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Novel 2, 5 - Disubstituted 1, 3, 4 - Oxadiazole derivatives act as potential anticancer agent against human liver cancer cell line hepG2 and hepatocellular carcinoma in rat model

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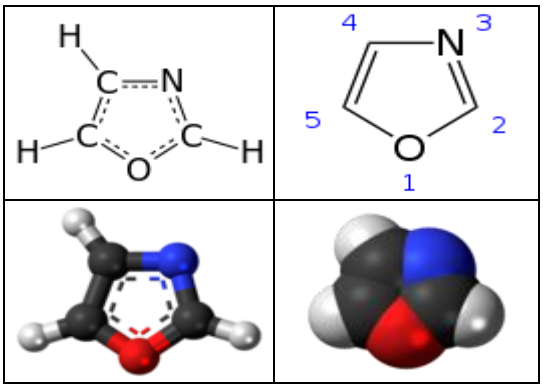
Abstract
Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer in adults, and is the most common cause of death in people with cirrhosis. It occurs in the setting of chronic liver inflammation, and is most closely linked to chronic viral hepatitis infection (hepatitis B or C) or exposure to toxins such as alcohol or aflatoxin. Certain diseases, such as haemochromatosis and alpha 1-antitrypsin deficiency, markedly increase the risk of developing HCC. Metabolic syndrome and NASH are also increasingly recognized as risk factors for HCC. Non-alcoholic fatty liver disease (NAFLD) is a condition in which fat builds up in your liver. Non-alcoholic steatohepatitis (NASH) is a type of NAFLD. If you have NASH, you have inflammation and liver cell damage, along with fat in your liver. As with any cancer, the treatment and prognosis of HCC vary depending on the specifics of tumour histology, size, how far the cancer has spread, and overall health. The *in vitro* anticancer activity of synthesized compounds was carried out by SRB assay where as *in vivo* anticancer activity of synthesized compounds was carried out against DEN and CCl_4 induced hepatocellular carcinoma in rat model. In the present study it was displayed that all the synthesized compounds (AB1-AB8) had the potential ability to inhibit the proliferation of HEPG2 cancer cell with the highest percentage of growth inhibition 93.92%, 92.23%, 89.53%,91.56%, 92.91%, 89.22%, 93.59%, 91.22%, etc. at dose 300 µg/ml and IC_{50} values of synthesized compounds were found to be 2.3 µg/ml, 3.1 µg/ml, 3.6 µg/ml, 3.4 µg/ml, 2.9 µg/ml, 3.9 µg/ml, 2.5 µg/ml, 3.8 µg/ml etc. and std. drug 5-FU (94.26%) found to be 2.2 µg/ml. The *in vivo* experimental data obtained from HCC in rat model displayed that all the synthesized compounds had the potential capability to restore normal hepatocellular status by inhibition of portal tract necrosis, centrilobular degeneration, fibrosis and anaplasia which was indicated by reduction in α-fetoprotein (AFP) by the synthesized compounds (AB1-AB8), a potential tumour marker raised in liver cancer and in addition it was also found that all the synthesized compounds restored the levels of SGOT, SGPT and ALP at the same dose. The apoptosis of the cancer cells caused by the synthesized compounds were also observed and it was indicated that compound AB1; AB7; AB5; AB2; and AB4 were able to significantly induce HEPG2 cells apoptosis among the eight synthesized compounds.

Keywords: Hepatocellular carcinoma; metabolic syndrome; NASH; SRB assay; anaplasia; α-fetoprotein etc.

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 therapeutic agents [3, 4]. One such medicinal important heterocyclic nucleus is oxadiazole moiety. Oxazole is the parent compound for a vast class of heterocyclic aromatic organic compounds. These are azoles with an oxygen and a nitrogen separated by one carbon Oxazoles are aromatic compounds but less so than the thiazoles. Oxazole is a weak base; its conjugate acid has a pKa of 0.8, compared to 7 for imidazole [5, 6]. Oxadiazoles are a class of heterocyclic aromatic chemical compound of the azole family; with the molecular formula $C_2H_2N_2O$. There are four isomers of oxadiazole depending on the position of nitrogen atom in the ring [7].

Table 1: Oxazole



IUPAC NAME: 1, 3-oxazole

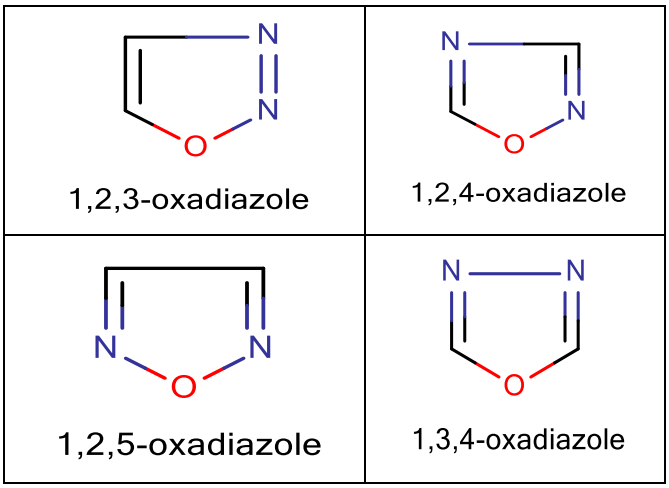
1.1 Chemistry of Oxadiazole

In chemistry, methine is a trivalent functional group =CH−, derived formally from methane. It consists of a carbon atom bound by two single bonds and one double bond, where one of the single bonds is to hydrogen. The group is also called methyne or methene; its IUPAC systematic name is methylidene or methylidene. Oxadiazole is derived from furan by replacement of two methine (-CH=) group by two pyridine type nitrogen (-N=) [8]. 1, 2, 4-Oxadiazole, 1, 2, 5-oxadiazole, and 1, 3, 4-oxadiazole are all known and appear in a variety of pharmaceutical drugs including raltegravir, butyl amine, fasiplon, oxolamine, and pleconaril. The 1, 2, 3-isomer is unstable and ring-opens to form the diazoketone tautomer. Oxadiazole, a very weak base due to inductive effect of the extra heteroatom. The replace of two -CH= groups in furan by two pyridine type (-N=) lowers aromaticity of resulting oxadiazole ring to an extent that the oxadiazole ring exhibit character of conjugated diene. The electrophilic substitutions in oxadiazole ring are extremely difficult at the carbon atom because, the relatively low electron density on the carbon atom which can be attributed to electron withdrawal effect of the pyridine type nitrogen atom. If oxadiazole ring is substituted with electron-releasing groups, the attack of electrophiles occurs at nitrogen. The ring is generally resistant to nucleophilic attack [8]. 1, 3, 4-oxadiazole is a five member 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 [3].

