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Synthesis and antiproliferative activity of benzophenone tagged pyridine analogues towards activation of caspase activated DNase mediated nuclear fragmentation in Dalton's lymphoma

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ABSTRACT

A series of benzophenones possessing pyridine nucleus **8a–l** were synthesized by multistep reaction sequence and evaluated for antiproliferative activity against DLA cells by *in vitro* and *in vivo* studies. The results suggested that, compounds **8b** with fluoro group and **8e** with chloro substituent at the benzoyl ring of benzophenone scaffold as well as pyridine ring with hydroxy group exhibited significant activity. Further investigation in mouse model suggests that compounds **8b** and **8e** have the potency to activate caspase activated DNase (endonuclease) which is responsible for DNA fragmentation, a primary hallmark of apoptosis and thereby inhibits the Dalton's lymphoma ascites tumour growth.

1. Introduction

Programmed cell death (PCD) or apoptosis, plays a very crucial role in the maintenance of tissue homeostasis by regulating cell death [1]. When there is a failure in the cell death, it leads to the malignant tumour [2,3]. During apoptosis, cells exhibit specific morphological changes. These include membrane blebbing, cytoplasmic condensation, chromatin condensation, nucleosomal fragmentation and apoptotic body formation [4]. The fragmentation of chromosomal DNA into nucleosomal units, producing small DNA fragments or DNA ladder, is considered as a prominent biochemical hallmark of apoptotic cell death [4,5]. The molecular characterization of this process identifies a specific DNase called Caspase activated DNase (CAD) or DNA fragmentation factor (DFF-40) that cleaves cellular DNA in a caspase-dependent manner that eradicates the cells in an appropriate way [6,7]. Apoptosis can be activated through awakening CAD by dissociating the inhibitor of CAD (ICAD) from CAD which normally heterodimerised with ICAD [8–10]. In this manner, the development of the active, selective and less toxic candidates, which targets the activation of CAD has become a key strategy for cancer treatment.

Pyridine is one of the most prevalent heterocyclic compounds in nature. For example, it is present in the coenzyme vitamin B_6 family and in numerous alkaloids, further it plays a central role as versatile building block in the synthesis of natural products as well as biologically active compounds. Further, pyridine bases are widely used in pharmaceutics as nicotinamides and nicotinic acid derivatives. The various therapeutic potential of pyridine derivatives have been reported in the treatment of cancers of diverse cells, by targeting angiogenesis [11,12], apoptosis [13,14] and by inhibiting wide range of tumour promoting factors like, FAK [14], CDK [13,16] and topoisomerase II [17]. Nevertheless, benzophenone derivatives are extensively used in medicine research for their recognized potencies against various pathological conditions including cancer [18–20]. In recent years, our group has reported a number of novel benzophenone conjugated analogues as potent inhibitors targeting angiogenesis [21–23] and apoptosis [24,25]. In continuation, this current study is based on the synthesis of benzophenone bearing pyridine nucleus and their evaluation for antiproliferative and apoptogenic properties against Dalton's lymphoma by both *in vitro* and *in vivo* analysis.

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2. Results and discussion

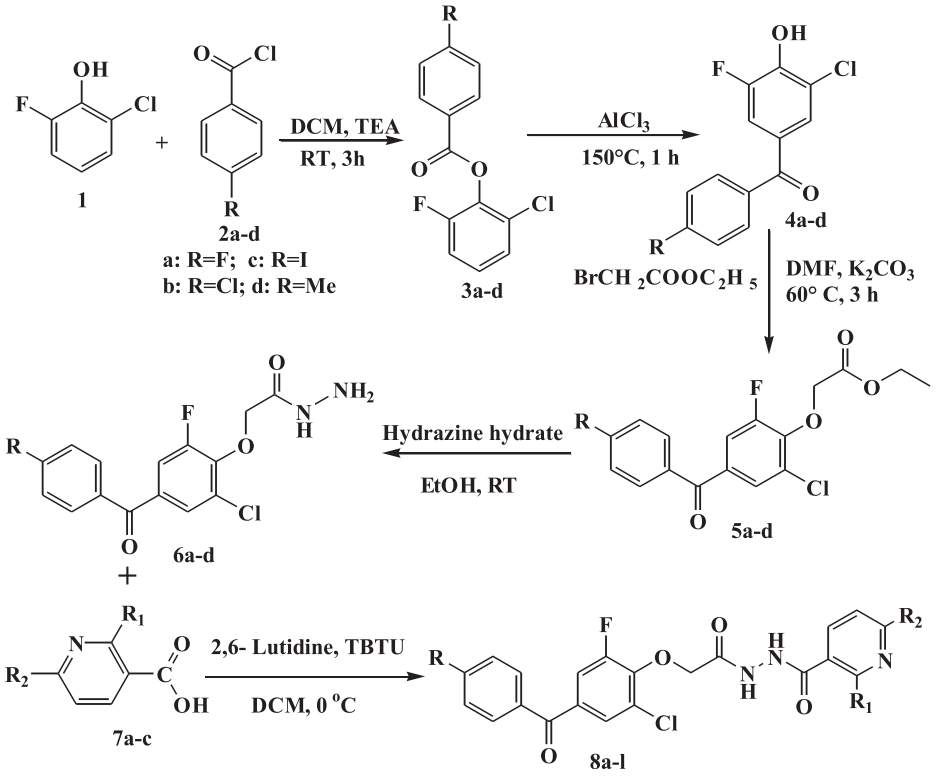
2.1. Chemistry

The reaction sequence for various title compounds **8a–l** was outlined in Scheme 1. Substituted phenyl benzoates **3a–d** were synthesized by stirring 2-chloro-6-fluoro phenol **1** with substituted acid chlorides **2a–d** in alkaline medium using triethylamine. The phenyl benzoates **3a–d** were subjected to Fries rearrangement to afford hydroxy benzophenones **4a–d.** Condensation of **4a–d** with ethyl chloroacetate in the presence of anhydrous potassium carbonate in dry acetone gave ethyl (2-aroyl-4-methylphenoxy) acetates **5a–d**, which on treatment with 99% hydrazine hydrate in ethanol gave 4-aryloylaryloxyacethydrazides **6a–d**. Finally, the title compounds **8a–l** were achieved in excellence yield by coupling **6a–d** with substituted nicotinic acids **7a–c** in the presence of 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) as coupling agent and 2,6-dimethyl pyridine (lutidine). The structures of the compounds were elucidated by IR, ^{1}H NMR and mass spectral studies and also by microanalyses.

