yield 72%; M.p. 218–220 °C; IR (KBr): 1640 (C=O), 1652 (amide, C=O), 1733 (ester, C=O), 3200–3300 cm^{-1} (NH–NH); ^1H NMR ($CDCl_3$): δ 1.3 (t, $J = 7$ Hz, 3H, CH_3 of ester), 2.28 (s, 3H, CH_3), 3.12 (s, 2H, CH_2), 4.16 (q, $J = 6$ Hz, 2H, CH_2 of ester), 4.75 (s, 2H, OCH_2), 6.76–7.75 (m, 8H, Ar–H), 9.1 (bs, 2H, NH); ^{13}C NMR ($CDCl_3$): δ 13.61, 20.92, 39.31, 59.21, 78.0, 113.71, 123.31, 128.2, 129.71, 130.1, 131.81, 132.2, 133.91, 137.8, 160.62, 170.32, 171.02, 187.02. EI-MS: *m/z* 398 (M^+, 48). Anal. Calcd. for $C_{21}H_{22}N_2O_6$ (398): C, 63.31; H, 5.52; N, 7.03. Found: C, 63.34; H, 5.55; N, 7.05%.

4.2.3.5. (N-{2-[2-(2-chloro-benzoyl)-4-methyl-phenoxy]-acetyl} hydrazinocarbonyl)-ethyl acetate ***4e***. Yield 70%; M.p. 202–204 °C; IR (KBr): 1660 (C=O), 1655 (amide, C=O), 1740 (ester, C=O), 3240–3330 cm^{-1} (NH–NH); ^1H NMR ($CDCl_3$): δ 1.32 (t, $J = 7$ Hz, 3H, CH_3 of ester), 2.3 (s, 3H, CH_3), 3.15 (s, 2H, CH_2), 4.2 (q, $J = 6$ Hz, 2H, CH_2 of ester), 4.8 (s, 2H, OCH_2), 6.8–7.7 (m, 8H, Ar–H), 9.2 (bs, 2H, NH); ^{13}C NMR ($CDCl_3$): δ 13.66, 20.80, 39.35, 59.25, 78.3, 113.74, 123.35, 128.5, 129.75, 130.15, 131.85, 132.24, 133.95, 137.85, 160.65, 170.35, 171.1, 187.08. EI-MS: *m/z* 432.5 (M^+, 48). Anal. Calcd. for $C_{21}H_{21}ClN_2O_6$ (432.5): C, 58.20; H, 4.85; N, 6.47. Found: C, 58.22; H, 4.87; N, 6.45%.

4.2.3.6. (N-{2-[2-(4-chloro-benzoyl)-4-methyl-phenoxy]-acetyl} hydrazinocarbonyl)-ethyl acetate ***4f***. Yield 75%; M.p. 210–211 °C; IR (KBr): 1665 (C=O), 1655 (amide, C=O), 1745 (ester, C=O), 3230–3320 cm^{-1} (NH–NH); ^1H NMR ($CDCl_3$): δ 1.35 (t, $J = 7$ Hz, 3H, CH_3 of ester), 2.4 (s, 3H, CH_3), 3.18 (s, 2H, CH_2), 4.3 (q, $J = 6$ Hz, 2H, CH_2 of ester), 4.6 (s, 2H, OCH_2), 6.9–7.6 (m, 8H, Ar–H), 9.3 (bs, 2H, NH); ^{13}C NMR ($CDCl_3$): δ 13.66, 20.80, 39.35, 59.25, 78.3, 113.74, 123.35, 128.5, 129.75, 130.15, 131.85, 132.24, 133.95, 137.85, 160.65, 170.35, 171.1, 187.08. EI-MS: *m/z* 432.5 (M^+, 48). Anal. Calcd. for $C_{21}H_{21}ClN_2O_6$ (432.5): C, 58.20; H, 4.85; N, 6.47. Found: C, 58.22; H, 4.87; N, 6.45%.