1.2 Physicochemical properties of Oxadiazole

1, 3, 4-oxadiazole is a liquid having boiling point 150 ºC. 2, 5-disubstituted-1, 3, 4-oxadiazole derivatives are colourless substances. The lower alkyl derivatives are liquids which distil. 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 substituent's 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 [9].

Table 2: Oxadiazole



1.3 Biological activity: Biologically active molecules containing oxadiazole moiety possessed a wide range of pharmacological activities such as antimicrobial, anticancer, anticonvulsant, anti-inflammatory and antiviral agents [10], antifungal [11], antimycobacterial [12] etc.

2. Experimental

2.1 Synthetic Chemistry

2.1.1 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 GC mate. 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.

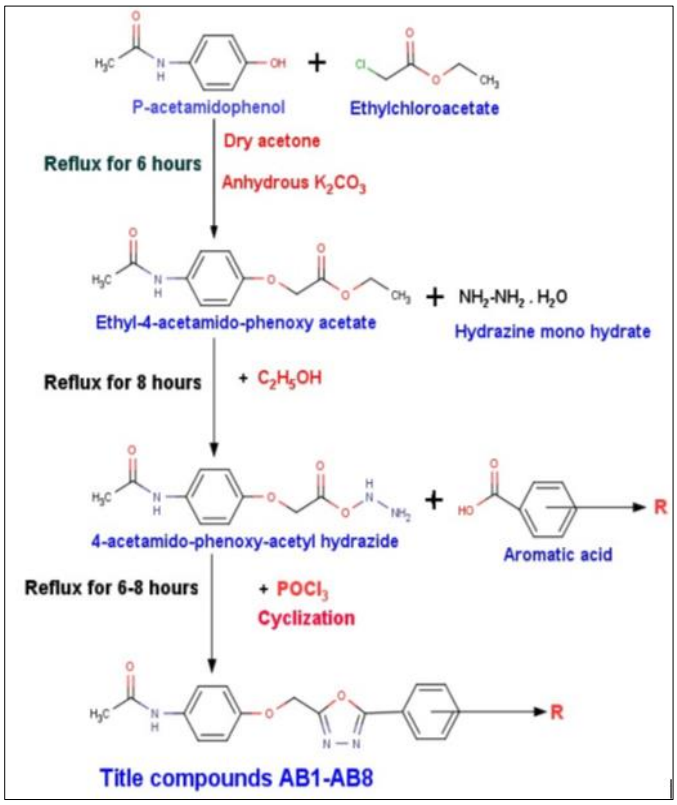
2.1.2 Steps involved in the synthesis of target compounds [13].

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 [14]: 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.

2.1.3 Synthetic scheme



2.1.4 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^{0}C	74.5 %.
2	AB2	$C_{17}H_{13}Cl_2N_3O_3$	378.209	0.74	180^{0}C	69.9%
3	AB3	$C_{17}H_{14}FN_3O_3$	327.309	0.75	189^{0}C	74%
4	AB4	$C_{17}H_{14}BrN_3O_3$	388.215	0.65	183^{0}C	69%
5	AB5	$C_{17}H_{13}BrN_4O_5$	433.213	0.64	166^{0}C	60%
6	AB6	$C_{17}H_{14}N_4O_5$	354.31	0.72	171^{0}C	64%
7	AB7	$C_{17}H_{13}N_5O_7$	399.31	0.68	204^{0}C	78%
8	AB8	$C_{17}H_{13}N_5O_8$	415.31	0.72	215^{0}C	68%

2.1.5 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).

2.2 Experimental Oncology
2.2.1 Cell culture
The cell cultures of human liver cancer cell line HEPG2 was provided by Amla Cancer Research Centre, Thrissur, Kerala and were 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.

2.2.2 Human liver cancer cell line HEPG2
HEPG2 (hepatoblastoma cell line HepG2) is a perpetual cell line which was derived from the liver tissue of a 15-year-old Caucasian American male with a well-differentiated hepatocellular carcinoma. These cells are epithelial in morphology, have a modal chromosome number of 55, and are not tumourigenic in nude mice [15]. The cells secrete a variety of major plasma proteins, e.g., albumin, transferring, and the acute-phase proteins fibrinogen, alpha 2-macroglobulin, alpha 1-antitrypsin, transferring, and plasminogen. They have been grown successfully in large-scale cultivation systems. Hepatitis B virus surface antigens have not been detected. HepG2 will respond to stimulation with human growth hormone.

2.2.3 Standard drug
5-FU was used as standard anticancer drug for the evaluation of *in vitro* anticancer activity.

2.2.4 Evaluation of *in vitro* anticancer activity
The *in vitro* anticancer activity of synthesized compounds was evaluated by SRB assay against liver cancer cell line (HEPG2 or hepatoblastoma cell line HepG2).

Principle: 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 [16]. The dye has maximal absorbance at 565 nm light and maximal fluorescence emission at 586 nm light [1]. It does not exhibit pH-dependent absorption or fluorescence over the range of 3 to 10 [17]. Sulforhodamine B is often used as a membrane-impermeable polar tracer [18] or used for cell density determination via determination of cellular proteins (Cytotoxicity assay) [19]**.** IUPAC name: 2-(3-diethylamino-6-diethylazaniumylidene-xanthen-9-yl)-5-sulfo-benzenesulfonate.
Sulforhodamine 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 [20, 21].