2.2. Biology

2.2.1. In vitro selection of the lead compound and their structure activity relationships

During the last decade, the researchers focused on benzophenone derivative and suggest that the benzophenone conjugated with various specific bioactive molecules such as benzimidazole [21], coumarin [22], thiazole [23], oxadiazole [24] and acetamide [25,26] has a promising pharmacological activity against various pathological conditions including cancer. While, pyridine or nicotinic acid is well known for its biological activity against carcinogenesis [14–19]. In the present investigation, compounds **8a–l** analogues were synthesized, by tagging the benzophenone with pyridine ring and evaluated for their antiproliferative and cytotoxic properties against Dalton's Lymphoma Ascites (DLA) cells by performing MTT, trypan blue and lactate dehydrogenase (LDH) release assays [Fig. 1]. In the series of compounds **8a–l,** the compounds **8b** with fluoro and **8e** with chloro group at the para position in the benzoyl ring of the benzophenone and also with hydroxy pyridine moiety exhibited a noticeable cytotoxic effect against DLA cells. The compounds **8b** and **8e** were found to be exhibiting a promising antiproliferative effect with IC_{50} of 8.5 μM and 9.3 μM respectively in MTT assay [Fig. 1A]. The synchronized results were obtained for both the compounds **8b** and **8e** in trypan blue dye exclusion assay with the cytotoxic effects at IC_{50} of 8.7 μM and 9.5 μM respectively [Fig. 1B]. The cellular integrity of the title compounds was evaluated using the LDH release assay [Fig. 1C], which is one of the gold standard methods to assess the cytotoxic effect of the compounds. The results obtained from LDH release assay showed that there was an increase in the release of LDH after the treatment with compounds **8b** and **8e,** which exhibited IC_{50} of 8.5 μM and 9.4 μM respectively. In the direction of structure and structure activity relationship (SAR), the compounds **8h** and **8k** with hydroxy pyridine moiety, but different substituents at the para position in the benzoyl ring of benzophenone exhibited moderate antiproliferative activity whereas, compounds **8b** and **8e** exhibited noticeable antiproliferative activity. Surprisingly, the compounds



Scheme 1. Synthesis of nicotinic acid N′-[2-(4-benzoyl-phenoxy)-acetyl]-hydrazides **8a–l**.

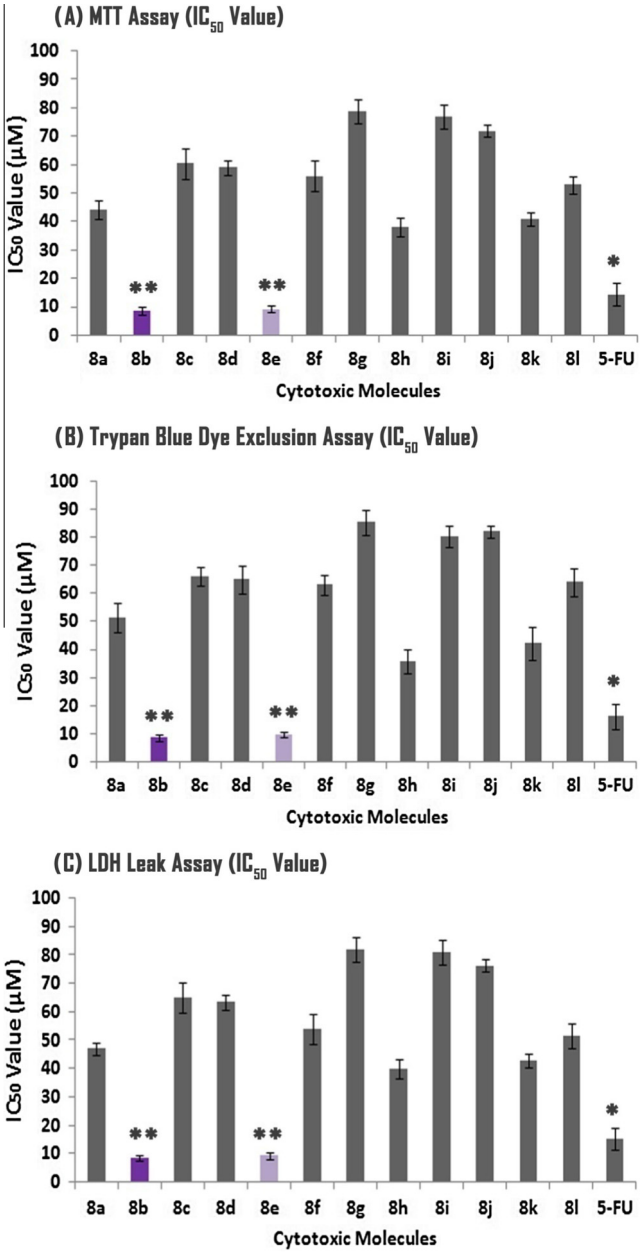


Fig. 1. *In vitro* selection of the active anti-mitogenic molecule. IC_{50} Value of cytotoxic compounds **8a–l** were determined against DLA cells by performing MTT, Trypan blue dye exclusion and LDH release assay (A) IC_{50} values of **8a–l** determined through (A) MTT assay (B) Trypan blue (C) LDH release assay**,** in DLA cells. In these series **8a–l**, compounds **8b** and **8e** which showed a minimal inhibitory concentration (IC_{50}) were chosen as lead compounds.

8a, 8c, 8d, 8f, 8g, 8i and **8j** lacking a hydroxy pyridine moiety showed a very negligible cytotoxicity against DLA cells, even though fluoro and chloro groups are present in benzophenone. These results highlight that the presence of hydroxy pyridine moiety is fundamental for the antiproliferative effect of the drugs and also fluoro and chloro groups in the benzophenone is responsible for the enhancement of the activity of the compounds which is evident from cytotoxic studies. Based on the cytotoxic assay results and SAR, it is clear that compounds **8b** and **8e** have the potency to exhibit the antioncogenic effect against DLA cells. For further validation of cytotoxic effect, DLA cells were treated with and without compounds **8b** and **8e** at 10 µM for 48 h and FACS were carried out for detecting whether it could be due to apoptosis. Results indicate that compounds **8b** and **8e** have increased the cell population undergoing apoptosis about ~21% and ~19% (including early apoptosis) respectively, compared to untreated [Supplementary 1]. Hence the compounds **8b** and **8e** were chosen as lead compounds and investigated further for their *in vivo* activity.

*2.2.2. Compounds **8b** and **8e** exhibit antioncogenic activity in ascites lymphoma*

The DLA tumour model is frequently adapted to secret ascites and is a widely used cell line to study the antiproliferative activity [27–29]. The ascitic tumour implantation induces a local inflammatory reaction, with increasing vascular permeability, which results in cell proliferation, cellular migration and a progressive ascitic fluid formation. Ascites fluid is the direct nutritional source for tumour cell growth and a rapid increase in the secretion of this fluid is an extremely important feature for tumour survival [28–32]. Therefore repressing the ascites secretion is one of the central parameters in reticence of the tumour growth. To study the *in vivo* antiproliferative effect of compounds **8b** and **8e,** DLA ascites tumour models were administered with the concentration of 75 mg/kg body weight intraperitoneally as determined from the LD_{50} studies. The results inferred that compounds **8b** and **8e** significantly regressed the tumour progression in mice, which is apparent from tumour volume [Fig. 2A]. Administration of compounds **8b** and **8e** significantly reduced the ascitic fluid secretion about 76.5% and 73.2%, respectively, compared to the control [Fig. 2B] and diminished the tumour growth by increasing the tumour inhibition at 91.24% and 86.10% respectively, which was clear from tumour inhibition [Fig. 2C]. An anticancer drug is considered reliable if it can prolong the life span of the animal. Interestingly, treatment of the compounds **8b** and **8e** have prolonged the life span of animals from the 10th day to 40th and >40th day, with three fold increase in Survivalism [Fig. 2D].

