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Evaluation of Anticancer Activity of Some Novel 2, 5– Disubstituted 1, 3, 4 – Oxadiazole Derivatives Against Colo-205 And Eac Mouse Tumour Models Followed By Molecular Docking Against Topoisomerase I

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Abstract: *The main aim and objective of the present research work was the synthesis, spectral characterization and evaluation of in vitro anticancer as well as in vivo antitumour activity of some novel oxadiazole derivatives followed by docking studies against topoisomerase I enzyme. The structural confirmations of the synthesized compounds were assigned by IR, NMR and Mass spectral analysis. Molecular docking studies were carried out by AUTO DOCK programme. The in vitro anticancer activity of the synthesized compounds (AB1-AB8) was performed by SRB assay. An investigational study was designed to evaluate the in vivo antitumor activity of synthesized compounds and study was carried out with EAC cell line induced malignant ascites on mouse tumour models. In silico molecular docking studies displayed the binding energies (k.cal/ml) of synthesized compounds (AB1-AB8) -1.20, -0.92, -3.24, -1.83, -1.04, -3.28, -3.22, -3.29 which indicated that the compound had high binding affinity towards the target protein Topoisomerase I with PDB id 1A36 and inhibit the Topoisomerase I function in comparison with standard drug topotecan (TTN: -2.06 k. cal/ml). In vitro SRB assay displayed that all the synthesized compounds (AB1-AB8) had the potential ability to inhibit the proliferation of COLO-205 with the highest percentage of growth inhibition AB8 = 92.92±0.0305% > AB6 = 92.32±0.036% > AB7 = 91.74±0.025% > AB3 = 91.59±0.015% > AB4 = 90.89±0.045% > AB1 = 90.6±0.001% > AB5 = 89.23±0.321% > AB2 = 88.9±0.015% etc at dose 300 µg/ml and IC_{50} values of synthesized compounds (AB1-AB8) were found to be 3.89±0.095 µg/ml, 4.133±0.057 µg/ml, 3.233±0.208 µg/ml, 3.767±0.152 µg/ml, 3.39±0.441 µg/ml, 2.433±0.577 µg/ml, 2.767±0.152 µg/ml, 2.0203±0.095 µg/ml and standard drug topotecan (TTN): 94.64±0.01%) found to be 1.973±0.0152 µg/ml. In vivo experimental data displayed that the synthesized compounds AB1-AB8 (100 mg/kg) significantly increased the PILS. While 5-FU increased the life span of 95.65%, and the PILS of synthesized compounds were found to be AB1= 52.17%, AB2= 45.65%, AB3= 65.21%, AB4= 56.52%, AB5= 50.00%, AB6= 82.60%, AB7= 77. 27% and AB8= 91. 30%.*

Key Words: *Anticancer; molecular docking; SRB; malignant ascites and PILS etc.*

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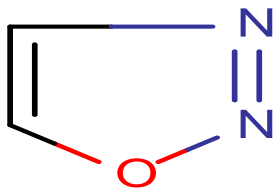
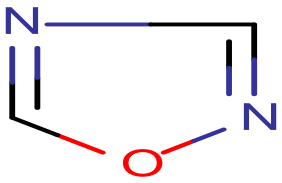
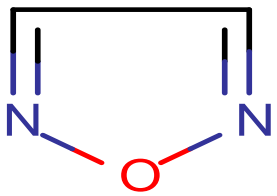
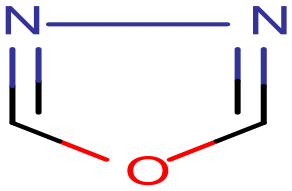
1. Introduction

The most numerous and important heterocyclic systems are those having five and six member rings having hetero atoms such as N, O, S, P, Si and B etc. Many heterocyclic compounds are employed in the treatment of infectious diseases due to their specific antimicrobial activity [1, 2]. Heterocyclic compounds have attracted the attention of medicinal chemists because of having broad spectrum of pharmacological activities and hence it continues to yield new medicinal agents [3, 4]. One such heterocyclic nucleus of medicinal importance is oxadiazole nucleus. Oxadiazoles are five member heterocyclic compounds with two nitrogen atoms and one oxygen atom. Depending on the position of hetero moiety includes antimicrobial, anticancer, anticonvulsant, anti-inflammatory and antiviral agents [5].

Table 1: Oxadiazole

 1,2,3-oxadiazole	 1,2,4-oxadiazole
 1,2,5-oxadiazole	 1,3,4-oxadiazole

2. Experimental Chemistry

2. 1. 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 ^{1}H-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, ^{1}H-NMR and MASS spectra were used to assign the structure of synthesized compounds.

2. 2. Steps involved in the synthesis of target compounds [6]

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 acetone in presence of anhydrous potassium carbonate (K_2CO_3) for 6hrs. 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 hrs. 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-Acetamidophenoxy methyl) -5-aryl substituted - 1, 3, 4-oxadiazole [7]: A mixture of 4-Acetamido phenoxy 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.

2. 3. Synthetic scheme

2. 4. Physicochemical properties

Table 2: Physicochemical properties of synthesized compounds

Sl. No.	Compounds code	M. F	M. Wt	R_f value	m. p	Yield
1.	AB1	$C_{17}H_{16}N_4O_3$	324.33	0.77	116^0C	74.5 %.
2.	AB2	$C_{17}H_{13}Cl_2N_3O_3$	378.209	0.74	180^0C	69.9%
3.	AB3	$C_{17}H_{14}FN_3O_3$	327.309	0.75	189^0C	74%
4.	AB4	$C_{17}H_{14}BrN_3O_3$	388.215	0.65	183^0C	69%
5.	AB5	$C_{17}H_{13}BrN_4O_5$	433.213	0.64	166^0C	60%
6.	AB6	$C_{17}H_{14}N_4O_5$	354.31	0.72	171^0C	64%
7.	AB7	$C_{17}H_{13}N_5O_7$	399.31	0.68	204^0C	78%
8.	AB8	$C_{17}H_{13}N_5O_8$	415.31	0.72	215^0C	68%

2. 5. Spectral data of synthesized compounds

Compound AB1

N-(4-{[5-(4-aminophenyl)-1, 3, 4-oxadiazol-2-yl]methoxy}phenyl) acetamide. IR (KBr) ν (cm^{-1}): 3393.16 cm^{-1} (Ar-NH), 1633.67 cm^{-1} (C=N), 1575.88 cm^{-1} (C=C), 1069.05 cm^{-1} (-C-O-C-), 3132.54 cm^{-1} (Ar-CH), 1249.43 cm^{-1}(Ar-NH_2), ^{1}H-NMR δ (ppm): 6.45-7.4 (s, 8H, Ar-H), 5.17 (s, 2H,-CH_2), 4.1(s, 2H, -NH_2), 2.05 (s,1H, -CH_3), 8.05 (s, 1H, -NH), Mass (m/e value) % relative abundance: 324.12 (M^+) (5.1), 310.87 (4) , 296.22 (8.25), 282.76 (2.2), 272.38(2.32), 262.6432 (7.3), 248.34 (11), 217.12 (15), 207.14 (7), 116.67 (18), 58.33(B).

Compound AB2

N-(4-{[5-(2,4-dichlorophenyl)-1, 3, 4-oxadiazol-2-yl]methoxy}phenyl) acetamide.. IR (KBr) ν (cm^{-1}): 3381.92 cm^{-1} (Ar-NH), 1673.42 cm^{-1} (C=N), 1545.03 cm^{-1} (C=C), 1085.04 cm^{-1} (-C-O-C-), 687.47 cm^{-1} (C-Cl), 3115.62 cm^{-1} (Ar-CH), ^{1}H-NMR δ (ppm): 6.6-7.82(s, 8H, Ar-CH), 2.5 (s, 3H, -CH_3), 8.03(s, 1H, -NH), 5.22(s, 2H, -CH_2), Mass (m/e value) % relative abundance: 377.03 (M^+) (2.8), 333.16 (1.5), 325.42 (2.7), 286.43 (2.6), 183.26 (6), 160.62 (7), 140.65 (16), 115.64 (33), 95.53 (B).

Compound AB3
N-(4-{[5-(4-flurophenyl)-1, 3, 4-oxadiazol-2-yl]methoxy}phenyl) acetamide. IR (KBr) ν (cm^{-1}): 3392.09 cm^{-1} (Ar-NH), 1617.53 cm^{-1} (C=N), 1528.16 cm^{-1} (C=C), 1093.52 cm^{-1} (-C-O-C-), 1371.78 cm^{-1} (C-F), 3114.61 cm^{-1} (Ar-CH), ^{1}H-NMR δ (ppm): 2.21 (s, 1H, -CH_3), 8.09 (s, 1H, -NH), 5.21(s, 1H, -CH_2), 6.7-8.01(m, 8H, Ar-CH), Mass (m/e value) % relative abundance: 327.10 (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 AB4
N-(4-{[5-(2-bromophenyl)-1, 3, 4-oxadiazol-2-yl]methoxy}phenyl) acetamide. IR (KBr) ν (cm^{-1}): 3286.82 cm^{-1} (Ar-NH), 1617.53 cm^{-1} (C=N), 1528.16 cm^{-1} (C=C), 1093.52 cm^{-1} (-C-O-C-), 687.47 cm^{-1} (C-Br), 3114.61 cm^{-1} (Ar-CH), ^{1}H-NMR δ (ppm): 2.21 (s, 1H, -CH_3), 8.09(s, 1H, -NH), 5.21(s, 1H, -CH_2), 6.7-8.01(m, 8H, Ar-CH), Mass (m/e value) % relative abundance: 387.02(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), 75.60 (B).