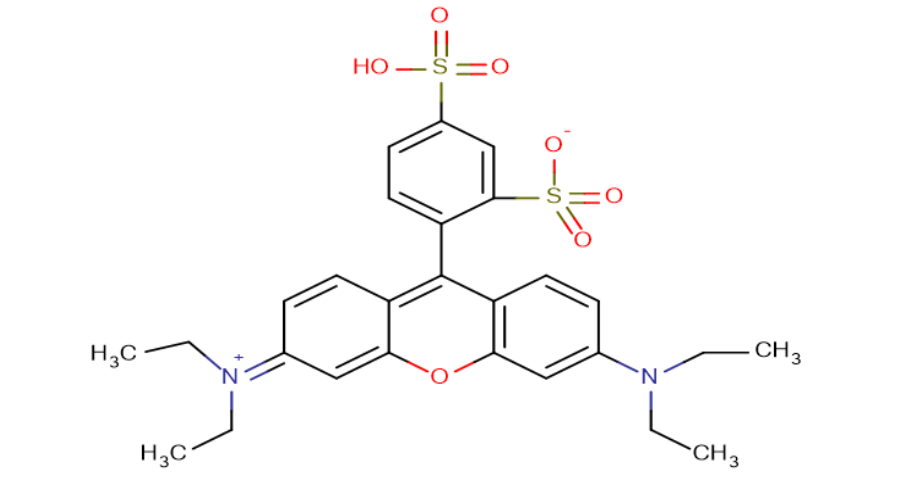


Fig 1: Structure of Sulforhodamine B

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).

Protocol
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 plates, 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 4^0c 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 [22]. 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 anticancer (Test): cells treated with a anticancer drug/chemical +SRB + solubilising buffer
Negative control for anticancer (control): cells left untreated + SRB + solubilising buffer.
Blank: medium without cells + SRB + solubilising buffer.

2.2.5 Experimental Histopathology
Protocol for H33342 staining [23]: Prepare the Hoechst dye stock solution by dissolving the contents of one vial (100 mg) in 10 ml of deionised water (diH_2O) to create a 10 mg/ml (16.23 mm) solution. Note: Hoechst dye has poor solubility in water, so sonicate as necessary to dissolve. The 10 mg/ml Hoechst stock solution may be stored at 2–6°C for up to 6 months or at –20°C for longer periods.

Labelling cells: H33342 staining was used to confirm whether the characteristic nuclear changes were associated with synthesized compounds in this assay. HEPG2 cells were cultured in 12-well plates (5×10^4 cells/well), which were placed in a humidified atmosphere for 24 h at 37 °C with 5% CO_2 and then each plate was treated with synthesized compounds making the final concentration of 300 µg/ml respectively. After 72 h, cell culture medium was discarded and the cells were washed twice with PBS. H33342 staining was then performed for 2 min and washed with water. In the end, the cells were observed and photographed under inverted phase-contrast microscope at a magnification of 200X.

2.2.6 Annexin V staining protocol for apoptosis [24, 25, 26]
A. Incubation of cells with annexin V-FITC
1. Induce apoptosis by desired method.
2. Collect 1–5 x 10^5 cells by centrifugation.
3. Resuspend cells in 500 µL of 1X binding buffer.
4. Add 5 µL of annexin V-FITC and 5 µL of propidium iodide (PI, optional).
5. Incubate at room temperature for 5 min in the dark.
6. Proceed to B or C below depending on method of analysis.

B. Quantification by flow cytometry
Analyze annexin V-FITC binding by flow cytometry (Ex = 488 nm; Em = 350 nm) using FITC signal detector (usually FL1) and PI staining by the phycoerythrin emission signal detector (usually FL2). For adherent cells, gently trypsinize and wash cells once with serum-containing media before incubation with annexin V-FITC (Steps A. iii–v).

C. Detection by fluorescence microscopy
1. Place the cell suspension from Step A.5 on a glass slide. Cover the cells with a glass cover slip. For analyzing adherent cells, grow cells directly on a cover slip. Following incubation (Step A. v), invert cover slip on a glass slide and visualize cells. The cells can also be washed and fixed in 2% formaldehyde before visualization. (Cells must be incubated with annexin V-FITC before fixation since any cell membrane disruption can cause non-specific binding of annexin V to PS on the inner surface of the cell membrane).
2. Observe the cells under a fluorescence microscope using a dual filter set for FITC and rhodamine. Cells that have bound annexin V-FITC will show green staining in the plasma membrane. Cells that have lost membrane integrity will show red staining (PI) throughout the nucleus and a halo of green staining (FITC) on the cell surface (plasma membrane).

2.3 Experimental Pharmacology
2.3.1 Experimental animals and standard drug
White female albino Wister rats weighing about 120-150 gm was used. They were obtained from the animal house of Anurag Pharmacy College, Kodad-508206 and Telangana State. They were kept under observation for about 7 days before onset of experiment to exclude any inter current infection, had free access to normal diet and water. The experimental protocol was approved by IAEC (Institutional Animal Ethics Committee) of CPCSEA: 1712/P0/a/13/CPCSEA. The standard drug 5-FU was purchased from retail local shop.

2.3.2 Evaluation of acute oral toxicity
In the present study acute oral toxicity of the synthesized compounds were performed by acute toxic class method according to OECD guideline-423 [27]. In this method the toxicity of the synthesized compounds were tested using a step wise procedure, each step using four female white albino rats of matched weight (120-150 g) for the study. The rats were fasted prior to dosing (food but water should be withheld) for three to four hours. Following the period of fasting the animal should be weighted and synthesized compounds were administered. The test compound was dissolved in 3% CMC, administered orally to the different groups with decreasing doses mg/kg b. w. Mortality was determined after 24 hours of treatment. 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 no mortality observed with the above dose. The dose at which 50% of rat survived was considered the LD_{50} value of the compound. Test compound dose reduced by specific intervals. The mortality was not observed at the dose 2000 mg / kg, i, e the LD_{50} cut of value mg/kg b. w. was found to be 2000. So 100 mg /kg body weight was selected for *in vivo* pharmacological evaluation. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC).

