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SYNTHESIS, CHARACTERIZATION AND EVALUATION OF HEPATOCYTES REGENERATOR POTENTIALITY OF SOME NOVEL OXADIAZOLE DERIVATIVES FOLLOWED BY MOLECULAR DOCKING AGAINST NF-KB

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Keywords:

NF-κB, transcription, IR, NMR, Hepatotoxin, Molecular docking etc

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ABSTRACT: NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that controls transcription of DNA, cytokine production and cell survival. Incorrect regulation of NF-κB has been linked to cancer, inflammatory and autoimmune diseases and improper immune development. The main objective of the present research work was the synthesis of N-(4-{[5-(substituted phenyl)-1,3,4-oxadiazol-2-yl] methoxy}phenyl) acetamide derivatives and to evaluate the hepatocytes regenerator potentiality by molecular docking with 2V2T-NF-KB and as well as *In vivo* methods and the synthesized compounds were characterized by IR, NMR, and Mass spectroscopy. The *in-vivo* Hepatoprotective activity was carried out by using albino rats where CCl_4 was used as a hepatotoxin. Molecular docking is performed to find out the binding affinity or molecular interaction energy (kcal/mol) of docked compounds. *In silico* molecular docking studies displayed the binding energies: -5.17, -5.52, -5.40, -4.60, -4.60, -4.87, -3.42, -3.85 k.cal/mol, of the synthesized compounds (AB1-AB8) which indicated that the compound had the high binding affinity towards the 2V2T-NF-KB protein and inhibit the NF-KB protein function in comparison with std. drug silymarin (-3.54 k.cal/mol). The in vivo experimental data displayed that the elevated levels of SGOT, SGPT, ALP and Sr. bilirubin were mainly due to CCl_4 intoxication, reduced significantly (*P<0.05) in rats, after treatment with synthesized compounds at dose of both 250 and 500 mg/kg body weight respectively. Although both the doses (250 and 500 mg/kg b. w.) of synthesized compounds executed *in vivo* hepatocytes regenerator potentiality, but the higher dose (500 mg/kg b. w.) was more effective and more significant.

INTRODUCTION: 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.

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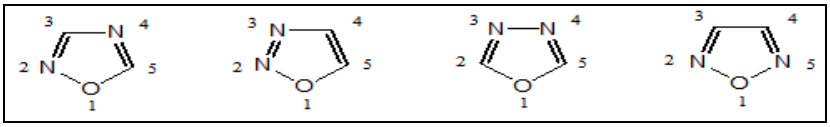
The group is also called methyne or methene; its IUPAC systematic name is methylylidene or methanylylidene. Oxadiazole is derived from furan by replacement of two methine (-CH=) group by two pyridine type nitrogen (-N=). There are four possible isomers of oxadiazole forms [1] (1, 2, 3, and 4) depending on the position of nitrogen atom in the ring and are numbered as shown in following figure.

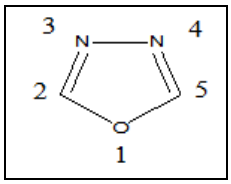
Oxadiazole, a very weak base due to inductive effect of the extra heteroatom. The replace of two -CH= groups in furan by two pyridine type (-N=)

lowers aromaticity of resulting oxadiazole ring to an extent that the oxadiazole ring exhibit character of conjugated diene. The electrophillic 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 [1].



1 2 3 4

1, 3, 4-oxadiazole is a five membered heterocyclic aromatic compound containing two nitrogen atom at position three and four and one oxygen atom present at position one. 1,3,4 oxadiazole is thermally stable than other oxadiazoles, these oxadiazole are very important compound in medicinal chemistry due to their biological activities, during last few years [2].



1, 3, 4-oxadiazole

The various literature survey were reported that 1, 3, 4-oxadiazoles a class of five member heterocyclic compounds possessed a wide range of biological activities such as antimicrobial [3], anti-inflammatory [4], antifungal [5], anticonvulsant [6], anthelmintics [7], analgesic [8], insecticidal [9], local anesthetic [10], antidiarrheal [1], anticancer [12], hypoglycaemic [13], protease inhibitor-resistant HIV-1 [14], vasorelaxant [15] etc.

2. Experimental:

2.1. Chemistry:

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

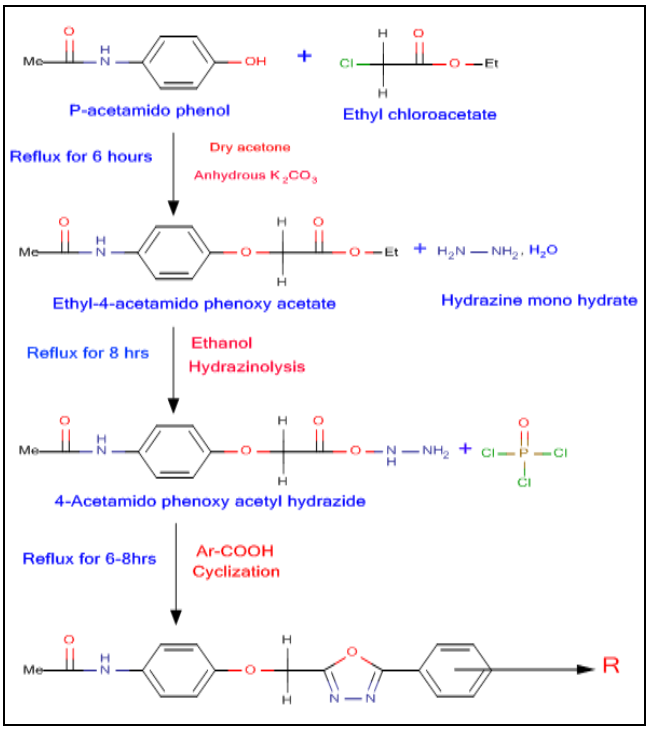
2.1.2. Steps involved in the synthesis of target compounds: [16]

Step 1: Ethyl-4-acetamido phenoxy acetate: A mixture of p-acetamido phenol (0.01mol) and ethyl chloroacetate (0.01mol) 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 [17]: A mixture of 4-Acetamido phenoxy acetyl hydrazide (0.01mol) and various aromatic acids (0.01mol) 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:



Title compounds (AB1-AB8)

2.1.4. Spectral and other physical data of synthesized compounds:

Compound AB1: N-(4-{[5-(4-aminophenyl)-1,3,4-oxadiazol-2-yl]methoxy}phenyl) acetamide. M.F- $C_{17}H_{16}N_4O_3$, M.W 324.33, m. p 116^{0}C, R_f – 0.77, Yield-74.5 %. 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.. M.F- $C_{17}H_{13}Cl_2N_3O_3$, M.W 378.209, m. p 180 ^{0}C, R_f –0.74, Yield-69.9 %. 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. M.F- $C_{17}H_{14}FN_3O_3$, M.W 327.309, m. p 189 ^{0}C, R_f – 0.75, Yield-74 %. 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. M.F- $C_{17}H_{14}BrN_3O_3$, M.W 388.215, m. p 183 ^{0}C, R_f – 0.65, Yield-69 %. 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 - nitro phenyl)-1,3,4-oxadiazol-2-yl] methoxy} phenyl) acetamide. M.F- $C_{17}H_{13}BrN_4O_5$, M.W 433.213, m. p 166 ^{0}C, R_f – 0.64, Yield-60 %. 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. M.F- $C_{17}H_{14}N_4O_5$, M.W 354.31, m. p 171 ^{0}C, R_f – 0.72, Yield-64%. 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. M.F- $C_{17}H_{13}N_5O_7$, M.W 399.31, m. p 204 ^{0}C, R_f – 0.68, Yield-78%. 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-dinitro phenyl) - 1,3,4 - oxadiazol-2-yl] methoxy} phenyl) acetamide. M.F- $C_{17}H_{13}N_5O_8$, M.W 415.31, m. p 215 ^{0}C, R_f – 0.72, Yield-68%. 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. Pharmacology:

2.2.1. Experimental animals and standard drug: White male albino Wister rats weighing about 200-250gm was used. They were obtained from the animal house of Anurag Pharmacy College, Kodad-508206, Telangana state.They were kept under observation for about 7 days before onset of experiment to exclude any intercurrent 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 silymarin was purchased from retail local shop.

2.2.2. Molecular docking [18]: 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.

2.2.3. Computational Analysis: For the present research work (Project Id: RSID001020/10/2015, PDB Code: 2V2T) the Crystalline structure of the target protein NF-KB with PDB id 2V2T 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 silymarin with respect to the target protein was evaluated by Autodock program and the best dock pose was selected based on the interaction study analysis.

