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Possible Antineoplastic Agents, Part XVII: Synthesis, Antitumor and Antiangiogenic Study of Few Bioisosteres of Thalidomide Metabolites

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Two series of bioisostere of thalidomide metabolites and their cyclized variants were designed, synthesized and characterized. *In vivo* antitumor activity was studied on EAC in swiss albino mice. Cytotoxicity study was carried out on HUVEC and Vero cell lines by MTT assay method. Antiangiogenic activity on Ehrlich ascites tumor (EAT) was investigated studying inhibition of formation of micro vessels by identification of CD31 antigen through immunohistochemistry. One molecule has exhibited both antitumor and antiangiogenic activity but there is no significant toxic effect on normal cell.

Key Words: Thalidomide, Glutamic acid, EAC, HUVEC, Vero cell, Ehrlich ascites tumor, Immunohistochemistry.

INTRODUCTION

Thalidomide (Fig. 1) is a potential chemotherapeutic agent used against multiple myeloma, myelo dysplastic syndrome, leprosy *etc*. It has multifarious mechanisms like inhibition of VEGF, TNF-α, GI growth factor, augmentation of apoptosis, proliferation of NK cells and stimulation of T cells[1,2].

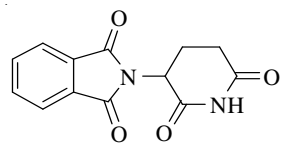


Fig. 1. Thalidomide

All the primarily formed metabolites[3,4] of thalidomide such as *N*-(*o*-carboxy benzoyl)-DL-glutamine, *N*-phthalyl-DL-glutamine, *N*-phthalyl isoglutamine *etc*., are converted into *N*-(*o*-carboxy benzoyl)-DL-glutamic acid (Fig. 2) in biological system.

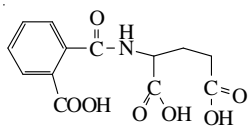


Fig. 2. *N*-(o-Carboxy benzoyl)-DL-glutamic acid

There is striking structural similarity between *N*-(*o*-carboxy benzoyl) glutamic acid and folic acid (Fig. 3). Considering the common parts of the two molecules, the general structure of the **Scheme-I** (Fig. 4) was designed with phthalimido substitution in the *para* position of the benzene ring. The amide linkage between benzene ring and glutaric acid is replaced by sulfonamide linkage and the acid groups of glutamic acid moiety are replaced by alkyl substituted amide groups because of similar electron distribution.

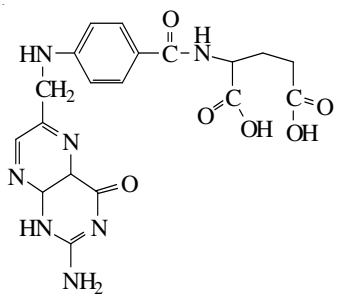


Fig. 3. Folic acid

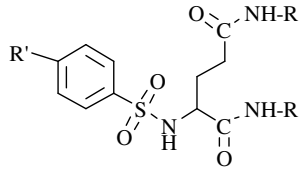
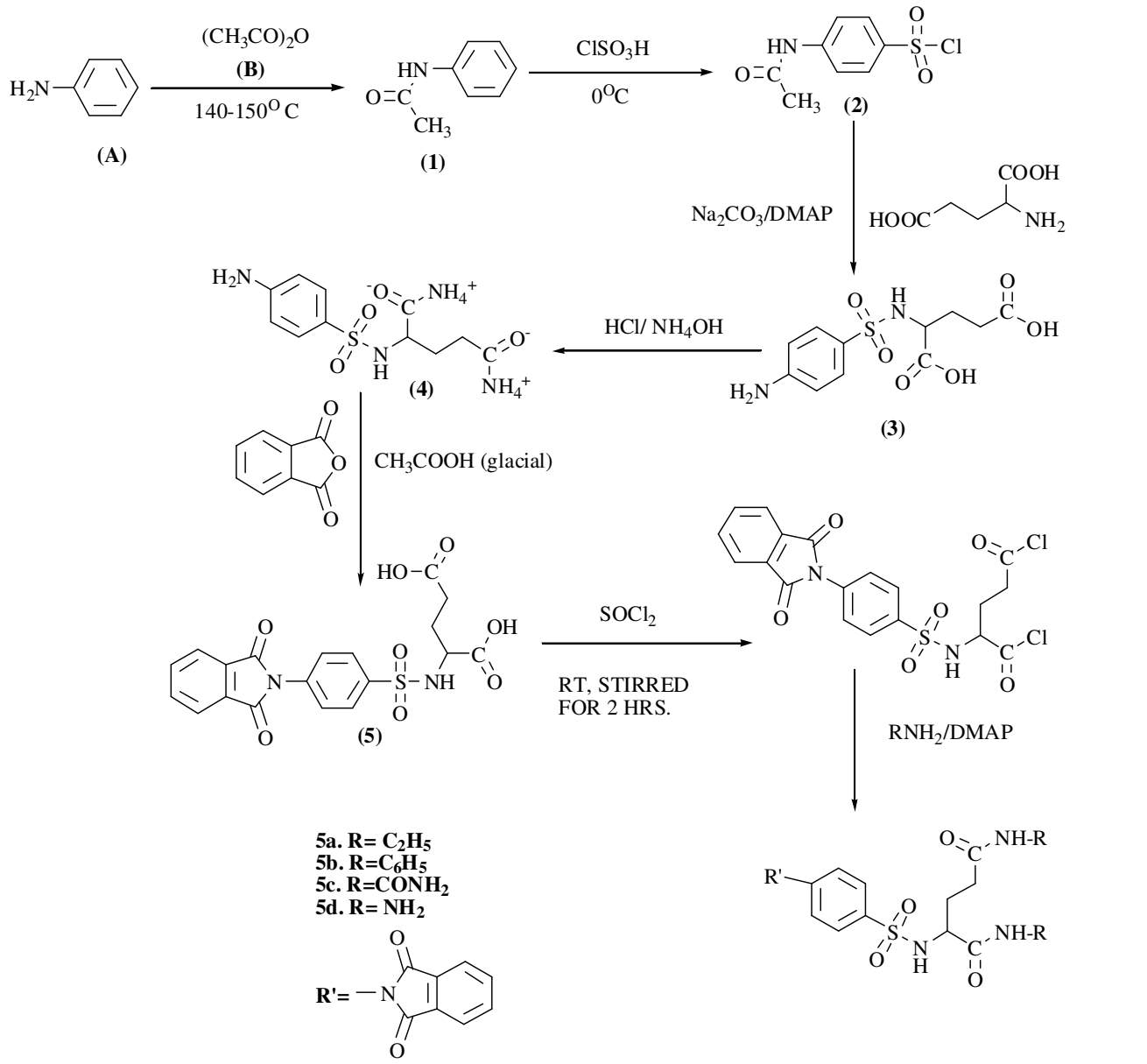


