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Research Article

SYNTHESIS AND EVALUATION OF ANTIANGIOGENIC AND ANTICANCER PROPERTIES OF NOVEL OXADIAZOLE ANALOGUES

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ABSTRACT
Oxadiazole, a heterocyclic nucleus has attracted a wide attention of the chemist in search for the new therapeutic molecules. Out of its four possible isomers, 1, 3, 4-oxadiazole is biologically active, synthetically useful and widely exploited for various applications. Various biological activities are reported to be associated with 1, 3, 4-oxadiazoles. Encouraged by these reports the present study has been undertaken. The newly synthesized compounds were confirmed from IR, ^{1}H NMR and mass spectral data. All the synthesized compounds were subjected for anti angiogenesis as well as anticancer activity. Compounds 7b and 10d showed very good anti angiogenesis and anticancer activity compared to all other analogues in the series.
Keywords: Anti angiogenesis, Anti cancer activity, 1, 3, 4-oxadiazoles, Synthesis.

INTRODUCTION

Numerous compounds with biological activity have been investigated, however many of them are not suitable for therapeutic use due to their toxic, carcinogenic and mutagenic properties. Nowadays, it is possible to make modifications of active chemical structures, in order to synthesize compounds with improved therapeutic activity and reduced toxicity. Apoptosis or programmed cell death has been characterized as a fundamental cellular activity occurring under a wide range of physiological and pathological conditions[1]. The various types of mature cells in the body have a specific life span. After the death of these cells, new cells are generated by proliferation and differentiation into different types of cells. Sometimes cells begin to divide in an uncontrolled manner and they no longer respond to the growth regulatory mechanisms. These cells divide and form a clump of cells producing a tumor or neoplasm[2]. About 200 types of cancers affecting major organs like lungs, brain, kidneys, colon, breasts and stomach have been identified. According to the National Cancer Institute, most can fit into categories of carcinoma, sarcoma, leukemia, lymphoma, and central nervous system cancers. The developments of efficient, selective and less toxic anti-cancer agents are a challenge in cancer research[3]. Five member nitrogen containing heterocycles with oxygen atom are an important class of compounds in medicinal chemistry. Oxadiazole derivatives have attracted much attention among five-membered oxygen containing heterocycles because of their biological and pharmacological properties like antibacterial[4-6], antimicrobial[7], fungicidal[8], anti-inflammatory[9], antipsychotic[10], anticonvulsant[11], antidepressant [12] and anticancer[13]. In the light of the above discussion we have investigated the effects of oxadiazoles related compounds on a panel of cell-lines. The most recent work involves three cell-lines, EAT, K562 and CEM[13-16]. Further, to achieve extensive applications in the field of cancer therapy herein we report the synthesis of different oxadiazole analogues 7a-d, 10a-d and 11a-b with the hope to get better antiangiogenic as well as anticancer agents.

MATERIALS AND METHODS

All solvents and reagents were purchased from Sigma Aldrich Chemicals Pvt Ltd. Melting points were determined on an electrically heated VMP-III melting point apparatus. The FT-IR spectra were recorded using KBr discs and Nujol on FT-IR Jasco 4100 infrared spectrophotometer. ^{1}H NMR spectra were recorded using Bruker DRX 400 spectrometer at 400 MHz with TMS as an internal standard. Mass spectra were recorded on LC-MS/MS (API-4000) mass spectrometer. Further elemental analysis of the compounds was performed on a Perkin Elmer 2400 elemental analyzer.

Chemistry

The synthesis of the hitherto unreported title compounds is as outlined in Scheme 1, 2 and 3. Methyl 4-nitrobenzoate (2) was achieved in excellent yield by treating 4-Nitro benzoic acid (1) with thinoyl chloride. Compound 2 on reaction with hydrazine hydrate afforded 4-nitrobenzoyl hydrazide (3) which on treatment with oxalyl chloride in presence of dry THF gave N,N-bis(4-nitrobenzoyl) oxalyl hydrazide (4). Further, compound 4 on treatment with phosphorous pentoxide yield 2,2' (4,4'-dinitro)diphenyl bis-1,3,4-oxadiazole (5). Condensation of one mole of compound 5 with two moles of 6a-d in the presence of potassium carbonate and DMF afforded compounds 7a-d (scheme-1). Besides, compound 1 on treatment with compound 3 in the presence of 2,6 lutidine, O-(benzotriazol-1-yl)- N,N,N0,N0-tetramethyluronium tetrafluoroborate (TBTU) and dichloromethane (DCM) afforded N,N(4.4'-dinitro)dibenzoyl hydrazide (8). Compound 8 on treatment with phosphorous pentoxide yield 2,5(4,4'-dinitro)diphenyl 1,3,4-oxadiazole (9). Compounds 2,5 (4,4'-dialkoxy)diphenyl -1,3,4-oxadiazole (10a-d) were furnished by the condensation of one mole of compound 9 with two moles of compounds 6a-d in the presence of potassium carbonate and DMF (scheme-2).

