Synthesis, Spectral and Molecular Characterization of Some Novel 2, 5-Disubstituted-1, 3, 4-Oxadiazole Derivatives and Evaluation of in vivo Antitumour Activity against HT 29 Cell Line

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ABSTRACT

Neoplasia is a type of abnormal and excessive growth of tissue. The growth of a neoplasia is uncoordinated with that of the normal surrounding tissue, and it persists growing abnormally, even if the original trigger is removed. This abnormal growth usually forms a mass. The main objective of the present research work was the synthesis, characterization and evaluation of in vivo antitumour activity of some novel 2, 5-disubstituted 1, 3, 4-oxadiazole derivatives. The in vivo antitumour activity of synthesized compounds was evaluated by HT 29 cell line induced malignant ascites on mouse model. The apoptosis of HT 29 cells was evaluated by using Giemsa and H33342 stain and the apoptosis ratios were analysed by FCM using AnnexinV-FITC/PI staining. The present experimental data displayed that the mortality was less in all groups except in tumour control group and all the synthesized compounds AB1-AB8 (100 mg/kg) significantly increased the PILS. While 5-FU increased the lifespan of 97.72%, and the PILS of synthesized compounds were found to be 45.45%, 59.09%, 68.18%, 56.81%, 38.63%, 84.09%, 77.27% and 90.90%. So the Synthesized compounds AB1-AB8 at the dose of 100 mg/kg significantly improved the overall survival of all treated animals and 5-FU was not significantly differed from each other in improving the overall survival of HT-29 cells. The apoptosis ratios of synthesized compounds were found as followed: AB1=26%; AB2=37.6%; AB3=43%; AB4=29%; AB5=24.1%; AB6=59.2%; AB7=48.2%; and AB8=63% respectively, while that of the Group-II (T. control) was 6.1%. When compared with standard drug 5-FU: 66.2%, it was indicated that compound AB8>AB6>AB7>AB3 were able to significantly induce HT-29 cells apoptosis.

Keywords: Neoplasia, antitumour, malignant ascites, HT 29, apoptosis, PILS.

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INTRODUCTION
Oxadiazoles are a class of heterocyclic aromatic chemical compound of the azole family; with the molecular formula C₃H₅N₂O. There are four isomers of oxadiazole depending on the position of nitrogen atom in the ring. [1] 1, 3, 4-oxadiazole is a five membered heterocyclic aromatic compound containing two nitrogen atom at position three and four and one oxygen atom present at position one. 1, 3, 4 oxadiazole is thermally stable than other oxadiazoles, these oxadiazole are very important compound in medicinal chemistry due to their biological activities, during last few years. [2] 1, 3, 4-oxadiazole is a liquid having boiling point 150°C. 2, 5-disubstituted-1, 3, 4-oxadiazole derivatives are colorless substances. The lower alkyl derivatives are liquids which distill without decomposition. Replacement of an alkyl residue by an aryl radical considerably raises the melting and boiling points. Usually the asymmetrical 1, 3, 4-oxadiazole derivatives melt and boil at lower temperature than the symmetrical compounds. The solubility of oxadiazoles in water varies with the substituents present: 2, 5-dimethyl-1, 3, 4-oxadiazole is miscible with water in all proportions whereas the solubility of 2, 5-diphenyl-1, 3, 4-oxadiazole in water is less. Electrophilic introduction of functional groups (for example nitro or sulphuric acid groups) into the nucleus is unusual. Electrophilic substitution occurs in aryl substituent. Halogenations are also difficult, but 2, 5-diaryl-1, 3, 4-oxadiazoles, afford complexes with halogens. A range of acylation and alkylation reactions of hydroxyl, thio and amino-1, 3, 4-oxadiazoles occur at the ring nitrogen. [3] Biologically active molecules containing oxadiazole moiety possessed a wide range of pharmacological activities such as antimicrobial, anticancer, anticonvulsant, anti-inflammatory and antiviral agents [4], antifungal [5], antimycobacterial [6] etc.

MATERIALS AND METHODS

Chemicals
The solvents and other chemicals which were used for the synthesis and purification of target compounds provided by institutional store and were of LR and AR grade.