2.3.3 Assessment of *in vivo* hepatocellular carcinoma (HCC) [28, 29, 30].
Animals and grouping: Sixty six female albino rats of matched weight (120-150 g) were taken for the study. Rats were maintained according to the standard guidelines of

Institutional Animal Care. Animals were fed a semi-purified diet that contained (g/kg): 200 casein, 555 sucrose, 100 cellulose, 100 fat blends, 35 vitamin mix, and 35 mineral mixes. The animals were divided equally into the following groups each group contained six animals:

Group-I: Normal control (3% CMC+ Vehicle).
Group-II: Negative control (50 mg of diethyl nitrosamine + 3ml of CCl_4)-HCC (hepatocellular carcinoma).
Group-III- X: AB1-AB8 [Group-IV: AB1, Group-V: AB2, Group-VI: AB3, Group-VII: AB4, Group-VIII: AB5, Group-IX: AB6, Group-X: AB7 and Group-XI: AB8 (50 mg of diethyl nitrosamine+3ml of CCl_4+ 100 mg of synthesized compounds)].
Group-XI-Standard: 100 mg of 5-FU+50 mg of diethyl nitrosamine + 3 ml of CCl_4.

2.3.4 Preparation of HCC model
Hepatocellular carcinogenesis was induced chemically in rats by injection of a single intra peritoneal dose of diethyl nitrosamine (DEN) at a dose of 50 mg/kg b. wt. followed by weekly subcutaneous injections of CCl_4 at a dose of 3 ml/kg. b. wt. for 6 weeks.

2.3.5 Collection of blood
On the 8th day, blood was collected by retro orbital puncture, under mild ether anaesthesia after 8 hr fasting. Blood samples were centrifuged at 3000 rpm for 20 mints. Serum was separated and stored at – 20 ^{0}C until biochemical estimation was carried out.

2.3.6 Biochemical Analysis
The Serum samples were analyzed for alanine aminotransferase (ALT) (SGPT); aspartate aminotransferase (AST) (SGOT); alkaline phosphatase (ALP); serum Bilirubin and α-feto protein (AFP). At the proper time of scarification, blood samples were assessed for α- fetoprotein (AFP) by ELISA (provided by Diagnostic Systems Laboratories, Inc., Web star, Texas, USA).

2.3.7 Histopathological Analysis
Samples of livers from control and experimental groups were fixed in 10% neutral buffered formalin. The standard method of dehydration, clearing in xylene, and paraffin embedding was used. Sections of 5-µm thickness were cut by a rotary microtome and stained with Masson's Trichrome (Bancroft and Gamble 2008). Sections were examined by light microscopy.

3. Results and Discussion
Evaluation of *in vitro* anticancer activity by SRB assay

Table 3: For percentage (%) of cell growth inhibition of Oxadiazole against HEPG2 at 100 µg/ml by SRB Assay

Compound Codes	Concentrations	Absorbance	PCGI (%)
AB1*	100 µg/ml	0.0637±0.0005	78.02±0.516
AB2*	100 µg/ml	0.077±0.0015	74.39±1.506
AB3	100 µg/ml	0.303±0.418	68.19±0.572
AB4	100 µg/ml	0.0807±0.005	73.33±0.968
AB5*	100 µg/ml	0.0752±0.0005	74.84±0.233
AB6	100 µg/ml	0.0832±0.0005	71.48±0.678
AB7*	100 µg/ml	0.0685±0.0005	76.85±0.219
AB8	100 µg/ml	0.0837±0.0005	71.16±0.671
5-FU***	75 µg/ml	0.0167±0.0005	94.27±0.014
Control		0.2965±0.00057	0

Table 4: For percentage (%) of cell growth inhibition of Oxadiazole against HEPG2 at 200 µg/ml by SRB Assay

Compound Codes	Concentrations	Absorbance	PCGI (%)
AB1*	200 µg/ml	0.0447±0.0005	84.68±0.169
AB2*	200 µg/ml	0.051±0.0008	82.88±0.134
AB3	200 µg/ml	0.0622±0.0009	78.35±0.523
AB4	200 µg/ml	0.0542±0.0012	81.86±0.134
AB5*	200 µg/ml	0.0485±0.001	83.62±0.24
AB6	200 µg/ml	0.061±0.0014	74.7±0.647
AB7*	200 µg/ml	0.048±0.0008	83.84±0.063
AB8	200 µg/ml	0.059±0.0014	81.23±0.134
5-FU***	75 µg/ml	0.0167±0.0005	94.27±0.014
Control		0.296±0.0008	0

Table 5: For percentage (%) of cell growth inhibition of Oxadiazole against HEPG2 at 300 µg/ml by SRB Assay

Compound Codes	Concentrations	Absorbance	PCGI (%)
AB1**	300 µg/ml	0.0172±0.0009	93.46±0.6505
AB2**	300 µg/ml	0.0225±0.0005	91.61±0.876
AB3	300 µg/ml	0.03±0.0008	89.22±0.438
AB4*	300 µg/ml	0.0255±0.0005	91.34±0.311
AB5**	300 µg/ml	0.0202±0.0009	92.85±0.0919
AB6	300 µg/ml	0.0997±0.1402	89.07±0.212
AB7**	300 µg/ml	0.018±0.0011	93.29±0.424
AB8*	300 µg/ml	0.025±0.0014	91.06±0.233
5-FU***	75 µg/ml	0.0162±0.0005	94.27±0.0141
Control		0.2955	0

Table 6: For IC_{50} values for the synthesized compounds at 300 (µg/ml) against HEPG2 cell line