Finally, 2,5 (4-nitro-4'-alkoxy)diphenyl-1,3,4-oxadiazole (11a-b) were achieved by condensation of equimolar mixture of compound 9 with compounds 6a-b in the presence of potassium carbonate and DMF (scheme-3).

Synthesis of methyl 4-nitrobenzoate (2)

To a cooled solution of 4-nitro benzoic acid (1, 10 g, 0.05 mol) in dry methanol (25 mL) thinoyl chloride (29.7 g, 0.25 mol) was added slowly at 0-5° C over a period of 30 minutes. The reaction mixture was first warmed to 25-30°C and then heated to 60°C for 12 h. Progress of the reaction was monitored by TLC using 7:3 petroleum ether: ethyl acetate solvent mixture. The reaction mixture was then cooled to 25-30°C and organic solvent was evaporated to achieve the crude product. Finally, the crude sample was recrystallized with alcohol to get pure compound 2 as a white solid. Yield: 90 %, M.P: 94-96°C; IR (KBr): 1740 cm^{-1} (COO); ^{1}H NMR (DMSO-d_6) δ: 3.8 (*s*, 3H, OCH_3), 8.1 (d, 2H, Ar-H), 8.3 (d, 2H, Ar-H). MS (EI): m/z (80 %): M^+ 181; Anal. Calcd. for $C_8H_7NO_4$ (181): C, 53.04; H, 3.89; N, 7.73. Found: C, 53.00; H, 3.91; N, 7.75 %.

Synthesis of 4-nitrobenzoyl hydrazide (3)

Hydrazine hydrate (6.4 g, 0.22 mol) was added to a cooled solution of methyl 4-nitrobenzoate (2, 10 g, 0.05 mol) in methanol (25 mL) and stirred for 30 minutes at 0-5°C. The reaction mass was heated to 60°C for 2 h. Progress of the reaction was monitored by TLC using 7:3 petroleum ether: ethyl acetate solvent mixture. The reaction mass was cooled, filtered and recrystallized with alcohol to achieve compound 3 as a white solid. Yield: 95 %, M.P: 107-109°C; IR (KBr): 1645 (C=O), 3110-3215 cm^{-1} (NH-NH_2); ^{1}H NMR (DMSO-d_6) δ: 4.35 (bs, 2H, NH_2), 8.1(d, 2H, Ar-H), 8.30 (d, 2H, Ar-H), 9.30 (bs, 1H, CO-NH). MS (EI): m/z (80 %): M^+ 181; Anal. Calcd. for $C_7H_7N_3O_4$ (181): C, 42.65; H, 3.58; N, 21.31; Found: C, 42.69.; H, 3.46; N, 21.30 %.

Synthesis of N,N- bis (4-nitrobenzoyl) oxalyl hydrazide (4)

Oxalyl chloride (6.5 g, 0.051 mol) was added to the cooled mixture of 4-nitrobenzoyl hydrazide (3, 10 g, 0.11 mol) in dry THF (15 mL) at 0-5° C and stirred for 30 minutes. The reaction mass was then warmed to 25°C and stirred for 2 h. Progress of the reaction was monitored by TLC using 5:5 petroleum ether: ethyl acetate solvent mixture. The reaction mixture was filtered and the crude solid was recrystallized with alcohol to afford compound 4 as brown solid. Yield: 60 %, M.P: 133-135°C; IR (KBr): 1630 (CONH), 1680 (CO-CO), 3250-3315 cm^{-1} (NH-NH); ^{1}H NMR (DMSO-d6) δ: 6.9-7.1 (d, 4H, Ar-H), 7.15-7.25 (d, 4H, Ar-H), 10.3 (bs, 2H, 2NH), 10.5 (bs, 2H, 2NH). MS (EI): m/z (86 %): M^+ 386; Anal. Calcd. for $C_{16}H_{10}N_4O_8$ (386) for: C, 49.75; H, 2.61; N, 14.50; Found: C, 49.72; H, 2.60; N, 14.40 %.