Further, the drug induced side effects were evaluated by injecting compounds **8b** and **8e** (75 mg/kg body weight per mice) consecutively for 10 days in normal swiss albino mouse model. Compounds **8b** and **8e** treated mice serum containing alkaline phosphatase, creatinine, urea, RBC and WBC levels reveal that the compounds **8b** and **8e** have no or very less adverse effects [Table 1]. These results clearly ascertain that the compounds **8b** and **8e** specifically act upon the progressive tumour cells and responsible for the tumour inhibition without much toxicological effect.

*2.2.3. Compounds **8b** and **8e** induce the CAD mediated DNA fragmentation in DLA cells*

The intracellular chemical mediators of programmed cell death have been relatively well characterized [33]. Activation of effective members of the caspase family of cysteine proteases is the key process in apoptotic death signalling [29]. ICAD is the major apoptotic endonuclease responsible for internucleosomal DNA cleavage and chromatin condensation, and their activation requires cleavage of ICAD primarily by caspase-3 through the dissociation of the CAD–ICAD complex [6,7,30]. This process allows the CAD to enter the nucleus and degrade chromosomal DNA into small DNA fragments which causes the apoptotic cell death and apoptotic body formation [5]. Besides, the tumour inhibitory mechanism of compounds **8b** and **8e** was verified by various assays such as immunoblot, endonuclease assay, DNA fragmentation assay and giemsa stain. Mechanism underlying the compound **8b** and **8e** exhibiting apoptosis studied by immunoblots and the results inferred that compounds **8b** and **8e** induces the activation of CAD by cleaving the ICAD/CAD complex through the expression of caspase-3 [Fig. 3A]. Cell fractionation (cytosolic and nuclear proteins) studies were performed for evaluating the nuclear translocation of active CAD and is suggested that compounds **8b** and **8e** promotes the nuclear localization of CAD where it can degrade the cellular DNAs

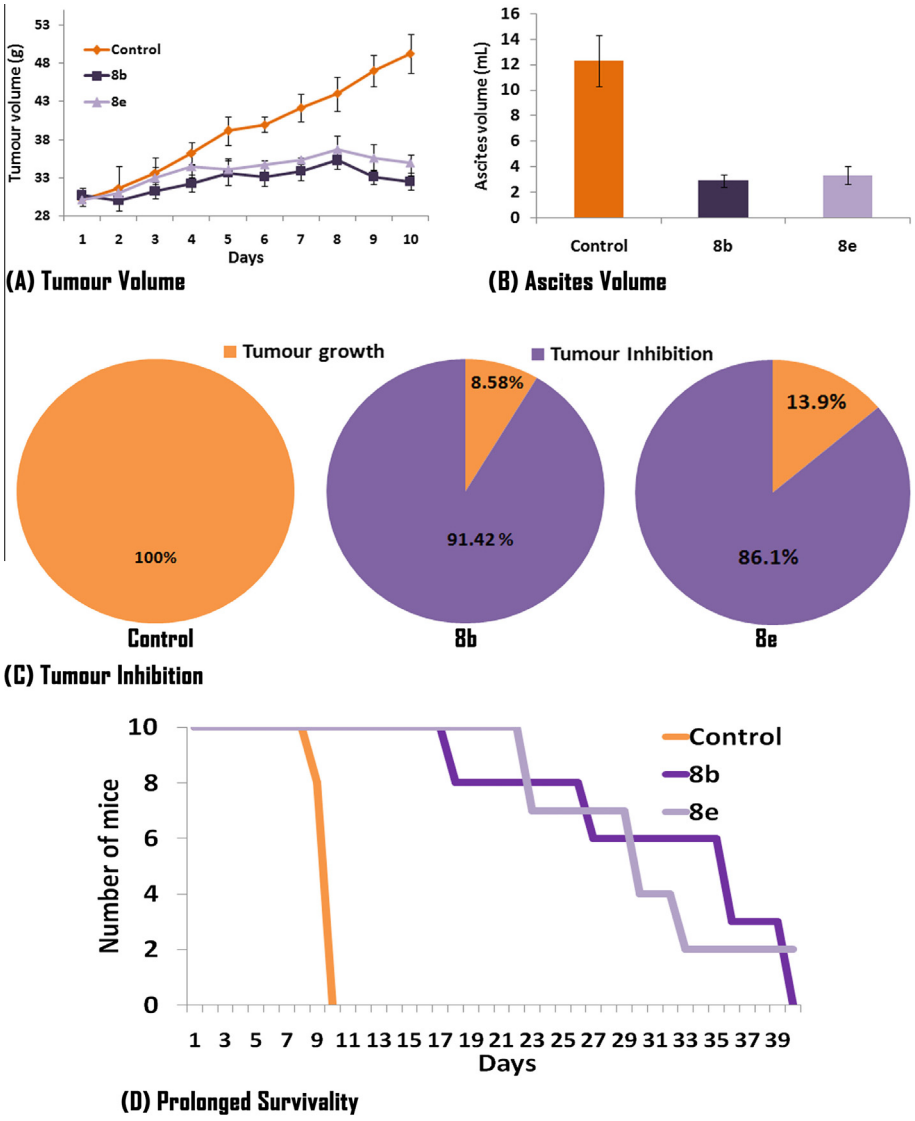


Fig. 2. Compound **8b** and **8e** has potency to show the inhibition in Dalton's lymphoma ascites (DLA) proliferation *in vivo*. Ascites tumour was induced in mice by culturing DLA cells in peritoneum and administered with 75 mg/kg (i.p) of compound **8b** and **8e** for three doses. Compound **8b** and **8e** has ability to exhibit (A) the reduction in tumour volume, (B) the decrease in ascites secretion, (C) the inhibition of tumour growth and (D) the extended survivalism compared to control.

Table 1
Hematological and serum profile of non tumour bearing mice following treatment with compounds **8b** and **8e** at day 10. Values are indicated in mean ± SEM.