Compound Codes	IC_{50} values (µg/ml)
AB1	2.237±0.1484***
AB2	3.08±0.05292**
AB3	3.517±0.132
AB4	3.33±0.06083*
AB5	2.803±0.1002**
AB6	3.827±0.06429
AB7	2.47±0.06083***
AB8	3.907±0.01528
5-FU	2.17±0.06083***

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

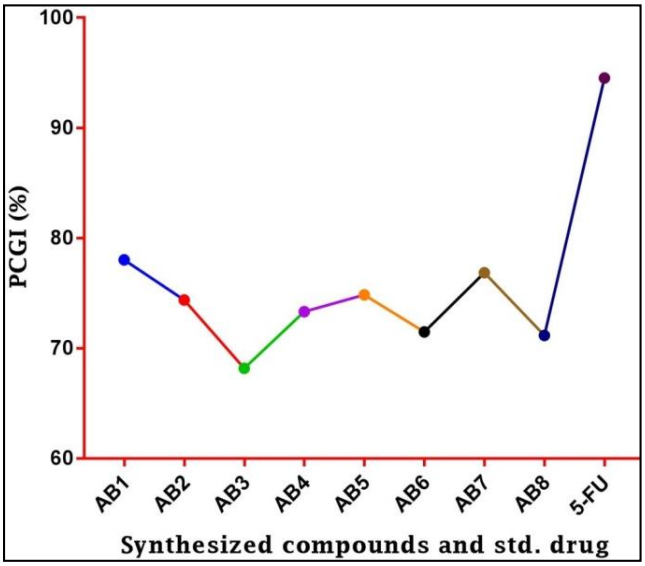


Fig 2: Percentage of cell growth inhibition at 100 µg/ml.

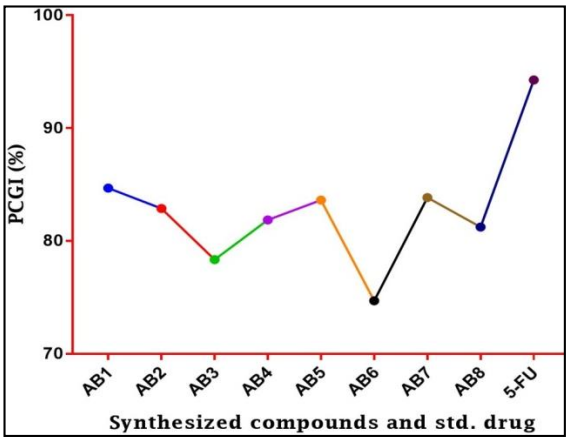


Fig 3: Percentage of cell growth inhibition at 200 µg/ml.

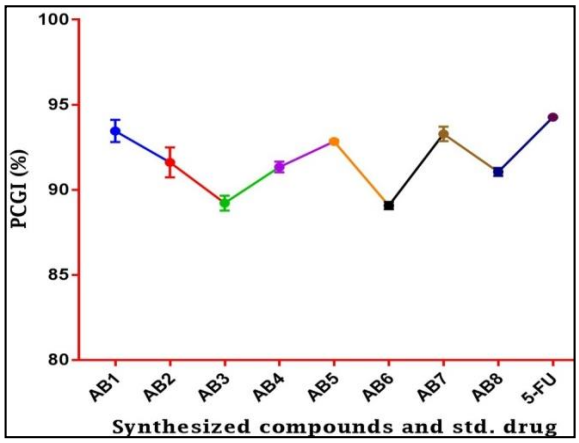


Fig 4: Percentage of cell growth inhibition at 300 µg/ml.

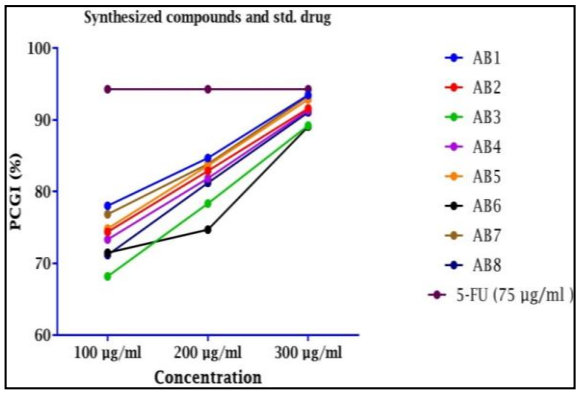


Fig 5: Comparison of percentage of cell growth inhibition by the synthesized Compounds and std. drug against HEPG2 cell line

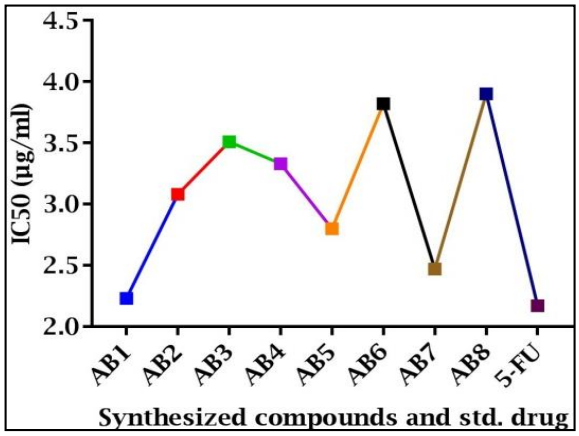


Fig 6: Comparison of IC_{50} values for the synthesized compounds

Apoptosis analysis

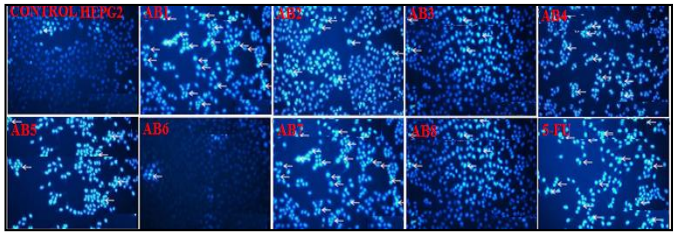


Fig 7: Hoechst 33342 staining assay of HEPG2 apoptosis. Nuclear condensation and/or fragmentation represent cell apoptosis. Images were taken at a magnification of 200X. Arrows marked the apoptotic cells for representing nuclear condensation/fragmentation. Annexin V-FITC/propidium iodide (PI) assay