4.2.3.7. (N-{2-[2-(3-bromo-benzoyl)-4-methyl-phenoxy]-acetyl} hydrazinocarbonyl)-ethyl acetate ***4g***. Yield 65%; M.p. 220–223 °C; IR (KBr): 1645 (C=O), 1664 (amide, C=O), 1738 (ester, C=O), 3208–3315 cm^{-1} (NH–NH); ^1H NMR ($CDCl_3$): δ 1.28 (t, $J = 7$ Hz, 3H, CH_3 of ester), 2.29 (s, 3H, CH_3), 3.13 (s, 2H, CH_2), 4.17 (q, $J = 6$ Hz, 2H, CH_2 of ester), 4.74 (s, 2H, OCH_2), 6.75–7.71 (m, 7H, Ar–H), 9.28 (bs, 2H, NH); ^{13}C NMR ($CDCl_3$): δ 13.62, 20.93, 39.32, 59.22, 78.02, 113.73, 123.32, 124.7, 127.2, 129.71, 131.5, 131.81, 132.3, 133.9, 134.4, 141.1, 160.62, 170.32, 171.02, 187.03. EI-MS: *m/z* 477 (M^+, 48). Anal. Calcd. for $C_{21}H_{21}BrN_2O_6$ (477): C, 52.83; H, 4.40; N, 5.87. Found: C, 52.85; H, 4.43; N, 5.85%.

4.2.3.8. (N-{2-[2-(4-bromo-benzoyl)-4-methyl-phenoxy]-acetyl} hydrazinocarbonyl)-ethyl acetate ***4h***. Yield 65%; M.p. 212–213 °C; IR (KBr): 1642 (C=O), 1660 (amide, C=O), 1740 (ester, C=O), 3212–3318 cm^{-1} (NH–NH); ^1H NMR ($CDCl_3$): δ 1.25 (t, $J = 7$ Hz, 3H, CH_3 of ester), 2.22 (s, 3H, CH_3), 3.15 (s, 2H, CH_2), 4.18 (q, $J = 6$ Hz, 2H, CH_2 of ester), 4.74 (s, 2H, OCH_2), 6.70–7.75 (m, 7H, Ar–H), 9.26 (bs, 2H, NH); ^{13}C NMR ($CDCl_3$): δ 13.62, 20.93, 39.32, 59.22, 78.02, 113.73, 123.32, 124.7, 127.2, 129.71, 131.5, 131.81, 132.3, 133.9, 134.4, 141.1, 160.62, 170.32, 171.02, 187.03. EI-MS: *m/z* 477 (M^+, 48). Anal. Calcd. for $C_{21}H_{21}BrN_2O_6$ (477): C, 52.83; H, 4.40; N, 5.87. Found: C, 52.85; H, 4.43; N, 5.85%.

4.2.3.9. (N-{2-[2-(4-fluoro-benzoyl)-4-methyl-phenoxy]-acetyl} hydrazinocarbonyl)-ethyl acetate ***4i***. Yield 78%; M.p. 202–204 °C; IR (KBr): 1666 (C=O), 1659 (amide, C=O), 1755 (ester, C=O), 3230–3320 cm^{-1} (NH–NH); ^1H NMR ($CDCl_3$): δ 1.35 (t, $J = 7$ Hz, 3H, CH_3 of ester), 2.4 (s, 3H, CH_3), 3.18 (s, 2H, CH_2), 4.3 (q, $J = 6$ Hz, 2H, CH_2 of ester), 4.6 (s, 2H, OCH_2), 6.9–7.6 (m, 8H, Ar–H), 9.3 (bs, 2H, NH); ^{13}C NMR ($CDCl_3$): δ 13.66, 20.80, 39.35, 59.25, 78.3, 113.74, 123.35, 128.5, 129.75, 130.15, 131.85, 132.24, 133.95, 137.85, 160.65, 170.35, 171.1, 187.08. EI-MS: *m/z* 416 (M^+, 48). Anal. Calcd. for $C_{21}H_{21}FN_2O_6$ (416): C, 60.57; H, 5.08; N, 6.73. Found: C, 60.59; H, 5.05; N, 6.77%.