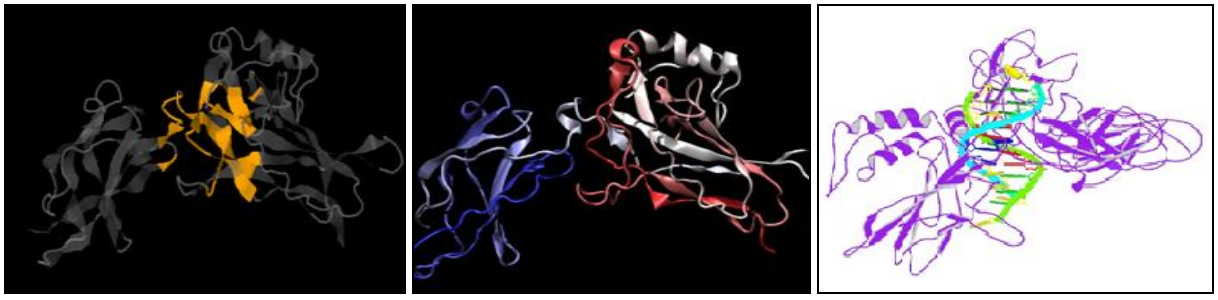


FIG. 1: STRUCTURE OF PROTEIN NF-KB

2.2.4. Evaluation of acute toxicity: In the present study acute oral toxicity of the synthesized compounds were performed by acute toxic class method according to OECD guideline - 423 [19]. In this method the toxicity of synthesized compounds were tested using a step wise procedure, each step using three rat of single sex (female/male). The rats 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 synthesized compound were administered orally at a dose 2000 mg/kg body weight.

Animals were observed individually after dosing at least once during the first 30min; 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 250 mg /Kg body weight (low dose) and 500 (high dose) were selected for their pharmacological evaluation.

2.2.5. Experimental protocol for Hepatoprotetive activity: [20] A total of 66 rats were taken and divided into 11 groups of 6 rats each.

Treatment Groups:
(A) Group I: Normal Control Group (only the vehicle (1 ml/kg/day of 1% CMC; p.o.)
(B) Group II: Negative Control CCl_4 1 ml/kg (1:1 of CCl_4 in olive oil) i.p.
(C) Group III-X (AB1-AB8): (i) Low Dose Group [CCl_4 1 mL/kg (1:1 of CCl_4 in olive oil) i. p + Synthesized compounds (250 mg/ kg b. w., p. o)]. Treatment was given daily for seven days orally and **(ii)** High Dose Group [CCl_4 1 mL/kg (1:1 of CCl_4 in olive oil) i.p + Synthesized compounds (500 mg/ kg b. w., p. o)]
(D) Group XI: Positive Control/Standard Group [CCl_4 1 ml/kg (1:1 of CCl_4 in olive oil) i.p.+ Standard Silymarin 100 mg/kg orally (p. o) for 7 days].

Collection of blood: On the 8th day, blood was collected by retro orbital puncture, under mild ether anesthesia after 8 hr fasting. Blood samples were centrifuged at 3000 rpm for 20 mins. Serum was separated and stored at – 20 °C until biochemical estimation was carried out.

Biochemical Analysis:
The Serum samples were analyzed for
(I) Alanine Aminotransferase (ALT) (SGPT)
(II) Aspartate Aminotransferase (AST) (SGOT)
(III) Alkaline Phosphatase (ALP)
(IV) Serum Bilirubin

Histopathological Analysis: The pathological status of hepatocytes of rat was done by FNAB [21].

3. RESULTS AND DISCUSSION:

3.1. 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.

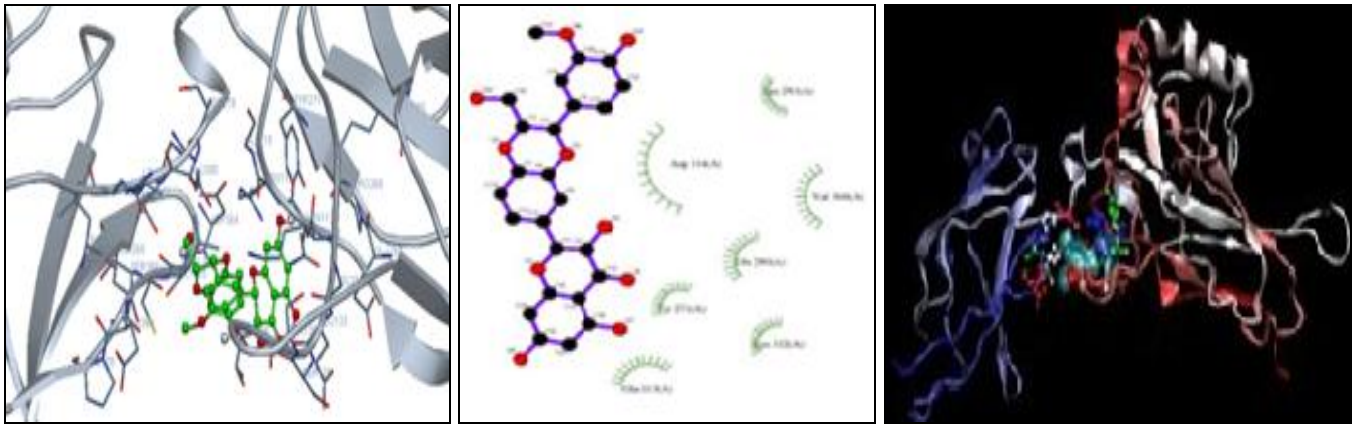
All the synthesized compounds having the following solubility profile: Insoluble in water, slightly soluble in chloroform, ethanol, and freely soluble in DMF, DMSO.

3.2. Molecular docking studies: 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 indicates high binding affinity with the target protein/compound.

In silico molecular docking studies displayed the binding energies **(Table 1 and Fig. 1- 6) :** -5.17, -5.52, -5.40, -4.60, -4.60, -4.87, -3.42, -3.85 k.cal/mol, of the synthesized compounds (AB1-AB8) which indicated that the compound had high binding affinity towards the 2V2T-NF-KB protein and inhibit the NF-KB protein function in comparison with standard drug silymarin (-3.54 k.cal/mol).

TABLE 1: FOR THE RESULTS OF MOLECULAR DOCKING OF SYNTHESIZED COMPOUNDS (AB1-AB8) & SILYMARIN WAS USED AS A STANDARD DRUG

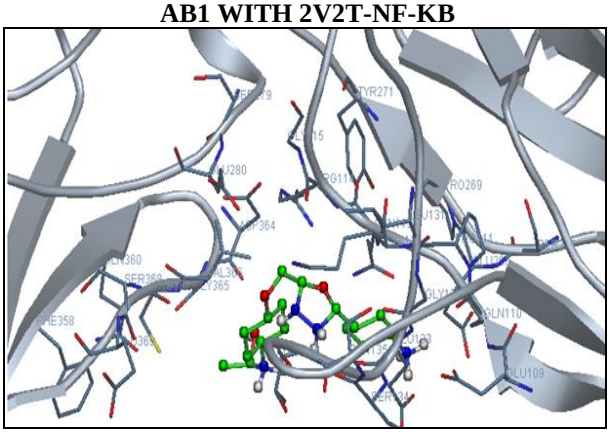
Comp. Code	EFB	EIC	Vdw-HB-DE	EE	Time	Fr	IS
Silymarin	-3.54 K.cal/mol	255 mM	-4.48 K.cal/mol	-0.08 K.cal/mol	-4.56 K.cal/mol	50%	724.115
AB1	-5.17 K.cal/mol	161.93 µM	-5.56 K.cal/mol	-1.37 K.cal/mol	-6.37 K.cal/mol	50%	632.611
AB2	-5.52 K.cal/mol	90.10 µM	-6.65 K.cal/mol	-0.61 K.cal/mol	-7.26 K.cal/mol	50%	683.582
AB3	-5.40 K.cal/mol	110.20 µM	-4.93 K.cal/mol	-2.13 K.cal/mol	-7.05 K.cal/mol	50%	518.24
AB4	-4.60 K.cal/mol	421.87 µM	-5.47 K.cal/mol	-0.93 K.cal/mol	-6.40 K.cal/mol	50%	629.76
AB5	-4.60 K.cal/mol	423.56 µM	-6.95 K.cal/mol	-0.62 K.cal/mol	-7.57 K.cal/mol	50%	655.286
AB6	-4.87 K.cal/mol	267.96 µM	-6.27 K.cal/mol	-0.21 K.cal/mol	-6.48 K.cal/mol	50%	612.497
AB7	-3.42 K.cal/mol	3.12 mM	-5.27 K.cal/mol	-0.23 K.cal/mol	-5.48 K.cal/mol	50%	646.458
AB8	-3.85 K.cal/mol	1.51 mM	-5.91 K.cal/mol	+0.01K.cal/mol	-5.90 K.cal/mol	50%	697.054



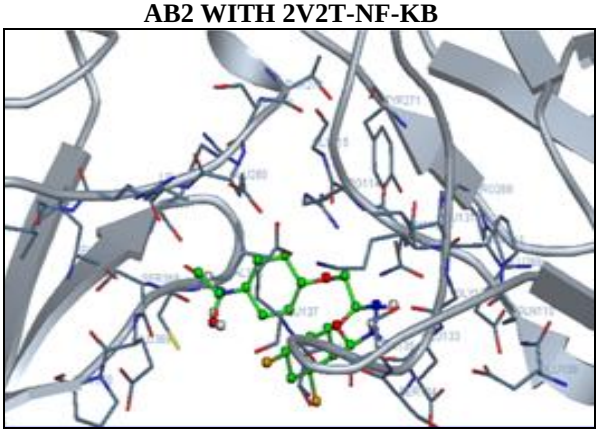
Silymarin with 2V2T-NF-KB Interaction Study Receptor Ligand Complex