Instrumentation
The melting points of the synthesized compounds were determined by open capillary tube method. The IR spectra of the synthesized compounds were recorded on ABB Bomen FT-IR spectrometer MB 104 IR spectra recorded with potassium bromide pellets. The ¹H-NMR spectra of synthesized compounds were recorded on instrument BRUKER NMR spectrometer in DMSO. The Mass spectra of synthesized compounds were recorded JEOL GCmate. TLC method was used to determine the progress of the reaction. TLC plates are Pre-coated Silica gel (HF254-200 mesh) aluminium plates using ethyl acetate: n-hexane are used as solvent and visualized under UV- chamber. The IR, ¹H-NMR and MASS spectra were used to assign the structure of synthesized compounds.

| Table 1: Oxadiazole |
|---------------------|
| ![1,2,3-oxadiazole](image1) |
| ![1,2,4-oxadiazole](image2) |
| ![1,2,5-oxadiazole](image3) |
| ![1,3,4-oxadiazole](image4) |

Steps involved in the synthesis of target compounds [7-9]

Step 1: Ethyl-4-acetamido phenoxy acetate
A mixture of p-acetamido phenol (0.01 mol) and ethyl chloroacetate (0.01 mol) was refluxed by using dry aceton in presence of anhydrous potassium carbonate (K₂CO₃) for 6 hours. The reaction mixture was cooled and then poured in to crushed ice. The solid product obtained, these product was filtered, dried and recrystallized using ethanol.

Step 2: 4-Acetamido phenoxy acetyl hydrazide
A mixture of ethyl-4-acetamido phenoxy acetate (0.01 mol), hydrazine hydrate (0.01 mol) in ethanol (15 ml) was refluxed for 5-8 hours. The reaction mixture was cooled and then poured in to crushed ice. The solid product was obtained; this product was filtered, dried and recrystallized from ethanol.
Step 3: 2-(4-Acetamidophenoxyl methyl) -5-aryl substituted - 1, 3, 4-oxadiazole

A mixture of 4-Acetamido phenoxyl acetyl hydrazide (0.01 mol) and various aromatic acids (0.01 mol) in phosphorus oxychloride (10 ml) was refluxed for 6-8 hours. The completion of the reaction process was monitored by TLC plates. The contents were cooled and poured into the crushed ice and then neutralized the reaction mixture with sodium bicarbonate solution and the solid product was obtained; the product was filtered, dried and recrystallized from ethanol. [10]

Table 2: Physicochemical properties of synthesized compounds

| S. No. | Compounds code | M. F. | M. Wt | Rf value | m.p. (°C) | Yield (%) |
|--------|----------------|-------|-------|----------|-----------|-----------|
| 1.     | AB1 C6H3N4O5  | 324.33| 0.77  | 116      | 74.5      |           |
| 2.     | AB2 C6H3ClN4O2| 378.20| 0.74  | 180      | 69.9      |           |
| 3.     | AB3 C6H3BrN4O3| 327.30| 0.75  | 189      | 74        |           |
| 4.     | AB4 C6H3BrN4O2| 398.21| 0.65  | 183      | 69        |           |
| 5.     | AB5 C6H3BrN3O2| 433.21| 0.64  | 166      | 60        |           |
| 6.     | AB6 C6H3N3O2  | 354.31| 0.72  | 171      | 64        |           |
| 7.     | AB7 C6H3N2O2  | 399.31| 0.68  | 204      | 78        |           |
| 8.     | AB8 C6H3N2O3  | 415.31| 0.72  | 215      | 68        |           |

3114.61 cm⁻¹ (Ar-CH), 1H-NMR δ (ppm): 2.21 (s, 1H, -CH3), 8.09 (s, 1H, -NH), 5.21 (s, 1H, -CH2), 6.7-8.01 (m, 8H, Ar-CH), Mass (m/e value) % relative abundance: 387.62 (M⁺) (6.3), 310.37 (2.3), 299.57 (3), 282.87 (3.9), 266.22 (5), 249.61 (1.2), 232.72 (4), 104.86 (8.1), 75.50 (B).