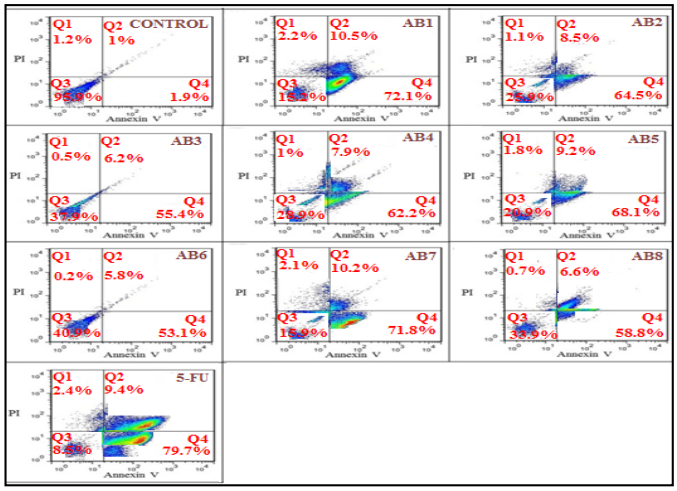


Fig 8: Apoptosis analysis by FCM using Annexin V-FITC/PI staining on the HEPG2 cells treated by synthesized compounds (AB1-AB8 at dose of 300 µg/ml); Tumour control without compound.

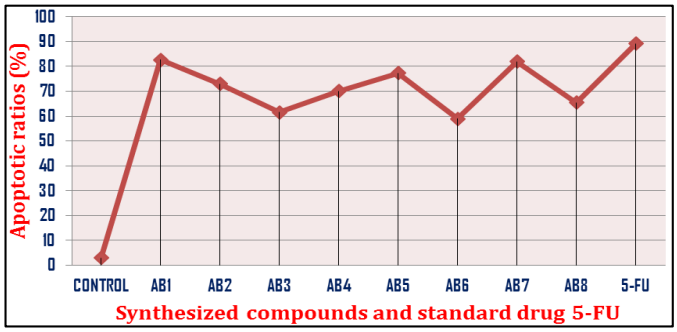


Fig 9: Percentage of HEPG2 cells death by synthesized compounds

SRB assay: These synthesized compounds (AB1-AB8) were evaluated for their *in vitro* anticancer activity by using SRB assay. A preliminary screening against human liver cancer cell line (HEPG2 displayed that the compounds AB1-AB8 (at concentration 300µg/ml) as well as standard drug 5-FU 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 HEPG2 cancer cell lines. 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 HEPG2 cancer cell with the highest

percentage of growth inhibition 93.92%, 92.23%, 89.53%,91.56%, 92.91%, 89.22%, 93.59%, 91.22%, etc at dose 300 µg/ml and IC_{50} values of synthesized compounds were found to be 2.3 µg/ml, 3.1 µg/ml, 3.6 µg/ml, 3.4 µg/ml, 2.9 µg/ml, 3.9 µg/ml, 2.5 µg/ml, 3.8 µg/ml etc. and std. drug 5-FU (94.26%) found to be 2.2 µg/ml.

H33342 staining on HEPG2 cells: In the tumour control, HEPG2 cell nuclei displayed a normal and complete blue appearance. By contrast, in HEPG2 cells treated with synthesized compounds AB1-AB8 and with standard drug 5-FU, the cells displayed cell shrinkage, the cells were smaller in size, the cytoplasm was dense and the organelles were more tightly packed, enhance pyknosis which was mainly due to the chromatin condensation and these were typical characteristic features associated with cellular apoptosis. When compared with standard drug 5-FU, nuclear pyknosis and fragmentation in HEPG2 cells were significantly increased by treatment with compounds AB1< AB7< AB5< AB2< and AB4 among the eight synthesized compounds.

Annexin V-FITC/propidium iodide (PI) assay: The effects of synthesized compounds on apoptosis in HEPG2 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 results were shown in Fig. below. The apoptosis ratios of synthesized compounds were found as followed: AB1=82.6%; AB2=73%; AB3=61.6%; AB4=70%; AB5=77.3%; AB6=58.9%; AB7=82% and AB8=65.4% respectively, while that of the control was 3%. When compared with standard drug 5-FU (89.1%) it was indicated that compound AB1>AB7>AB5>AB2>AB4 were able to significantly induce HEPG2 cells apoptosis.

Evaluation of *in vivo* anticancer activity against HCC in rat model

Table 7: For the assessment of Biochemical parameters

Treatment Group	AST(SGOT) IU/L	ALT(SGPT) IU/L	ALP(SALP) IU/L	Sr. bilirubin mg/dL	Sr. AFP µg/ml
Group I	51.15±0.022	46.65±0.264	50.35±0.238	0.71±0.014	15.11±0.083
Group II	202.5±0.365	195.6±0.388	285.2±0.086	8.85±0.057	55.14±0.043
Group III (AB1)	78.93±0.492	75.32±0.383	83.23±0.330	2.395±0.005	32.8±0.081
Group IV (AB2)	90.18±0.25	88.43±0.359	89.07±0.098	4.35±0.238	34.72±0.166
Group V (AB3)	87.79±1.318	91.14±0.209	90.13±0.031	6.238±0.106	37.09±0.059
Group VI (AB4)	91.32±0.196	96.6±0.58	99.13±0.090	5.75±0.238	38.65±0.042
Group VII (AB5)	85.01±0.416	83.49±0.329	87.02±0.053	3.8±0.081	34.61±0.085
Group VIII (AB6)	93.16±0.238	90.51±0.118	90.55±0.11	5.325±0.221	36.05±0.06
Group IX (AB7)	81.5±1.008	79.82±0.167	84.76±0.083	3.45±0.057	33.01±0.017
Group X (AB8)	90.46±0.289	89.37±0.104	92.75±0.129	5.3±0.081	36.83±0.602
Group XI (5-FU)	67.9±0.588	58.6±0.294	63.28±0.206	2.025±0.32	25.13±0.095