Compound AB5
N-(4-{[5-(2-bromo,4-nitrophenyl)-1, 3, 4-oxadiazol-2-yl]methoxy}phenyl) acetamide. IR (KBr) ν (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), ^{1}H-NMR δ (ppm): 6.74-8.36(m, 7H, Ar-CH) ,5.31(s, 2H, -CH_2),2.31 (s, 1H, -CH_3), 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-yl]methoxy}phenyl)acetamide. IR (KBr) ν (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), ^{1}H-NMR δ (ppm): 6.41-7.8(m, 8H, Ar-CH), 2.42 (s, 3H, -CH_3), 8.13(s, 1H, -NH), 5.21(s, 2H, CH_2), 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-yl]methoxy}phenyl) acetamide. IR (KBr) ν (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 asym cm^{-1} (N=O), 3117.5 cm^{-1} (Ar-CH), ^{1}H-NMR δ (ppm): 6.83-8.42(m, 8H, Ar-CH), 5.35(s, 2H,-CH_2), 2.07 (s, 1H,- CH_3), 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-yl]methoxy}phenyl) acetamide. IR (KBr) ν (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 asym. cm-1 (N=O),1090.01 cm^{-1} (-C-O-C-), 3118.84 cm^{-1} (Ar-CH), 3382.83 cm^{-1}(Ar-OH), ^{1}H-NMR δ (ppm): 6.7-7.6(s, 6H, Ar-CH), 2.11 (s, H, -CH_3), 8.00(s, 1H, -NH), 5.12(s, 1H, -CH_2), 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), 182.2587 (8), 134.4966 (32), 116.55 (B).

3. Computational Chemistry

3. 1. Molecular docking

Molecular docking is defined as an optimization problem, which would describe the "best-fit" orientation of a ligand that binds to a particular protein of interest. During the course of the process, the ligand and the protein adjust their conformation to achieve an overall "best-fit" and this kind of conformational adjustment resulting in the overall binding is referred to as "induced fit. The aim of the molecular docking to achieve an optimized conformation for both the protein and the ligand and to achieve relative orientation between protein and ligand such that free energy of overall system is minimized. The application of docking are the hit identification – docking combined with a scoring function can be used to quickly screen large databases of potential drugs in silico to identify molecules that are likely to bind to protein target of interest and the lead optimization – docking can be used to predict in where and in which relative orientation a ligand binds to a protein. This information may in turn be used to design more potent and selective analogues [8].

3. 2. Scoring functions

In the fields of computational chemistry and molecular modelling, scoring functions are fast approximate mathematical methods used to predict the strength of the non-covalent interaction (also referred to as binding affinity) between two molecules after they have been docked. Structure-based drug design attempts to use the structure of proteins as a basis for designing new ligands by applying the principles of molecular recognition. Selective high affinity binding to the target is generally desirable since it leads to more efficacious drugs with fewer side effects. Thus, one of the most important principles for designing or obtaining potential new ligands is to predict the binding affinity of a certain ligand to its target (and known antitargets) and use the predicted affinity as a criterion for selection [9].

One early general-purposed empirical scoring function to describe the binding energy of ligands to receptors was developed by Böhm [10, 11]. This empirical scoring function took the form:

$\Delta G_{bind} = \Delta G_0 + \Delta G_{hb} \sum \mathrm{n} - \mathrm{bonds} + \Delta G_{ionic} \sum_{ionic\ -int} + \Delta G\ \mathrm{lipophilic}\ |A| + \Delta G_{rot}\ \mathrm{NROT}$

Where

ΔG_0: Empirically derived offset that in part corresponds to the overall loss of translational and rotational entropy of the ligand upon binding.

ΔG_{hb}: Contribution from hydrogen bonding.

ΔG_{ionic}: Contribution from ionic interactions.

ΔG_{lip}: Contribution from lipophilic interactions where $|A_{lipo}|$ is surface area of lipophilic contact between the ligand and receptor.

ΔG_{rot}: Entropy penalty due to freezing a rotatable in the ligand bond upon binding.

A more general thermodynamic "master" equation is as follows:

$$G_{bind} = -\mathrm{RTlnK_d}$$

$$K_d = \frac{[\mathrm{Ligand}][\mathrm{Receptor}]}{[\mathrm{Complex}]}$$

$$\Delta G_{bind} = \Delta G_{desolvation} + \Delta G_{motion} + \Delta G_{configuration} + \Delta G_{interaction}$$

Where

Desolvation: Enthalpic penalty for removing the ligand from solvent

Motion: Entropic penalty for reducing the degrees of freedom when a ligand binds to its receptor

Configuration: Conformational strain energy required to put the ligand in its "active" conformation

Interaction: Enthalpic gain for "resolvating" the ligand with its receptor

3. 3. Computational analysis of synthesized compounds

Type of Work: Computational Analysis**; Project Id:** RSID001142/11/2015**; PDB Code:** 1A36.

Structure of protein topoisomerase I

Crystalline structure of the target protein Topoisomerase – I with PDB id 1A36 was retrieved from protein data bank and protein clean-up process was done and essential missing hydrogen atom were been added. Different orientation of the lead molecules AB1 to AB8 along with standard drug Topotecan with respect to the target protein was evaluated by Autodock program and the best dock pose was selected based on the interaction study analysis.

In molecular biology Type I topoisomerases are enzymes that cut one of the two strands of double-stranded DNA, relax the strand, and reanneal the strand. They are further subdivided into two structurally and mechanistically distinct topoisomerases: type IA and type IB. Type IA topoisomerases change the linking number of a circular DNA strand by units of strictly 1 Type IB topoisomerases change the linking number by multiples of 1 (n). Historically, type IA topoisomerases are referred to as prokaryotic topo I, while type IB topoisomerases are referred to as eukaryotic topoisomerase. This distinction, however, no longer applies as type IA and type IB topoisomerases exist in all domains of life. Functionally, these subclasses perform very specialized functions. Prokaryotic topoisomerase I (topo IA) can only relax negative supercoiled DNA, whereas eukaryotic topoisomerase I (topo IB) can introduce positive supercoils, separating the DNA of daughter chromosomes after DNA replication, and relax DNA [12].

Fig 1: Structure of Topoisomerase I

4. Experimental Oncology

4. 1. Cell culture

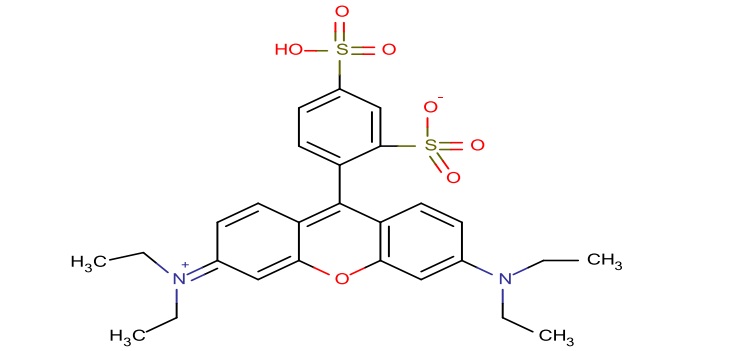
The cell culture human colon adenocarcinoma cell line COLO-205 was provided by Amla Cancer Research Centre, Thrissur, Kerala and was grown in Eagles Minimum Essential Medium (EMEM) which contained 10% fetal bovine serum (FBS). All cells were maintained at 37°C, 100% relative humidity, 5% CO2, 95% air and the culture medium was changed twice a week.

4. 2. Drugs: The standard drug topotecan (TTN) was provided by central drug store of institute.

4. 3. Methods

The in vitro anticancer activity of synthesized compounds was evaluated by SRB assay.

SRB: Sulforhodamine B or Kiton Red 620 ($C_{27}H_{30}N_2O_7S_2$) is a fluorescent dye with uses spanning from laser-induced fluorescence (LIF) to the quantification of cellular proteins of cultured cells. This red solid dye is very water-soluble [13]. The dye has maximal absorbance at 565 nm light and maximal fluorescence emission at 586 nm light [13] It does not exhibit pH-dependent absorption or fluorescence over the range of 3 to 10 [14]. Sulforhodamine B is often used as a membrane-impermeable polar tracer or used for cell density determination via determination of cellular proteins (Cytotoxicity assay) [15**].**



2-[6-(diethylamino)-3-(diethyliminiumyl)-3H-xanthen-9-yl]-5-sulfobenzene-1-sulfonate

Fig 2: Structure of sulforhodamine B

Principle: Sulforodamine B (SRB) is a bright pink aminoxanthine dye with two sulfonic acid group. Under mild acidic conditions SRB dye binds to basic amino acid residues in trichloro acetic acid (TCA) fixed cells to provide a sensitive index of cellular protein content that is linear over a cell density range of visible at least two order of magnitude [16, 17].

Reagents:

1. PBS (Phosphate buffer saline)
2. 40-50% TCA
3. 1% acetic acid solution
4. Sulforhodamine B (0.4% in 1% TCA)
5. 10 Mm Tris (P^H = 10.5).

Procedure: The monolayer cell culture was trypsinized and the cell count was adjusted to 0.5-1.0 x10^5 cells/ml using medium containing 10% new born sheep serum. To each well of the 96 well micro titre plate, 0.1 ml of the diluted cell suspension (approximately) 10,000 cells was added. After 24 hrs, when a partial monolayer was formed, the supernatant was flicked off, washed once and100 µg/ml, 200 µg/ml and 300 µg/ml of different concentration synthesized compounds were added to the cell in micro titre plate. The plates were incubated at 37

^{0}c for 72 hrs in 5% CO2 incubator, microscopic examination was carried out and observations were recorded every 24 hrs. After 72 hrs, 25µl of 50% TCA was added to wells gently such that it forms a thin layer over the synthesized compounds to form overall concentrations 10%. The plates were incubated at 4^{0}c for 1 hr. The plates were flicked and washed five times with tap water to remove traces of medium sample and serum and were then air dried. The air dried plates were stained with 100 µl SRB and kept for 30 mints at room temperature. The unbound dye was removed by rapidly washing four times with 1% acetic acid. The plates were then air dried. 100 µl of 10 mM Tris base was then added to the wells to solubilise the dye [7]. The plates were shaken vigorously for 5 mints. The absorbance was measured using micro plate reader at a 540 nm. The % growth inhibition was calculated by the following formula:

% cell growth inhibition = 100-{(At-Ab/Ac-Ab)}x 100

At = Absorbance value of test compound, Ab = Absorbance value of blank, Ac = Absorbance value of control.