Fig. 4. General structure of **Scheme-I**

As a bioisostere, glutamic acid (Fig. 4) is replaced by 5-oxo pyrrolidine-2-carboxylic acid and the general structure



Scheme-I: Route of synthesis of 2-(4-phthalimidobenzenesulphonamido)glutaric acid substituted amides

of the **Scheme-II** (Fig. 5) is designed. This modification in structure is bolstered up by the fact that tenuazonic acid[5] and azatoxin[6] are the examples of the potential anticancer agents containing oxopyrrolidine ring.

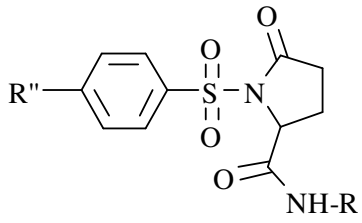
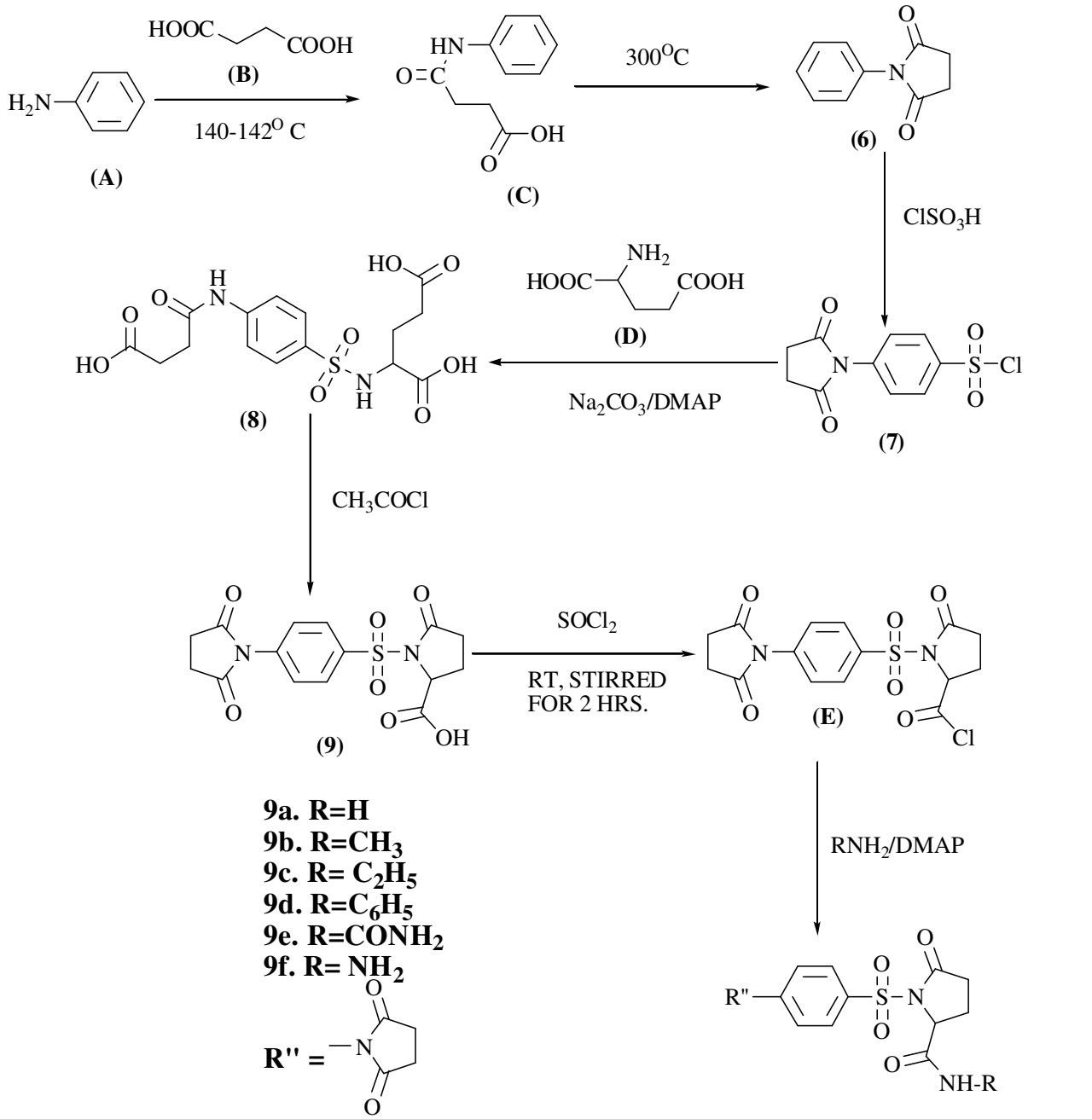


Fig. 5. General structure of **Scheme-II**

Synthesis, anticancer activity and QSAR study of many series of glutamic acid analogs were performed by the previous authors[7-12]. Few of the synthesized molecules have shown good activity against different cancer strains.