Table 8: For the declined percentage of Biochemical parameters

Treatment Group	AST(SGOT) %	ALT(SGPT) %	ALP(SALP) %	Bilirubin %	AFP %
Group III (AB1)	59.41	68.40	69.98	50.72	40.29
Group IV (AB2)	55.80	52.74	67.77	46.37	36.66
Group V (AB3)	62.48	55.30	68.47	52.17	32.84
Group VI (AB4)	55.46	50.23	65.9	43.47	29.94
Group VII (AB5)	55.90	56.84	69.49	65.21	37.20
Group VIII (AB6)	60.89	57.25	70.96	62.31	35.22
Group IX (AB7)	61.29	55.40	69.53	36.66	40.10
Group X (AB8)	57.68	54.27	67.28	63.76	34.30
Group XI (5-FU)	66.13	69.81	77.91	72.46	54.44

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

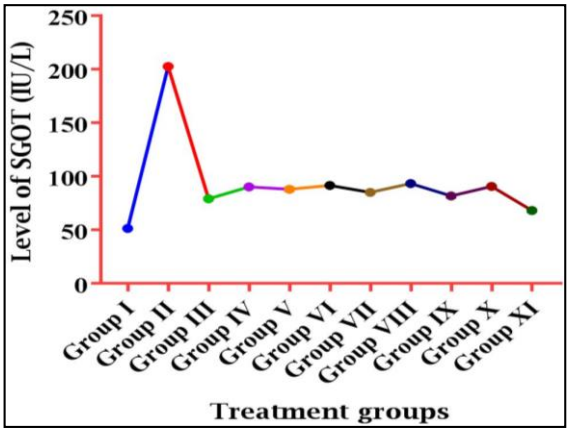


Fig 10: Comparison of SGOT in different treatment groups

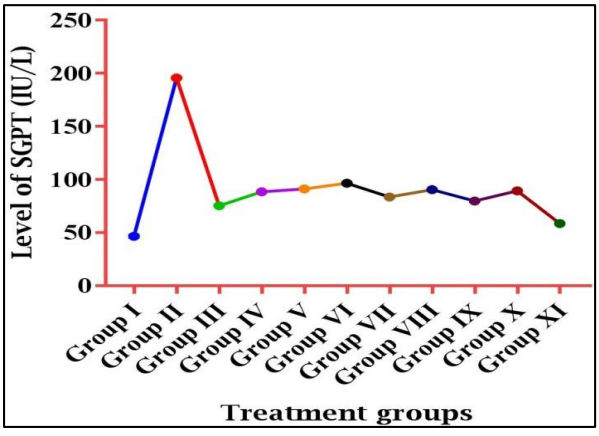


Fig 11: Comparison of SGPT in different treatment groups

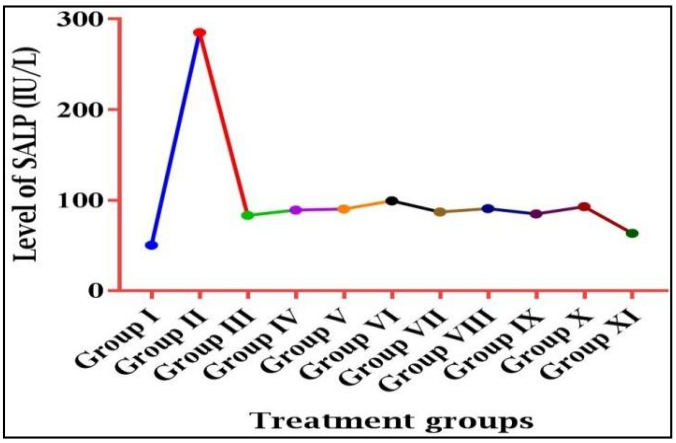


Fig 12: Comparison of serum SAP in different treatment groups

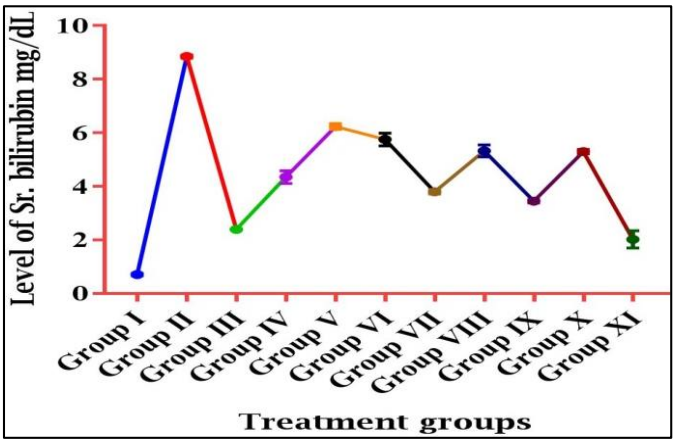


Fig 13: Comparison of serum bilirubin in different treatment groups

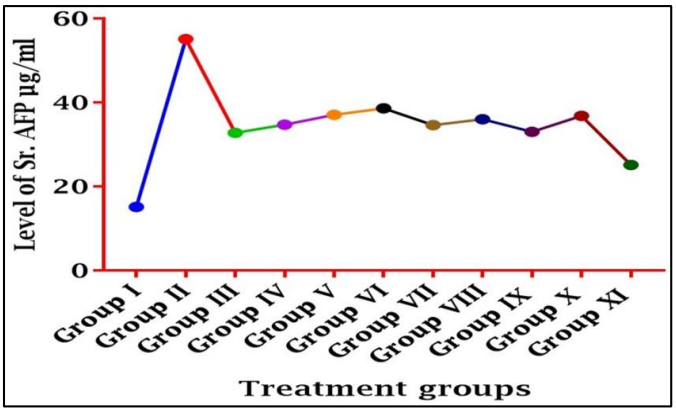


Fig 14: Comparison of serum AFP in different treatment groups

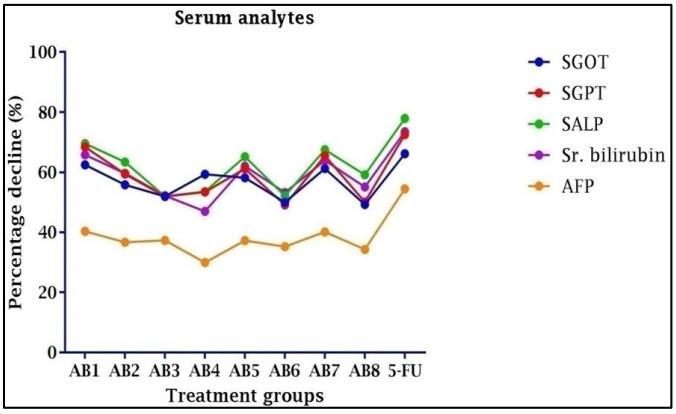


Fig 15: Comparison of % decline of serum analytes in different treatment groups

Histopathological analysis

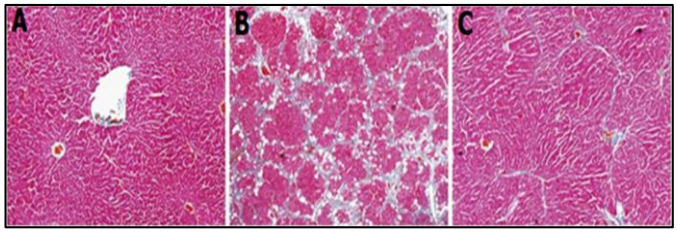


Fig 16: Masson Trichrome examinations (original magnification X40) of liver sections in rats. A: Normal group (I); B: DEN+CCl_4-treated group (II); C: DEN+CCl_4+100 mg of 5-FU-treated group (XI).

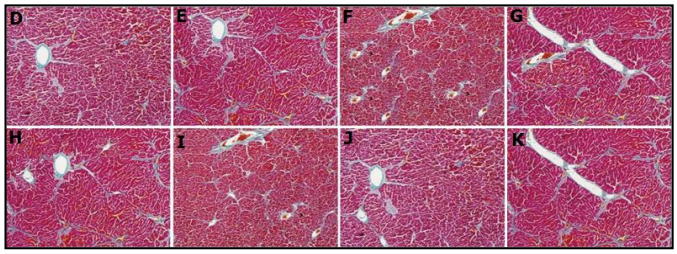


Fig 17: Masson Trichrome examinations (original magnification X40) of liver sections in rats. D: DEN+CCl_4+AB1-treated group (III); E: DEN+CCl_4+AB2-treated group (IV); F: DEN+CCl_4+AB3-treated group (V); G: DEN and CCl_4+AB4-treated group (VI); H: DEN+CCl_4+AB5-treated group (VII); I: DEN+CCl_4+AB6-treated group (VIII); J: DEN+CCl_4+AB7-treated group (IX) and K: DEN+CCl_4+AB8-treated group (X) etc.

In HCC rat model: The activity was evaluated by administration of synthesized compounds at 100 mg /kg body weight in albino Wistar rats by oral route against DEN and CCl_4 induced hepatocellular carcinoma and all the compounds were displayed potential capability to restore normal hepatocellular status by inhibition of portal tract necrosis, centrilobular degeneration, fibrosis and anaplasia which was indicated by reduction in α-fitorotein (AFP) by the synthesized compounds (AB1-AB8), a potential tumour marker raised in liver cancer and in addition it was also found that all the synthesized compounds restored the levels of SGOT, SGPT and ALP at the same dose.