Hematological and serum profile parameters	Control mice	**8b** treated mice	**8e** treated mice
Alkaline phosphatase (IU/L)	129.55 ± 2.95	135.65 ± 5.6	132.05 ± 4.7
Creatinine (mg/dl)	0.59 ± 1.05	0.63 ± 0.335	0.55 ± 0.25
Urea (mg/dl)	45 ± 2.78	46 ± 1.9	39 ± 2.8
RBC (10^6/μl)	5.28 ± 1.45	5.08 ± 1.05	5.67 ± 1.85
WBC (10^6/μl)	3.32 ± 1.6	3.21 ± 0.40	3.10 ± 0.56

[Fig. 3B&C]. Endonuclease assay was performed for studying the activation of CAD, and the results showed that compounds **8b** and **8e** have promoted the endonuclease (CAD) activation as visualized by DNA lysis zone. Increased concentration of **8b** and **8e** treated cytosolic fractions (400 and 600 μg) exhibit enlarged DNA lysis zone (**8b** with 0.4–0.6 cm DNA lysis and **8e** with 0.3–0.5 cm of DNA lysis) compared to control, which is evident for endonuclease (CAD) activation [Fig. 3D]. Eventually the activated CAD degrades the DNA as observed by DNA fragmentation assay, which is concurrent to immunoblot and endonuclease assay results [Fig. 3E]. The cell morphology analysed by Giemsa staining further inferred that compounds **8b** and **8e** treated cells posed cell shrinkage, membrane blebbing and irregular shape with apoptotic bodies which are major features of apoptotic hallmarks [Fig. 3F]. These results confirm that compounds **8b** and **8e** specifically induce the CAD mediated apoptotic DNA fragmentation in DLA tumour.

3. Conclusion

In summary, a series of benzophenone tagged pyridine analogous **8a–l** were synthesized and evaluated for antiproliferative activity against DLA cells by *in vitro* and *in vivo* studies. From the present investigation, structural activity relationship of these compounds suggests that hydroxy pyridine moiety and fluoro and chloro group at the benzoyl ring of the benzophenone scaffold as in compounds **8b** and **8e** are significant for activity. Further investigation in mouse model suggests that compounds **8b** and **8e** have the potency to activate CAD which is responsible for

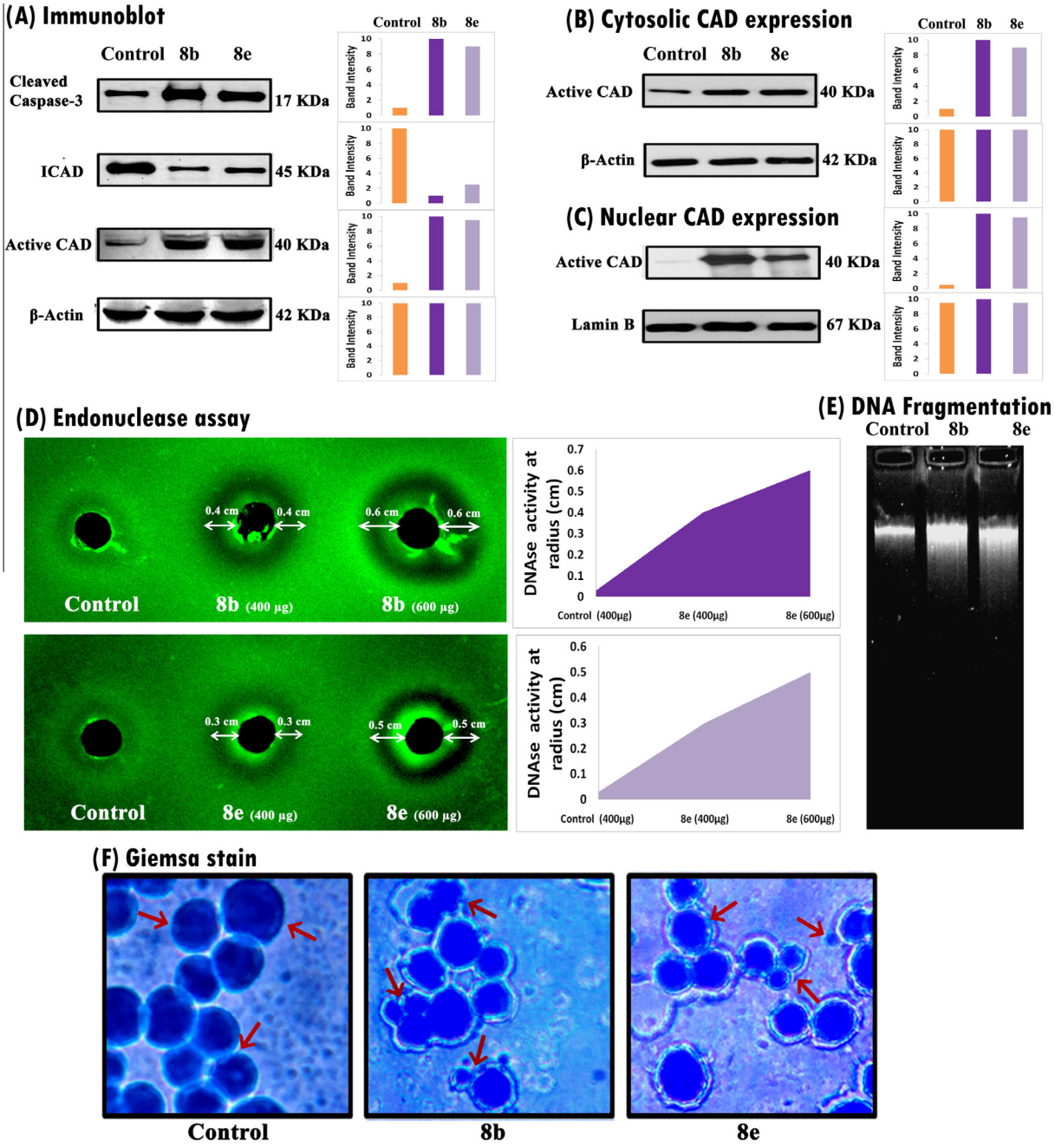


Fig. 3. Compound **8b** and **8e** exhibits CAD mediated DNA fragmentation in DLA cells *in vivo*. (A) Immunoblot photograph shows that compound **8b** and **8e** have induced the activation of CAD through cleaved casspase-3 and inhibits the ICAD *in vivo*. (B) Cytosolic expression of active CAD (C) Compound **8b** and **8e** promotes the nuclear translocation of active CAD. (D) The endonuclease assay images illustrates that the DNA degrading potentiality (DNA lysis zone) of compound **8b** and **8e** through activated endonuclease (DNase) in concentration dependant manner (400 and 600 µg/well) and respective graphs shows the increased DNase activity in DNA lysis zone. (E) DNA fragmentation profile from control and compound **8b** & **8e** treated samples. (F) Giemsa stain photograph depicting that regular shape of the cells, none of apoptotic bodies in control and irregular shape and membrane blebbing, apoptotic bodies in Compound **8b** and **8e** treated cells.

DNA fragmentation, a primary hallmark of apoptosis. Elucidation of compounds **8b** and **8e** induced upstream signalling is under investigation for detailed studies.