Compound AB5

N-(4-[5-(2-bromo-4-nitrophenyl)-1,3,4-oxadiazol-2-ylmethoxy]phenyl) acetamide. IR (KBr) v (cm⁻¹): 3381.95 cm⁻¹ (Ar-NH), 1684.44 cm⁻¹ (C=N), 1586.2 cm⁻¹ (C=C), 1064.25 cm⁻¹ (-C-O-C-), 1365.57 cm⁻¹ (N=O), 619.89 cm⁻¹ (C-Br), 3130.43 cm⁻¹ (Ar-CH), 1H-NMR δ (ppm): 6.74-8.36 (m, 7H, Ar-CH), 5.31 (s, 2H, -CH2), 2.31 (s, 1H, -CH3), 8.16 (s, 1H, -NH), Mass (m/e value) % relative abundance: 432.00 (M⁺) (4), 388.71 (8.1), 362.27 (4.2), 233.28 (5), 217.31 (8.9), 182.52 (5), 96.79 (7), 78.82 (B).

Compound AB6

N-(4-[5-(4-nitrophenyl)-1,3,4-oxadiazol-2-ylmethoxy]phenyl)acetamide. IR (KBr) v (cm⁻¹): 3382.43 cm⁻¹ (Ar-NH), 1703.01 cm⁻¹ (C=N), 1592.32 cm⁻¹ (C=C), 1088.54 cm⁻¹ (-C-O-C-), 1378.11 cm⁻¹ (N=O), 3112.69 cm⁻¹ (Ar-CH), 1H-NMR δ (ppm): 6.41-7.8 (m, 8H, Ar-CH), 2.42 (s, 3H, -CH3), 8.13 (s, 1H, -NH), 5.21 (s, 2H, CH2), Mass (m/e value) % relative abundance: 354.09 (M⁺) (3.8), 335.16 (4.8), 302.39 (3.1), 287.43 (3.7), 249.58 (7.1), 226.00 (5.8), 204.96 (6.7), 127.56 (13.1), 103.69 (9), 89.93 (B).

Compound AB7

N-(4-[5-(3,5-dinitrophenyl)-1,3,4-oxadiazol-2-ylmethoxy]phenyl)acetamide. IR (KBr) v (cm⁻¹): 3382.02 cm⁻¹ (Ar-NH), 1677.79 cm⁻¹ (C=N), 1530.6 cm⁻¹ (C=C), 1089.68 cm⁻¹ (-C-O-C-), 1372.45 cm⁻¹ (N=O), 1523.12 asim cm⁻¹ (N=O), 3117.5 cm⁻¹ (Ar-CH), 1H-NMR δ (ppm): 6.83-8.42 (m, 8H, Ar-CH), 5.35 (s, 2H, -CH2), 2.07 (s, 1H, -CH3), 8.24 (s, 1H, -NH), Mass (m/e value) % relative abundance: 399.08 (M⁺) (5), 388.76 (13), 380.25 (8), 261.63 (8), 182.52 (5), 167.62 (17), 156.56 (19), 81.97 (B).

Compound AB8

N-(4-[5-(2-hydroxy-3,5-dinitrophenyl)-1,3,4-oxadiazol-2-ylmethoxy]phenyl) acetamide. IR (KBr) v (cm⁻¹): 3118.84 cm⁻¹ (Ar-NH), 1654.42 cm⁻¹ (C=N), 1541.89 cm⁻¹ (C=C), 1368.45 cm⁻¹ (N=O), 1528.45 asim. cm⁻¹ (N=O),1090.01 cm⁻¹ (-C-O-C-), 3118.84 cm⁻¹ (Ar-CH), 3282.83 cm⁻¹ (Ar-OH), 1H-NMR δ (ppm): 6.7-7.6 (6H, 6H, Ar-CH), 2.11 (s, 1H, -CH3), 8.00 (s, 1H, -NH), 5.12 (s, 1H, -CH2), Mass (m/e value) % relative abundance: 415.07 (M⁺) (11.1), 318.68 (16), 292.76 (7), 276.89 (20), 249.99 (8.2), 236.0277 (28.1), 203.2266 (76), 182.2858 (78), 134.4966 (32), 116.55 (B).