FIG. 2: SILYMARIN WITH 2V2T-NF-KB INTERACTION

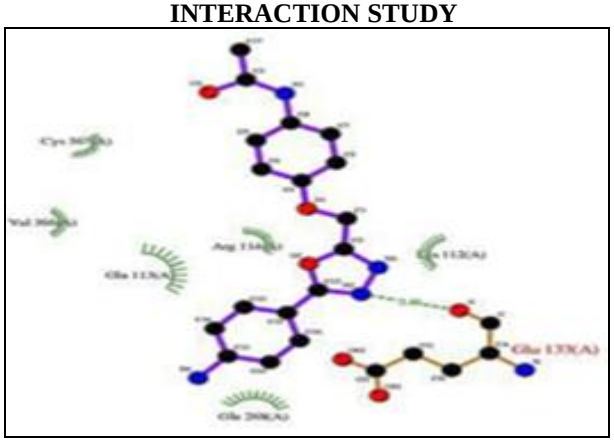
AB1 WITH 2V2T-NF-KB



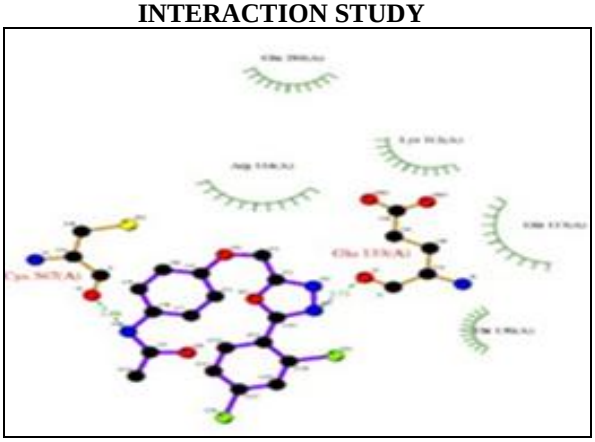
AB2 WITH 2V2T-NF-KB



INTERACTION STUDY



INTERACTION STUDY



RECEPTOR LIGAND COMPLEX **RECEPTOR LIGAND COMPLEX**

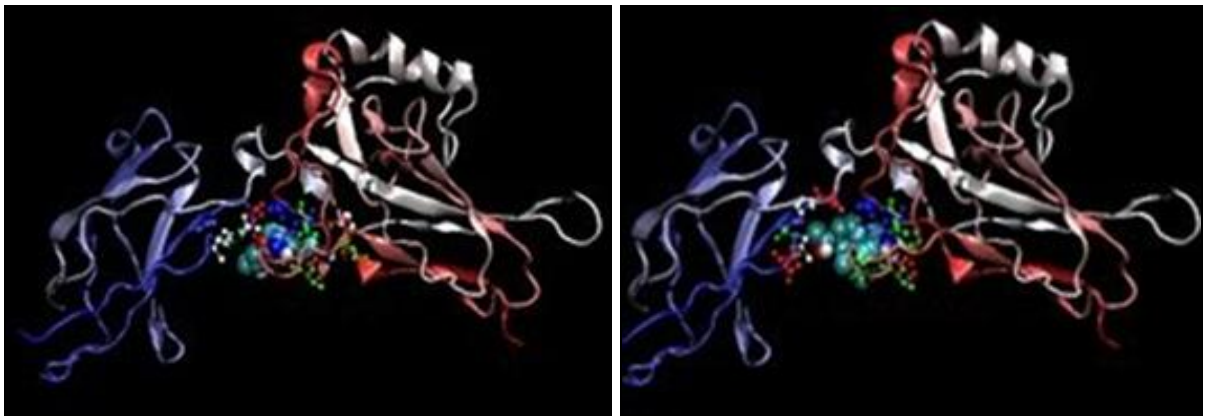
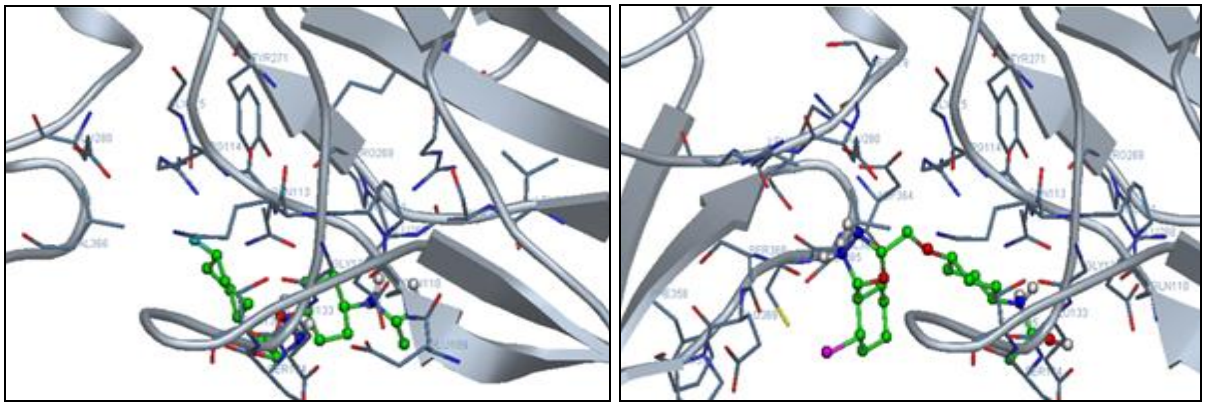
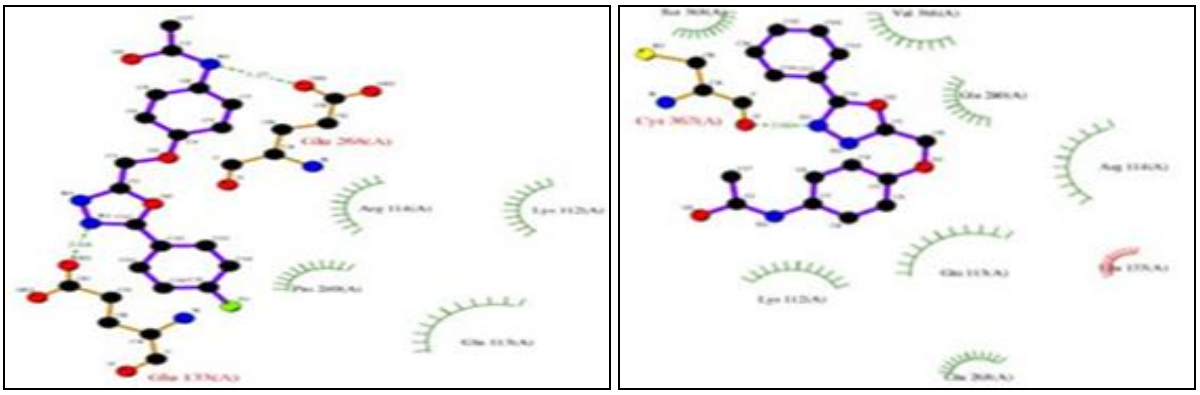


FIG. 3: COMPOUND AB1 AND AB2 WITH 2V2T-NF-KB INTERACTION

AB3 WITH 2V2T-NF-KB **AB4 WITH 2V2T-NF-KB**



INTERACTION STUDY **INTERACTION STUDY**



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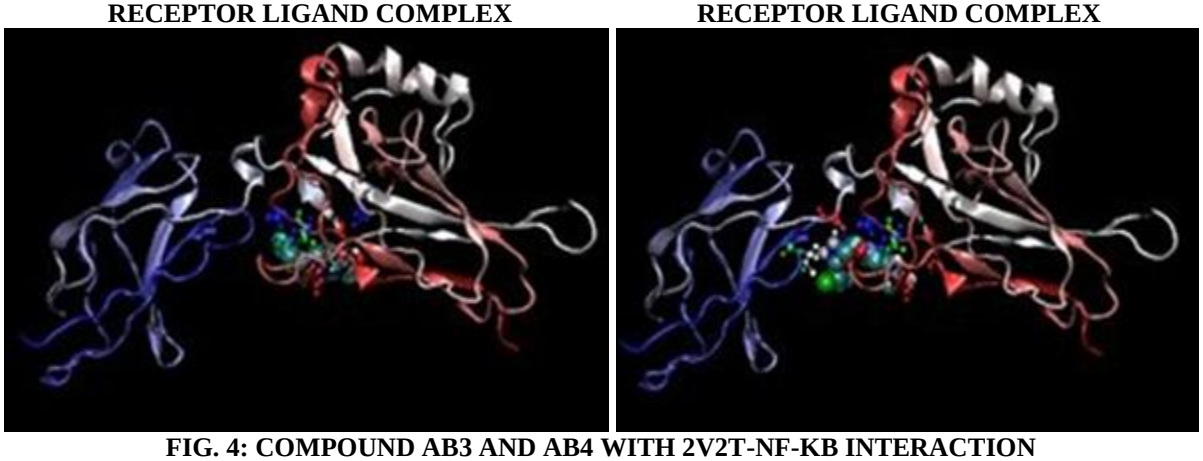
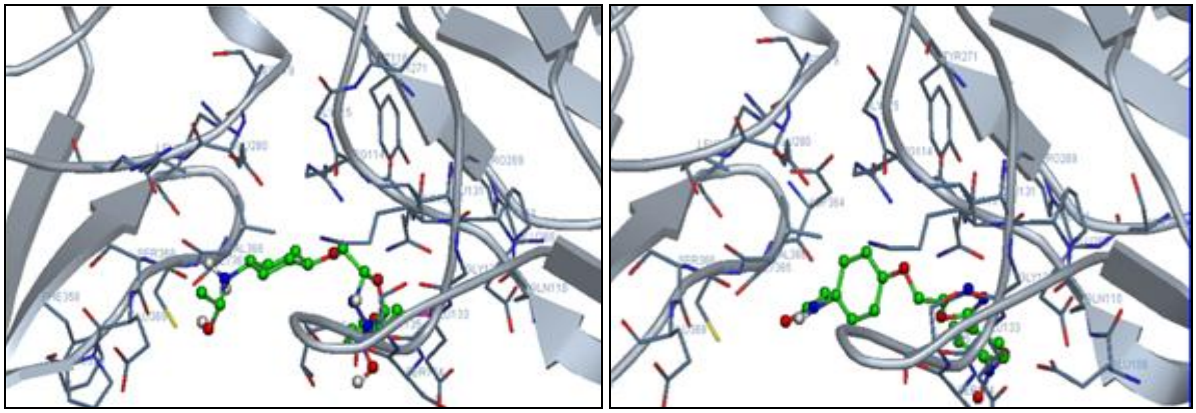