EXPERIMENTAL

All chemicals for synthesis were purchased either from Merk (India) or Loba Chemie (India). The reagents used for cytotoxicity study and immunohistochemical investigations were purchased from BD biosciences, Vector laboratories and Sigma-Aldrich. Melting points were determined in open capillary tubes and are uncorrected. ^{1}H NMR (300 MHz) spectra and ^{13}C NMR (75 MHz) spectra were recorded on a Bruker DPX 300 MHz spectrometer using $CDCl_3$ and DMSO-d_6 as solvent and TMS as an internal standard. IR spectra were recorded on a Perkin Elmer Spectrum RX1 spectrometer using KBr (IR grade). Elemental analysis of the synthesized compounds was carried out on Carlo Erba 1108 analyzer. Mass spectral analysis was carried out with MICROMASS Q-T of micro™. The compounds were purified by flash column chromatography using 230-400 mesh Silica gel (Sigma-Aldrich make), ethyl acetate and benzene (at different ratio).



Scheme-II: Synthetic route of *N*-(4-succinimidobenzenesulphonyl)pyrroglutamic acid substituted amides

Synthetic procedures and detection of the compounds

General synthetic description of Scheme-I. Aniline (**A**) was acetylated with acetic anhydride (**B**) to yield acetanilide (**1**). Acetanilide was chlorosulphonated with chlorosulphonic acid to yield 4-acetamido benzenesulphonyl chloride (**2**). 4-Acetamido benzenesulphonyl chloride was treated with L-glutamic acid in alkaline medium to yield N^2-(4-acetamido benzenesulphonyl)-L-glutamic acid (**3**). This dibasic acid thus formed was hydrolyzed with dilute hydrochloric acid and then made ammoniacal with liquid ammonia to produce ammonium N^2-(4-amino benzenesulphonyl)-L-glutamate (**4**). The ammonium salt was condensed with phthalic anhydride in presence of glacial acetic acid to yield N^2-(4-pthalimido benzenesulphonyl)-L-glutamic acid (**5**). This dibasic acid thus formed was treated with thionyl chloride to form the corresponding 2-(4-phthalimido benzenesulphonamido)glutaric acid chloride, which on treatment with different amines yielded the desired target compounds, 2-(4-phtalimido benzenesulphonamido) glutaric acid substituted amides in presence of DMAP.

Synthesis of acetanilide: 4-Acetamidobenzene (1): In a beaker, water (500 mL), conc. HCl (18.3 mL) and aniline (A) (20 mL, 20.5 g, 220 mmol) were taken and to it redistilled acetic anhydride (25.6 mL, 27.7 g, 1.7 mol) was added, stirred to solution and immediately poured in a solution of sodium acetate (33 g, 402 mmol) in 100 mL water. It was stirred vigorously and cooled in ice. Solid was washed with little water and recrystallized with methanol. Yield-77.30 %, 45 g; m.p. 113-114 ºC.

Synthesis of 4-acetamidobenzenesulphonyl chloride (2): To a stirred solution of chlorosulphonic acid (82.5 mL, 145 g, 1.245 mol), acetanilide (3.75 g, 250 mmol) was added gradually maintaining temperature at 12-15 ºC. After addition the mixture was heated to 60 ºC for 2 h with stirring. The content was poured on crushed ice. The solid appeared was filtered, dried and recrystallized from benzene. Yield-81 %, 95 g; m.p. 145-147 ºC.

Synthesis of N^2-(4-acetamidobenzenesulphonyl)-L-glutamic acid (3): 4-Acetamido benzenesulphonyl chloride (36.5 g, 156 mmol) was added in portion to an alkaline solution (made with Na_2CO_3) of L-glutamic acid (23 g, 156 mmol). After complete addition, 40 mg of DMAP in aqueous solution was added to the reaction medium. 2 h later, the content was acidified to pH-3.2, extracted with ethyl acetate and dried over anhydrous magnesium sulphate overnight. The extract was distilled out. Yield- 92.94 %, 50 g; m.p. 141-143 ºC. Anal. calcd. for $C_{13}H_{16}N_2O_7S$: C, 45.35; H, 4.68; N, 8.14 % found: C, 45.36; H, 4.56; N, 8.02 %.

Synthesis of ammonium N^2-(4-acetamidobenzenesulphonyl)-L-glutamate (4): N^2-(4-acetamidobenzenesulphonyl)-L-glutamic acid (25 g, 73 mmol) was hydrolyzed with dil HCl. (2 N, 50 mL) and refluxed on an oil bath for 1 h. The mass was allowed to cool to room temperature and made ammoniacal with liquor ammonia. The mass was bone dried on a rotary film evaporator and used for next reaction without further purification. The substance softens at 148-150 ºC and decomposes at 180 ºC.

Synthesis of N^2-(4-phthalimidobenzenesulphonyl)-L-glutamic acid; 2[4-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)benzenesulphonylamino)pentanedioic acid (5): Ammonium N^2-(4-acetamidobenzenesulphonyl)-L-glutamate (47.5 g, 138 mmol), phthalic acid (20.36 g, 138 mmol) and glacial acetic acid (330 mL) was refluxed on an oil bath for 6 h. The content was filtered in hot condition and it solidifies when cooled to room temperature. Product was recrystallized from water. Yield- 74.15 %, 45 g; m.p. 226 ºC, dec at 230 ºC. IR (KBr, ν_{max}, cm^{-1}): 3285 (N-H str, $-SO_2NH-$), 1709 (C=O str, -COOH), 1348 (S=O str. asym, $-SO_2-$), 1168 (S=O str sym, $-SO_2-$). ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.324 (br, 2H, -COOH), 8.1853 (d, 2H, *J* = 8.4 Hz, Ar-H), 7.9954 (d, 2H, *J* = 7.4 Hz, Ar-H), 7.7356 (d, 2H, *J* = 8.4 Hz, Ar-H), 7.7186 (d, 2H, *J* = 7.4 Hz, Ar-H), 7.563 (s, 1H, $-SO_2NH-$), 3.5671 (m, 1H, -NH-C<u>H</u>-), 2.3121 (t, 2H, *J* = 8.7 Hz, $-CH_2C\underline{H}_2COOH$), 1.8971 (m, 2H, -NH-CH-C<u>H</u>$_2$-), ^{13}C NMR (DMSO-d_6): δ 25.14, 30.46, 58.29, 121.86, 123.74, 129.44, 132.01, 132.27, 135.92, 140.14, 167.24, 174.71, 176.46. Mass (*m/z*) ESI TOF: 433.3698 (M+H), Anal. calcd. for $C_{19}H_{16}N_2O_8S$ (432.4039): C, 52.78; H, 3.73; N, 6.48 %. Found: C, 52.88; H, 3.51; N, 6.69 %.