Synthesis of 2,2' (4,4'-dinitro)diphenyl bis-1,3,4-oxadiazole (5)

The mixture of compound 4 (10 g, 0.025 mol) and phosphorous pentoxide (29.32 g, 0.103 mol) was heated to 240°C for 12 h. The progress of the reaction was monitored by TLC using 1:1 petroleum ether: ethyl acetate solvent mixture. The reaction mixture was cooled to 40°C and quenched carefully with ice cold water (200 mL) and stirred at 25°C for 1 h. The solid precipitated was filtered and recrystallized with alcohol to yield compound 5 as brown solid. Yield: 69 %, M.P: 153-154°C; IR (KBr): 1625 cm^{-1} (C=N); ^{1}H NMR (DMSO-d_6) δ: 7.05-7.15 (d, 4H, Ar-H), 7.2-7.28 (d, 4H, Ar-H). MS (EI): m/z (81 %): M^+ 380; Anal. Calcd. for $C_{16}H_8N_6O_6$ (380): C, 50.54; H, 2.12; N, 22.01; Found: C, 50.58; H, 2.10; N, 22.04 %.

Synthesis of 2,2' (4,4'-dialkoxy)diphenyl bis-1,3,4-oxadiazoles (7a-d)

To a solution of compound 5 (0.5 g, 0.013 mol) in dry DMF(10 mL) potassium carbonate (0.72 g, 0.005 mol) was added followed by diols 6a-c (0.0039 mol). Then the reaction mixture was heated to 145°C with constant stirring for 18 h. Completion of the reaction was monitored by TLC using 9.5:0.5 dichloromethane: methanol solvent mixture. After cooling, the reaction mass was diluted with ethyl acetate (10 mL), potassium carbonate was filtered off and the bed was washed with ethyl acetate (20 mL). The organic layer was washed with water (3 × 30 mL), brine (2 × 40 mL), dried over sodium sulfate, filtered and concentrated. The crude product was re-crystallized twice with ether to yield compounds 7a-c. Compound 7d was also synthesized analogously using 2-(2-methoxy-ethoxy)ethanol (6d). 7a: Yield: 45 %; M.P. 113-115°C; IR (KBr): 1645 cm^{-1} (C=N); ^{1}H NMR (DMSO-d6) δ: 1.8-2.1 (m, 4H, $2CH_2$), 3.50 (t, 4H, $2CH_2OH$), 4.1 (t, 4H, $2OCH_2$), 4.5 (bs, 2H, 2OH), 7.0-7.15 (d, 4H, Ar-H), 7.2-7.25 (d, 4H, Ar-H). MS (EI): m/z (90 %): M^+ 438; Anal. Calcd. for $C_{22}H_{22}N_4O_6$ (438): C, 60.27; H, 5.06; N, 12.78. Found: C, 60.23; H, 5.10; N, 12.81 %. 7b: Yield: 34 %; M.P.130-132 °C; IR (KBr):1635 cm^1 (C=N); ^{1}H NMR (DMSO-*d6*) δ: 1.6-1.72 (m, 4H, $2CH_2$), 1.73-1.75 (m, 4H, $2CH_2$), 3.55 (t, 4H, $2CH_2OH$), 4.3 (t, 4H, $2OCH_2$), 4.45 (bs, 2H, 2OH), 6.9-7.05 (d, 4H, Ar-H), 7.1-7.2 (d, 4H, Ar-H). MS (EI): m/z (91 %): M^+ 466. Anal. Calcd. for $C_{24}H_{26}N_4O_6$ (466): C, 61.79; H, 5.62; N, 12.01. Found: C, 61.86; H, 5.68; N, 12.07 %. 7c: Yield: 35 %; M.P. 148-150 °C; IR (KBr): 1650 cm^{-1} (C=N); ^{1}H NMR (DMSO-*d6*) δ: 1.5-1.9 (m, 16H, $2CH_2$), 3.55 (t, 4H, $2CH_2OH$), 4.4 (t, 4H, $2OCH_2$), 4.6 (bs, 2H, 2OH), 7.0-7.15 (d, 4H, Ar-H), 7.2-7.25 (d, 4H, Ar-H). MS (EI): m/z (90 %): M^+ 522. Anal. Calcd. for $C_{28}H_{34}N_4O_6$ (522): C, 64.13; H, 6.56; N, 10.72. Found: C, 64.19; H, 6.59; N, 10.78 %. 7d: Yield: 40.3 % ; M.P. 148-150 °C; IR (KBr): 1655 cm^{-1} (C=N); ^{1}H NMR (DMSO-*d6*) δ: 3.25 (s, 6H, $2OCH_3$), 3.5 (t, 8H, $2OCH_2CH_2O$), 4.1 (t, 8H, $2OCH_2CH_2$), 6.95-7.15 (d, 4H, Ar-H), 7.2-7.3 (d, 4H, Ar-H). MS (EI): m/z (92 %): M^+ 512. Anal. Calcd. for $C_{26}H_{30}N_4O_6$ (512): C, 58.59; H, 5.51; N, 10.93 Found: C, 58.62; H, 5.55; N, 10.98 %.