4. Material and methods

4.1. Chemistry

Chemicals were procured from Sigma Aldrich Chemical Co. TLC was performed on aluminium-backed silica plates and visualized by UV-light. Melting points were measured on a Thomas Hoover capillary melting point apparatus with a digital thermometer. IR spectra were recorded on FT-IR Shimadzu 8300 spectrophotometer, ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 MHz NMR spectrophotometer using TMS as an internal standard and DMSO-d_6 as solvent. Chemical shifts are given in parts per million downfield from tetramethylsilane. The mass spectra were obtained with a VG70-70H spectrophotometer and the elemental analysis (C, H, and N) was performed on Elementar Vario EL III elemental analyzer. The results of elemental analyses were within ±0.4% of the theoretical values.

*4.1.1. General procedure for the synthesis of aryl benzoates (**3a–d**)*

2-Chloro-6-fluoro phenol (**1**, 0.20 mol) was dissolved in dichloro methane (DCM) and triethylamine (TEA, 0.45 mol) was added to it. Then the reaction mixture was cooled to 0 °C. Further, a solution of substituted benzoyl chloride **2a–d** (0.21 mol) in DCM was slowly added to the reaction mixture and stirred for 3 h and the completion of the reaction was monitored by TLC using 4:1 n-hexane: ethyl acetate solvent mixture. Then the reaction mass was diluted with DCM (100 ml), washed with 10% sodium hydroxide solution (3 × 40 ml), followed by water (3 × 30 ml). The organic layer was dried over sodium sulphate and the solid obtained after evaporation of the solvent was recrystallized from ethanol to give compounds **3a–d.** [21] Compound (**3a**) is taken as a representative example to explain physical and characterization data.

*4.1.1.1. 2-Chloro-6-fluorophenyl-4-fluorobenzoate (**3a**).* Yield: 92%; m.p. 52–54 °C; FT-IR (KBr, cm^{-1}) 1738 (C=O); ^1H NMR (DMSO-d_6): δ 7.42–8.28 (m, 7H, Ar—H); LC–MS *m/z* 268 (M^+) and 270 (M +2). Anal. Calcd. for $C_{13}H_7ClF_2O_2$: C, 58.12; H, 2.63; Cl, 13.20; F, 14.14. Found: C, 58.22; H, 2.43; Cl, 13.30; F, 14.29%.

*4.1.2. General procedure for the synthesis of (4-hydroxy aryl) aryl methanones (**4a–d**)*

Substituted 4-hydroxy benzophenones **4a–d** were synthesized by Fries rearrangement. Compounds **3a–d** (0.063 mol) was blended with aluminium chloride (0.126 mol) and the mixture was heated to 150 °C and this temperature was maintained for 2 h and the completion of the reaction was monitored by TLC using 4:1 n-hexane: ethyl acetate solvent mixture. Then the reaction mixture was cooled to room temperature and quenched with 6 N hydrochloric acid and ice water. The reaction mixture was stirred for about 1 h, the obtained solid was filtered and recrystallized with methanol to obtain desired compounds **4a–d.** [23] Compound (**4a**) is taken as a representative example to explain physical and characterization data.

*4.1.2.1. (3-Chloro-5-fluoro-4-hydroxyphenyl)-4-fluorophenyl methanone (**4a**).* Yield: 70%; m.p. 146–147 °C; FT-IR (KBr, cm^{-1}): 1671 (C=O), 3545–3635 (OH); ^1H NMR (DMSO-d_6): δ 7.36–7.82 (m, 6H, Ar—H), 11.64 (bs, 1H, OH); LC–MS *m/z* 268 (M^+) and 270 (M +2). Anal. Calcd. for $C_{13}H_7ClF_2O_2$: C, 58.12; H, 2.63; Cl, 13.20; F, 14.14. Found: C, 58.21; H, 2.52; Cl, 13.20; F, 14.25%.

*4.1.3. General procedure for the synthesis of ethyl 4-aryloylaryloxyacetates (**5a–d**)*

To a solution of compounds **4a–d** (0.038 mol) in dry DMF (70 ml), potassium carbonate (0.076 mol) and ethyl chloroacetate (0.057 mol) were added and the reaction mass was heated to 60 °C for 3 h, the progress of the reactions was monitored by TLC using 2:1 n-hexane: ethyl acetate. The reaction mass was diluted with ethyl acetate (60 ml), potassium carbonate was filtered off and the bed was washed with ethyl acetate (40 ml). Finally, the organic layer was washed with water (3 × 30 ml), brine (2 × 40 ml), dried over sodium sulphate and evaporated to dryness to obtain crude solid, which, on recrystallization with ethanol afforded desired compounds **5a–d**. [34] Compound (**5a**) is taken as a representative example to explain physical and characterization data.

*4.1.3.1. Ethyl [2-(4-fluorobenzoyl)-2-chloro-6-fluorophenoxy]acetate (**5a**).* Yield: 95%; FT-IR (Nujol, cm^{-1}): 1660 (C=O), 1730 (ester, C=O); ^1H NMR (DMSO-d_6): δ 1.20 (t, *J* = 7.0 Hz, 3H, CH_3 of ester), 4.17 (q, *J* = 7.50 Hz, 2H, CH_2), 5.02 (s, 2H, OCH_2), 7.64–7.86 (m, 6H, Ar—H); LC–MS *m/z* 354 (M^+) and 356 (M+2). Anal. Calcd. for $C_{17}H_{13}ClF_2O_4$: C, 57.56; H, 3.69; Cl, 9.99; F, 10.71. Found: C, 57.41; H, 3.52; Cl, 9.79; F, 10.88%.

*4.1.4. General procedure for the synthesis of 4-aryloyl aryloxy acethydrazides (**6a–d**)*

Hydrazine hydrate (0.018 mol) was added to a solution of compounds **5a–d** (0.018 mol) in ethanol (30 ml) and continuously stirred for 2 h at room temperature. After the completion of the reaction, mointered by TLC using 2:1 chloroform: ethyl acetate as a mobile phase. A white solid was separated out, which was quenched with water (50 ml), filtered and washed with water (50 ml). The solid was dried under vacuum and the product was recrystallized from ethanol to obtain compounds **6a–d** with good yield [35]. Compound (**6a**) is taken as a representative example to explain physical and characterization data.

*4.1.4.1. [2-Chloro-6-fluoro-4-(4-fluoro-benzoyl)-phenoxy]acetic acid hydrazide (**6a**).* Yield: 76%; m.p. 107–109 °C; FT-IR (KBr, cm^{-1}): 1610 (C=O), 1645 (amide, C=O), 3100–3205 (NH—NH_2); ^1H NMR (DMSO-d_6): δ 4.35 (bs, 2H, NH_2), 4.69 (s, 2H, OCH_2), 7.20–7.86 (m, 6H, Ar—H), 9.32 (bs, 1H, NH); LC–MS *m/z* 340 (M^+) and 342 (M+2). Anal. Calcd. for $C_{15}H_{11}ClF_2N_2O_3$: C, 52.88; H, 3.25; Cl, 10.41; F, 11.15; N, 8.22. Found: C, 52.75; H, 3.38; Cl, 10.29; F, 11.24; N, 8.11%.