FIG. 4: COMPOUND AB3 AND AB4 WITH 2V2T-NF-KB INTERACTION

AB5 WITH 2V2T-NF-KB **AB6 WITH 2V2T-NF-KB**



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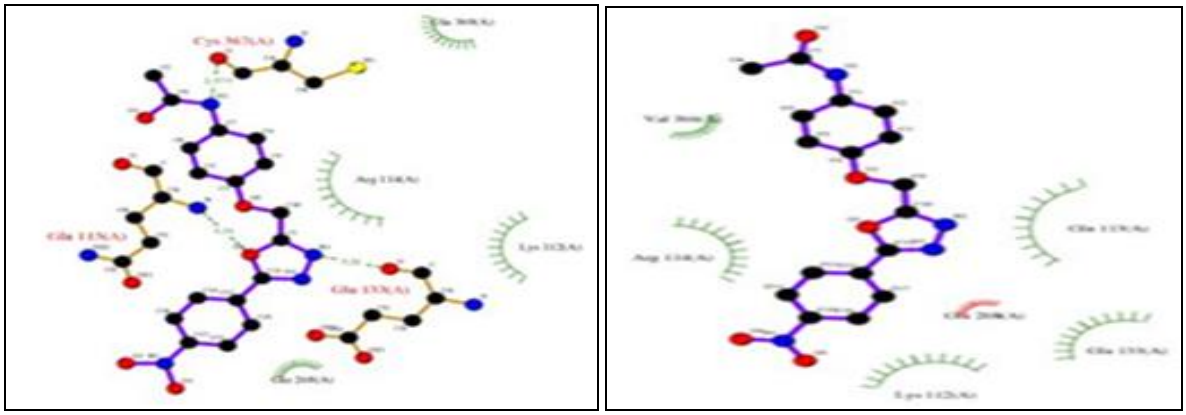

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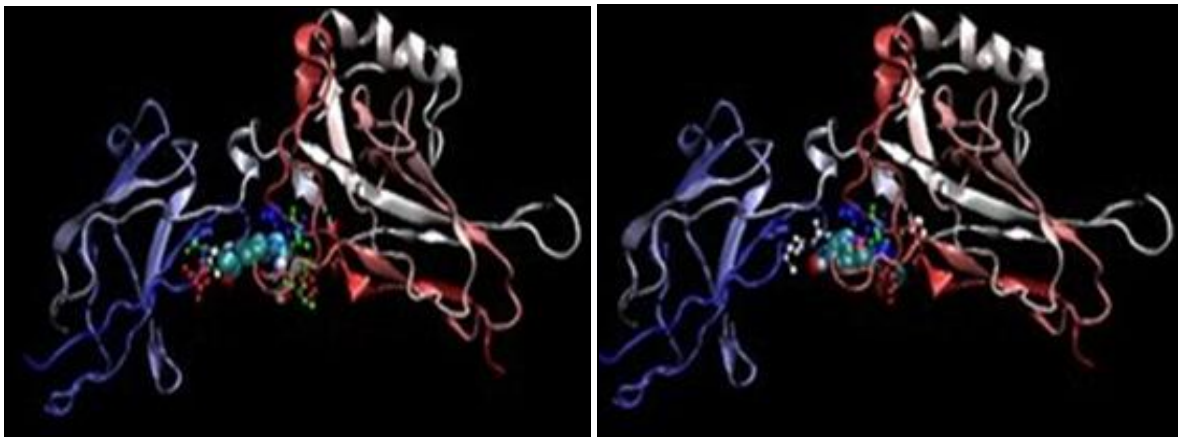
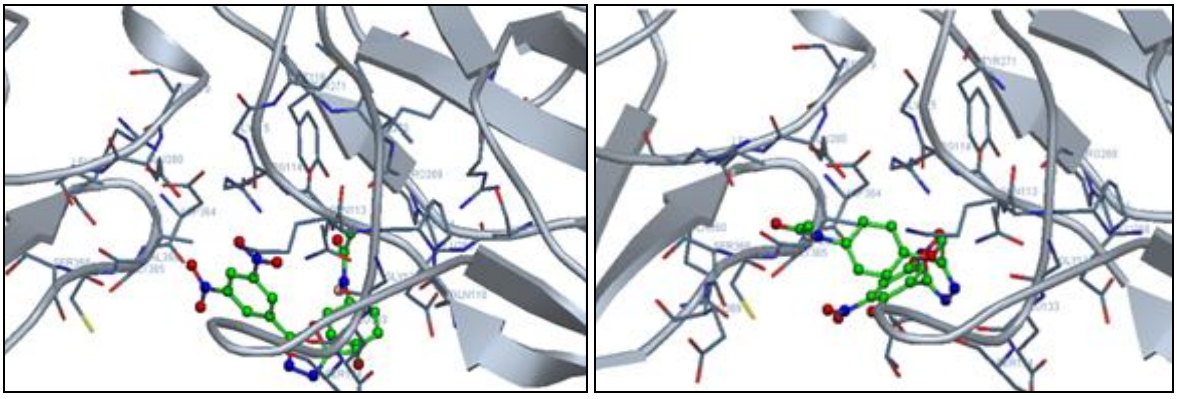


FIG. 5: COMPOUND AB5 AND AB6 WITH 2V2T-NF-KB INTERACTION

AB7 WITH 2V2T-NF-KB **AB8 WITH 2V2T-NF-KB**



INTERACTION STUDY **INTERACTION STUDY**

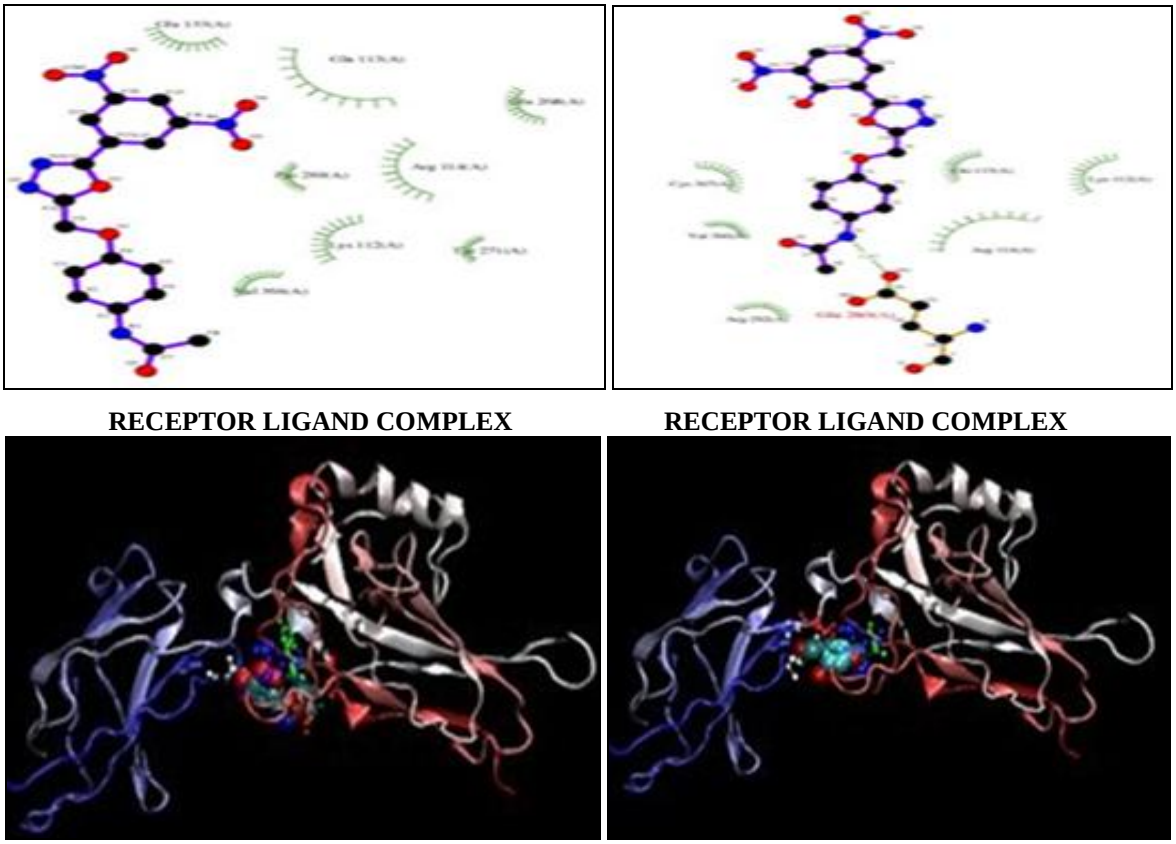


FIG. 6: COMPOUND AB7 AND AB8 WITH 2V2T-NF-KB INTERACTION

3.3. Evaluation of hepatoprotective activity:

Statistical analysis: The data **(Table 2)** were expressed as mean ± SD. Statistical differences at *P < 0.05 between the groups were analyzed by one-way ANOVA followed by Dunnett's Multiple Comparison Test using Graph Pad Prism 5.04 Instate software package. The data's were compared with group 2 *i.e.* Negative Control group.