4.2.3.10. (N-{2-[2-(4-methyl-benzoyl)-4-methyl-phenoxy]-acetyl} hydrazinocarbonyl)-ethyl acetate ***4j***. Yield 78%; M.p. 210–212 °C; IR (KBr): 1640 (C=O), 1660 (amide, C=O), 1738 (ester, C=O), 3215–3300 cm^{-1} (NH–NH); ^1H NMR ($CDCl_3$): δ 1.26 (t, $J = 7$ Hz, 3H, CH_3 of ester), 2.4 (s, 3H, CH_3), 2.5 (s, 3H, CH_3), 3.14 (s, 2H, CH_2), 4.18 (q, $J = 6$ Hz, 2H, CH_2 of ester), 4.7 (s, 2H, OCH_2), 6.9–7.8 (m, 7H, Ar–H), 9.5 (bs, 2H, NH); ^{13}C NMR ($CDCl_3$): δ 13.6, 20.9, 39.3, 56.0, 59.2, 78.0, 113.7, 113.8, 123.3, 129.7, 130.1, 131.1, 131.8, 133.9, 160.6, 165.7, 170.3, 171.0, 187.0. EI-MS: *m/z* 412 (M^+, 48). Anal. Calcd. for $C_{22}H_{24}N_2O_6$ (412): C, 64.07; H, 5.87; N, 6.79. Found: C, 64.04; H, 5.89; N, 6.76%.

*4.2.4. General procedure for synthesis of N-[2-(2-benzoyl-4-methylphenoxy)-acetyl]-hydrazide methanone coumarins (**5a**–**j**)*

To a solution of *o*-hydroxy benzaldehyde (2 mmol) in alcohol (20 ml), compounds **4a**–**j** (1.16 mmol) were added and the mixture was refluxed for 4–5 h in the presence of catalytic amount of acetic acid. The mixture was cooled and poured into ice-cold water, the solid separated was filtered, dried and recrystallized from alcohol to obtain compounds **5a**–**j**.

4.2.4.1. N-{2-[2-(4-methoxybenzoyl)-4-methylphenoxy]-acetyl}-hydrazide methanone coumarin ***5a***. Yield 69%; M.p. 200–202 °C; IR (KBr): 1640 (C=O), 1660 (amide, C=O), 1733 (ring C=O), 3250–3340 cm^{-1} (NH–NH); ^1H NMR ($CDCl_3$): δ 2.3 (s, 3H, CH_3), 3.86 (s, 3H, OCH_3), 4.7 (s, 2H, OCH_2), 6.9–7.65 (m, 11H, Ar–H), 8.72 (s, 1H, =CH), 9.1 (bs, 2H, NH); ^{13}C NMR ($CDCl_3$): δ 20.9, 59.01, 78.02, 113.71, 113.82, 121.32, 123.31, 124.4, 125.2, 128.1, 129.71, 130.11, 131.1, 131.8,

133.91, 150.8, 151.4, 160.62, 162.0, 165.71, 165.9, 170.3, 187.01. EI-MS: *m/z* 486 (M^+, 48). Anal. Calcd. for $C_{27}H_{22}N_2O_7$ (486): C, 66.66; H, 4.52; N, 5.76. Found: C, 66.64; H, 4.55; N, 5.96%.

4.2.4.2. N-{2-[2-(3-chlorobenzoyl)-4-methylphenoxy]-acetyl}-hydrazide methanone coumarin ***5b***. Yield 70%. M.p. 210–212 °C; IR (KBr): 1645 (C=O), 1664 (amide, C=O), 1735 (ring C=O), 3255–3345 cm^{-1} (NH–NH); ^1H NMR ($CDCl_3$): δ 2.25 (s, 3H, CH_3), 4.72 (s, 2H, OCH_2), 6.85–7.65 (m, 11H, Ar–H), 8.71 (s, 1H, =CH), 9.2 (bs, 2H, NH); ^{13}C NMR ($CDCl_3$): δ 20.92, 78.01, 113.7, 121.31, 123.3, 124.41, 125.21, 126.6, 127.8, 128.1, 128.2, 129.6, 129.7, 130.5, 131.8, 132.6, 133.5, 133.9, 139.2, 150.81, 151.41, 160.61, 162.01, 165.9, 170.31, 187.02. EI-MS: *m/z* 490 (M^+, 48). Anal. Calcd. for $C_{26}H_{19}ClN_2O_6$ (490.5): C, 63.60; H, 3.87; N, 5.70. Found: C, 63.62; H, 3.85; N, 5.73%.