Experimental Pharmacology

Requirements

Carboxymethyl cellulose sodium (CMC) was provided by institutional store. The standard drug 5-FU was purchased from local retail shop Apollo pharmacy, Jyothinagar, OMR road, Chennai. Female Swiss albino mice (20–30 g) were obtained from the central animal house of C. L. Baird Metha College of pharmacy, Jyothinagar, OMR, Chennai and they were maintained.

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under standard laboratory conditions throughout the study. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC) reference number: IAEC/XXIX/10/2017.

| Table 3A: Molecular properties of synthesized compounds |
|------------------------------------------------------|
| Compound codes | Molecular a (XX) | Polarity b | a (YY) | a (ZZ) | Conf.1 | Conf.2 | Conf.3 | Conf.4 |
|----------------|-----------------|------------|--------|--------|--------|--------|--------|--------|
| AB1            | 35.00           | 19.00      | 30.00  | 55.2   | 59.94  | 56.59  | 58.16  | 58.35  |
| AB2            | 37.89           | 21.96      | 33.05  | 58.66  | 59.01  | 69.11  | 60.66  | 60.72  |
| AB3            | 33.54           | 18.46      | 29.65  | 52.52  | 55.29  | 55.42  | 55.85  | 38.13  |
| AB4            | 36.32           | 51.74      | 32.75  | 24.47  | 57.31  | 57.62  | 58.25  | 59.42  |
| AB5            | 38.96           | 56.70      | 35.70  | 24.49  | 64.29  | 64.51  | 65.36  | 66.64  |
| AB6            | 35.90           | 32.78      | 20.88  | 84.03  | 62.01  | 62.63  | 63.65  | 63.79  |
| AB7            | 38.32           | 35.03      | 23.00  | 56.14  | 69.64  | 69.92  | 70.56  | 71.87  |
| AB8            | 38.71           | 36.06      | 25.11  | 54.95  | 73.62  | 73.94  | 75.98  | 76.27  |

Conf: Conformer

| Table 3B: Molecular geometry of synthesized compounds |
|------------------------------------------------------|
| Compounds codes | DE (kcal/mol) | MMFF94 | Min. pa | Max. pa | Min.pr | Max.pr | Vdw.vol | Lp.max.a | Lp.min.a |
|-----------------|---------------|--------|---------|---------|--------|--------|---------|----------|----------|
| AB1             | 57.23         | 99.59  | 40.44   | 103.65  | 4.17   | 10.08  | 280.38  | 5.55     | 19.92    |
| AB2             | 59.01         | 98.71  | 35.35   | 109.22  | 4.46   | 10.74  | 297.21  | 6.67     | 21.4     |
| AB3             | 55.42         | 105.21 | 34.19   | 102.79  | 4.03   | 10.48  | 274.33  | 5.69     | 20.77    |
| AB4             | 57.31         | 110.43 | 34.34   | 105.77  | 4.17   | 10.00  | 287.02  | 5.70     | 19.91    |
| AB5             | 64.51         | 148.45 | 34.38   | 112.98  | 4.18   | 10.79  | 310.7   | 5.75     | 21.57    |
| AB6             | 62.53         | 156.94 | 37.72   | 107.67  | 4.62   | 10.66  | 292.24  | 7.19     | 21.16    |
| AB7             | 69.64         | 172.16 | 41.05   | 115.79  | 4.94   | 10.61  | 315.35  | 6.14     | 21.12    |
| AB8             | 73.62         | 186.39 | 42.39   | 118.30  | 4.97   | 10.58  | 324.00  | 6.18     | 21.10    |

DE: Dredging energy (kcal/mol); MMFF94 energy (kcal/mol); Min. Pa: Minimal projection area; Max. Pa: Maximal projection area; Min.pr: Minimal projection radius; Max.pr: Maximal projection radius; Lp.max.a: Length perpendicular to the max area; Lp. mina: Length perpendicular to the min; Van der Waals volume