Positive control for Cytotoxicity (Test): cells treated with a cytotoxic drug/chemical +SRB + solubilizing buffer

Negative control for cytotoxicty (control): cells left untreated + SRB + solubilizing buffer.

Blank: medium without cells + SRB + Solubilizing buffer.

4. 4. Trypan blue exclusion assay for the determination of cell viability [20]

Trypan blue is one of several stains recommended for use in dye exclusion procedures for viable cell counting. This method is based on the principle that live (viable) cells do not take up certain dyes, whereas dead (non-viable) cells do. Staining facilitates the visualization of cell morphology.

Trypan Blue has a greater affinity for serum proteins than for cellular protein. If the background is too dark, cells should be pelleted and resuspended in protein-free medium or salt solution prior to counting.

Trypan Blue Protocol:

1. Prepare a cell suspension in a balanced salt solution (e.g., Hanks' Balanced Salts [HBSS], Cat. No. H9269).
2. Transfer 0.5 ml of 0.4% Trypan Blue solution (w/v) to a test tube. Add 0.3 ml of HBSS and 0.2 ml of the cell suspension (dilution factor = 5) and mix thoroughly. Allow to stand for 5 to 15 minutes.
3. With the cover-slip in place, use a Pasteur pipette or other suitable device to transfer a small amount of Trypan Blue-cell suspension mixture to both chambers of the hemacytometer. Carefully touch the edge of the cover-slip with the pipette tip and allow each chamber to fill by capillary action. Do not overfill or underfill the chambers.
4. Starting with chamber 1 of the hemacytometer, count all the cells in the 1 mm center square and four 1 mm corner squares. Non-viable cells will stain blue. Keep a separate count of viable and non-viable cells. Count cells on top and left touching middle line of the perimeter of each square. Do not count cells touching the middle line at bottom and right sides.
5. Repeat this procedure for chamber 2.
6. Withdraw a second sample and repeat count procedure to ensure accuracy.

Calculations

Cell Counts: Each square of the haemocytometer, with cover-slip in place, represents a total volume of 0.1 mm^3 or 10-4 cm^3. Since 1 cm^3 is equivalent to approximately 1 ml, the subsequent cell concentration per ml (and the total number of cells) will be determined using the following calculations

Ca**lculations for compound AB1:**

Cells Per mL = the average count per square × dilution factor × 10^4 (count 10 squares)

The average counts per square are 43 cells × 5 × 10^4 = (2.15 ×10^2× 10^4) = 2.15×10^6 cells/ml

Total Cells = cells per ml × the original volume of fluid from which cell sample was removed.

2.15×10^6 (cells/ml) × 10 ml (original volume) = 2.15 × 10^7 total cells.

Cell Viability (%) = [total viable cells (unstained) ÷ total cells (stained and unstained)] × 100.

The average count per square of unstained (viable) cells is 31.3, the total viable cells = [31.3 × 5 × 10^4] viable cells/ml × 10 ml (original volume) = 1.565 × 10^7 viable cells. Cell viability (%) = 1.565 × 10^7 (viable cells) ÷ 2.15 × 10^7 (total cells) × 100 = 72.79 % viability.

5. Experimental Pharmacology

5. 1. Cancer cell lines: The initial inoculums of the EAC cells were provided by the Amala Cancer Research Centre, Thrissur, Kerala, India. The EAC cells were then propagated in our laboratory biweekly through intraperitoneal transplantation of 2 × 10^6 cells per mouse (freshly drawn from a donor Swiss albino mouse, bearing 6- to 7-day-old ascites tumor cells).

5. 2. Chemicals and drug: Carboxymethyl cellulose sodium (CMC), phosphate buffered saline (PBS) Diethyl ether and topotican (standard antitumour drug) were used for the study.

5. 3. Animals: Female Swiss albino mice were obtained from the central animal house of C. L. Baid Metha College of pharmacy, Jyothinagar, OMR, Chennai and they were maintained under standard laboratory conditions throughout the study. The animals were fed with standard rodent pellet feed (Hindustan lever, Bangalore) and water ad libitum. Adult mice weighing 20–30 g were used for the experiments. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC, reference number:

IAEC/XXIX/10/2017) and all the animal experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

5. 4. Determination of median lethal doses (LD_{50})

In the present study acute oral toxicity of the synthesized compounds were performed by acute toxic class method according to OECD guideline-423 [21]. In this method the toxicity of synthesized compounds were tested using a step wise procedure, each step using three mice of single sex (female/male). The Swiss albino mice were fasted prior to dosing (food but water should be with held) for three to four hours. Following the period of fasting the animal should be weighted and the test compounds were dissolved in 3% CMC, administered intraperitoneally to the different groups with 2000 mg / kg body weight. Animals were observed individually after dosing at least once during the first 30 min; periodically during the first 24 h with special attention giving during the first 4 h and daily thereafter, for total of 14 days. As know mortality observed with the above dose. Test compound dose reduced by specific intervals. The mortality was not observed at the dose 2000 mg / Kg. So 100 mg /Kg body weight was selected for their pharmacological evaluation (since LD_{50} cut of value is > 2000). Mortality was determined after 24 hours of treatment. The dose at which 50% of the mice survived was considered the LD_{50} value of the compound.

5. 6. Study design

An investigational study was designed to evaluate the *in vivo* antitumor activity of synthesized compounds (AB1-AB8) on EAC mouse tumour models. Study was carried out with EAC 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 five different groups as follows:

The animals were divided into eleven different 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 orallay + EAC = 2×10^6 i. p.)

C. Group III: Standard (EAC = 2×10^6 i. p + 5-FU 25 mg/ml inj.)

D. Group IV-XI: AB1-AB8 (EAC = 2×10^6 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 3: Designing of experiment

Days	**Activity was carried out**	**No. of mice / group (6)**
Day 1	Collection of 0.3 ml of blood sample	Group-I-XI
Day 2	Tumor cell injection, EAC = 2×10^6 i. p.	Group-II-XI
Day 3-12	Treatment of CMC	Group-II
	Treatment of std. drug 5-FU	Group-III
	Treatment of Synthesized compounds (AB1-AB8)	Group-IV -XI
Day 15	Collection of 0.3 ml of blood sample	Group-II-XI
Day 16-35 follow up	Observed till death/35^{th} day	Group-II-XI

5. 7. Experimental procedure [22]

On day 1, blood collection from retro orbital plexus was carried out and the samples (0.3 ml) in EDTA were used for the assessment of haematological parameters such as haemoglobin (Hb) content, red blood cell (RBC) count, total white blood cell (WBC) count, DLC and platelet count. On day 2, tumour fluid was withdrawn from the stock animals for EAC and the tumour cell count was done using Neubauer chamber under the light microscope. The PBS was added to make a concentration of 1×10^6 cells in 0.1 ml. For tumour induction in study each experimental animal (Group-II to Group-XI) was injected with 2×10^6 EAC cells i.e. 0.2 ml intra peritoneal route. After 24 h of the tumour cells inoculation, the animals were treated according to their respective groups once daily for next 10 days. On day 15, the retro-orbital blood collection was done again for haematological assessment, if the animal was alive. The animals were followed till death or up to 35 days. The parameters for antitumor activity in study were recorded as followed. Determination of the percentage increase in life span (PILS): It is calculated from the mean survival time (MST) values [23]. 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 (% ILS) 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. A group of four normal mice was studied for assessing their haematological parameters. These normal (control) values were used for comparisons. The tumour bearing animals alive at the end of the study were sacrificed by cervical dislocation.

6. Results And Discussion

Molecular docking

Table-4: Docking results analysis

C.C	EFEB (k.cal/ml)	EIC (Ki) mM	vdw+H-bond+de (k.cal/ml)	EE (k.cal/ml)	TIME (k.cal ml)	Fr (%)	IS
AB1	**-1.20**	**131.78**	**-5.39**	**+1.67**	**-3.72**	**50**	**719.96**
AB2	**-0.92**	**211.41**	**-4.23**	**+1.56**	**-2.67**	**50**	**761.341**
AB3	**-3.24**	**4.24**	**-5.09**	**+0.94**	**-4.15**	**50**	**640.468**
AB4	**-1.83**	**45.53**	**-4.43**	**+1.10**	**-3.33**	**50**	**665.095**
AB5	**-1.04**	**174.17**	**-3.70**	**+0.80**	**-2.89**	**50**	**565.908**
AB6	**-3.28**	**3.99**	**-4.68**	**-0.23**	**-4.92**	**50**	**678.391**
AB7	**-3.22**	**4.36**	**-5.29**	**-0.12**	**-5.41**	**50**	**719.469**
AB8	**-3.29**	**3.87**	**-5.07**	**-0.13**	**-5.19**	**50**	**707.469**
Topotecan	**-2.06**	**30.81**	**-4.38**	**+0.40**	**-3.38**	**50**	**602.191**

C.C = Compounds code. EFEB = Est. Free Energy of Binding. EIC = Est. Inhibition Constant. Vdw + H-bond + dE = Vender walls + Hydrogen bond + Dessolve energy. EE = Electrostatic Energy. TIME = Total Intermolecular Energy. Fr = Frequency. IS = Interaction Surface.