N^2-(4-Phthalimidobenzenesulphonyl)-L-glutamic acid dichloride: N^2-(4-phthalimidobenzenesulphonyl)-L-glutamic acid (3 g, 7 mmol) was stirred with thionyl chloride (10 mL) at room temperature for 2 h. The content was distilled with benzene (20 × 3 mL). The compound was taken for next step without any further purification.

Synthesis of 2-(4-phthalimidobenzenesulphonamido)-glutaric acid ethylamide; 2[4-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)benzenesulphonylamino]pentanedioic acid bis-*N*-ethylamide (5a): From the above step the content was dissolved in anhydrous benzene and dry ethyl amine gas was passed through it for 0.5 h. 20 mg of DMAP was dissolved in the reaction medium and kept overnight. The solid compound was filtered and washed with dilute acid and with water. Yield-74.2 %, 2.5 g; m.p. 246-247 ºC. IR (KBr, ν_{max}, cm^{-1}): 3275 (N-H str, $-SO_2NH-$), 3265 (NH str, -CONH-), 1723, 1701.46, 1698.88 (C=O str), 1667 (Amide-I), 1555 (Amide-II), 1352 (S=O str asym, $-SO_2-$), 1179 (S=O str sym, $-SO_2-$). ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.1793 (d, 2H, *J* = 8.36 Hz, Ar-H), 8.103-8.076 (o, 2H, -CONH-), 7.9815 (d, 2H, *J* = 7.4 Hz, Ar-H), 7.7279 (d, 2H, *J* = 8.35 Hz, Ar-H), 7.7129 (d, 2H, *J* = 7.4 Hz, Ar-H), 7.5541 (s, 1H, $-SO_2NH-$), 3.5723 (m, 1H, -NH-C<u>H</u>-), 3.2412 (q, 2H, *J* = 8.7 Hz, -CONH-C<u>H</u>$_2$-), 3.1314 (m, 2H, -CONH-C<u>H</u>$_2$-), 2.2981 (m, 2H, $-CH_2-C\underline{H}_2$-CONH-), 1.8665 (m, 2H, $-SO_2NH-CH-C\underline{H}_2$), 0.981 (t, 3H, *J* = 7.2 Hz, $-CONHC\underline{H}_2CH_3$), 0.9034 (t, 3H, *J* = 7.2 Hz, $-CONHC\underline{H}_2CH_3$). ^{13}C NMR (DMSO-$d_6$): δ 15.24, 15.31, 26.87, 32.44, 34.03, 34.17, 56.39, 122.13, 124.05, 129.49, 132.47, 132.77, 135.89, 140.74, 167.16, 171.29, 172.96. Mass (*m/z*) ESI TOF: 487.3989 (M+H), Anal. calcd. for $C_{23}H_{26}N_4O_6S$ (486.5407): C, 56.78; H, 5.39; N, 11.52 %. Found: C, 56.90; H, 5.45; N, 11.65 %.

Synthesis of 2-(4-phthalimidobenzenesulphonamido)glutaric acid phenyl amide; 2[4-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)benzenesulphonylamino)-pentanedioic acid bis-*N*-phenylamide (5b): The same process was repeated using 2 mL of Aniline and was refluxed for 15 min. Yield-79.20 %, 3.2 g; m.p. 199-201 ºC. IR (KBr, ν_{max}, cm^{-1}): 3271 (N-H str, $-SO_2NH-$), 3267 (NH str, -CONH-), 1721, 1702, 1693 (C=O str), 1665 (Amide-I), 1559 (Amide-II), 1351.77 (S=O str asym, $-SO_2-$), 1180 (S=O str sym, $-SO_2-$). ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.1126 (d, 2H, *J* = 8.7 Hz, Ar-H), 7.8212 (d, 2H, *J* = 7.6 Hz, ArH), 7.7342 (d, 2H, *J* = 8.7 Hz, Ar-H), 7.7019 (d, 2H, *J* = 7.6 Hz, Ar-H), 7.5342-7.5212 (o, 5H, Ar-H, $-SO_2NH-$), 7.311-7.2992 (o, 6H, -CONH-, Ar-H), 3.6093 (m, 1H, -NH-C<u>H</u>-), 2.2176 (t, 2H, *J* = 8.2 Hz, $-CH_2C\underline{H}_2CONH-$), 1.9234 (m, 2H, $-CH_2C\underline{H}_2CONH-$). ^{13}C NMR (DMSO-$d_6$): δ 26.90, 30.19, 55.92, 121.64, 121.99, 123.75, 128.08, 128.87, 129.68, 132.11, 132.24, 136.15, 138.50, 140.44, 166.79, 172.64, 176.85. Mass (*m/z*) ESI TOF: 583.4987 (M+H), Anal. calcd. for $C_{31}H_{26}N_4O_6S$ (582.6263): C, 63.91; H, 4.5; N, 9.62 %. Found: C, 63.95; H, 4.72; N, 9.43 %.

Synthesis of 2-(4-phthalimidobenzenesulphonamido)-glutaric acid ureid; 4-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)-*N*-(4-oxo-4-ureido-1-ureidocarbonylbutyl)benzenesulphonamide (5c): The same process was repeated using 1.3 g of crushed urea and was refluxed for 0.5 h. Yield-78.2 %, 2.8 g; m.p. 148-150 ºC. IR (KBr, ν_{max}, cm^{-1}): 3273 (N-H str, $-SO_2NH-$), 3265 (NH str, -CONH-), 1719, 1701, 1691, (C=O str), 1669 (Amide-I), 1557 (Amide-II), 1356 (S=O str asym, $-SO_2-$), 1179 (S=O str sym, $-SO_2-$). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.692 (s, 1H, -CONH-), 10.1592 (s, 1H, -CONH-), 8.0293 (d, 2H, *J* = 8.7 Hz, Ar-H), 7.8234 (d, 2H, *J* = 7.74 Hz, ArH), 7.7281 (d, 2H, *J* = 8.7 Hz, Ar-H), 7.93 (d, 2H, *J* = 7.74 Hz, Ar-H), 7.5341 (s, 1H,-SO_2NH-), 5.591 (br, 4H, $-CONH_2-$, Ar-H), 3.642 (t, 1H, *J* = 7.8Hz, -NH-C<u>H</u>-), 2.2091 (m, 2H, $-CH_2C\underline{H}_2CONH-$), 1.7592 (m, 2H, - $CH_2C\underline{H}_2CONH-$). ^{13}C NMR (DMSO-$d_6$): δ 25.88, 29.46, 55.30, 121.80, 123.69,