*4.1.5. General procedure for the synthesis of nicotinic acid N′-[2-(4-benzoyl-phenoxy)-acetyl]-hydrazides (**8a–l**)*

4-Aryloyl aryloxy acethydrazides **6a–d** (0.0010 mol) in dry DCM (20 ml) was stirred at 25–30 °C, and then lutidine (0.0015 mol) was added, followed by the addition of nicotinic acid derivatives **7a–c** (0.0010 mol). The reaction mixture was stirred at the same temperature for 30 min, then cooled to 0–5 °C and TBTU (0.0015 mol) was added over a period of 30 min maintaining the temperature below 5 °C. The reaction mass was stirred overnight and monitored by TLC using chloroform: methanol (9:1) as the mobile phase. The solvent was evaporated at reduced pressure, quenched by the addition of crushed ice and the obtained solid was filtered, dried and recrystallized from ethanol to afford compounds **8a–l** in good yield.

*4.1.5.1. 2,6-Dichloro-nicotinic acid N′-{2-[2-chloro-6-fluoro-4-(4-fluoro-benzoyl)-phenoxy]-acetyl}-hydrazide (**8a**).* Yield: 85%; m.p. 140–142 °C; FT-IR (KBr, cm^{-1}): 1640 (C=O), 1665 (amide, C=O), 3240–3350 (NH—NH); ^1H NMR (DMSO-d_6): δ 4.91 (s, 2H, CH_2), 7.35–8.06 (m, 8H, Ar—H), 10.79 (bs, 2H, 2NH); ^{13}C NMR (DMSO-d_6): δ 175.6, 169.3, 164.2, 162.3, 158.8, 153.4, 147.3, 138.4, 135.1, 132.4, 131.5, 130.3, 129.2, 128.4, 127.5, 115.6, 113.9, 108.5, 68.3; LC–MS *m/z* 514 (M^+), 516 (M+2) 518 (M+4) and 520 (M+6). Anal. Calcd. for $C_{21}H_{12}Cl_3F_2N_3O_4$: C, 49.0; H, 2.35; Cl, 20.66; F, 7.38; N, 8.16. Found: C, 49.30; H, 2.16; Cl, 20.52; F, 7.26; N, 8.47%.

*4.1.5.2. 6-Hydroxy-nicotinic acid N′-{2-[2-chloro-6-fluoro-4-(4-fluoro-benzoyl)-phenoxy]-acetyl}-hydrazide (**8b**).* Yield: 75%; m.p. 132–134 °C; FT-IR (KBr, cm^{-1}): 1640 (C=O), 1670 (amide, C=O), 3270–3370 (NH—NH), 3545–3635 (OH); ^1H NMR (DMSO-d_6): δ 4.93 (s, 2H, CH_2), 7.28–7.93 (m, 9H, Ar—H), 12.4 (s, 1H, OH), 10.47 (bs, 2H, 2NH); ^{13}C NMR (DMSO-d_6): δ 175.3, 169.3, 164.3, 163.4, 162.3, 158.8, 144.3, 136.4, 134.4, 132.4, 131.5, 130.3, 129.2, 120.4, 115.8, 114.5, 113.9, 108.5, 68.3; LC–MS *m/z* 462 (M^+) and 464 (M+2). Anal. Calcd. for $C_{21}H_{14}ClF_2N_3O_5$: C, 54.62; H, 3.06; N, 9.10. Found: C, 54.38; H, 3.20; N, 8.96%.

*4.1.5.3. 2-Amino-nicotinic acid N′-{2-[2-chloro-6-fluoro-4-(4-fluoro-benzoyl)-phenoxy]-acetyl}-hydrazide (**8c**).* Yield: 82%; m.p. 127–129 °C; FT-IR (KBr, cm^{-1}): 1640 (C=O), 1655 (amide, C=O), 3220–3385 (broad NH—NH and NH_2); ^1H NMR (DMSO-d_6): δ 4.86

(s, 2H, CH_2), 5.73 (s, 2H, NH_2), 7.31–8.02 (m, 9H, Ar—H), 10.63 (bs, 2H, 2NH); ^{13}C NMR (DMSO-d_6): δ 175.6, 169.3, 164.2, 162.7, 162.3, 158.8, 147.3, 137.4, 135.8, 133.0, 131.5, 130.3, 129.2, 118.4, 116.4, 114.5, 113.9, 108.5, 68.3; LC–MS *m*/*z* 461 (M^+) and 463 (M+2). Anal. Calcd. for $C_{21}H_{15}ClF_2N_4O_4$: C, 54.73; H, 3.82; N, 12.16. Found: C, 55.08; H, 3.74; N, 12.12%.

*4.1.5.4. 2,6-Dichloro-nicotinic acid N′-{2-[2-chloro-4-(4-chloro-benzoyl)-6-fluoro-phenoxy]-acetyl}-hydrazide (**8d**).* Yield: 87%; m.p. 135–137 °C; FT-IR (KBr, cm^{-1}): 1635 (C=O), 1665 (amide, C=O), 3240–3350 (NH—NH); ^{1}H NMR (DMSO-d_6): δ 4.92 (s, 2H, CH_2), 7.26–7.93 (m, 8H, Ar—H), 10.75 (bs, 2H, 2NH); ^{13}C NMR (DMSO-d_6): δ 175.6, 169.3, 164.2, 158.8, 153.4, 147.3, 138.4, 136.7, 134.4, 132.4, 131.5, 130.3, 129.2, 128.4, 127.5, 114.1, 113.9, 108.5, 68.3; LC–MS *m*/*z* 529 (M^+), 531 (M+2), 533 (M+4) and 535 (M+6). Anal. Calcd. for $C_{21}H_{12}Cl_4FN_3O_4$: C, 47.49; H, 2.28; N, 7.91. Found: C, 47.29; H, 2.14; N, 7.76%.

*4.1.5.5. 6-Hydroxy-nicotinic acid N′-{2-[2-chloro-4-(4-chloro-benzoyl)-6-fluoro-phenoxy]-acetyl}-hydrazide (**8e**).* Yield: 77%; m.p. 137–139 °C; FT-IR (KBr, cm^{-1}): 1635 (C=O), 1670 (amide, C=O), 3270–3370 (NH—NH), 3540–3635 (OH); ^{1}H NMR (DMSO-d_6): δ 4.88 (s, 2H, CH_2), 7.27–8.0 (m, 9H, Ar—H), 10.67 (bs, 2H, 2NH), 12.6 (s,1H, OH); ^{13}C NMR (DMSO-d_6): δ 175.3, 169.3, 164.3, 163.4, 158.8, 144.3, 136.5, 134.4, 133.0, 132.4, 131.5, 130.3, 129.2, 120.4, 115.8, 114.5, 113.9, 108.5, 68.3; LC–MS *m*/*z* 478 (M^+), 480 (M+2) and 482 (M+4). Anal. Calcd. for $C_{21}H_{14}Cl_2FN_3O_5$: C, 52.74; H, 2.95; N, 8.79. Found: C, 52.38; H, 3.25; N, 8.76%.