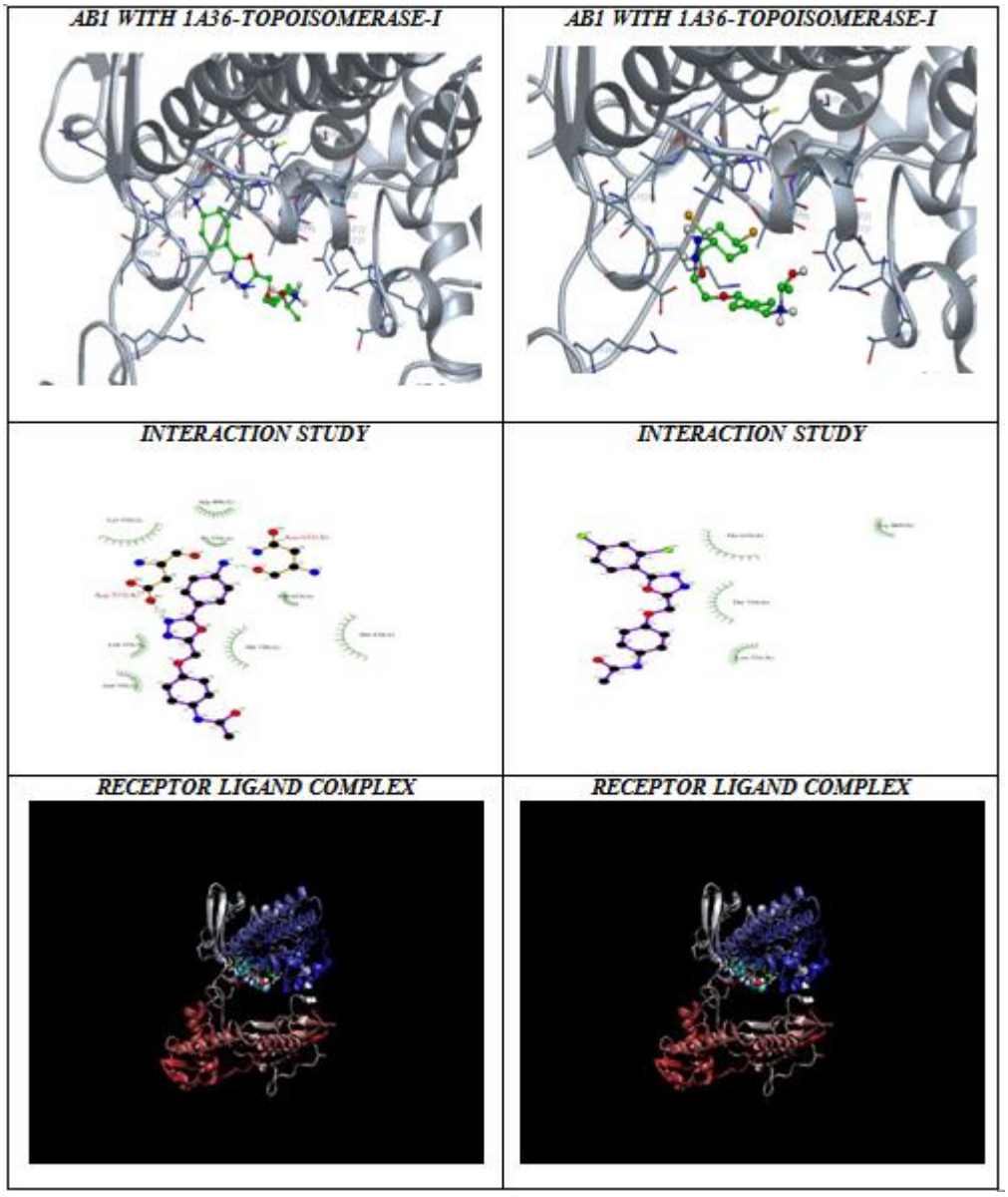


Fig 3: Compound AB1 and AB2 with topoisomerase I interaction

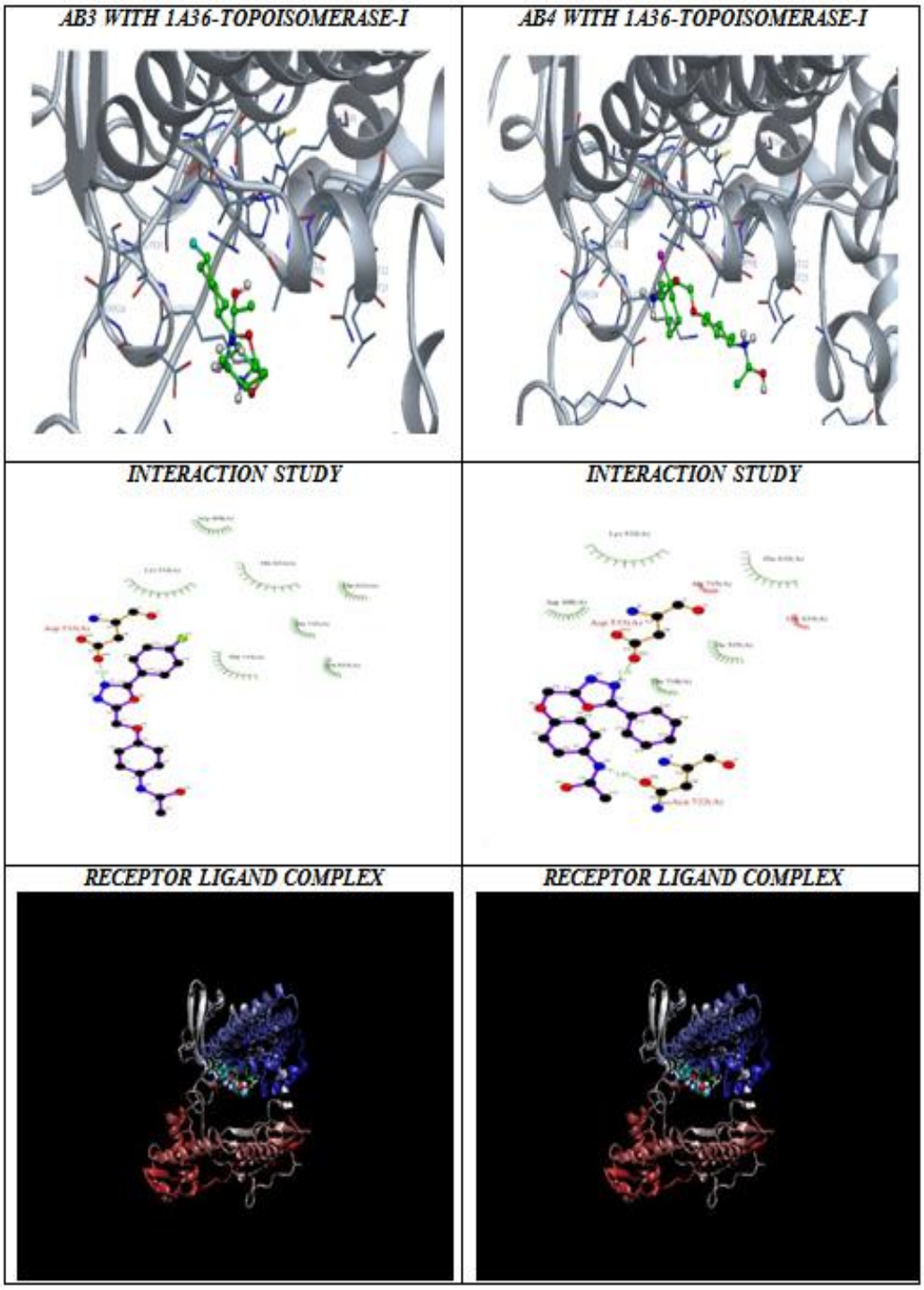


Fig 4: Compound AB3 and AB4 with topoisomerase I interaction

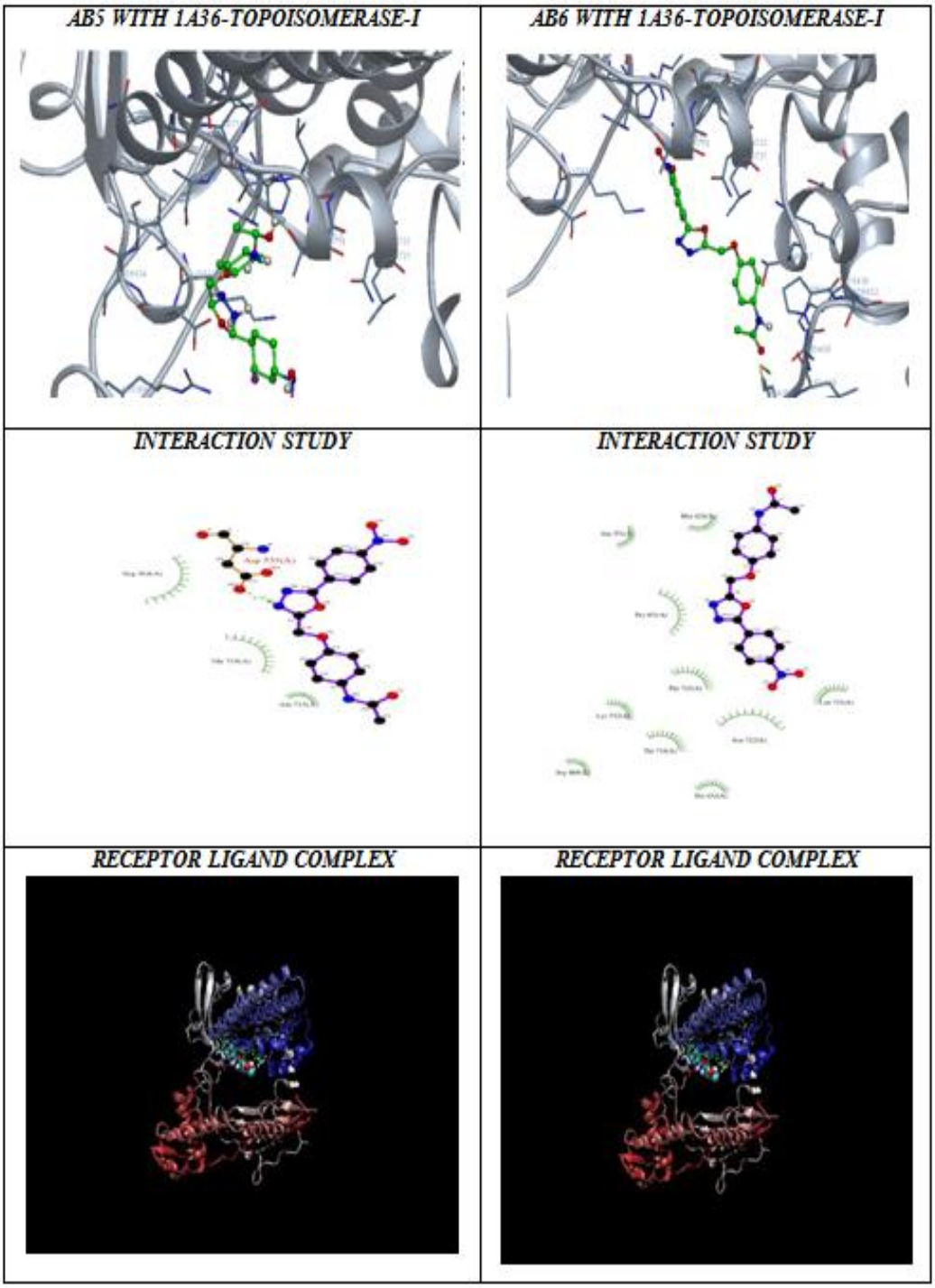


Fig 5: Compound AB5 and AB6 with topoisomerase I interaction

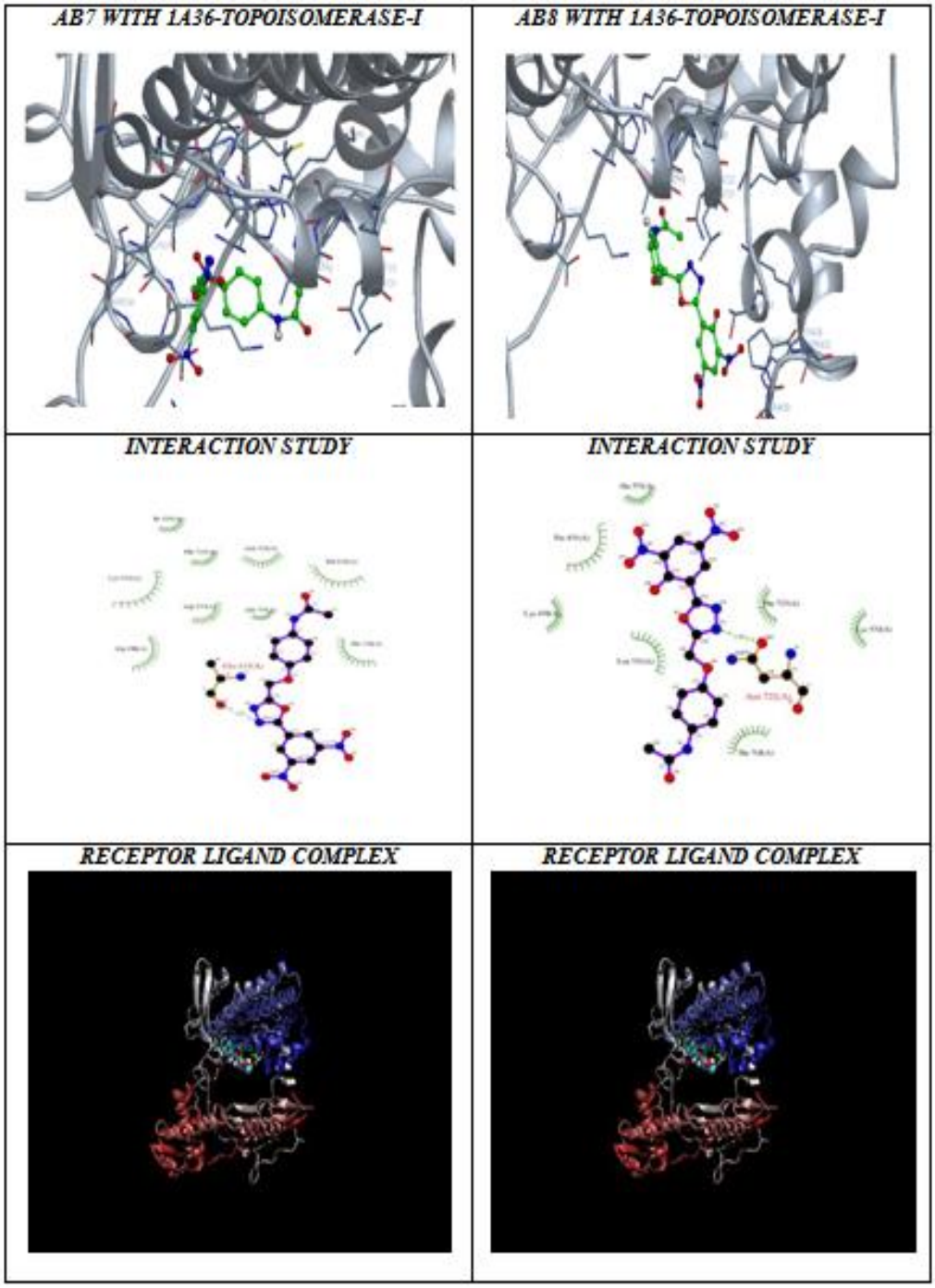


Fig 6: Compound AB7 and AB8 with topoisomerase I interaction

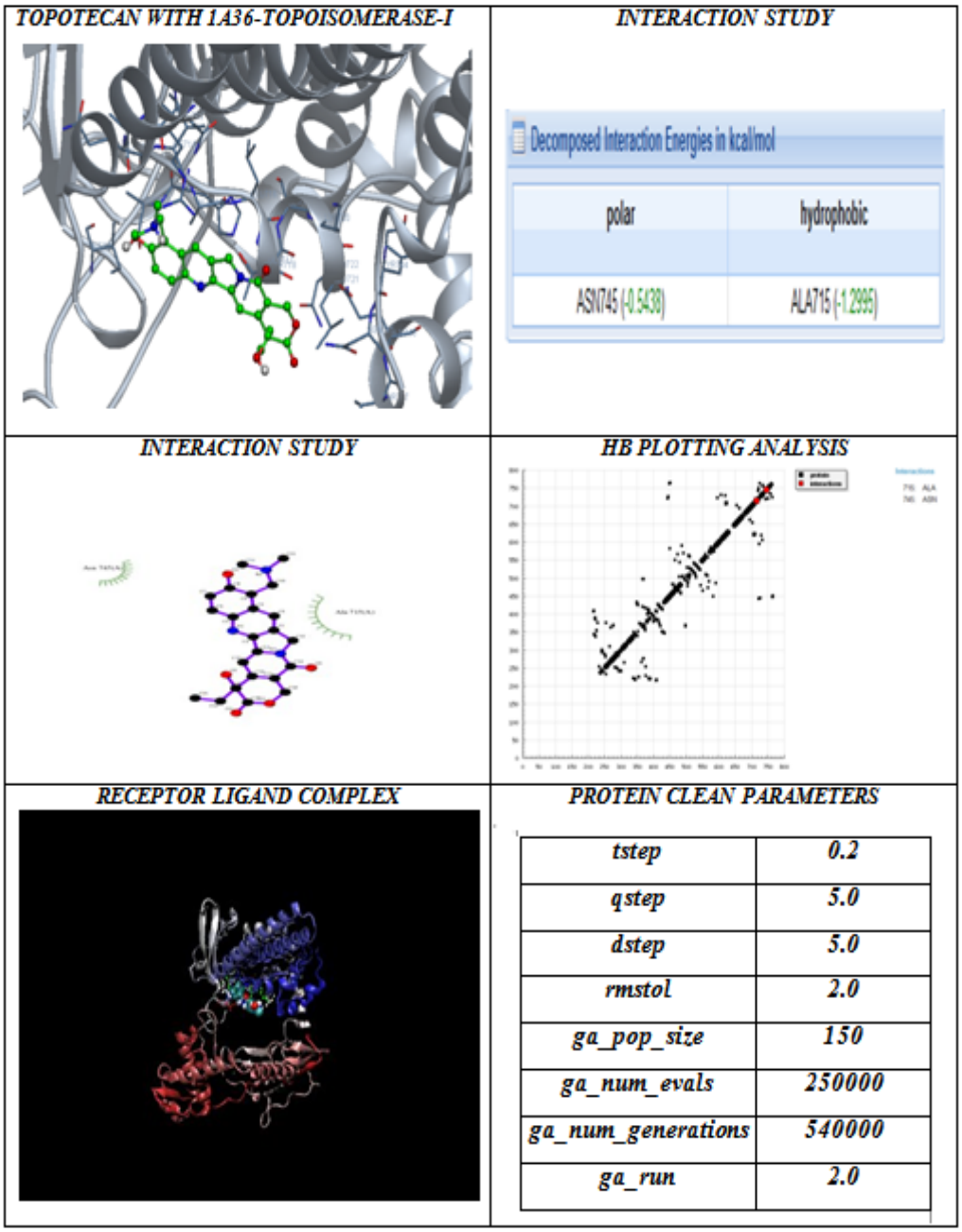


Fig 7: Standard drug topotecan with topoisomerase I interaction

EXPERIMENTAL ONCOLOGY

Table-5: For percentage (%) of cell growth inhibition by synthesized compounds at 100 µg/ml analysed by SRB Assay

Compound Codes	Concentrations	Absorbance (MEAN±SD)	PCGI (MEAN±SD) %
AB1	100 µg/ml `	0.088±0.001	70.48±0.065
AB2	100 µg/ml	0.092±0.001	69.38±0.4504
AB3	**100 µg/ml**	**0.082±0.0057****	**72.18±0.035****
AB4	100 µg/ml	0.086±0.001*	71.35±0.3905*
AB5	100 µg/ml	0.008±0.0005	70.32±0.033
AB6	**100 µg/ml**	**0.077±0.001****	**74.86±0.025****
AB7	**100 µg/ml**	**0.081±0.0005****	**72.87±0.045****
AB8	**100 µg/ml**	**0.074±0.001****	**78.87±0.045****
TTN	**75 µg/ml**	**0.015±0.0005*****	**94.64±0.0208*****
Control		0.297±0.0005	0