4.2.4.3. N-{2-[2-(2-bromobenzoyl)-4-methylphenoxy]-acetyl}-hydrazide methanone coumarin ***5c***. Yield 68%; M.p. 195–197 °C; IR (KBr): 1650 (C=O), 1670 (amide, C=O), 1740 (ring C=O), 3260–3350 cm^{-1} (NH–NH); ^1H NMR ($CDCl_3$): δ 2.28 (s, 3H, CH_3), 4.7 (s, 2H, OCH_2), 6.75–7.63 (m, 11H, Ar–H), 8.7 (s, 1H, =CH), 9.15 (bs, 2H, NH); ^{13}C NMR ($CDCl_3$): δ 20.93, 78.03, 113.7, 121.32, 122.8, 123.31, 124.4, 125.2, 126.61, 127.81, 128.11, 129.1, 129.72, 130.4, 131.82, 133.4, 133.9, 135.5, 140.0, 150.8, 151.42, 160.62, 162.02, 165.91, 170.32, 187.03. EI-MS: *m/z* 535 (M^+, 48). Anal. Calcd. for $C_{26}H_{19}BrN_2O_6$ (535): C, 58.31; H, 3.55; N, 5.23. Found: C, 58.30; H, 3.52; N, 5.25%.

4.2.4.4. N-[2-(2-benzoyl-4-methylphenoxy)-acetyl]-hydrazide methanone coumarin ***5d***. Yield; 70.5%; M.p. 215–217 °C; IR (KBr): 1642 (C=O), 1665 (amide, C=O), 1738 (ring C=O), 3240-3345 cm^{-1} (NH–NH); ^1H NMR ($CDCl_3$): δ 2.2 (s, 3H, CH_3), 4.65 (s, 2H, OCH_2), 6.7–7.6 (m, 12H, Ar–H), 8.66 (s, 1H, =CH), 9.12 (bs, 2H, NH); ^{13}C NMR ($CDCl_3$): δ 20.93, 78.0, 113.7, 121.3, 123.3, 124.4, 125.21, 126.6, 127.8, 128.1, 128.2, 129.7, 130.1, 131.8, 132.2, 133.9, 137.8, 150.8, 151.41, 160.6, 162.0, 165.9, 170.3, 187.0. EI-MS: *m/z* 456 (M^+, 48). Anal. Calcd. for $C_{26}H_{20}N_2O_6$ (456): C, 68.42; H, 4.38; N, 6.14. Found: C, 68.40; H, 4.40; N, 6.16%.

4.2.4.5. N-{2-[2-(2-chlorobenzoyl)-4-methylphenoxy]-acetyl}-hydrazide methanone coumarin ***5e***. Yield 70%. M.p. 215–217 °C; IR (KBr): 1648 (C=O), 1668 (amide, C=O), 1730 (ring C=O), 3250–3340 cm^{-1} (NH–NH); ^1H NMR ($CDCl_3$): δ 2.26 (s, 3H, CH_3), 4.76 (s, 2H, OCH_2), 6.82–7.68 (m, 11H, Ar–H), 8.75 (s, 1H, =CH), 9.6 (bs, 2H, NH); ^{13}C NMR ($CDCl_3$): δ 20.92, 78.01, 113.7, 121.31, 123.3, 124.41, 125.21, 126.6, 127.8, 128.1, 128.2, 129.6, 129.7, 130.5, 131.8, 132.6, 133.5, 133.9, 139.2, 150.81, 151.41, 160.61, 162.01, 165.9, 170.31, 187.02. EI-MS: *m/z* 491 (M^+, 48). Anal. Calcd. for $C_{26}H_{19}ClN_2O_6$ (491): C, 63.60; H, 3.87; N, 5.70. Found: C, 63.62; H, 3.85; N, 5.73%.