Histopathological analysis: Histopathology of liver tissues treated with the synthesized compounds (Figure: 16 and 17) confirmed its anticancer activity. Histopathological analysis displayed following microscopical features. A: Normal hepatocytes with poorly defined classical hepatic lobules and central veins hexagonal hepatocyte with central nucleus and prominent nucleolus arranged in strands around the central vein and blood sinusoids B. Characterized by large anaplastic carcinoma cells with eosinophilic cytoplasm, large hyperchromatic nuclei and prominent nucleoli. The normal trabecular structure of the liver is distorted. C, D, E, H and J displayed no nodularity & liver cells and lobules appear normal with ballooning degeneration normal portal tracts, no fibrosis and no inflammation where as F, G, I and K indicated prominent nucleoli, mild eosinophilic cytoplasm, necrotic hepatocytes, inflammation with ballooning degeneration and the normal trabecular structure of the liver is moderately restored.

There was a good correlation between *in vitro* and *in vivo* anticancer activity of synthesized compounds. The results obtained from *in vitro* anticancer activity of synthesized

compounds matched with *in vivo* anticancer activity, it displayed that those compounds (compounds AB1, AB2, AB5 and AB7) showed a potential cell growth inhibition and apoptosis of human liver cancer cell line HEPG2 and same results obtained from *in vivo* experiments i, e compounds AB1, AB2, AB5 and AB7 inhibit the hepatocellolar carcinoma also caused by CCl_4 and DES in rat model and it was proved by analysis of tumour marker α-fetoprotein from serum of rat model and this marker raised only in case of hapatocellular degeneration, hepatic necrosis, cirrhosis etc.

4. Conclusion

The experimental data obtained from *in vitro* anticancer studies by SRB assay revealed that compounds AB1, AB2, AB5 and AB7 potentially inhibit the growth and proliferation of human liver cancer cell line HEPG2 and also caused apoptosis of HEPG2 cell line and apoptosis analysis was carried out by FCM using Annexin V-FITC/PI and the *in vivo* anticancer activity which was carried out against carbon tetra chloride and diethyl nitrosamine induced rat model also displayed that the compounds AB1, AB2, AB5, and AB7 possessed potential anticancer activity against hepatocellular carcinoma among the all synthesized compounds.

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