| Table 3C: Molecular properties of synthesized compounds |
|------------------------------------------------------|
| Compounds codes | PSA(2D) | Vdw.SA(3D) | H-bonding | Refractivity |
|-----------------|---------|------------|-----------|-------------|
| AB1             | 103.27  | 445.73     | 03        | 02         | 06        | 05       | 102.05  |
| AB2             | 77.25   | 461.95     | 01        | 01         | 05        | 04       | 106.96  |
| AB3             | 77.25   | 437.77     | 01        | 01         | 05        | 04       | 97.57   |
| AB4             | 77.25   | 449.66     | 01        | 01         | 05        | 06       | 104.97  |
| AB5             | 123.07  | 489.50     | 01        | 01         | 09        | 06       | 112.30  |
| AB6             | 123.07  | 470.58     | 01        | 01         | 09        | 06       | 104.68  |
| AB7             | 168.89  | 510.43     | 01        | 01         | 13        | 08       | 112.00  |
| AB8             | 189.12  | 519.03     | 01        | 01         | 13        | 08       | 113.98  |

PSA(2D): Polar surface area; Vdw.SA(3D): Van der Waals surface area; Ds: Donor sites; Dc: Donor count; As: Acceptor sites; Ac: Acceptor count.

Determination of median lethal doses (LD₅₀)

In the present study acute oral toxicity of the synthesized compounds were performed by acute toxic class method according to OECD guideline-423. [9]

Cell culturing

HT 29 human colorectal cancer cells were purchased from Amala Cancer Research Centre, Thrissur, and Kerala.

Study design [10]

An investigational study was designed to evaluate the in vivo antitumor activity of synthesized compounds (AB1-AB8) on mouse tumour models. Study was carried out with HT 29 cell line induced malignant ascites on mouse models. The dose of synthesized compounds 100 mg/kg were chosen based on the results of a toxicity study done previously. The animals were divided into eleven groups (each group contain 6 mice) as follows:

A. Group I: Normal Control Group [only the vehicle (1 ml/kg/day of 1% CMC orally)]
B. Group II: T. Control (1% CMC orally + HT 29 = 2×10⁶ i.p.)
C. Group III: Standard (HT 29 = 2×10⁶ i.p. + 5-FU 25 mg/ml inj.)
D. Group IV-XI: AB1-AB8 (HT 29 = 2×10⁶ i.p. + 100 mg/kg orally)

(Group IV: AB1, Group V: AB2, Group VI: AB3, Group VII: AB4, Group VIII: AB5, Group IX: AB6, Group X: AB7, Group XI: AB8)

| Table 4: Designing of experiment [11] |
|---------------------------------------|
| Days   | Activity carried out |
| Day 1  | Collection of 0.3 ml of blood sample |
| Day 2  | Tumour cell injection, HT 29 = 2×10⁶ i.p. |
| Day 3-12 | Treatment of CMC |
| Day 15 | Treatment of standard drug 5-FU |
| Day 16-35 | Treatment of synthesized compounds (AB1-AB8) |
| Observed till death/35th day |

B. Group II-XI: Group-II-XI
C. Group II-XI: Group-II-XI
D. Group II-XI: Group-II-XI

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Table 5A: The assessment of haematological parameters

| Group | Treatment | Hb (g/dl) (MEAN ± SEM) | RBCs (1 × 10^6/mm³) (MEAN ± SEM) | WBCs (1 × 10^9/mm³) (MEAN ± SEM) |
|-------|-----------|------------------------|----------------------------------|-------------------------------|
| I     | N. Control| 13.62 ± 0.213          | 9.405 ± 0.666                    | 6.032 ± 0.007                 |
| II    | T. Control| 7.275 ± 0.259          | 6.19 ± 0.096                     | 8.455 ± 0.348                 |
| III   | 5-FU***   | 12.7 ± 0.204           | 9.353 ± 0.183                    | 6.17 ± 0.001                  |
| IV    | AB1       | 7.825 ± 0.301          | 8.1 ± 0.105                      | 6.44 ± 0.024                  |
| V     | AB2**     | 10.35 ± 0.246          | 8.55 ± 0.0108                    | 6.416 ± 0.029                 |
| VI    | AB3***    | 10.65 ± 0.184          | 8.683 ± 0.027                    | 6.294 ± 0.024                 |
| VII   | AB4       | 8.05 ± 0.225           | 8.365 ± 0.022                    | 6.725 ± 0.195                 |
| VIII  | AB5       | 7.625 ± 0.239          | 8.45 ± 0.167                     | 6.957 ± 0.295                 |
| IX    | AB6***    | 11.88 ± 0.946          | 8.843 ± 0.028                    | 6.173 ± 0.019                 |
| X     | AB7***    | 11.53 ± 0.149          | 8.755 ± 0.0202                   | 6.284 ± 0.032                 |
| XI    | AB8***    | 12.43 ± 0.179          | 8.955 ± 0.055                    | 6.168 ± 0.007                 |