TABLE 2: FOR THE ASSESSMENT OF BIOCHEMICAL PARAMETERS

Treatment Group	AST(SGOT) IU/L	ALT(SGPT) IU/L	ALP(SALP) IU/L	Sr. bilirubin mg/dL
Group I	52.25±0.356	48.11±0.1533	51.95±0.1531	0.7368±0.00673
Group II	202.5±0.3038***	209.4±0.1065***	390.9±1.499***	7.383±0.1014***
		Low Dose		
Group III (AB1)	180.7±0.2176***	160.6±0.08021***	351.1±2.629***	4.665± 0.03977***
Group IV (AB2)	184.8±0.3201***	160.5±0.2836***	355.6±1.562***	4.216± 0.04606***
Group V (AB3)	184.1±0.1520***	161.5±0.3068***	350.6±0.4546***	3.998± 0.02858***
Group VI (AB4)	188.4±0.1184***	159.6±0.08492***	351.0±0.5089***	4.671± 0.06393***
Group VII (AB5)	183.1±0.2110***	160.8±0.08124***	354.4±0.2036***	4.573± 0.06113***
Group VIII (AB6)	189.1±0.1533***	161.5±0.09425***	349.9±0.1698***	4.261± 0.05351***
Group IX (AB7)	178.9±0.4359***	161.2±0.08625***	357.4±0.2007***	4.677±0.06992***
Group X (AB8)	186.7±0.1707***	159.3±0.06532***	356.5±0.1116***	4.552±0.1193***
		High Dose		
Group III (AB1)	151.2±0.1461***	120.9±0.6005***	302.8±0.1769***	3.397±0.04347***
Group IV (AB2)	152.5±0.09995***	122.1±0.5848***	302.3±0.09098***	3.312±0.01184***
Group V (AB3)	152.2±0.07968***	121.5±0.1256***	302.6±0.2044***	3.140±0.05241***
Group VI (AB4)	154.2±0.08871***	120.7±0.07638***	303.5±0.05834***	3.124±0.1242***
Group VII (AB5)	152.5±0.2031***	122.4±0.1478***	303.5±0.0966***	3.432±0.03542***
Group VIII (AB6)	154.4±0.07149***	122.5±0.07424***	302.7±0.08570***	3.261±0.05351***
Group IX (AB7)	149.4±0.1400***	122.7±0.6640***	303.7±0.09988***	3.319±0.04237***
Group X (AB8)	153.5±0.1282***	120.6±0.1176***	303.6±0.03464***	3.407±0.09574***
Group XI	91.55±0.1502***	109.7±0.1453***	154.8±0.1229***	1.631±0.2323***

Biochemical analysis: The present study displayed that synthesized compounds were possessed a significant hepatoprotective activity. The declining of plasma enzyme level **(Fig. 7-16)** is a prognostic status of the hepatoprotective action of the drug. Protection of hepatic damage caused by carbon tetrachloride administration was observed by recording SGOT, SGPT, SALP and Serum bilirubin levels in different groups. The transport function of the hepatocytes is disturbed in hepatic injury, causing the leakage of enzymes due to altered membrane permeability. Although both the doses (250 and 500 mg/kg b. w.) of synthesized compounds executed hepatoprotective activity, the higher dose (500 mg/kg b. w.) is more effective and more significant. The *in vivo* experimental data displayed that the elevated levels of SGOT, SGPT, ALP and Sr. bilirubin were mainly due to CCl_4 intoxication, reduced significantly (*P<0.05) in rats, after treatment with synthesized compounds.