129.78, 132.09, 132.62, 135.89, 140.27, 157.30, 157.52, 166.90, 173.24, 173.66. Mass (*m/z*) ESI TOF: 517.2521 (M+H), Anal. calcd. for $C_{21}H_{20}N_6O_8S$ (516.4839): C, 48.84; H, 3.90 N, 16.27 %. Found: C, 48.88; H, 4.11; N, 16.06 %.

Synthesis of 2-(4-phthalimidobenzenesulphonamido)glutaric acid hydrazide; *N*-[1,3-*bis*-*N*-(hydrazinecarbonyl)propyl]-4-(1,3-dioxo-1,3-dihydro-isoindol-2-yl) benzenesulphonamide (5d): The same process was repeated using 1.5 mL of hydrazine hydrate (80 % w/w) and was refluxed for 10 min. White solid, Yield-75.12 %, 2.4 g; m.p. > 300 ºC. IR (KBr, ν_{max}, cm^{-1}): 3276 (N-H str, -SO_2NH-), 3268 (NH str, -CONH-), 1718, 1702, 1692, (C=O str), 1670 (Amide-I), 1559 (Amide-II), 1354 (S=O str asym, -SO_2-), 1178 (S=O str sym, -SO_2-). ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.0652 (s, 1H, -CONHNH$_2$-), 8.9126 (s, 1H, -CONHNH$_2$-), 8.123 (d, 2H, *J* = 8.6 Hz, Ar-H), 7.8562 (d, 2H, *J* = 7.69 Hz, Ar-H), 7.7392 (d, 2H, *J* = 8.6 Hz, Ar-H), 7.7153 (d, 2H, *J* = 7.6 Hz, Ar-H), 7.529 (s, 1H, -SO_2NH-), 4.122 (br, 4H, -CONHNH$_2$-, Ar-H), 3.5427 (m, 1H, *J* = 7.8 Hz, -NH-CH-), 2.192 (m, 2H, -CH$_2$CH$_2$CONHNH$_2$-), 1.8335 (m, 2H, -CH$_2$CH$_2$CONHNH$_2$). ^{13}C NMR (DMSO-d_6): δ 27.25, 33.19, 58.92, 121.84, 123.70, 129.64, 132.07, 132.82, 135.95, 140.10, 167.33, 170.49, 176.66. Mass (m/z) ESI TOF: 461.3344 (M+H), Anal. calcd. for $C_{19}H_{20}N_6O_6S$ (460.4637): C, 49.56; H, 4.38 N, 18.25 %. Found: C, 49.78; H, 4.10; N, 18.33 %.

General synthetic description of Scheme-II: Aniline (**A**) was heated with succinic acid (**B**) at 140-142 ºC to give succinic acid monoanilide (**C**), which on distillation at 330 ºC at normal atmospheric pressure yielded *N*-phenylsuccinimide (**6**). Chlorosulphonation of compound (**6**) with chlorosulphonic acid at 80 ºC yielded 4-succinimido benzenesulphonyl chloride (**7**). Reaction of (**7**) with L-glutamic acid (**D**) in presence of sodium hydroxide at 60 ºC resulted in opening of the succinimido ring yielding 2-[4-(3-carboxypropionylamino)-benzene sulphonylamino] pentane dioic acid (**8**), a tricarboxylic acid. Refluxing this tri-acid with acetyl chloride for 4 h resulted in cyclization of both the succinic acid moiety and the glutamic acid moiety to succinimido ring and pyrroglutamic acid moiety respectively to yield 1-(4-succin-imidobenzene sulphonyl)-5-oxopyrrolidine-2-carboxylic acid (**9**), a mono carboxylic acid. This monocarboxylic acid was treated with thionyl chloride to yield the corresponding 1-(4-succinimidobenzene sulphonyl)-5-oxopyrrolidine-2-carboxylic acid chloride (E), which was aminated with appropriate amines or amino compounds in presence of DMAP (dimethyl amino pyridine) as catalyst to yield the desired target compounds *N*-(4-succinimidobenzenesulphonyl) pyrroglutamic acid substituted amides.

Synthesis of *N*-phenyl succinimide: 4-(2,5-dioxopyrrolidine-1-yl)benzene (6). A mixture of freshly distilled aniline (34 mL, 370 mmol) and pulverized succinic acid (43.7 g, 370 mmol) was placed in an round bottom flask and heated on an oil bath at 145-150 ºC for 4 h. The reaction mass was distilled at atmospheric pressure and at 330 ºC, which is necessary to convert the succinic acid monoanilide to *N*-phenyl succinimide. The distillate was recrystallized from 95 % ethanol. Yield-85.73 %, 28.6 g; m.p. 155-156 ºC. (reported m.p. 156-158 ºC).

Synthesis of 4-succinimido benzenesulphonyl chloride: 4-(2, 5-dioxo pyrrolidin-1-yl)benzene sulphonyl chloride (7): Chlorosulphonic acid (25 mL, 260 mmol), was added drop wise to *N*-phenyl succinimide (15 g, 120 mmol) and was heated at 80-85 ºC for 2 h. The reaction mass was poured on crushed ice with continuous stirring. The product obtained was filtered and washed with water and recrystallized with acetone. Yield-79.35 %, 18.60 g; m.p. 194-195 ºC (reported m.p. 195-198 ºC).