Synthesis of N,N(4.4'-dinitro)dibenzoyl hydrazide (8)

To a solution of 4-nitro benzoic acid (1, 2.03 g, 0.012 mmol) in dichloromethane (20 mL), 2,6-lutidine (3.9 g, 0.364 mmol) and TBTU (3.99 g, 0.121 mmol) were added at room temperature. Finally 4-nitrobenzoyl hydrazide (3, 2 g, 0.11 mmol) was added and stirred for 3 h. The reaction mixture was quenched with 10 % sodium bicarbonate solution and stirred for 30 minutes. The solid precipitated was filtered, washed with water, dried and recrystallized with alcohol to yield compound 8 as white solid. Yield: 75 %; M.P. 143-151°C; IR (KBr): 1638 (CONH), 3250-3315 (NH-NH); ^{1}H NMR (DMSO-d6) δ: 7.1-7.25 (d, 4H, Ar-H), 7.3-7.4 (d, 4H, Ar-H), 10.8 (bs, 2H, NH-NH). MS (EI): m/z (80 %): M^+ 386; Anal. Calcd. for; $C_{16}H_{10}N_4O_8$ (386) for: C,50.91; H, 3.65; N 16.96 Found: C, 50.72; H, 3.12; N, 16.85 %.

Synthesis of 2,5(4,4'-dinitro)diphenyl 1,3,4-oxadiazole (9)

A mixture of compound (8, 2 g, 6.1 mmol) and phosphorous pentoxide (3.44 g, 24.2 mmol) were heated to 240°C for 12 h.

The progress of the reaction was monitored by TLC using 1:1 petroleum ether: ethyl acetate solvent mixture. The reaction mixture was cooled to 40°C and quenched carefully with ice cold water and the mixture was stirred at 25°C for 1 h. The separated solid was filtered, dried and recrystallized with alcohol to attain compound 9 as white solid. Yield: 82 %, M.P: 156-157 °C; IR (KBr): 1645 (C=N) cm^{-1}; ^1H NMR (DMSO-d_6) δ: 7.1-7.2 (d, 4H, Ar-H), 7.25-7.35 (d, 4H, Ar-H). MS (EI): m/z (95 %): M^+ 312. Anal. Calcd. for $C_{14}H_8N_4O_5$ (312): C, 53.85; H, 2.58; N,17.94 Found: C, 53.89; H, 2.53; N, 17.98 %.

Synthesis of 2,5 (4,4'-dialkoxy)diphenyl -1,3,4-oxadiazole (10a-d)

To a solution of compound 9 (0.4 g, 0.013 mol) in dry DMF (10 mL) potassium carbonate (0.72 g, 0.005 mol) was added followed by diols 6a-c (0.0039 mol). Then the reaction mixture was heated to 145°C with constant stirring for 18 h. Completion of the reaction was monitored by TLC using 9.5:0.5 dichloromethane: methanol solvent mixture. After cooling, the reaction mass was diluted with ethyl acetate (10 mL), potassium carbonate was filtered off and the bed was washed with ethyl acetate (20 mL). The organic layer was washed with water (3 × 30 mL), brine (2 × 40 mL), dried over sodium sulfate, filtered and concentrated. The crude product was re-crystallized twice with ether to yield compounds 10a-c. Compound 10d was also synthesized analogously using 2-(2-methoxy-ethoxy)ethanol (6d). 10a: Yield: 44 %; M.P. 104-104 °C; IR (KBr): 1615 (C=N) cm^{-1}; ^1H NMR (DMSO-d6) δ: 1.75-1.95 (m, 4H, $2CH_2$), 3.51 (t, 4H, $2CH_2OH$), 4.2 (t, 4H, $2OCH_2$), 4.55 (bs, 2H, 2OH), 7.15-7.25 (d, 4H, Ar-H), 7.3-7.4 (d, 4H, Ar-H). MS (EI): m/z (92 %): M^+ 370; Anal. Calcd. for $C_{20}H_{22}N_2O_5$ (370): C, 64.85; H, 5.99; N, 7.56. Found: C, 64.86; H, 6.00; N, 7.60 %. 10b: Yield: 35 %; M.P. 113-114 °C; IR (KBr):1615 (C=N) cm^{-1}; ^1H NMR (DMSO-d6) δ: 1.55-1.62 (m, 4H, $2CH_2$), 1.7-1.75 (m, 4H, $2CH_2$), 3.5 (t, 4H, $2CH_2OH$), 4.2 (t, 4H, $2OCH_2$), 4.4 (bs, 2H, 2OH), 6.95-7.1 (d, 4H, Ar-H), 7.1-7.2 (d, 4H, Ar-H). MS (EI): m/z (91 %): M^+ 398. Anal. Calcd. for $C_{22}H_{26}N_2O_5$ (398): C, 66.32; H, 6.58; N, 7.03. Found: C, 66.38; H, 6.55; N, 7.06 %. 10c: Yield: 40 %; M.P. 133-134.°C; IR (KBr): 1612 (C=N) cm^{-1}; ^1H NMR (DMSO-d6) δ: 1.4-1.8 (m, 16H, $2CH_2$), 3.5 (t, 4H, $2CH_2OH$), 4.5 (t, 4H, $2OCH_2$), 4.6 (bs, 2H, 2OH), 6.95-7.1 (d, 4H, Ar-H), 7.15-7.25 (d, 4H, Ar-H). MS (EI): m/z (92 %): M^+ 454. Anal. Calcd. for $C_{26}H_{34}N_2O_5$ (454): C, 68.70; H, 7.54; N, 6.16. Found: C, 68.74; H, 7.55; N, 6.20 %. 10d: Yield: 27 %; M.P. 126-127 °C; IR (KBr): 1620 (C=N) cm^{-1}; ^1H NMR (DMSO-d6) δ: 3.15 (s, 6H, $2OCH_3$), 3.6 (t, 8H, $2OCH_2CH_2O$), 4.15 (t, 8H, $2OCH_2CH_2$), 7.2-7.3 (d, 4H, Ar-H), 7.35-7.4 (d, 4H, Ar-H). MS (EI): m/z (93 %): M^+ 458. Anal. Calcd. for $C_{24}H_{30}N_2O_7$ (458): C, 62.87; H, 6.60; N, 6.11. Found: C, 62.86; H, 6.70; N, 6.10 %.