Table 5B: The assessment of haematological parameters

| Group | Treatment | Neutrophils (%) (MEAN ± SEM) | Lymphocytes (%) (MEAN ± SEM) | Platelets (1 × 10^9/mm³) (MEAN ± SEM) |
|-------|-----------|------------------------------|------------------------------|----------------------------------------|
| I     | N. Control| 14.1 ± 0.294                 | 13.33 ± 0.125                | 4.499 ± 0.0009                          |
| II    | T. Control| 86.24 ± 0.745                | 7.856 ± 0.021                | 12.85 ± 0.248                           |
| III   | 5-FU***   | 14.47 ± 0.211                | 12.14 ± 0.016                | 4.574 ± 0.006                           |
| IV    | AB1       | 15.83 ± 0.058                | 8.325 ± 0.047                | 5.431 ± 0.032                           |
| V     | AB2*      | 15.13 ± 0.243                | 10.1 ± 0.238                 | 5.177 ± 0.004                           |
| VI    | AB5*      | 14.72 ± 0.072                | 10.32 ± 0.235                | 5.017 ± 0.028                           |
| VII   | AB4       | 15.07 ± 0.125                | 8.52 ± 0.033                 | 5.253 ± 0.003                           |
| VIII  | AB5      | 15.24 ± 0.099                | 8.538 ± 0.209                | 5.367 ± 0.005                           |
| IX    | AB6**     | 14.78 ± 0.346                | 11.25 ± 0.159                | 4.943 ± 0.004                           |
| X     | AB7**     | 14.82 ± 0.32                 | 10.87 ± 0.141                | 4.973 ± 0.007                           |
| XI    | AB8**     | 14.56 ± 0.164                | 11.74 ± 0.436                | 4.704 ± 0.002                           |

Table 6: Comparison PILS (%) in different treatment groups

| Group | Treatment | MST (days) (MEAN ± SEM) | PILS (%) |
|-------|-----------|-------------------------|----------|
| II    | T. Control| 4.4 ± 0.04              | -----≤----- |
| III   | 5-FU***   | 8.725 ± 0.025           | 97.72    |
| IV    | AB1       | 6.4 ± 0.04              | 45.45    |
| V     | AB2*      | 7.05 ± 0.064            | 59.09    |
| VI    | AB3*      | 7.45 ± 0.028            | 68.18    |
| VII   | AB4       | 6.8 ± 0.04              | 56.81    |
| VIII  | AB5       | 6.125 ± 0.025           | 38.63    |
| IX    | AB6**     | 8.25 ± 0.064            | 84.09    |
| X     | AB7**     | 7.75 ± 0.028            | 77.27    |
| XI    | AB8***    | 8.475 ± 0.047           | 90.90    |

Assessment of haematological parameters

Effect on the haematological parameters

The present experimental data displayed that the mortality was less in all groups except in tumour control group. The haemoglobin and RBCs count were significantly lower in tumour control group compared to normal control group and significantly raise nearly to normal in all treatment groups when compared with control group. The WBC counts were significantly increased in tumour control and it came down to nearly normal range in all treatment groups. The neutrophils were increased and lymphocytes were decreased significantly in tumour control groups and significantly decreased neutrophils and increased lymphocytes in all treatment groups. The platelet count was significantly increased in tumour control (except Group-III to XI) group compared to normal group.

Determination of the percentage increase in life span (PILS): It is calculated from the mean survival time (MST) values. The MST for each group was calculated as: MST (days) = Total number of days survived by all animals in the group/Number of animals in the group. For each group, Percent increase of lifespan (%IILS) was determined by the following formula: PILS (%) = [(MST of treated group/MST of control group) –1] × 100. The haematological parameters of all surviving animals such as haemoglobin, RBC, WBC, neutrophils, lymphocytes and platelets were assessed for all.