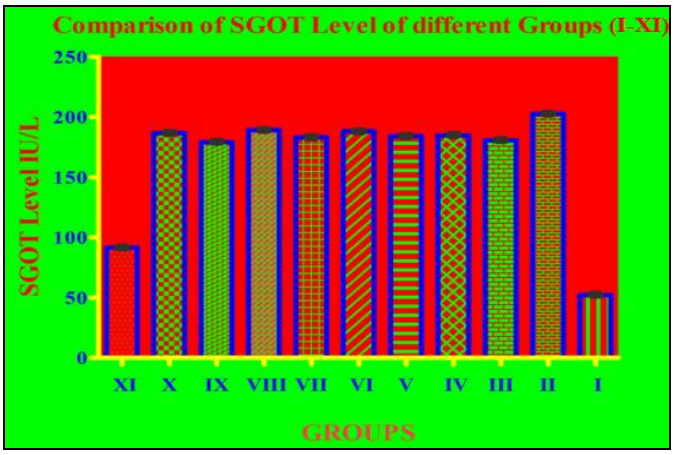


FIG. 7: SGOT LEVEL (IU/L) IN DIFFERENT GROUPS AFTER TREATED WITH LOW DOSE OF TEST COMPOUNDS

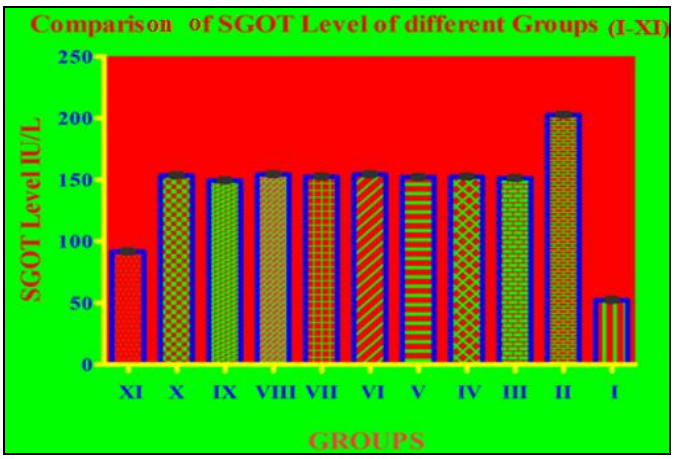


FIG. 8: SGOT LEVEL (IU/L) IN DIFFERENT GROUPS AFTER TREATED WITH HIGH DOSE OF TEST COMPOUNDS

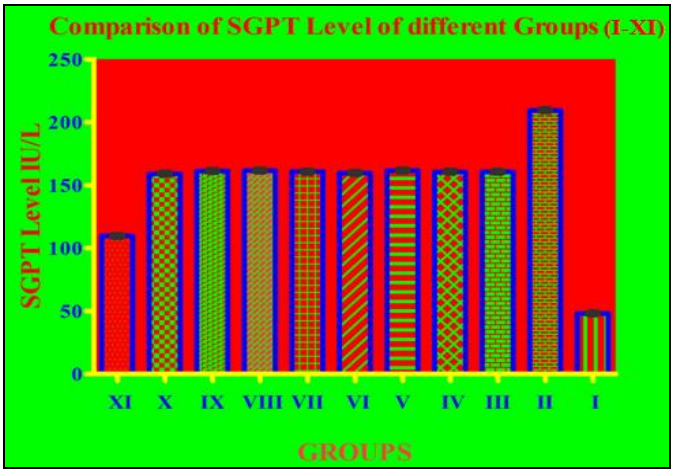


FIG. 9: SGPT LEVEL (IU/L) IN DIFFERENT GROUPS AFTER TREATED WITH LOW DOSE OF TEST COMPOUNDS

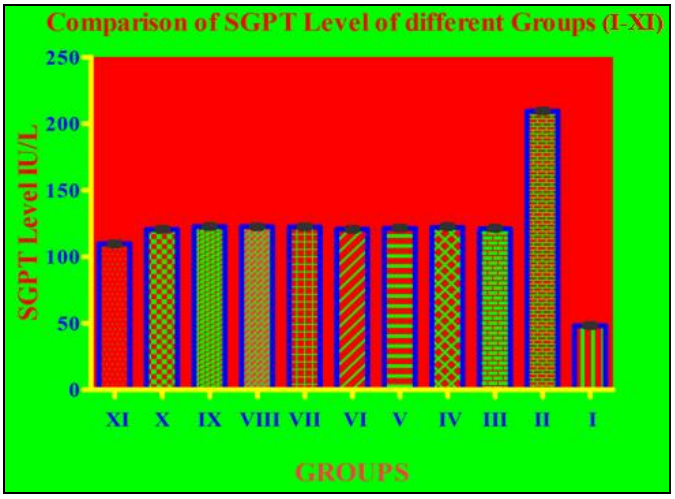


FIG. 10: SGPT LEVEL (IU/L) IN DIFFERENT GROUPS AFTER TREATED WITH HIGH DOSE OF TEST COMPOUNDS

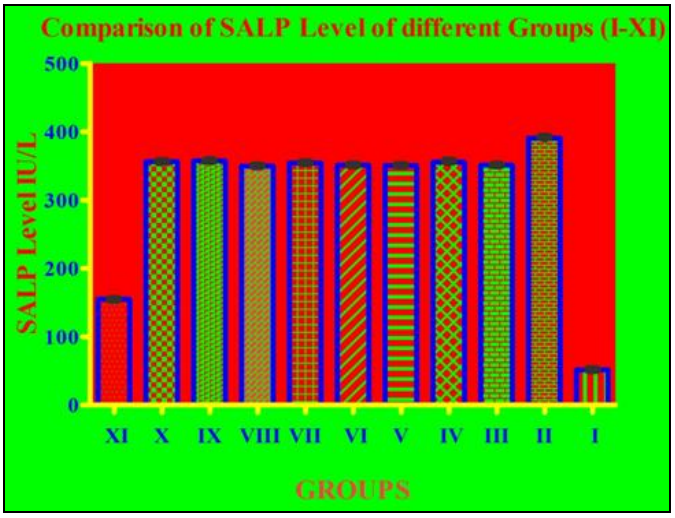


FIG. 11: SALP LEVEL (IU/L) IN DIFFERENT GROUPS AFTER TREATED WITH LOW DOSE OF TEST COMPOUNDS

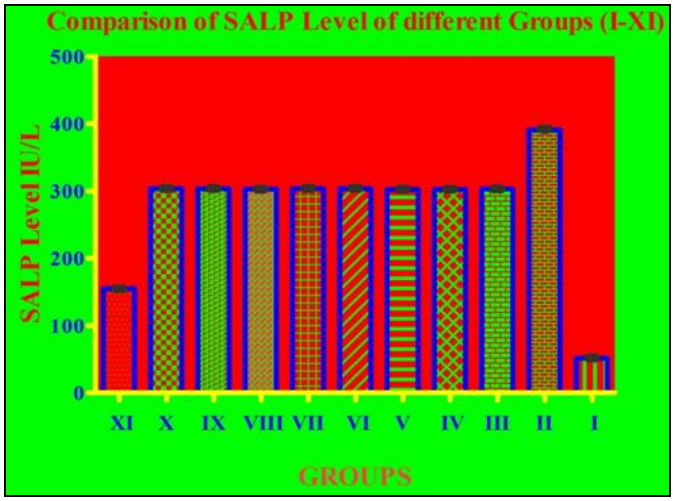


FIG. 12: SALP LEVEL (IU/L) IN DIFFERENT GROUPS AFTER TREATED WITH HIGH DOSE OF TEST COMPOUNDS

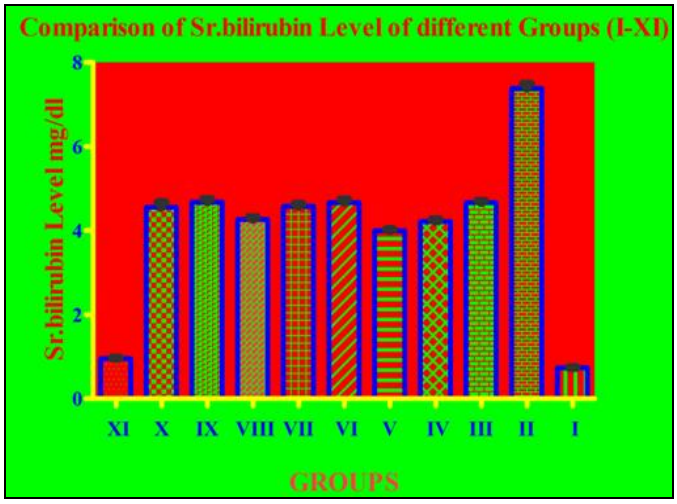


FIG. 13: SR. BILIRUBIN (mg/dl) IN DIFFERENT GROUPS AFTER TREATED WITH LOW DOSE OF TEST COMPOUNDS

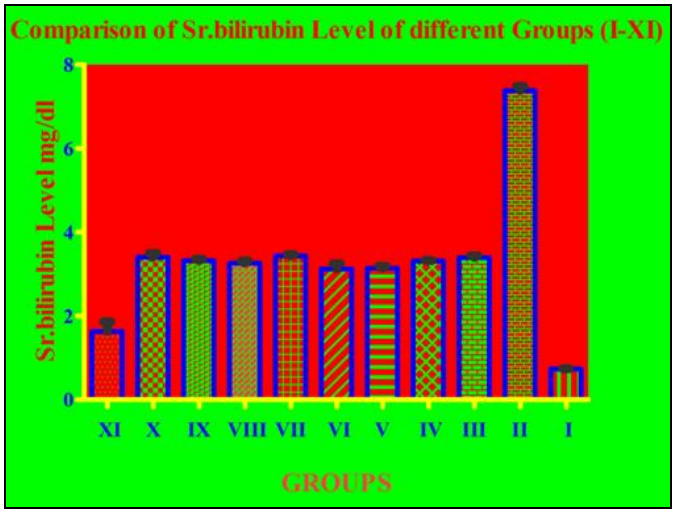


FIG. 14: SR. BILIRUBIN (mg/dl) IN DIFFERENT GROUPS AFTER TREATED WITH HIGH DOSE OF TEST COMPOUNDS

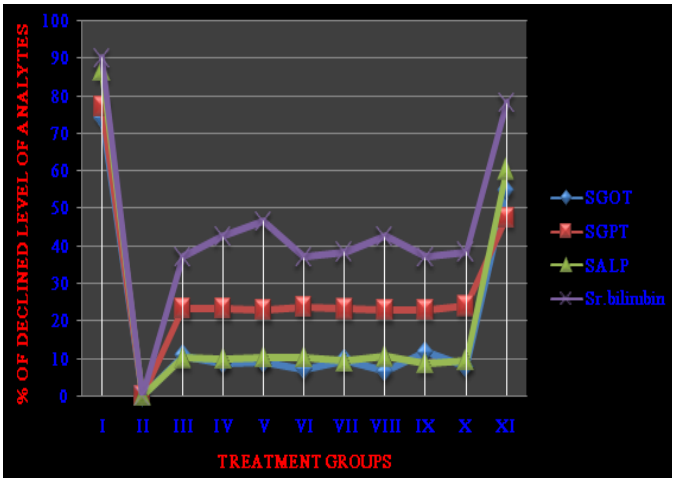


FIG. 15: GRAPHICAL REPRESENTATION OF PERCENTAGE (%) OF DECLINED LEVEL OF ANALYTES IN DIFFERENT GROUPS AFTER TREATED WITH LOW DOSE OF TEST COMPOUNDS

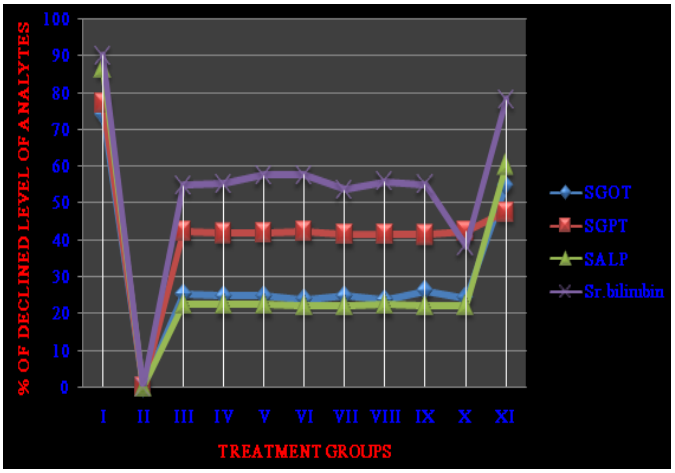


FIG. 16: GRAPHICAL REPRESENTATION OF PERCENTAGE (%) OF DECLINED LEVEL OF ANALYTES IN DIFFERENT GROUPS AFTER TREATED WITH HIGH DOSE OF TEST COMPOUNDS

Treatment with a synthesized compounds (AB1-AB8) at a dose of 250 mg/kg b. w. decreased the SGOT: 10.76%, 8.74%, 9.08%, 7.16%, 9.58%, 6.61%, 11.65%, 7.80%, SGPT: 23.30%, 23.35%, 22.87%, 23.78%, 23.20%, 22.87%, 23.01%, 23.92%, ALP: 10.18%, 9.92%, 10.30%, 10.20%, 9.33%, 10.56%, 8.80%, 9.56% and Serum bilirubin levels by 36.98%, 42.46%, 46.57%, 36.98%, 38.35%, 42.46%, 36.98%, 38.35%, (significantly) respectively, while at higher dose of 500 mg/kg b. wt. was more effective, causing a reduction of SGOT: 25.33%, 24.69%, 24.83%, 23.85%, 24.69%, 23.75%, 26.22%, 24.19%, SGPT: 42.26%, 41.69%, 41.97%, 42.39%, 41.54%, 41.49%, 41.40%, 42.40%, SALP: 22.66%, 22.58%, 22.58%, 22.35%, 22.35%, 22.56%, 22.30%, 22.33%, and Sr.

bilirubin: 54.79%, 55.10%, 57.46%, 57.68%, 53.51%, 55.83%, 55.04%, 53.85%. Silymarin is used as standard drug showed a significant reduction of level of SGOT: 54.79%, SGPT: 47.61%, SALP: 60.39% and Sr. bilirubin: 78.08% respectively receiving CCl_4 alone.