Synthesis of 2,5 (4-nitro-4'-alkoxy)diphenyl-1,3,4-oxadiazole (11a-b)

To a solution of compound 9 (0.4 g, 0.013 mol) in dry DMF (10 mL) potassium carbonate (0.72 g, 0.005 mol) was added followed by diols 6a-b (0.013 mol). Then the reaction mixture was heated to 145°C with constant stirring for 18 h. Completion of the reaction was monitored by TLC using 9.5:0.5 dichloromethane: methanol solvent mixture. After cooling, the reaction mass was diluted with ethyl acetate (10 mL), potassium carbonate was filtered off and the bed was washed with ethyl acetate (20 mL). The organic layer was washed with water (3 × 30 mL), brine (2 × 40 mL), dried over sodium sulfate, filtered and concentrated. The crude product was re-crystallized twice with ether to yield compounds 11a-b. 11a. Yield: 23 %, M.P. 124-125 °C; IR (KBr): 1620 cm^{-1} (C=N); ^1H NMR (DMSO-d6) δ: 1.6-1.75 (m, 2H, CH_2), 3.4 (t, 2H, $2H_2OH$), 4.3 (t, 2H, OCH_2), 4.55 (bs, 1H, OH), 7.1-7.2 (d, 4H, Ar-H), 7.3-7.4 (d, 4H, Ar-H). MS (EI): m/z (93 %): M^+ 342 Anal. Calcd. for $C_{17}H_{15}N_3O_5$ (342): C, 59.82; H, 4.43; N, 12.31 Found: C, 59.87; H, 4.45; N, 12.36 %. 11b. Yield: 40 %, M.P. 144-146 °C; IR (KBr): 1625 cm^{-1} (C=N); ^1H NMR (DMSO-d6) δ: 1.5-1.6 (m, 2H, CH_2), 1.65-1.72 (m, 2H, CH_2), 3.4 (t, 2H, CH_2OH), 4.1 (t, 2H, OCH_2), 4.3 (bs, 1H, OH), 6.9-7.12 (d, 4H, Ar-H), 7.2-7.25 (d, 4H, Ar-H). MS (EI): m/z (92 %): M^+ 356 Anal. Calcd. for $C_{18}H_{17}N_3O_5$ (356): C, 60.84; H, 4.82; N, 11.83. Found: C, 60.89; H, 4.87; N, 11.88 %.

Biology

Cell culture and in-vitro compounds treatment

EAT cells were used for the present study and cultured as reported earlier.[13] The cells were treated with varying concentration of compounds 7a-d, 10a-d and 11a-b (0, 10, 20, 50, 100 μM in DMSO) for various time intervals (0-48 h) and further used for experiments. Appropriate vehicle control was used and each experiment was repeated for a minimum of three independent times.