4.2.4.6. N-{2-[2-(4-chlorobenzoyl)-4-methylphenoxy]-acetyl}-hydrazide methanone coumarin ***5f***. Yield 72%. M.p. 219–221 °C; IR (KBr): 1642 (C=O), 1665 (amide, C=O), 1735 (ring C=O), 3255–3345 cm^{-1} (NH–NH); ^1H NMR ($CDCl_3$): δ 2.26 (s, 3H, CH_3), 4.76 (s, 2H, OCH_2), 6.82–7.68 (m, 11H, Ar–H), 8.75 (s, 1H, =CH), 9.6 (bs, 2H, NH); ^{13}C NMR ($CDCl_3$): δ 20.92, 78.01, 113.7, 121.31, 123.3, 124.41, 125.21, 126.6, 127.8, 128.1, 128.2, 129.6, 129.7, 130.5, 131.8, 132.6, 133.5, 133.9, 139.2, 150.81, 151.41, 160.61, 162.01, 165.9, 170.31, 187.02. EI-MS: *m/z* 491 (M^+, 48). Anal. Calcd. for $C_{26}H_{19}ClN_2O_6$ (491): C, 63.60; H, 3.87; N, 5.70. Found: C, 63.62; H, 3.85; N, 5.73%.

4.2.4.7. N-{2-[2-(3-bromobenzoyl)-4-methylphenoxy]-acetyl}-hydrazide methanone coumarin ***5g***. Yield 66%; M.p. 190–192 °C; IR (KBr): 1652 (C=O), 1678 (amide, C=O), 1745 (ring C=O), 3265–3354 cm^{-1} (NH–NH); ^1H NMR ($CDCl_3$): δ 2.29 (s, 3H, CH_3), 4.8 (s, 2H, OCH_2), 6.78–7.66 (m, 11H, Ar–H), 8.8 (s, 1H, =CH), 9.12 (bs, 2H, NH); ^{13}C NMR ($CDCl_3$): δ 20.93, 78.03, 113.7, 121.32, 122.8, 123.31, 124.4, 125.2, 126.61, 127.81, 128.11, 129.1, 129.72, 130.4, 131.82, 133.4, 133.9, 135.5, 140.0, 150.8, 151.42, 160.62, 162.02, 165.91, 170.32, 187.03. EI-MS: *m/z* 535 (M^+, 48). Anal. Calcd. for $C_{26}H_{19}BrN_2O_6$ (535): C, 58.31; H, 3.55; N, 5.23. Found: C, 58.30; H, 3.52; N, 5.25%.

4.2.4.8. N-{2-[2-(4-bromobenzoyl)-4-methylphenoxy]-acetyl}-hydrazide methanone coumarin ***5h***. Yield 68%; M.p. 197–199 °C; IR (KBr): 1652 (C=O), 1678 (amide, C=O), 1745 (ring C=O), 3265–3354 cm^{-1} (NH–NH); ^1H NMR ($CDCl_3$): δ 2.24 (s, 3H, CH_3), 4.6 (s, 2H, OCH_2), 6.75–7.68 (m, 11H, Ar–H), 8.6 (s, 1H, =CH), 9.14 (bs, 2H, NH); ^{13}C NMR ($CDCl_3$): δ 20.93, 78.03, 113.7, 121.32, 122.8, 123.31, 124.4, 125.2, 126.61, 127.81, 128.11, 129.1, 129.72, 130.4, 131.82, 133.4, 133.9, 135.5, 140.0, 150.8, 151.42, 160.62, 162.02, 165.91, 170.32, 187.03. EI-MS: *m/z* 535 (M^+, 48). Anal. Calcd. for $C_{26}H_{19}BrN_2O_6$ (535): C, 58.31; H, 3.55; N, 5.23. Found: C, 58.30; H, 3.52; N, 5.25%.

4.2.4.9. N-{2-[2-(4-fluorobenzoyl)-4-methylphenoxy]-acetyl}-hydrazide methanone coumarin ***5i***. Yield 76%. M.p. 205–207 °C; IR (KBr): 1645 (C=O), 1662 (amide, C=O), 1734 (ring C=O), 3252–3344 cm^{-1} (NH–NH); ^1H NMR ($CDCl_3$): δ 2.22 (s, 3H, CH_3), 4.74 (s, 2H, OCH_2), 6.81–7.69(m, 11H, Ar–H), 8.78 (s, 1H, =CH), 9.4 (bs, 2H, NH); ^{13}C NMR ($CDCl_3$): δ 20.92, 78.01, 113.7, 121.31, 123.3, 124.41, 125.21, 126.6, 127.8, 128.1, 128.2, 129.6, 129.7, 130.5, 131.8, 132.6, 133.5, 133.9, 139.2, 150.81, 151.41, 160.61, 162.01, 165.9, 170.31, 187.02. EI-MS: *m/z* 474 (M^+, 48). Anal. Calcd. for $C_{26}H_{19}FN_2O_6$ (474): C, 65.82; H, 4.04; N, 5.90 . Found: C, 65.86; H, 4.07; N, 5.94%.