Table-6: For percentage (%) of cell growth inhibition by synthesized compounds at 200 µg/ml analysed by SRB Assay

Compound Codes	Concentrations	Absorbance (MEAN±SD)	PCGI (MEAN±SD) %
AB1	200 µg/ml	0.062±0.001*	79.55±0.026*
AB2	200 µg/ml	0.067±0.001	77.5±0.015
AB3	**200 µg/ml**	**0.056±0.001****	**80.86±0.015****
AB4	200 µg/ml	0.057±0.0005*	79.55±0.025*
AB5	200 µg/ml	0.064±0.0011	78.49±0.0404
AB6	**200 µg/ml**	**0.0506±0.0005****	**82.9±0.015****
AB7	**200 µg/ml**	**0.053±0.001****	**82.24±0.015****
AB8	**200 µg/ml**	**0.048±0.001****	**83.56±0.01****
TTN	**75 µg/ml**	**0.015±0.0005*****	**94.63±0.01*****
Control		0.297±0.00057	0

Table-7: For percentage (%) of cell growth inhibition by synthesized compounds at 300 µg/ml analysed by SRB Assay

Compound Codes	Concentrations	Absorbance (MEAN±SD)	PCGI (MEAN±SD) %
AB1	300 µg/ml	0.029±0.001*	90.6±0.001*
AB2	300 µg/ml	0.033±0.0005	88.9±0.015
AB3	**300 µg/ml**	**0.026±0.001*****	**91.59±0.015*****
AB4	300 µg/ml	0.027±0.001**	90.89±0.045*
AB5	300 µg/ml	0.031±0.002	89.23±0.321
AB6	**300 µg/ml**	**0.022±0.001*****	**92.32±0.036*****
AB7	**300 µg/ml**	**0.024±0.001*****	**91.74±0.025*****
AB8	**300 µg/ml**	**0.021±0.001*****	**92.92±0.0305*****
TTN	**75 µg/ml**	**0.011±0.0005*****	**94.64±0.01*****
Control		0.297±0.0005	0

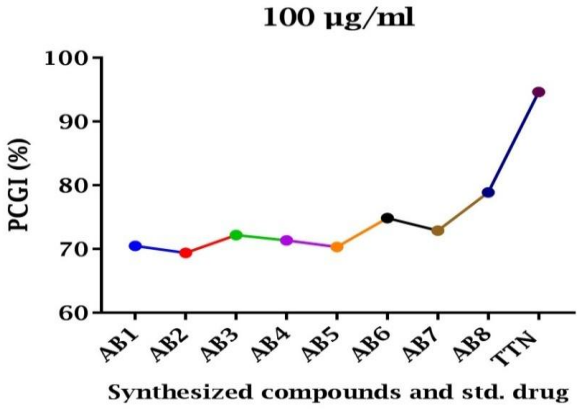


Fig 8: PCGI (COLO-205) at 100 µg/ml.

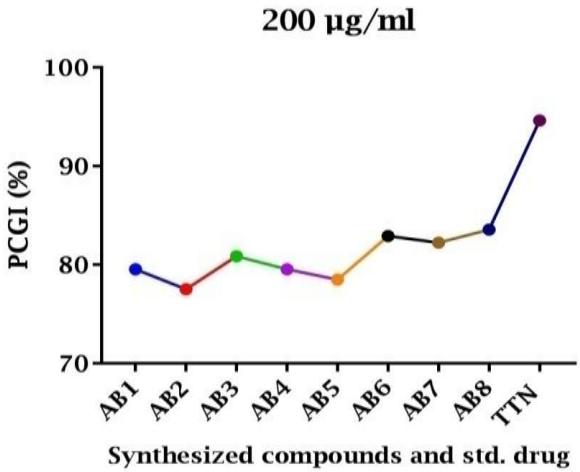


Fig 9: PCGI (COLO-205) at 200 µg/ml.

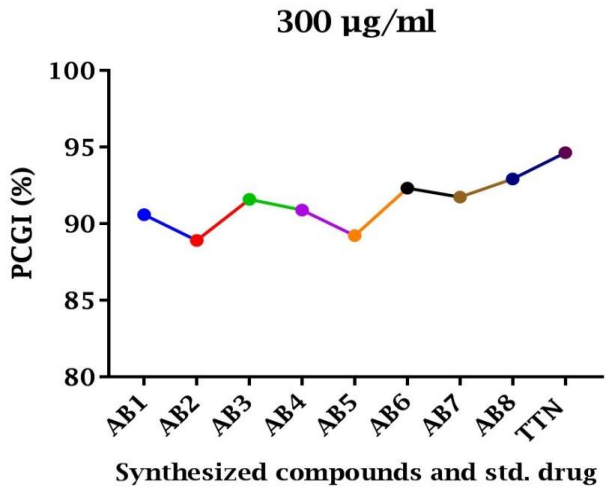


Fig 10: PCGI (COLO-205) at 300 µg/ml.

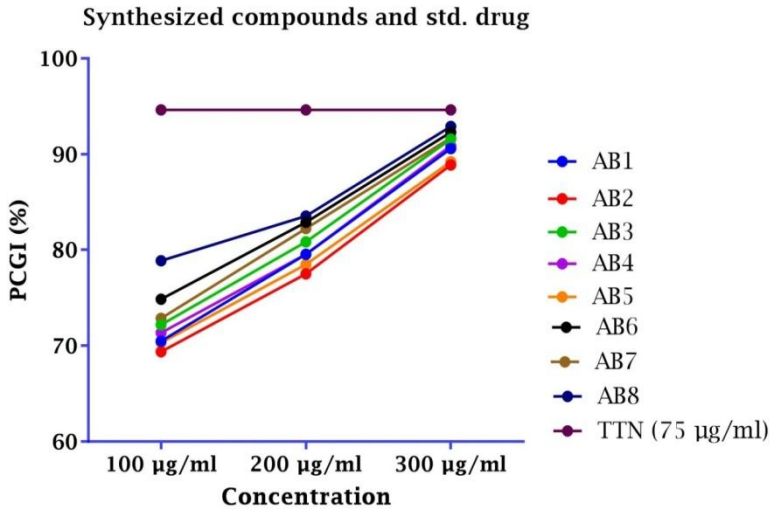


Fig 11: Comparison of PCGI (COLO-205) at different concentration of synthesized compounds.

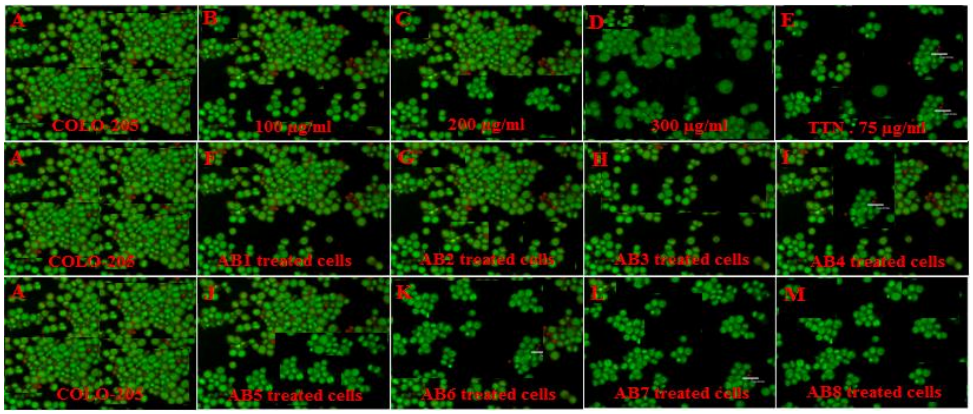


Fig 12: The growth of COLO-205 cells was inhibited by the synthesized compounds AB1-AB8. COLO-205 cells were treated with synthesized compounds (100 µg, 200 µg and 300 µg). Where A= Control, B= 100 µg/ml of synthesized compound treated cells, C= 200 µg/ml of synthesized compound treated cells, D= 300 µg/ml of synthesized compound treated cells, E= 75 µg/ml of topotecan (std. drug-TTN) treated cells and F-M= 300 µg/ml of synthesized compounds (AB1-AB8) treated cells. Photographs were taken under a fluorescence microscope (200 X original magnifications) and Cells were observed by using Hoechst 33342 staining.

Table -8: For IC_{50} Values of synthesized compounds

Compound Codes	IC_{50} (µg/ml)
AB1	3.89±0.095*
AB2	4.133±0.057
AB3	3.233±0.208**
AB4	3.767±0.152*
AB5	3.39±0.441*
AB6	2.433±0.577***
AB7	2.767±0.152**
AB8	2.0203±0.095***
TTN	1.973±0.0152***

TTN>AB8>AB6>AB7>AB3>AB4>AB1>AB5>AB2

P<0.001= *, highly significant. P<0.01= **, moderate significant P<0.05= *, significant. P>0.05= ns. Values are expressed as MEAN ±SD of sixet. The data were statistically analysed by ONE WAY ANOVA followed by Tukey Kramer multiple comparison test.**

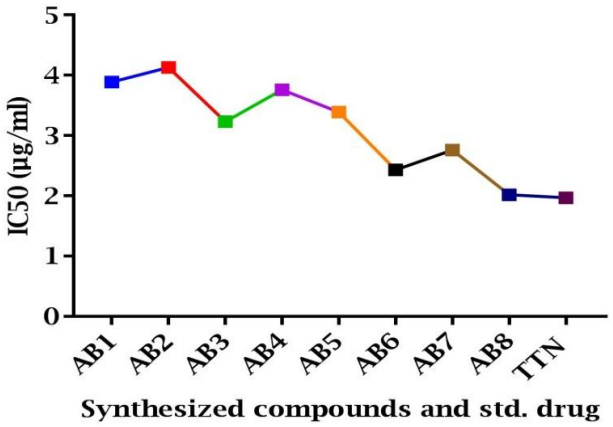


Fig 13: IC_{50} value of synthesized compounds against COLO-205 cell line

Trypan blue exclusion assay for the determination of cell viability

Table-9: For cell viability assay

Compound Codes	Cell viability (%)
AB1	72.79
AB2	73.48
AB3	65.34
AB4	72.32
AB5	73
AB6	50
AB7	58.6
AB8	44.18
TTN	34.88