Apoptosis analysis

Cardinal morphological features of apoptotic cells are determined by Gimsa staining technique, annexin V staining by fluorescence microscopy.

RESULTS AND DISCUSSION

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Effect on the survival

All the synthesized compounds AB1-AB8 (100 mg/kg) significantly increased the PILS. While 5-FU increased the life span of 97.72%, and the PILS of synthesized compounds were found to be 45.45%, 59.09%, 68.18%, 56.81%, 38.63%, 84.09%, 77.27% and 90.90%. So the Synthesized compounds AB1-AB8 at the dose of 100 mg/kg significantly improved the overall survival of all treated animals and 5-FU was not significantly differed from each other in improving the overall survival of HT-29 cells.
Fig. 9: Morphological changes observation of HT-29 cells by Giemsa staining (X200): Group-I: Tumour control; Group-III: Treated with standard drug and Group-IV-XI: Treated with 100 mg of synthesized compounds AB1-AB8. The cell morphology was observed and photographed under inverted phase-contrast microscope after Giemsa staining.

Fig. 10: Morphological changes observation of HT-29 cells by H33342 staining (X200): Group-I: T. Control; Group-III: Treated with standard drug 5-FU and Group-IV to Group-XI: Animals are treated with synthesized compounds (AB1-AB8).
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Fig. 11: Apoptosis analysis by FCM using Annexin V-FITC/PI staining on the HT-29 cells treated by synthesized compounds; Group-II: Tumour control without compound and Group-III: Treated with standard drug 5-FU and Group-IV-XI: Treated with 100 mg of synthesized compounds: AB1-AB8.

Apoptosis analysis
Giemsa staining
To confirm whether the apoptotic morphological changes could be associated with the synthesized compounds, HT-29 cells were stained with Giemsa. The morphology changes were observed and photographed under inverted phase-contrast microscope at a magnification of 200X. With the treatment of tests compounds, the process of cell loss, nuclei lysis, chromatin condensation and cytoplasmic shrinkage were aggravated.

H33342 staining on HT-29 cells
In the group-II i.e. tumour control group, HT 29 cell nuclei displayed a normal and complete blue
appearance. By contrast, in HT 29 cells treated with standard drug 5-FU (group-III), synthesized compounds (AB1-AB8), the cells displayed enhanced fragmentation or pyknosis of the nuclei, which were typical changes associated with cellular apoptosis. When compared with standard drug 5-FU, nuclear pyknosis and fragmentation in HT 29 cells were significantly increased by treatment with compounds AB8< AB6< AB7< AB3< AB2 and AB4 among the eight synthesized compounds.

**Annexin V-FITC/propidium iodide (PI) assay**

The effects of synthesized compounds on apoptosis in HT-29 cells were further determined by flow cytometric analysis. Cells were stained with both annexin V-FITC and PI. The flow cytometry observed four quadrant images: the Q1 area represented necrotic cells, the Q2 area represented late apoptotic cells, the Q3 area represented intact cells and the Q4 area represented the early apoptotic cells. The apoptosis ratios of different treatment groups were found as followed: Group-IV=26%; Group-V=37.6%; Group-VI=43%; Group-VII=29%; Group-VIII=24.1%; Group-IX=59.2%; Group-X=48.2%; and XI=63% respectively, while that of the Group-II (T. control) was 6.1%. When compared with Group-III (standard drug 5-FU: 66.2%), it was indicated that compound AB8> AB6> AB7> AB3 were able to significantly induce HT-29 cells apoptosis.

**Fig. 12:** Percentage of HT 29 cells death in different treatment groups.

*In vivo* experimental data showed that all the synthesized compounds possessed a mild to good antitumor activity against human colon cancer cell line HT29. Here it was found that among the all eight synthesized compounds the following compounds executed excellent antitumor activity against HT 29 cell line: AB2, AB3, AB6, AB7 and AB8 and apoptosis of HT 29 cell lines caused by synthesized compounds was evaluated by Giemsa staining and H33342 staining and cells death of HT 29 was further confirmed by Annexin V-FITC/propidium iodide (PI) assay.

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