4.2.4.10. N-{2-[2-(4-methylbenzoyl)-4-methylphenoxy]-acetyl}-hydrazide methanone coumarin ***5j***. Yield 70%; M.p. 202–204 °C; IR (KBr): 1645 (C=O), 1664 (amide, C=O), 1733 (ring C=O), 3252–3343 cm^{-1} (NH–NH); ^1H NMR ($CDCl_3$): δ 2.2 (s, 3H, CH_3), 2.26 (s, 3H, CH_3), 4.6 (s, 2H, OCH_2), 6.8–7.68 (m, 11H, Ar–H), 8.76 (s, 1H, =CH), 9.8 (bs, 2H, NH); ^{13}C NMR ($CDCl_3$): δ 20.9, 59.01, 78.02, 113.71, 113.82, 121.32, 123.31, 124.4, 125.2, 128.1, 129.71, 130.11, 131.1, 131.8, 133.91, 150.8, 151.4, 160.62, 162.0, 165.71, 165.9, 170.3, 187.01. EI-MS: *m/z* 470 (M^+, 48). Anal. Calcd. for $C_{27}H_{22}N_2O_6$ (470) : C, 68.93; H, 4.71; N, 5.95 . Found: C, 68.95; H, 4.76; N, 5.97; %.

4.3. Biology

4.3.1. Cell culture and in vitro treatment

EAC and DLA cells were used for the present study and cultured as described earlier [19,23]. The cells were treated using increasing concentrations of compounds **5a**–**j** (0, 10, 20, 50, 100 μM in DMSO) at various time intervals (0–48 h) and further used for experiments. Appropriate vehicle control and 5-fluorouracil as positive control were used and each experiment was repeated a minimum of 3 independent times.

4.3.2. Trypan blue dye exclusion assay

The effects of compounds **5a**–**j** on EAC and DLA cells were determined by Trypan blue dye exclusion assay [19]. EAC and DLA cells were cultured and treated with or without compounds were collected after 48 h. The viable cells were counted by resuspending the cells in 0.4% Trypan blue and the IC_{50} values were estimated.

4.3.3. MTT assay

The MTT assay was performed as described earlier to evaluate the effect of compounds **5a**–**j** on cell proliferation of EAC and DLA cells [19]. Cells were treated with or without compounds and incubated for 48 h. MTT reagent (5 mg/mL) was added and the color change due to proliferating cells was estimated.

4.3.4. LDH release assay

Lactate dehydrogenase (LDH) assay was performed to assess the LDH release following the treatment with compound **5a–j** (0, 10, 20, 50, 100 μM) on both EAC and DLA cells after 48 h of incubation as described earlier [24]. The cells were lysed using 0.1% Triton-X 100 in PBS. The amount of LDH released in both culture media and cell lysate was measured at 490 nm using an ELISA reader (Robotronics). The percentage of LDH release was calculated as LDH release in media/(LDH release in media + intracellular LDH release) × 100.

4.3.5. Animal models and ethics

Swiss albino female mice weighing 25–28 g were housed under standard laboratory conditions with food and water *ad libitum*. All procedures for animal experimentation used were approved by the Institutional Animal Ethics Committee, National College of Pharmacy, Shimoga, India, in accordance with the CPCSEA guidelines for laboratory animal facility (NCP/IAEC/CL/101/05/2012-13).

*4.3.6. Determination of LD_{50} and evaluation of side effects of compound **5c** in normal animals*

The short term acute toxicity studies of the compound **5c** was performed in non-tumor bearing Swiss albino mice divided into 5 groups ($n = 6$) by injecting intraperitoneally (i.p.) and LD_{50} was determined as per the standard CPCSEA guidelines.