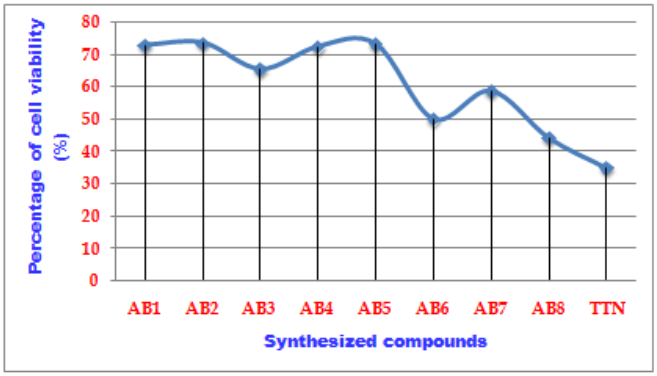


Fig 14: Percentage (%) of cell viability of COLO-205 at 300 µg/ml of synthesized compounds and 75 µg/ml of TTN

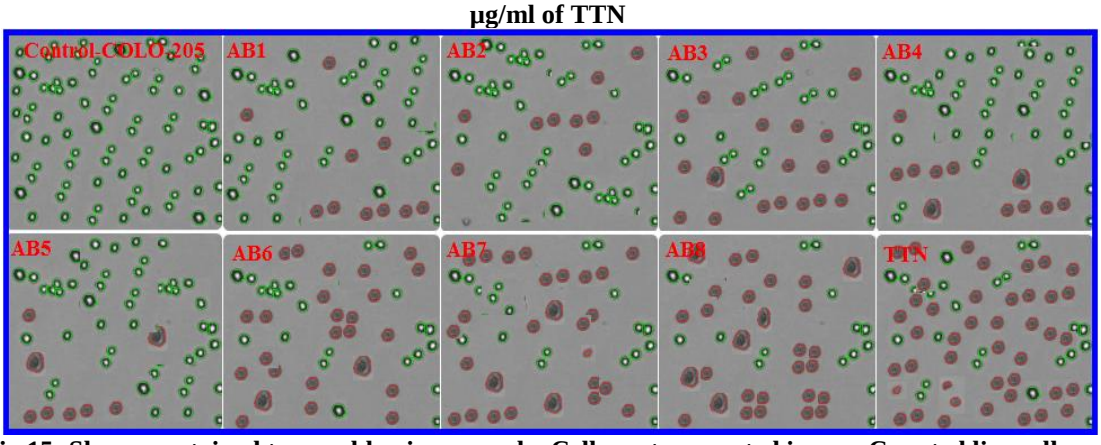


Fig 15: Shown, a stained trypan blue image and a Cello meter counted image. Counted live cells are outlined in green while the dead trypan blue positive cells are outlined in red caused by the synthesized compounds at 300 µg.

EXPERIMENTAL PHARMACOLOGY

Table -10-A: The assessment of haematological parameters

Treatment group	Hb (g/dl) (MEAN±SD)	RBCs (1×10^6/mmm^3) (MEAN±SD)	WBCs (1×10^3/mm^3) (MEAN±SD)	Platelets (1×10^5/mm^3) (MEAN±SD)
I **(N. Control)**	14.1± 0.182	9.438±0.015	6.313±0.017	4.51±0.0175
II **(T. Control)**	9.1±0.141	6.415±0.012	29.09±0.056	12.05±0.0421
III (5-FU)	**13.23±0.15*** **	**9.328±0.009****	**6.538±0.009*** **	**4.465±0.0584*** **
IV (AB1)	9.725±0.095*	6.89±4.507*	7.155±0.005**	**5.276±0.0346***
V (AB2)	9.25±0.057*	9.8±0.081*	7.258±0.009*	**4.864±0.0435****
VI (AB3)	**11.5±0.081****	**9.193±0.012****	**6.88±0.0081*** **	**4.749±0.0119*** **
VII (AB4)	10.07±0.098*	9.16±0.008*	6.98±0.008**	5.365±0.0685****
VIII (AB5)	9.5±0.081*	9.118±0.005*	7.403±0.005*	5.656±0.022****
IX (AB6)	**12.45±0.173*** **	**9.258±0.009****	**6.688±0.005*** **	**4.624±0.0505*** **
X (AB7)	**11.58±0.095****	**9.23±0.011****	**6.758±0.005*** **	**4.714±0.0612*** **
XI (AB8)	**12.68±0.05*** **	**9.298±0.015****	**6.608±0.005*** **	**4.571±0.0565*** **

Table -10-B: The assessment of haematological parameters

Treatment group	Neutrophils (%) (MEAN±SEM)	Lymphocytes (%) (MEAN±SEM)
I (N. Control)	**13.02±0.0028**	**88.13±0.3965**
II (T. Control)	**82.78±0.075**	**13.1±0.2677**
III (5-FU)	**13.4±0.0408*** **	**90.78±1.309****
IV (AB1)	**13.36±0.00408****	**58.06±7.382***
V (AB2)	**13.45±0.0047****	**65.4±4.798***
VI (AB3)	**13.25±0.0047*** **	**74.53±2.353****
VII (AB4)	**13.31±0.0062****	**61.55±6.573***
VIII (AB5)	**13.42±0.0028****	**57.38±7.576***
IX (AB6)	**13.12±0.0062*** **	**83.68±0.7268****
X (AB7)	**13.19±0.0062*** **	**80.13±0.5308****
XI (AB8)	**13.8±0.0408*** **	**86.39±0.3787****

Values are expressed as MEAN ±SD and MEAN±SEM of 6 animals. The data were statistically analysed by ONE WAY ANOVA followed by Tukey Kramer multiple comparison test.

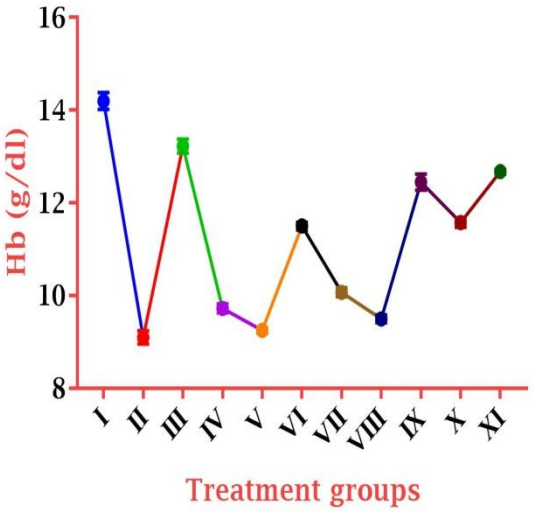


Fig 16: Comparison of Hb level between different groups received EAC tumour cells.

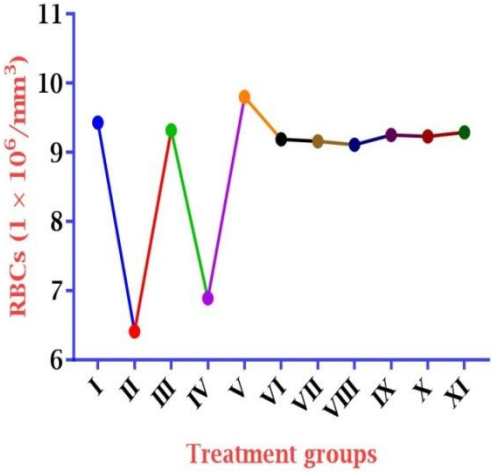


Fig 17: Comparison of RBCs level between different groups received EAC tumour cells.

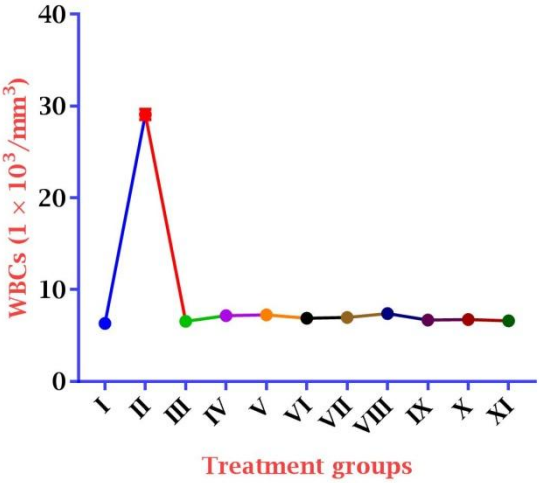


Fig 18: Comparison of WBCs level between different groups received EAC tumour cells.

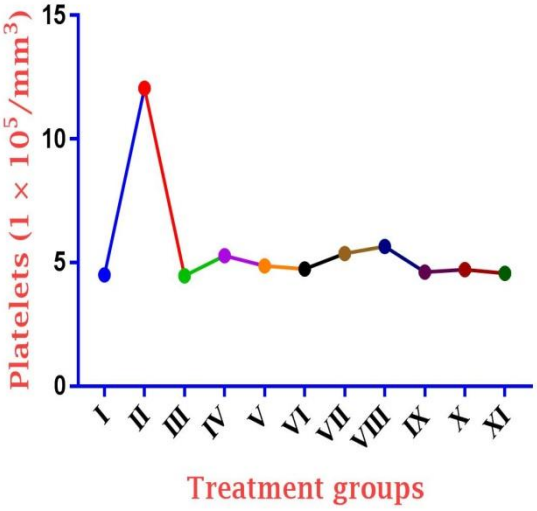


Fig 19: Comparison of platelets count between different groups received EAC tumour cells.