Synthesis of 2-[4-(3-carboxypropionyl amino) benzene sulphonylamino] pentanedioic acid (8): 4-Succinimido benzenesulphonyl chloride (35 g, 130 mmol) was added in portion to an alkaline solution (made with Na_2CO_3) of L-glutamic acid (19 g, 130 mmol). After complete addition, 40 mg of DMAP in aqueous solution was added to the reaction medium. 2 h later, the content was acidified to pH-3, extracted with ethyl acetate and dried over anhydrous magnesium sulphate overnight. The extract was distilled. Yield-57 %, 31.1 g; m.p. 185-188 ºC.

Synthesis of 1-[4-(2, 5-dioxo pyrrolidin-1-yl)benzene sulphonyl]-5-oxopyrrolidin-2-carboxylic acid (9): Compound 2-[4-(3-carboxypropionyl amino)benzene sulphonyl amino]pentanedioic acid (32 g, 79 mmol) was cyclized by refluxing with acetyl chloride (70 mL) in benzene (40 mL) as solvent at 100 ºC for 4 h. After completion the benzene was distilled off and the residue was cooled, poured on crushed ice and kept overnight. The content was filtered and washed with water and recrystallized with ethanol water mixture. Yield-94.53 %, 27.5 g; m.p. 234-238 ºC. IR (KBr, ν_{max}, cm^{-1}): 3281 (N-H str, -SO_2NH-), 1734, 1687 (C=O str), 1351 (S=O str asym, -SO_2-), 1171 (S=O str sym, -SO_2-). ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.298 (br, 1H, -COOH), 8.1259 (d, 2H, *J* = 8.3 Hz, Ar-H), 7.5578 (d, 2H, *J* = 8.3 Hz, Ar-H), 4.8 (m, 1H, -N-CHCOOH), 2.8121 (s, 4H, -COCH$_2$CH$_2$CO-), 2.496 (m, 2H, -CH$_2$CHCOOH), 2.0723 (m, 2H, -COCH$_2$CH$_2$CHCOOH). ^{13}C NMR (DMSO-d_6): δ 21.22, 27.60, 28.45, 46.88, 121.90, 127.56, 135.39, 138.61, 174.78, 175.10, 176.55. Mass (m/z) ESI TOF: 367.2894 (M+H), Anal. calcd. for $C_{15}H_{14}N_2O_7S$ (366.3459): C, 49.18; H, 3.85; N, 7.65 %. Found: C, 49.28; H, 3.63; N, 7.82 %.

1-[4-(2, 5-Dioxo pyrrolidine-1-yl)benzenesulphonyl]-5-oxopyrrolidine-2-carboxylic acid amide (9a): Thionyl chloride (10 mL) was added to 2 g, (5 mmol) of compound (**4**), stirred for 2 h in an inert atmosphere and distilled with dry benzene (3 × 20 mL). Yellowish semi-solid mass appeared and the compound was used for next step without purification. The compound recovered from the above reaction was dissolved in dry benzene and it was taken for amidation while DMAP (20 mg in dry benzene) was added to the content and dry ammonia gas was passed through the solution in an inert atmosphere.

Solid precipitated out, after an hour, was filtered, dried and purified. The final product was purified by flash chromatography using ethyl acetate and benzene in 9 : 1 ratio and recrystallized using ethanol and water mixture. Yield-50.14 %, 1 g; m.p. 193-195 ºC. IR (KBr, ν_{max}, cm^{-1}): 3285 (N-H str, -SO_2NH-), 3154 (NH str, -CONH-), 1706 (C=O str, -COOH), 1652 (Amide-I), 1553 (Amide-II), 1350 (S=O str asym, -SO_2-), 1173 (S=O str sym, -SO_2-). ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.0888 (d, 2H, *J* = 6.3 Hz, Ar-H), 7.5572 (d, 2H, *J* = 6.3 Hz, Ar-H), 7.3922 (s, 2H, -CONH$_2$), 4.8254 (m, 1H, -N-CHCONH$_2$), 2.8157 (s, 4H, -COCH$_2$CH$_2$CO-), 2.4876 (m, 2H, -COCH$_2$CH$_2$

$CHCONH_2$), 2.0348 (m, 2H, -$COCH_2C\underline{H}_2CHCONH_2$). ^{13}C NMR (DMSO-d_6): δ 21.89, 27.63, 28.20, 47.59, 122.18, 127, 64, 135.32, 138.67, 175.30, 176.52, 176.79. Mass (*m/z*) ESI TOF: 366.2314 (M+H), Anal. calcd. for $C_{15}H_{15}N_3O_6S$ (365.3611): C, 49.31; H, 4.14; N, 11.5 %. Found: C, 49.10; H, 3.09; N11.74 %.

Synthesis of 1-[4-(2, 5-dioxopyrrolidin-1-yl)]-5-oxopyrrolidine-2-carboxylic acid methyl amide (9b): The same process was repeated by passing dry methyl amine gas. Yield 53.14 %, 1.06 g; m.p. 264-267 ºC (dec). IR (KBr, ν_{max}, cm^{-1}): 3292 (N-H str, -SO_2NH-), 3149 (NH str, -CONH-), 1730, 1700 (C=O str, -COOH), 1655 (Amide-I), 1555 (Amide-II), 1346 (S=O str asym, -SO_2-), 1169 (S=O str sym, -SO_2-). ^1H NMR (300 MHz, DMSO-d_6): δ 8.112 (d, 2H, *J* = 8.27 Hz, Ar-H), 7.9324 (s, 1H, -CONH-), 7.5361 (d, 2H, *J* = 8.24 Hz, Ar-H), 4.744 (t, 1H, *J* = 7.2 Hz, -NC$\underline{H}CH_2$), 2.989 (s, 3H, -CONHC$\underline{H}_3$), 2.8136 (s, 4H, -COC$\underline{H}_2C\underline{H}_2$CO-), 2.3946 (m, 2H, $COCH_2C\underline{H}_2$CH-), 1.964 (m, 2H, -COC$\underline{H}_2CH_2$ CH-). ^{13}C NMR (DMSO-d_6): δ 21.1, 26.38, 27.72, 28.25, 45.19, 121.92, 127.56, 135.23, 138.62, 171.44, 174.91, 176.42. Mass (*m/z*) ESI TOF: 380.3121 (M+H), Anal. calcd .for $C_{16}H_{17}N_3O_6S$ (379.3877): C, 50.65; H, 4.52; N, 11.08 %. Found: C, 50.68; H, 4.50; N 11.67 %.