Histopathological analysis: The results of FANB of liver cells of rats of control, CCl_4 treated and treated with synthesized compounds (AB1-AB8) were represented in **(Fig. 17-26).** FANB of liver cells of rats revealed that the liver treated with CCl_4 showed a high degree of damage characterized by piecemeal necrosis and portal tract necrosis, interface hepatitis due to expanded portal tract by infiltration of lymphocytes, plasma cells and macrophages, fulminant necrosis which is characterized by submassive and massive necrosis.

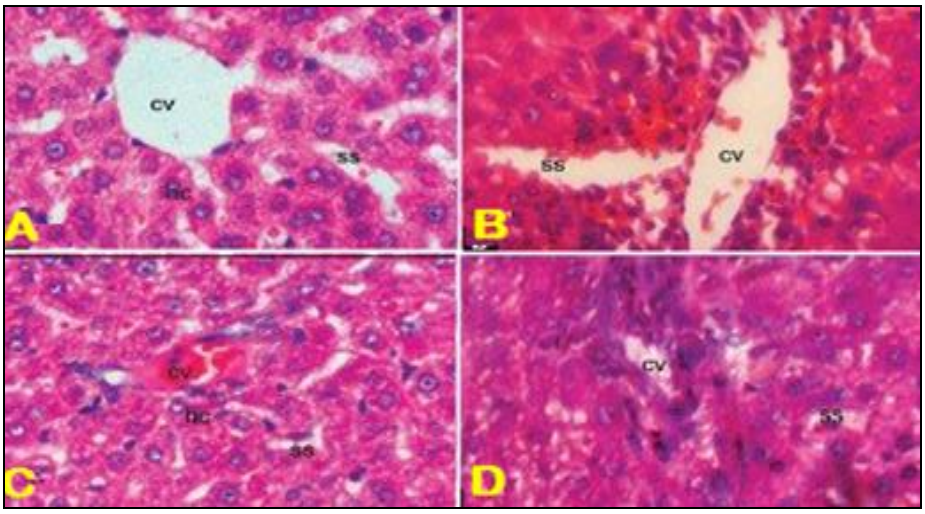


FIG. 17: HEMATOXYLIN AND EOSIN STAINING, X 400: (A) Normal control rat liver section **(Group-I)**: Normal histological findings in the liver parenchyma; **(B)** Liver section of the rat intoxicated with Carbon tetrachloride **(Group-II)**: Piecemeal and portal tract necrosis and mixed inflammatory infiltration; **(C)** Liver section of the rat treated with silymarin and intoxicated with carbon tetrachloride, showing mild necrotic debris. **(Group-XI) (D)** Liver section of the rat treated with 500 mg/kg of synthesized compound **AB1** and intoxicated with carbon tetrachloride **(Group-III):** Showing healing of hepatic damage without necrosis. C.V: Central vein, V.C: Vacuole, S.S: Sinusoidal spaces, H.C: Hepatocytes

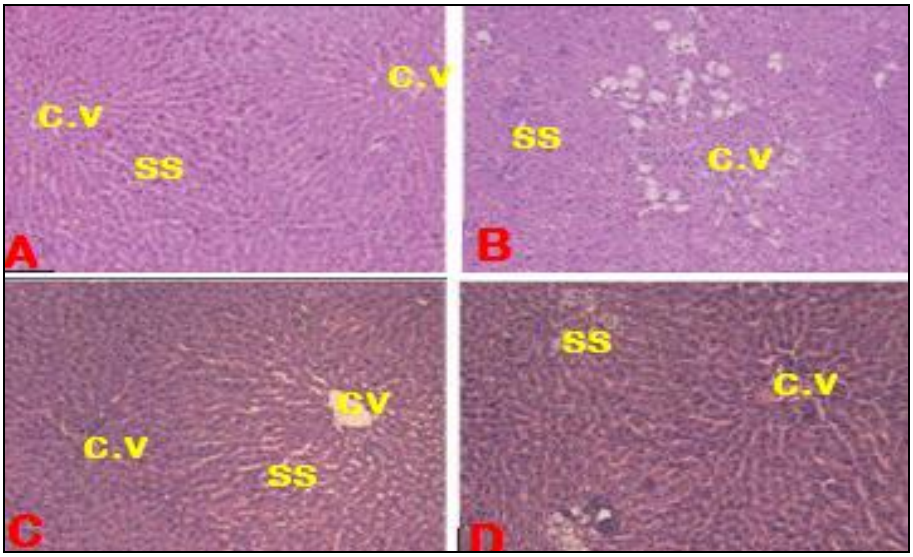


FIG. 18: HEMATOXYLIN AND EOSIN STAINING, X40: (A) Normal control **(Group-I)**: Showing normal histological findings in the liver parenchyma. **(B)** Liver section of the rat intoxicated with Carbon tetrachloride **(Group-II)**: Showing submassive confluent necrosis, portal tract necrosis and mixed inflammatory infiltration. **(C)** Liver section of the rat treated with silymarin and intoxicated with carbon tetrachloride **(Group-XI):** Indicated reduced perivenular microvesicular steatosis without necrosis. **(D)** Liver section of the rat treated with 500 mg/kg of synthesized compound **AB2** and intoxicated with carbon tetrachloride **(Group-IV):** Showing regeneration of hepatocytes and disappearance of necrotic debris.

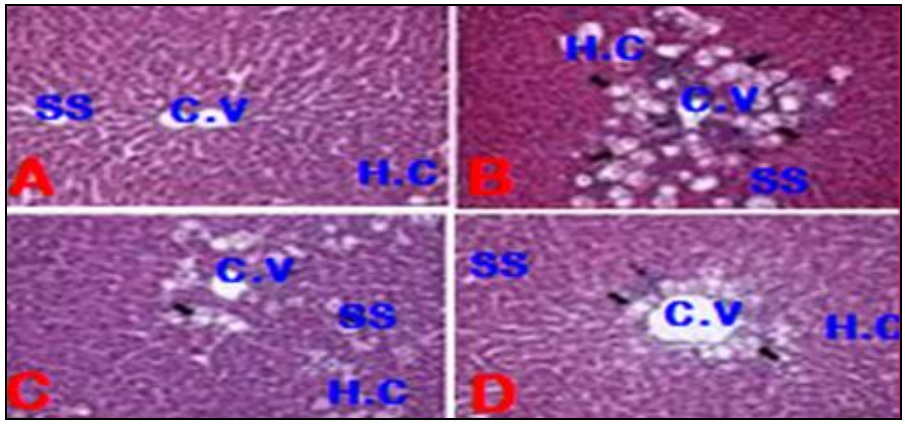


FIG. 19: STAINED WITH HEMATOXYLIN AND EOSIN(original magnification×100, calibration bar = 100µm). (A) Normal control **(Group-I)**: Showing normal histological findings in the liver parenchyma. **(B)** Liver section of the rat intoxicated with Carbon tetrachloride **(Group-II)**: Showing intralobular Nectrotic debris, degeneration of portal vessel and inflammatory infiltration. **(C)** Liver section of the rat treated with silymarin and intoxicated with carbon tetrachloride **(Group-XI):** Indicated regeneration in sub massive necrosis is more orderly and may result in restoration of normal architecture. **(D)** Liver section of the rat treated with 500 mg/kg of synthesized compound **AB3** and intoxicated with carbon tetrachloride **(Group-V):** Showing restoration of normal hepatic architecture

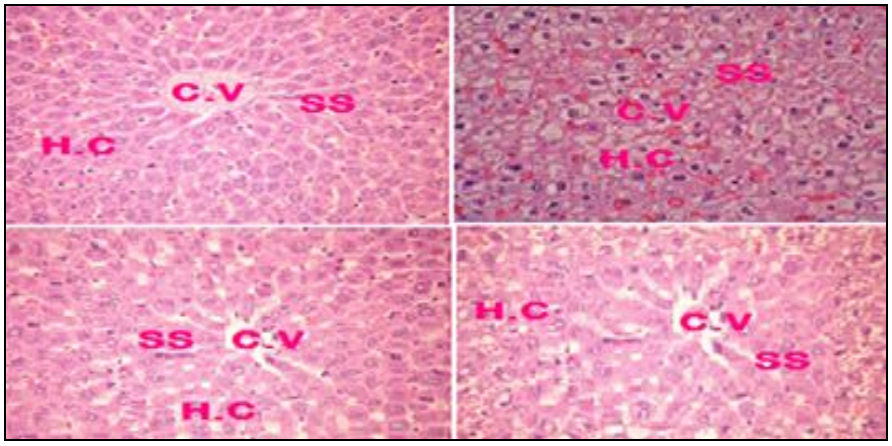


FIG. 20: HEMATOXYLIN AND EOSIN STAINING, X 400: (A) Normal control rat liver section **(Group-I)**: Normal histological findings in the liver parenchyma; **(B)** Liver section of the rat intoxicated with Carbon tetrachloride **(Group-II)**: Indicated variable degree of bridging necrosis of hepatocytes, most marked in centrilobular and mononuclear cellular infiltrate in the lobule. Mild degree of liver cell necrosis is seen as ballooning degeneration **(C)** Liver section of the rat treated with silymarin and intoxicated with carbon tetrachloride **(Group-XI) (D)** Liver section of the rat treated with 500 mg/kg of synthesized compound AB4 and intoxicated with carbon tetrachloride **(Group-VI):** Showing centrilobular regeneration with restoration of c.v, ss and hepatocytes with mild necrosis. C.V: Central vein, V.C: Vacuole, S.S: Sinusoidal spaces, H.C: Hepatocytes.