Trypan blue dye exclusion assay

The effect of compounds 7a-d, 10a-d and 11a-b on cell viability of EAT was determined by trypan blue dye exclusion assay[14]. EAT cells treated with or without compounds were harvested and re suspended in 0.4 % trypan blue and the viable cells were counted using haemocytometer. IC_{50} value was estimated after 48 h of treatment.

MTT assay

The effect of compounds 7a-d, 10a-d and 11a-b on cell proliferation of EAT cells was determined by MTT assay as described previously[14]. Cells were treated with or without compounds were incubated for 48 h. MTT reagent (5 mg/mL) was added and the color change due to proliferating cells was estimated.

Choriallanotoic Membrane (CAM) assay

Inhibition of neovessels formation was analyzed following with the treatment of compounds 7a-d, 10a-d and 11a-b (10 μM) in fertilized egg CAM[14] and changes in the micro vessels density was photographed by using a Nikon D3200 camera.

Giemsa and acridine orange - ethidium bromide staining

EAT cells were obtained from the JSS college of pharmacy, Mysore, India. The cells were centrifuged at 3000 rpm for 5 minutes and the packed cells were diluted 1:6 times with Phosphate buffer saline. 2 mL of the diluted cells were treated with the synthesized compounds 7a-d, 10a-d and 11a-b and incubated for 4 h at 37ºC. Untreated EAT cells served as control. At the end of 4 hours, the samples were centrifuged and smears were made from the cell pellet obtained, fixed with methanol-acetic acid (3:1) and the morphological features of the cells were observed using different stains. Batches of both test and control smears were stained with Giemsa's stain and acridine orange-ethedium bromide stain that highlights the apoptotic morphology of the

cells when observed under bright field microscope and fluorescent microscope respectively[14].

DNA fragmentation assay

DNA was isolated from both compounds treated and untreated cells after the incubation of EAT cells with compounds for 4 hour. Reactions were terminated using ice cold Hank's Balanced Salt Solution (HBSS) and the supernatant was discarded after centrifugation. Cells were lysed with 50 mM Tris-HCl buffer, pH 8.0 and 0.5 % SDS which was incubated for 30 minutes at 37 °C. The cell lysate was subjected to 8 M potassium acetate precipitation and left for 1 hour at 4°C. The supernatant was subjected to phenol: chloroform: isoamyl alcohol (25:24:1) extraction and chloroform extraction. DNA was precipitated by adding 1:2 volumes of ice-cold ethanol. The precipitated DNA was dissolved in 50 µL of TE buffer (pH 8.0). The DNA was digested with 20 µl/mL of RNase at 37°C for 1 hour. The DNA was quantified and equal concentration of DNA (50 µg) was resolved on 1.5 % agarosegel, viewed under UV light and documented using Bio-RAD.[14]

RESULT AND DISCUSSION

Preliminary Screening of compounds 7a-d, 10a-d and 11a-b using trypan blue and MTT assay

In the present investigation the cytotoxic effect of compounds 7a-d, 10a-d and 11a-b was evaluated on EAT cancer cell lines following 48 hour exposure using trypan blue and MTT. In this screening, tamoxifen was used as a standard and DMSO as negative control and the results are reported in Table 1. In the trypan blue assay the analogues 7b and 10d reveal excellent cell growth inhibition when compared to the standard with IC_{50} of 18, 15 and 11.4 µM respectively. Further the effect of compounds 7a-d, 10a-d and 11a-b on cell proliferation was tested using MTT assay. The results observed in MTT assay were synchronized with trypan blue assay (Table 1). Among the synthesized compounds 7b and 10d showed maximum cytotoxicity with an IC_{50} value of 20 and 18 respectively compared to standard drug tamoxifen (IC_{50} 11.4 µM). It is clear from the data that compound 7b with two oxadiazole rings carrying phenyl rings with butoxy alcoholic group at the para position exhibited good activity against EAT cells in trypan blue (IC_{50} 18 µM) and MTT (assay IC_{50} 20 µM) compared to the standard drug (IC_{50} 11.4 µM). Besides, compound 10d with ethoxy (2-methoxy) ethyl ether group at the para position of phenyl ring attached to second and fifth position of 1,3,4-oxadiazole ring exhibited excellent activity against EAT cells in trypan blue (IC_{50} 15 µM) and MTT (assay IC_{50} 18 µM) compared to the standard drug (IC_{50} 11.4 µM).

Angiogenesis in CAM model

To examine the antiangiogenic activity of compounds 7a-d, 10a-d and 11a-b in *in-vivo* models, all the synthesized compounds 7a-d, 10a-d and 11a-b were subjected to CAM angiogenesis assay. In this model the compounds showed moderate to good avasculture zone formation in the developing embryos. Among all the tested compounds, compound 7b and 10d were more clearly shown regression of newly formed micro vessels which were found around the area of disc implanted compared to the other compounds in the same series (Figure 1A). This regression of micro vessels density in the CAM model is highly evident for the tumor inhibition and it correlates to our *in-vitro* results more accurately.