Synthesis of 1-[4-(2,5-dioxopyrrolidin-1-yl)]-5-oxopyrrolidine-2-carboxylic acid ethyl amide (9c): The same process was repeated by passing dry ethyl amine gas. Yield 77.35 %, 1.5 g; m.p. 288-290 ºC. IR (KBr, ν_{max}, cm^{-1}): 3292 (N-H str, -SO_2NH-), 3147 (NH str, -CONH-), 1692, 1661 (C=O str), 1653 (Amide-I), 1556 (Amide-II), 1346 (S=O str asym, -SO_2-), 1161 (S=O str sym, -SO_2-). ^1H NMR (300 MHz, DMSO-d_6): δ 8.0820 (d, 2H, *J* = 8.42 Hz, Ar-H), 7.925 (s, 1H, -CONH-), 7.5361 (d, 2H, *J* = 8.42 Hz, Ar-H), 4.737 (m, 1H, -NC$\underline{H}CONH_2$), 3.233 (q, 2H, -CONHC$\underline{H}_2$-), 2.8334 (s, 4H, -COC$\underline{H}_2C\underline{H}_2$CO-), 2.4692 (m, 2H, $COCH_2CH_2$CH-), 1.9831 (m, 2H, -COC$\underline{H}_2CH_2$CH-), 1.2059 (q, 3H, -NHCH$_2$C$\underline{H}_3$). ^{13}C NMR (DMSO-d_6): δ 15.17, 22.13, 27.85, 28.07, 34.22, 45.3, 121.93, 127.55, 135.38, 138.69, 171.11, 175.40, 176.22. Mass (*m/z*) ESI TOF: 394.3876 (M+H), Anal. calcd. for $C_{17}H_{19}N_3O_6S$ (393.4143): C, 51.90; H, 4.87; N, 10.68 %. Found: C, 51.88; H, 4.77; N 10.32 %.

Synthesis of 1-[4-(2,5-dioxopyrrolidin-1-yl)]-5-oxopyrrolidine-2-carboxylic acid phenyl amide (9d): The same process was repeated by adding aniline (1 mL). Yield-95.80 %, 1.90 g; m.p. 240-241 ºC. IR (KBr, ν_{max}, cm^{-1}): 3287 (N-H str, -SO_2NH-), 3276 (NH str, -CONH-), 1692, 1723, 1700, 1676 (C=O str), 1658 (Amide -I), 1550 (Amide-II), 1346 (S=O str asym, -SO_2-), 1161 (S=O str sym, -SO_2-). ^1H NMR (300 MHz, DMSO-d_6): δ 8.0972 (d, 2H, *J* = 8.4 Hz, Ar-H), 7.7623 (d, 2H, *J* = 8.4 Hz, Ar-H), 7.649 (d, 2H, *J* = 7.6 Hz, Ar-H), 7.4276 (d, 2H, *J* = 7.6 Hz, Ar-H), 7.243 (s, 1H, -CONH-), 7.1817 (m, 1H, Ar-H), 4.633 (m, 1H, -NC$\underline{H}CONH_2$), 2.8129 (s, 4H, -COC$\underline{H}_2C\underline{H}_2$CO-), 2.5623 (m, 2H, $COCH_2C\underline{H}_2$CH-), 2.187 (m, 2H, COC$\underline{H}_2CH_2$CH-). ^{13}C NMR (DMSO-d_6): δ 22.15, 27.43, 28.02, 44.97, 121.60, 121.82, 124.49, 127.56, 129.11, 135.26, 138.36, 138.59, 172.30, 175.45, 176.44. Mass (m/z) ESI TOF: 442.3246 (M+H), Anal. calcd. for $C_{21}H_{19}N_3O_6S$ (441.4571): C, 57.14; H, 4.34; N, 9.52 %. Found: C, 57.25; H, 4.39; N 9.67 %.

Synthesis of {1-[4-(2, 5-dioxopyrrolidin-1-yl)]-5-oxopyrrolidine-2-carbonyl}-urea (9e): The same process was repeated by adding 600 mg of crushed urea. Yield-91.0 %, 1.8 g; m.p. 215-217 ºC (dec). IR (KBr, ν_{max}, cm^{-1}): 3288 (N-H str, -SO_2NH-), 3274 (NH str, -CONH-), 1702 (C=O str), 1659 (Amide-I), 1553 (Amide-II), 1346 (S=O str asym, -SO_2-), 1176 (S=O str sym, -SO_2-). ^1H NMR (300 MHz, DMSO-d_6): δ 10.7823 (s, 1H, -CONH-), 7.9812 (d, 2H, *J* = 8.73 Hz, Ar-H), 7.8192 (d, 2H, *J* =7.69 Hz, Ar-H), 5.4672 (br, 2H, -$CONH_2$), 4.7499 (m, 1H, -NC$\underline{H}CH_2$-), 2.6729 (s, 4H, -COC$\underline{H}_2C\underline{H}_2$CO-), 2.5344 (m, 2H, -$COCH_2C\underline{H}_2$CH-), 2.0644 (m, 2H, -COC$\underline{H}_2CH_2$CH-). ^{13}C NMR (DMSO-d_6): δ 21.44, 28.09, 28.14, 44.39, 121.89, 127.54, 135.20, 138.79, 158.29, 173.71, 175.54, 176.75. Mass (*m/z*) ESI TOF: 409.2457 (M+H), Anal. calcd. for $C_{16}H_{16}N_4O_7S$ (408.3858): C, 47.06; H, 3.95; N, 13.72 %. Found: C, 47.36; H, 4.01; N 13.41 %.