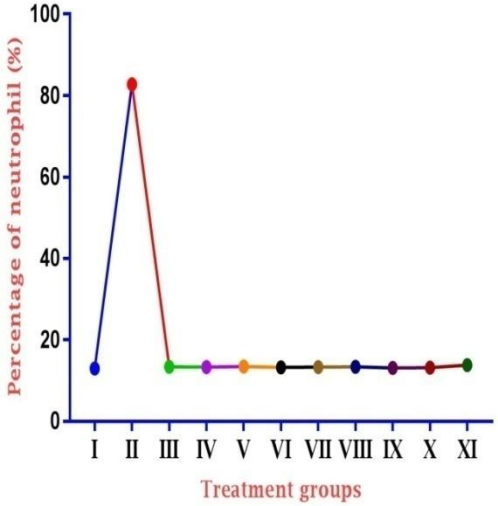


Fig 20: Comparison of % of neutrophil between different groups received EAC tumour cells.

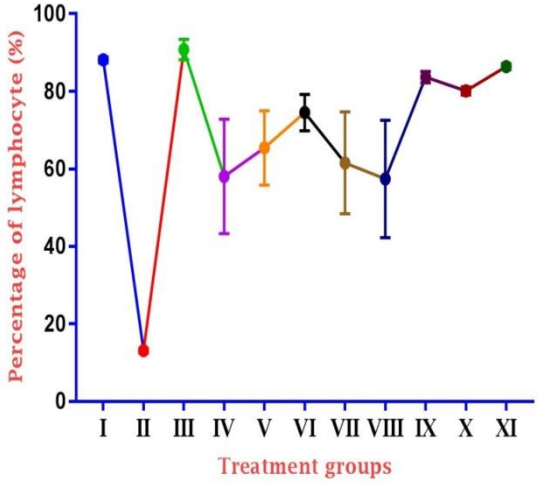


Fig 21: Comparison of % of lymphocyte between different groups received EAC tumour cells

Table-11: Comparison PILS (%) in different treatment groups

Treatment group	MST(days) (MEAN±SEM)	PILS (%)
II (T. Control)	4.65±0.0645	----
III (5-FU)	8.5±0.2887***	95.65
IV (AB1)	7.00±0.4082*	52.17
V (AB2)	6.625±0.2175	45.65
VI (AB3)	7.625±0.0629**	65.21
VII (AB4)	7.1±0.0577*	56.52
VIII (AB5)	6.85±0.0288	50.00
IX (AB6)	8.425±0.025**	82.60
X (AB7)	7.7±0.0577**	77.27
XI (AB8)	8.725±0.0478**	91.30

Values are expressed as MEAN ±SEM of 6 animals. The data were statistically analysed by ONE WAY ANOVA followed by Tukey Kramer multiple comparison test.

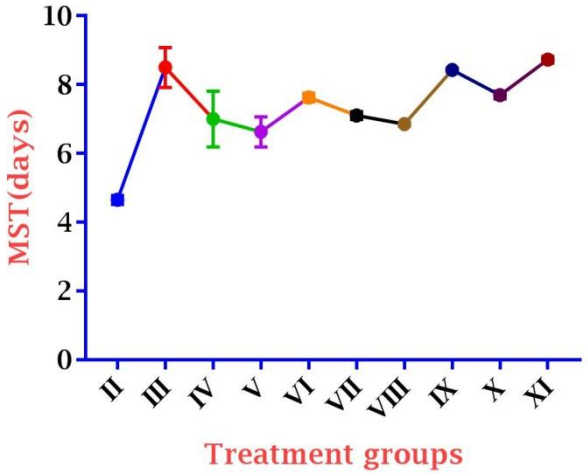


Fig 22: Comparison of MST between different groups received EAC tumour cells

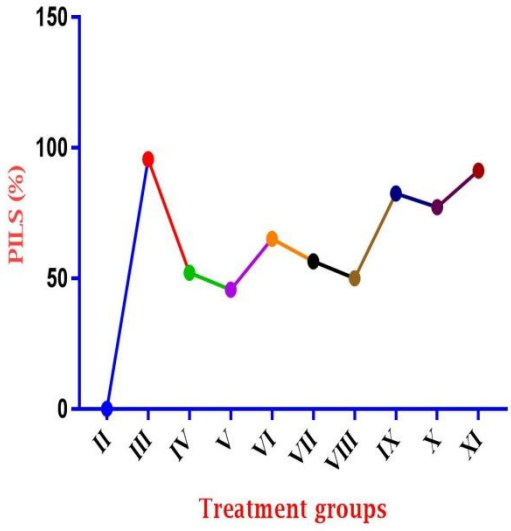


Fig 23: Comparison of PILS between different groups received EAC tumour cells.

Experimental Chemistry

The synthesis of target compounds (AB1-AB8) N-(4-{[substituted phenyl)-1, 3, 4-Oxadiazole-2-yl]methoxy}phenyl) acetamide were carried out by reacting Para acetamidophenol, ethylchloro acetate, hydrazine monohydrate and various aromatic acids. The synthesized compounds were characterized by IR,

NMR, and Mass spectroscopy. The progress of the reaction was monitored by TLC using solvent systems of different polarities. TLC plates are pre-coated silica gel (HF254-200 mesh) aluminium and spots were visualized under U.V chamber and the proposed structures of the synthesized compounds were ascertained by spectral data. All the synthesized compounds having the following solubility profile: Insoluble in water, slightly soluble in chloroform, ethanol, and freely soluble in DMF, DMSO.

Computational Chemistry

Most of the scoring functions in molecular docking are physics based molecular mechanics force fields that estimate the energy of the binding pose; a low (negative) energy indicates a stable system and thus a likely binding interaction. Molecular docking is performed to find out the binding affinity or molecular interaction energy (kcal/mol) of docked compounds. Lowest (negative value) energy of docked molecule indicated high binding affinity with the target protein or compound. In silico molecular docking studies displayed the binding energies (k.cal/ml) of synthesized compounds (AB1-AB8) -1. 20 , -0.92, -3.24, -1.83, -1.04, -3.28, -3.22, -3.29 which indicated that the compound had high binding affinity towards the target protein Topoisomerase I with PDB id 1A36 and inhibit the Topoisomerase I function in comparison with standard drug topotecan (TTN: -2.06 k. cal/ml).

Experimental Oncology

These synthesized compounds (AB1-AB8) were evaluated for their *in vitro* anticancer activity by using SRB assay. A preliminary screening against human colon cancer cell line COLO-205 displayed that the compounds AB1-AB8 (at concentration 300µg/ml) as well as standard drug topotecan at concentration 75 µg/ml were able to inhibit the proliferation of more than 50% cells. It was found that compounds AB1-AB8 displayed anticancer activity with IC_{50} values below 4 µg/ml against COLO-205. In the USNCI screening program a compound is generally considered to have in vitro anticancer activity, if the IC_{50} value following incubation between 48 hrs and 72 hrs is less than 4 µg/ml.

In the present study it was displayed that all the synthesized compounds (AB1-AB8) had the potential ability to inhibit the proliferation of COLO-205 with the highest percentage of growth inhibition AB8 = 92.92±0.0305% > AB6 = 92.32±0.036% > AB7 = 91.74±0.025% > AB3 = 91.59±0.015% > AB4 = 90.89±0.045% > AB1 = 90.6±0.001% > AB5 = 89.23±0.321% > AB2 = 88.9±0.015% etc at dose 300 µg/ml and IC_{50} values of synthesized compounds (AB1-AB8) were found to be 3.89±0.095 µg/ml, 4.133±0.057 µg/ml, 3.233±0.208 µg/ml, 3.767±0.152 µg/ml, 3.39±0.441 µg/ml, 2.433±0.577 µg/ml, 2.767±0.152 µg/ml, 2.0203±0.095 µg/ml and std. drug topotecan (TTN): 94.64±0.01%) found to be 1.973±0.0152 µg/ml.

Experimental Pharmacology

Effect on the haematological parameters: All 6 animals were in each group. The present experimental data displayed that the mortality was less in all groups. The Hb 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.

Effect on the survival: Synthesized compounds AB1-AB8 (100 mg/kg) significantly increased the PILS. While 5-FU increased the life span of 95.65%, and the PILS of synthesized compounds were found to be 52.17%, 45.65%, 65.21%, 56.52%, 50.00%, 82.60%, 77. 27% and 91. 30%. 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 EAC.

7. Conclusion

The *in vitro* experimental data of SRB assay displayed that all the synthesized compounds (AB1-AB8) had the potential ability to inhibit the growth and proliferation of human colon cancer cell line COLO-205, but among these all eight compounds AB3, AB6, AB7 and AB8 possessed potential anticancer activity in vitro assay. The activity of the synthesized compounds increased on dose dependent manner, the compounds displayed the highest inhibition of human colon cancer cell line COLO-205 at 300 µg/ml and it was also proved by percentage of cell viability carried out by Trypan blue exclusion assay. Molecular docking studies also revealed that all the synthesized compounds interact with different amino acids residues of target protein with PDB id 1A36 Topoisomerase I and executed overall binding affinity towards 1A36 Topoisomerase I and data displayed compounds AB3, AB6, AB7 and AB8 had higher binding affinity and hydrogen bonding interaction with target protein than the other compounds and known to be inhibitor Topoisomerase I.

In *vivo* experimental data of EAC mouse model displayed that all the synthesized compounds (AB1-AB8) had the potential ability to inhibit the growth and proliferation of cancer or tumour cells and the highest activity was shown by compounds AB3, AB6, AB7 and AB8 among all eight synthesized compounds which was proved by the assessment of haematological parameters of blood samples of mice and survival time of each mouse.

There was correlation between in vitro anticancer activity and in vivo anti tumour activity of the synthesized compounds. Those compounds executed potential *in vitro* anti cancer activity against human colon cancer cell line COLO-205 and the same compounds possessed well anti tumour activity in EAC mouse model. From this experiment we concluded that the compounds AB3, AB6, AB7 and AB8 were possessed potential anticancer and anti tumour activity among the eight synthesized compounds.

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