To evaluate the side effects of compound **5c**, normal Swiss albino mice were injected with the compound (75 mg/kg body weight, i.p.) for 10 days. Control and treated groups consisted of six mice each. The physiological functions of mice of both treated and untreated group were evaluated by collecting blood after the treatment with compound **5c**. Serum was separated from the blood and used for liver and kidney function tests by comparing the levels of alkaline phosphatase (ALP), creatinine and urea. The blood count was performed by collecting plasma and the number of RBC and WBC were noted down [25]. Values obtained were presented as mean ± SEM.

4.3.7. Animal tumor models and treatment

The antitumor efficacy of the compound **5c** was tested against EAC and solid DL cells *in vivo*. The DLA cells and EAC cells were the kind gift from Dr. Sathish Raghavan from the Indian Institute of Science (IISc), Bangalore, India. Both the cell lines were maintained separately in the peritoneal cavity of mice by injecting 0.2 ml of ascitic fluid containing 5×10^6 cells/mouse for every 10 days. Ascitic tumor cell counts were done in a Neubauer hemocytometer using the trypan blue dye exclusion method. Cell viability was always found to be 95% or more. Tumor cell suspensions were prepared in phosphate buffer saline (PBS).

Murine EAC cells were cultured *in vivo* and administered with **5c** (75 mg/kg body weight i.p.) 3 doses on every alternate day after the onset of tumor on 4th day was carried out as reported earlier [26].

Solid DL tumor induction was developed by adopting reported procedure before with slight modification [27]. In brief, the DLA cells were cultured *in vivo* by injecting the cells (5×10^6 cells/mouse) into peritoneum cavity of mice to develop murine ascites tumor and allowed to multiply. After the onset of tumor the cells were withdrawn from donor mice and re-injected into the right thigh of the experimental animals subcutaneously to develop solid tumor. The experimental animals grouped separately and administered with the compound **5c** (75 mg/kg body weight i.p., 10 doses) after the onset of visible solid tumor, i.e. from 10th day of the tumor implantation. The body weights of the mice were noted down and the tumor volume was measured using Vernier calipers on every alternative day. At the end of the 45th day from tumor implantation animals from each group were sacrificed and the tumor tissue along with liver and spleen was collected and analyzed.

4.3.8. Peritoneal angiogenesis assay and H&E staining for MVD

The restraint of neovessels' formation in peritoneum of mice bearing EAC treated with or without compound **5c** were photographed and further formalin fixed peritoneum was processed for H&E staining for measurement of MVD using Lawrence and Mayo Lynx Reg microscope as reported earlier [20].

4.3.9. Chorioallanotoic membrane (CAM) assay

The *in vivo* angioprevention effect induced by $rVEGF_{165}$ was analyzed following treatment with **5c** (10 μM) in 12 days fertilized egg CAM as described earlier [19] and changes in the MVD was photographed using Sony steady shot DSC-W610 camera.

To reconfirm the angioprevention effect of compound **5c**, the *ex vivo* shell less CAM assay was performed with minor modifications [27]. In brief the two days old fertilized incubated eggs were cracked out and the contents were poured on to a sterilized condiment cup wrapped with serine wrap. The egg preparations were covered with a sterilized Petridish and re-incubated at humidified condition in 37 °C. On day 4th the egg preparations were impregnated with filter discs containing $rVEGF_{165}$ following the treatment with compound **5c**. After 72 h of incubation the change in the vascularization in both treated and untreated egg preparations was photographed using Sony steady shot DSC-W610 camera.

4.3.10. Geimsa stain and DNA fragmentation assay

Geimsa stain was performed as described earlier [28]. In brief, the EAC cells either treated or untreated with **5c** (75 mg/kg body weight *in vivo*) were harvested and smeared on glass slide, fixed with methanol and acetic acid (3:1). Then the cells were hydrated with PBS and stained using Geimsa solution (0.1%). The cells were washed with PBS and viewed under Lawrence and Mayo Lynx Reg microscope. Simultaneously the genomic DNA from EAC cells of either treated or untreated were isolated as described previously [27]. The DNA was resolved on 1.5% agarose gel and documented using Bio-rad Gel Documentation™ XR + Imaging System.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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