*4.1.5.6. 2-Amino-nicotinic acid N′-{2-[2-chloro-4-(4-chloro-benzoyl)-6-fluoro-phenoxy]-acetyl}-hydrazide (**8f**).* Yield: 80%; m.p. 123–125 °C; FT-IR (KBr, cm^{-1}): 1635 (C=O), 1660 (amide, C=O), 3235–3385 (broad NH—NH and NH_2); ^{1}H NMR (DMSO-d_6): δ 4.86 (s, 2H, CH_2), 5.83 (s, 2H, NH_2), 7.14–8.08 (m, 9H, Ar—H), 10.39 (bs, 2H, 2NH); ^{13}C NMR (DMSO-d_6): δ 175.6, 169.3, 164.2, 162.7, 158.8, 147.3, 144.3, 137.4, 136.3, 132.4, 131.5, 130.3, 129.2, 118.4, 116.3, 114.5, 113.9, 108.5, 68.3; LC–MS *m*/*z* 477 (M^+), 479 (M+2) and 481 (M+4). Anal. Calcd. for $C_{21}H_{15}Cl_2FN_4O_4$: C, 52.85; H, 3.17; N, 11.74. Found: C, 52.50; H, 3.07; N, 11.70%.

*4.1.5.7. 2,6-Dichloro-nicotinic acid N′-{2-[2-chloro-6-fluoro-4-(4-iodo-benzoyl)-phenoxy]-acetyl}-hydrazide (**8g**).* Yield: 83%; m.p. 156–158 °C; FT-IR (KBr, cm^{-1}): 1630 (C=O), 1660 (amide, C=O), 3245–3345 (NH—NH); ^{1}H NMR (DMSO-d_6): δ 4.91 (s, 2H, CH_2), 7.15–8.00 (m, 8H, Ar—H), 10.37 (bs, 2H, 2NH); ^{13}C NMR (DMSO-d_6): δ 175.6, 169.3, 164.2, 162.3, 158.8, 153.4, 147.3, 137.8, 133.0, 131.5, 130.3, 129.2, 128.4, 127.5, 115.2, 113.9, 108.5, 102.3, 68.3; LC–MS *m*/*z* 620 (M^+), 622 (M+2), 624 (M+4) and 626 (M+6). Anal. Calcd. for $C_{21}H_{12}Cl_3FIN_3O_4$: C, 40.51; H, 1.94; N, 6.75. Found: C, 40.16; H, 1.92; N, 6.75%.

*4.1.5.8. 6-Hydroxy-nicotinic acid N′-{2-[2-chloro-6-fluoro-4-(4-iodo-benzoyl)-phenoxy]-acetyl}-hydrazide (**8h**).* Yield: 81%; m.p. 106–108 °C; FT-IR (KBr, cm^{-1}): 1630 (C=O), 1665 (amide, C=O), 3265–3375 (NH—NH), 3535–3630 (OH); ^{1}H NMR (DMSO-d_6): δ 4.90 (s, 2H, CH_2), 7.42–7.73 (m, 9H, Ar—H), 10.67 (bs, 2H, 2NH), 12.3 (s, 1H, OH); ^{13}C NMR (DMSO-d_6): δ 175.6, 169.3, 164.2, 163.4, 162.3, 158.8, 144.3, 136.5, 132.4, 131.5, 130.3, 129.2, 120.4, 115.6, 114.5, 113.9, 108.5, 102.3, 68.3; LC–MS *m*/*z* 570 (M^+), 572 (M+2). Anal. Calcd. for $C_{21}H_{14}ClFIN_3O_5$: C, 44.27; H, 2.48; N, 7.38. Found: C, 44.64; H, 2.55; N, 7.34%.

*4.1.5.9. 2-Amino-nicotinic acid N′-{2-[2-chloro-6-fluoro-4-(4-iodo-benzoyl)-phenoxy]-acetyl}-hydrazide (**8i**).* Yield: 78%; m.p. 108–110 °C; FT-IR (KBr, cm^{-1}): 1630 (C=O), 1665 (amide, C=O), 3235–3395 (broad NH—NH and NH_2); ^{1}H NMR (DMSO-d_6): δ 4.94 (s, 2H, CH_2), 5.76 (s, 2H, NH_2), 7.21–8.07 (m, 9H, Ar—H), 10.63 (bs, 2H, 2NH); ^{13}C NMR (DMSO-d_6): δ 175.6, 169.3, 164.2, 162.7, 158.8, 147.3, 136.3, 133.0, 132.4, 131.5, 130.3, 129.2, 118.4, 115.9, 114.5, 113.9, 108.5, 102.3, 68.3; LC–MS *m*/*z* 569 (M^+), 571 (M+2). Anal. Calcd. for $C_{21}H_{15}ClFIN_4O_4$: C, 44.35; H, 2.66; N, 9.85. Found: C, 44.59; H, 2.56; N, 9.65%.

*4.1.5.10. 2,6-Dichloro-nicotinic acid N′-{2-[2-chloro-6-fluoro-4-(4-methyl-benzoyl)-phenoxy]-acetyl}-hydrazide (**8j**).* Yield: 88%; m.p. 127–129 °C; FT-IR (KBr, cm^{-1}): 1635 (C=O), 1660 (amide, C=O), 3220–3320 (NH—NH); ^{1}H NMR (DMSO-d_6): δ 2.50 (s, 3H, CH_3), 4.91 (s, 2H, CH_2), 7.25–8.12 (m, 8H, Ar—H), 10.79 (bs, 2H, 2NH); ^{13}C NMR (DMSO-d_6): δ 165.2, 163.3, 152.2, 151.3, 148.0, 144.1, 143.7, 142.0, 134.0, 133.8, 133.5, 130.3, 129.7, 128.2, 127.4, 124.9, 124.1, 119.5, 110.0, 21.6; LC–MS *m*/*z* 511 (M^+), 513 (M+2), 515 (M+4) and 517 (M+6) Anal. Calcd. for $C_{22}H_{15}Cl_3FN_3O_4$: C, 51.74; H, 2.96; N, 8.23. Found: C, 51.39; H, 2.92; N, 8.21%.

*4.1.5.11. 6-Hydroxy-nicotinic acid N′-{2-[2-chloro-6-fluoro-4-(4-methyl-benzoyl)-phenoxy]-acetyl}-hydrazide (**8k**).* Yield: 80%; m.p. 132–134 °C; FT-IR (KBr, cm^{-1}): 1635 (C=O), 1665 (amide, C=O), 3260–3360 (NH—NH), 3545–3630 (OH); ^{1}H NMR (DMSO-d_6): δ 2.40 (s, 3H, CH_3), 4.90 (s, 2H, CH_2), 7.17–8.09 (m, 9H, Ar—H), 10.44 (bs, 2H, 2NH), 12.4 (s,1H, OH); ^{13}C NMR (DMSO-d_6): δ 168.2, 163.4, 152.5, 152.3, 148.1, 144.5, 143.4, 142.2, 134.8, 133.6, 132.6, 130.7,130.1, 127.8, 127.2, 125.2, 124.4, 120.3, 109.7, 21.5; LC–MS *m*/*z* 458 (M^+), 460 (M+2). Anal. Calcd. for $C_{22}H_{17}ClFN_3O_5$: C, 57.71; H, 3.74; N, 9.12. Found: C, 57.54; H, 3.86; N, 9.09%.