Synthesis of 1-[4-(2, 5-dioxopyrrolidin-1-yl)benzenesulphonyl]-2-(hydrazinocarbonyl)-5-oxopyrrolidine (9f): The same process was repeated by adding hydrazine hydrate (1mL, 80 % w/w). Yield-67.42 %, 1.48 g; m.p. 136-138 ºC (dec). IR (KBr, ν_{max}, cm^{-1}): 3279 (N-H str, -SO_2NH-), 3271 (NH str, -CONH-), 1701 (C=O str), 1661 (Amide-I), 1559 (Amide-II), 1350 (S=O str asym, -SO_2-), 1180 (S=O str sym, -SO_2-). ^1H NMR (300 MHz, DMSO-d_6): δ 8.9234 (S, 1H, -CONH NH_2), 8.0966 (d, 2H, *J* = 8.24 Hz, Ar-H), 7.5623 (d, 2H, *J* = 8.27 Hz, Ar-H), 4.7196 (t, 1H, *J* = 7.34Hz, -NC$\underline{H}CH_2$-), 2.8224 (s, 4H, -COC$\underline{H}_2C\underline{H}_2$CO-), 2.437 (m, 2H, -$COCH_2C\underline{H}_2$CH-), 1.8989 (m, 2H, -COC$\underline{H}_2CH_2$CH-). ^{13}C NMR (DMSO-d_6): δ 22.41, 27.59, 29.05, 47.18, 122.13, 128.04, 135.29, 139.03, 170.56, 175.34, 176.52. Mass (*m/z*) ESI TOF: 381.2314 (M+H), Anal. calcd. for $C_{15}H_{16}N_4O_6S$ (380.3757): C, 47.36; H, 4.24; N, 14.73 %. Found: C, 47.10; H, 4.02; N 14.59 %.

Biological evaluation

Antineoplastic activity: The compounds (**5-5d and 9-9f**) were screened *in vivo* for percentage inhibition of ascitic cell against EAC and the results are depicted in Table-1. Mitomycin C was used as standard drug where cell inhibition is 100 %. Biological experiments were carried out as described in the literature[7].

TABLE-1
PERCENTAGE INHIBITION OF CELL GROWTH OF EAC (% INHIBITION) AND CYTOTOXICITY STUDY (IC_{50}) REPORT

Entry	Inhibition on EAC (%)	IC_{50}[a] (μ molar) (Vero cell line)	IC_{50}[a] (μ molar) (HUVEC cell line)
5	44.79	75	60
5a	60.00	20	67
5b	89.77	66	12
5c	10.40	58	61
5d	77.60	45	72
9	55.64	80	55
9a	45.76	82	50
9b	92.54	76	5
9c	53.39	55	56
9d	66.09	92	53
9e	96.27	40	14
9f	50.00	25	54
Standard	100 (Mitomycin C)	4.5 (Doxorubicin)	0.5 (Doxorubicin)

[a]50 % Inhibitory concentration

The study was conducted on Swiss albino mice having average weight of 18-20 g. On day 1 approximately, 2×10^6 numbers of cells were inoculated, intraperitonially. One control group and test groups (each group contained six mice)

were taken. A dose of 0.1 mmol/Kg body weight was administered for 7 days. On day 9 animals were sacrificed and ascitic cells were counted for all the groups. Percentage inhibition of ascitic cell was calculated with respect to control group. The results are reported below. This Animal experimentation is approved by Animal Ethical Committee bearing Registration no. 1170/ac/08/CPCSEA.

Cytotoxicity study of compounds against HUVEC/ Vero cell lines: Cytotoxicity study was carried out according to the procedures mentioned in our previous publication[13].

Antiangiogenic activity study (immunohistochemistry): Ehrlich ascites tumor was developed in female Swiss albino mice by transplantation of 2.5×10^6 EAC cells (i.m.) in the muscle of upper portion of right leg. Six animals were taken in each of the test and control group. The test group was treated (11 days after inoculation) with i.p injection of the molecule **9b** every 24 h (dose: 0.1 mM per Kg body wt.) for 7 days while the control group was treated with vehicle. On nineteenth day the tumors were removed and sliced tumor samples were fixed in 4 % paraformaldehyde, rinsed in PBS, transferred to 30 % sucrose in PBS at 4 ºC and frozen in OCT compound. Immunohistochemical experiment was performed on frozen tissue sections using antibody against CD31 (1:50). Microvessel density was quantitated by analyzing 10 random fields/sections[14].

RESULTS AND DISCUSSION

Table-1 shows that the compounds **5b**, **9b** and **9e** have considerably high antitumor activity and among these three molecules only **9b** is found to have remarkable cytotoxic activity on HUVEC cells (a primary antiangiogenic activity study). This finding has prompted the necessity of performance of immunohistochemical experiment of the molecule **9b** by evaluation of its inhibitory effect on microvessel formation by detection of CD31 antigen on the microvessels and the result shows that the molecule **9b** has striking antiangiogenic activity (Fig. 6).

Conclusion

To sum up, three molecules (**5b, 9b, 9e**) have significant antitumor activity and **9b** has both antitumor and antineoplastic activity *i.e.*, it can wage two prongs attack on some specific tumor. On top of that there is no significant toxic effect of these molecules on Vero cells (normal cell). Subsequent 2D and 3D QSAR studies are being carried out in quest of more potential molecule.

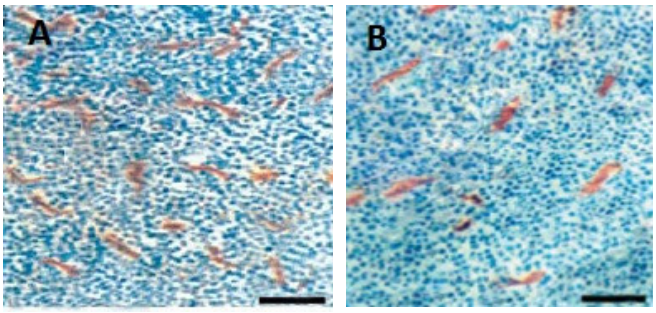


Fig. 6. Evaluation of microvessel density by immunohistochemical analysis of CD31+ in EAT. Significant expression of CD31+ cells in vehicle treated tumor tissue (**A**). Molecule **9b** treated tumor tissue showed marked reduction in CD31+ cells (**B**). Microvessel density was measured by counting the number of microvessels in 10 randomly chosen high power microscopic fields within the sections, which showed that microvessel density was reduced (*, $p < 0.05$) in molecule **9b**-administered tumor-bearing animals (**B**).

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