Giemsa and Ethidium bromide/acridine orange staining

To study the morphological features of the cells compounds 7a-d, 10a-d and 11a-b were subjected for Giemsa and ethidium bromide / acridine orange staining. In this experimental batch, both test and control smears were stained with Giemsa and ethidium bromide / acridine orange stain which highlighted the apoptotic morphology of the cells when observed under florescent microscope. The apoptotic morphology clearly indicated the potentiality of the compounds 7b and 10d and the same were photographed (Figure 1B and C).

DNA fragmentation assay

DNA fragmentation was performed for elucidating the mode of action of the investigated compounds, especially with respect to induction of oligonucleosomal DNA fragmentation (DNA ladder), which is a characteristic feature of the programmed cell death or apoptosis. Apoptotic degradation of DNA was analyzed by agarose gel electrophoresis. Among compounds 7a-d, 10a-d and 11a-b, the fragmented DNA observed under UV light clearly demonstrated the *in-vivo* apoptotic effect of compounds 7b and 10d only. In the clearly documented Bio-RAD image (Figure 2) the lane one indicates control and lane two indicates the marker and lane 3 and 4 indicate the apoptotic effect of compounds 7b and 10d respectively. These results have greater resemblance to our other *in-vitro* and *in-vivo* results. Further by observing these facts we can conclude that the compounds 7b and 10d have more proapoptotic properties when compared to all other analogues in the series.

Table 1: **IC_{50} values of compounds 7a-d, 10a-d and 11a-b were calculated based on trypan blue and MTT assay at 48h in EAT cells; Based on the IC_{50} values of compounds 7b and 10d were very significant and chosen as lead compounds**

S. No	Tryphan Blue assay IC_{50} value (µM)	MTT assay IC_{50} value (µM)
Control	-	-
7a	95	90
7b	18	20
7c	48	52
7d	87	87
10a	43	38
10b	>100	>100
10c	34	30
10d	15	18
11a	32	30
11b	>100	>100
Std	11.4	11.4

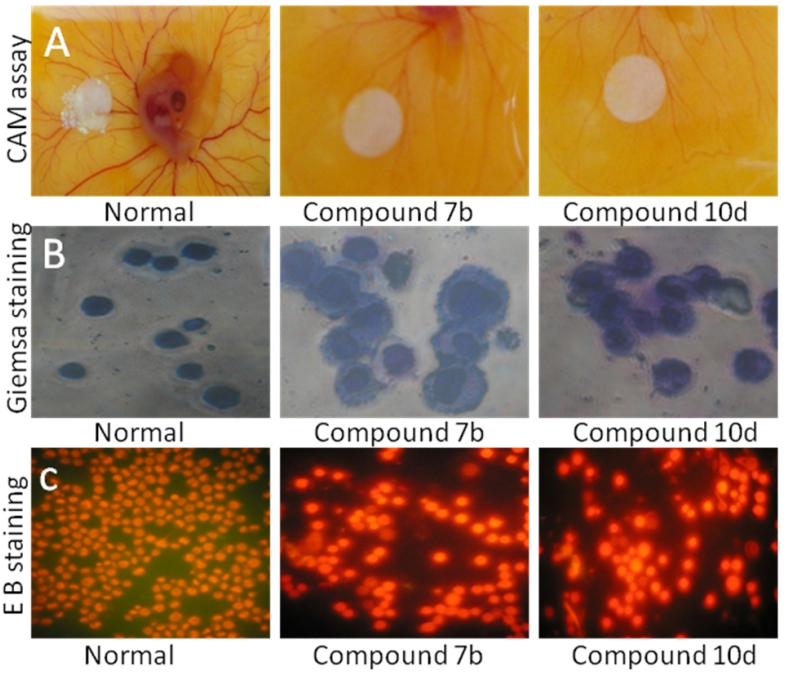


Figure 1. Angioprevention effect of compounds 7b and 10d (A) CAM Photos illustrates the formation of blood vessels in normal and fallowed by regression in compounds treated 7b and 10d, Inhibition of angiogenesis is evident, (B) Giemsa staining of normal cells and cells treated with compounds 7b and 10d highlights the apoptotic morphology of the cells, (C) Representative photograph of cells treated with compounds 7b and 10d stained with ethidium bromide (EB) - acridine orange stain in this apoptotic morphology were clearly indicates