*4.1.5.12. 2-Amino-nicotinic acid N′-{2-[2-chloro-6-fluoro-4-(4-methyl-benzoyl)-phenoxy]-acetyl}-hydrazide (**8l**).* Yield: 84%; m.p. 116–118 °C; FT-IR (KBr, cm^{-1}): 1630 (C=O), 1655 (amide, C=O), 3230–3380 (broad NH—NH and NH_2); ^{1}H NMR (DMSO-d_6): δ 2.30 (s, 3H, CH_3), 4.86 (s, 2H, CH_2), 5.70 (s, 2H, NH_2), 7.13–7.96 (m, 9H, Ar—H), 10.51 (bs, 2H, 2NH); ^{13}C NMR (DMSO-d_6): δ 167.6, 163.2, 152.6, 152.3, 148.2, 144.7, 143.7, 141.9, 134.6, 133.5, 133.1, 130.7, 129.5, 127.8, 127.5, 125.3, 123.6, 119.4, 109.1, 22.2; LC–MS *m*/*z* 457 (M^+), 459 (M+2). Anal. Calcd. for $C_{22}H_{18}ClFN_4O_4$: C, 57.84; H, 3.97; N, 12.26. Found: C, 57.49; H, 4.11; N, 12.39%.

4.2. Biology

The Dalton's lymphoma Ascites (DLA) cells were selected for assessing the cytotoxicity of newly synthesized compounds by MTT assay, trypan blue dye exclusion assay, and LDH release assay and for studying the *in vivo* antitumour effect of the potent compounds by FACS, Immunoblot, Endonuclease assay, DNA fragmentation assay and Giemsa stain.

4.2.1. Cell Culture and in vitro treatment

The DLA cells were grown in DMEM medium (Gibco-Invitrogen, USA), supplemented with 10% FBS (In vitrogen, USA), Penicillin–Streptomycin (Sigma–aldrich, USA) and $NaHCO_3$ (0.37%) in a humidified CO_2 incubator at 37 °C with 5% CO_2. The cells were treated with six different concentrations of compounds **8a–j** (0, 5, 10, 25, 50, 100 μM in DMSO) and reincubated at 37 °C for 45 h. MTT assay, trypan blue dye exclusion assay, and LDH release assays were performed as reported earlier for cytotoxicity analysis [21].

4.2.2. Animal ethics and determination of LD_{50} value

Swiss female albino mice weighing between 28–30 g were used throughout the study. The mice were grouped and housed in polyacrylic cages with not more than ten animals per cage with adequate food and water supply. All procedures described were reviewed and approved by the National College of Pharmacy Ethical Committee, Shimoga, India, in accordance with the CPCSEA

guidelines for laboratory animal facility (NCP/IAEC/CL/101/05/2012-13). LD_{50} of the compounds **8b** and **8e** were evaluated as described earlier and their adverse effects were studied by injecting 75 mg/kg body weight, intraperitoneally (i.p) to healthy Swiss albino mice continuously for 10 days [22]. The serum profile such as alkaline phosphatase (ALP), creatinine, urea, number of RBC and WBC from the blood of both treated and untreated animals were evaluated.

4.2.3. Animal tumour models and treatment

DLA tumours were maintained as ascites by intraperitoneal serial transplantation in mice. The tumour cells were aspirated from the tumour bearing mice aseptically and washed thrice in phosphate-buffered saline. A DLA tumour model was developed by injecting 5×10^6 cells/ mouse intraperitoneally. After the onset of the tumour development (4th day), the mice bearing DLA were administered with or without compounds **8b** and **8e** (75 mg/kg body weight i.p) 3 doses on every alternate day. On day 10, tumour parameters such as tumour volume, ascites secretion, tumour inhibition and survivality were evaluated.

4.2.4. Cell Sorting (FACS)

DLA cells were cultured *in vitro* and after 24 h, the cells were treated with compounds **8b** and **8e** at 10 μM for 48 h. The harvested cells were stained with propidium iodide for determining the cell death [25]. Compounds **8**b and **8**e induced cell death were analysed by WinMDI 2.9 software and documented.

4.2.5. Lysate/protein fraction preparation and Immunobloting

The whole cell lysates of plus or minus compounds **8b** and **8e** *in vivo* treated DLA cells were prepared (by using RIPA buffer with PMSF and Protease inhibitor cocktail) and their concentration was determined by using biospectrophotometer (Eppendorf nanodrop). Cytosolic and nuclear fractions from compounds **8b** and **8e** treated and untreated cells were prepared by using buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.05% NP40 and protease inhibitors) and buffer B (5 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 26% glycerol (v/v), protease inhibitors) respectively, for determining the nuclear translocation of CAD. Immunoblot analysis was carried out for Caspase Activated DNase (CAD), ICAD, Lamin B (Santa cruz), active caspase-3 and β-actin (BD Bioscience) as mentioned earlier [25].

4.2.6. Endonuclease assay

Endonuclease assay was performed to assess the compound induced DNase activation [36]. In brief, cytosolic extract of DLA cells treated with or without compounds **8b** and **8e** were prepared in lysis buffer (10 mM Tris–HCl, pH 8.0, 0.1 mM EGTA and 0.1% β-mercaptoethanol and protease inhibitors). One percent agarose gel was prepared with salmon sperm DNA (50 μg/ml) and Ethidium bromide (10 μg/ml) in the clean petri dish and wells were made by suction pressure. Cytosolic extract from treated at increasing concentration (400 and 600 μg) and untreated (400 μg) were loaded and incubated at 37 °C for 18 h with 80% humidity. The DNA lysis zone was visualized under SYBR green view of Bio-rad Gel Documentation™ XR + Imaging System.

4.2.7. DNA fragmentation assay and Giemsa stain

Genomic DNA was isolated by phenol: chloroform method from DLA cells of both treated and untreated groups, then obtained genomic DNA was resolved in 1% agarose gel and fragmentation of treated DNA was observed and documented [22]. Giemsa stain was performed as reported earlier [22]. Morphology and apoptotic body formations were evaluated by control versus compound treated and then photographed.

4.2.8. Statistical analysis

Values were expressed as mean ± standard error (SEM). Statistical significance (5%) was evaluated by one-way analysis of variance (ANOVA) followed by Student's *t*-test ($^{*}p < 0.05$) and ($^{**}p < 0.01$).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2016.02.001.

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