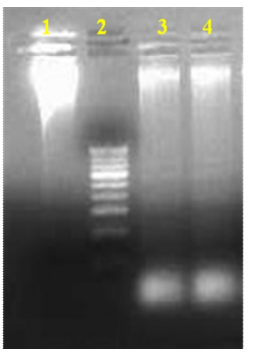
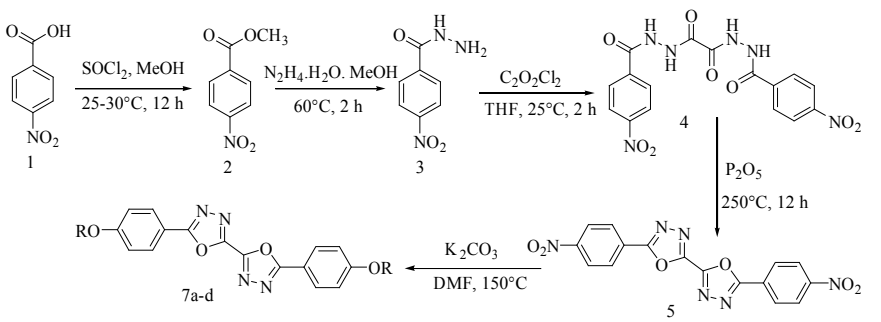


Figure 2: DNA Fragmentation assay; the fragmented DNA observed under UV light, it clearly demonstrated the *in-vivo* apoptotic effect of compounds, Lane 1 and 2 are Control and Marker, lane 3 and 4 are Compounds 7b and 10d respectively

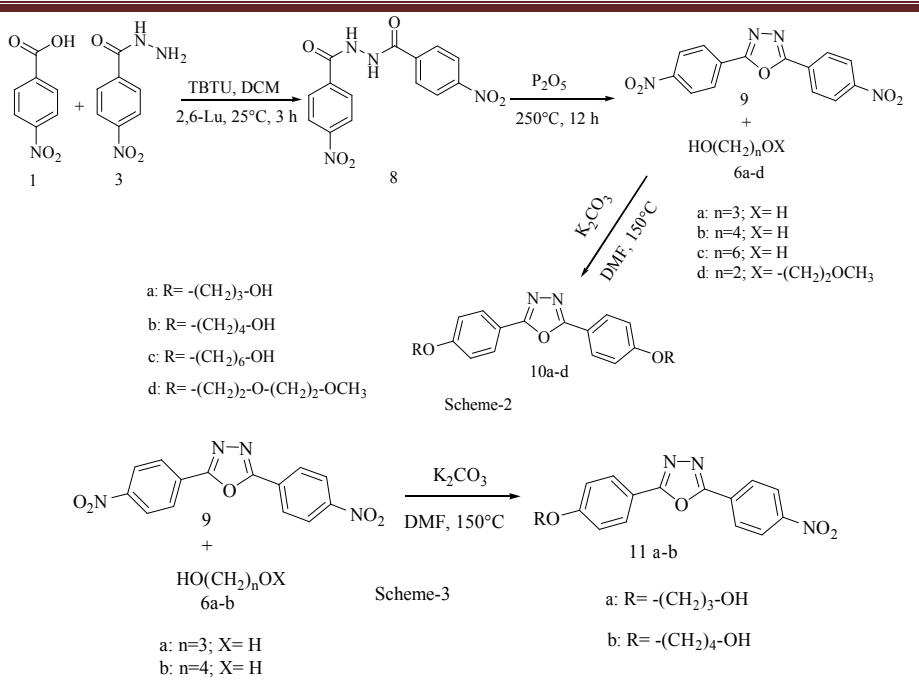


a: R= -$(CH_2)_3$-OH
b: R= -$(CH_2)_4$-OH
c: R= -$(CH_2)_6$-OH
d: R= -$(CH_2)_2$-O-$(CH_2)_2$-OCH_3

$HO(CH_2)_nOX$

a: n=3; X= H
b: n=4; X= H
c: n=6; X= H
d: n=2; X= -$(CH_2)_2OCH_3$

Scheme-1



Scheme 1, 2 and 3: Synthesis of 1,3,4-Oxadiazole analogues

CONCLUSION

In order to develop potent anticancer as well as antiangiogenic molecules we have designed and synthesized a new bioactive oxadiazole analogues 7a-d, 10a-d and 11a-b and tested them for anticancer as well as antiangiogenic properties. The anticancer activity of these compounds was tested against EAT cell lines in different methods. Compounds 7b and 10d exhibited potent anticancer as well as antiangiogenic properties among the series. Further *in-vivo* investigations are in progress in our laboratory. By observing the present results the compounds 7b and 10d can be considered as potential anticancer drugs for future chemotherapy.

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