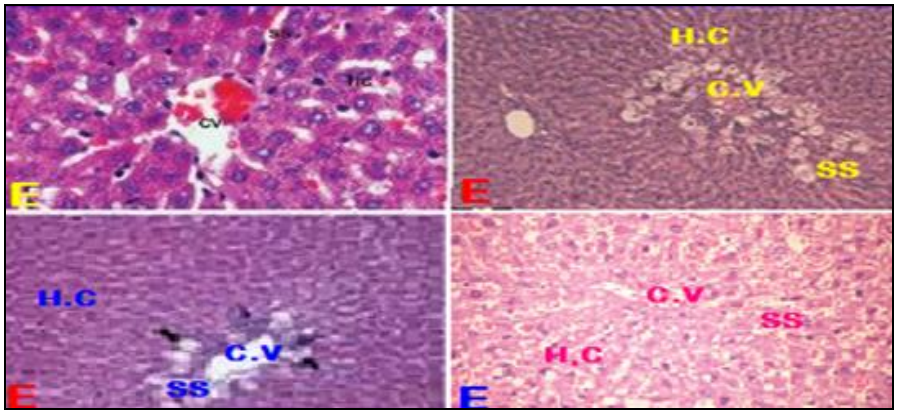


FIG. 21: STAINED WITH HEMATOXYLIN AND EOSIN: (E) Liver section of the rat treated with 250mg/kg of synthesized compound AB1, AB2, AB3 and AB4 (Group: III, IV, V and VI) and intoxicated with carbon tetrachloride.

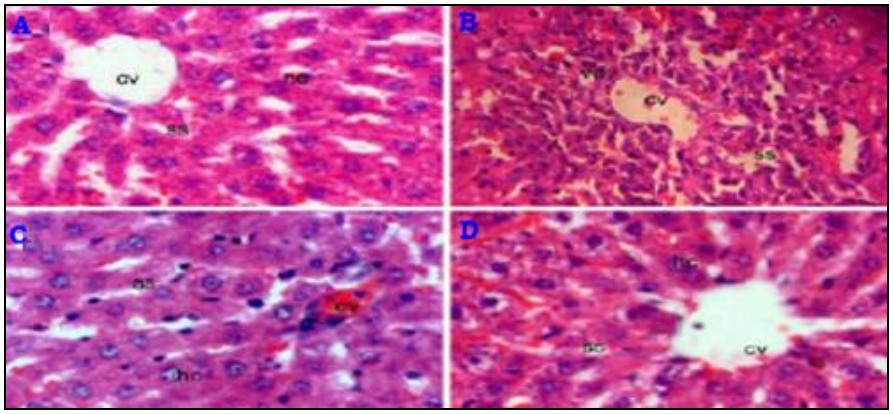


FIG. 22: HEMATOXYLIN AND EOSIN STAINING, X400: (A) Normal control rat liver section **(Group-I)**: Normal histological findings in the liver parenchyma; **(B)** Liver section of the rat intoxicated with Carbon tetrachloride **(Group-II)**: Indicated focal of necrosis of hepatocytes, most marked in centrilobular and mononuclear cellular infiltrate in the lobule with varying portal tract inflammation.. Mild degree of liver cell necrosis is seen as ballooning degeneration; **(C)** Liver section of the rat treated with silymarin and intoxicated with carbon tetrachloride **(Group-XI); (D)** Liver section of the rat treated with 500 mg/kg of synthesized compound AB5 and intoxicated with carbon tetrachloride **(Group-VII):** Showing centrilobular regeneration with restoration of c.v, ss and hepatocytes with mild to moderate necrotizing cells. C.V: Central vein, V.C: Vacuole, S.S: Sinusoidal spaces, H.C: Hepatocytes.

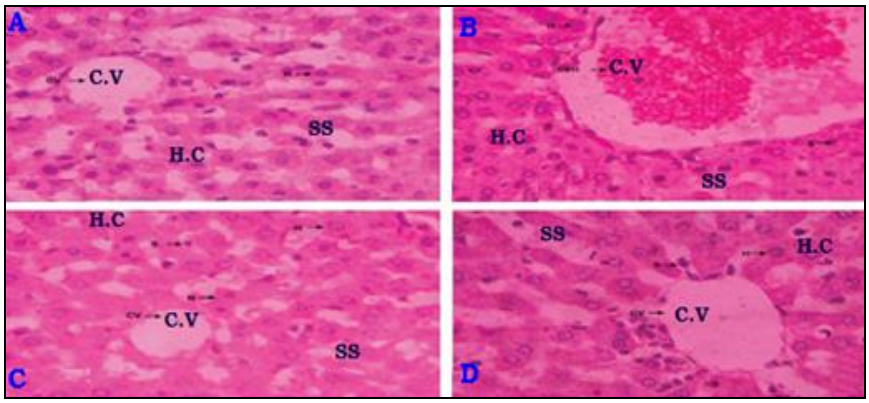


FIG. 23: HISTOPATHOLOGY REPORT. (A) NORMAL CONTROL RAT LIVER SECTION (Group-I): Showed a normal lobular architecture of the liver; **(B)** Liver section of the rat intoxicated with Carbon tetrachloride **(Group-II)**: Showed moderate panacinar necrosis and inflammation and neutrophil infiltration also observed in the centrilobular region with portal triaditis; **(C)** Liver section of the rat treated with silymarin and intoxicated with carbon tetrachloride **(Group-XI):** Showed minimal inflammation and hepatic congestion with moderate portal triditis and their lobular architecture was normal; **(D)** Liver section of the rat treated with 500 mg/kg of synthesized compound **AB6** and intoxicated with carbon tetrachloride **(Group-VIII):** Showed minimal inflammation with moderate portal triditis, mild necrosis and their lobular architecture was normal.

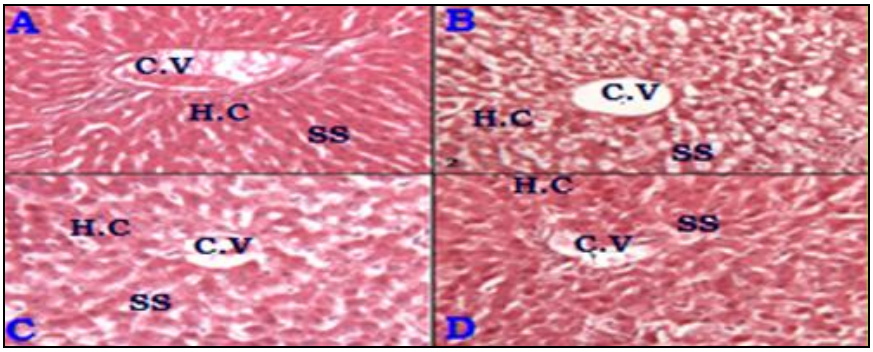


FIG. 24: HISTOPATHOLOGY REPORT. (A) NORMAL CONTROL RAT LIVER SECTION (Group-I): Showed a normal structure of the liver; **(B)** Liver section of the rat intoxicated with Carbon tetrachloride **(Group-II)**: Showed portal inflammation and panacinar necrosis necrosis; **(C)** Liver section of the rat treated with silymarin and intoxicated with carbon tetrachloride **(Group-XI):** Showed minimal portal inflammation and their lobular architecture was normal; **(D)** Liver section of the rat treated with 500 mg/kg of synthesized compound **AB7** and intoxicated with carbon tetrachloride **(Group-IX):** Showed minimal inflammation with moderate portal triditis, moderate necrotic debris and their lobular architecture was normal.

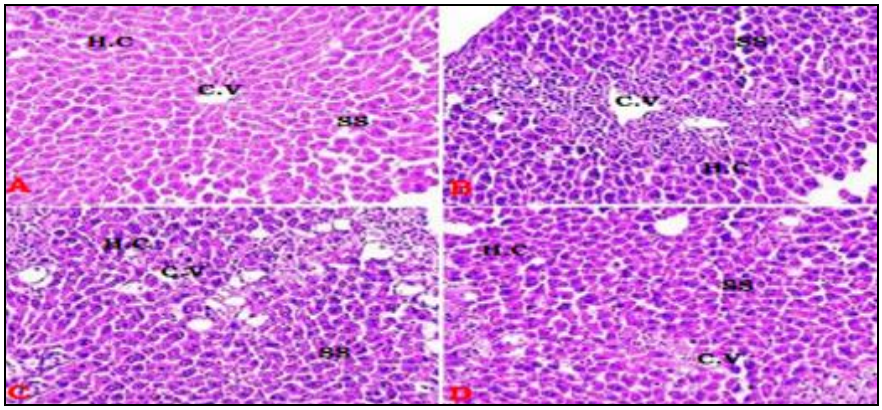


FIG. 25: AT A MAGNIFICATION OF 200 X OF HEMATOXYLIN AND EOSIN-STAINED LIVER SECTIONS. **(A)** Normal control rat liver section **(Group-I)**: No noticeable histological changes; **(B)** Liver section of the rat intoxicated with Carbon tetrachloride **(Group-II)**: Showed portal inflammation and confluent necrosis; **(C)** Liver section of the rat treated with silymarin and intoxicated with carbon tetrachloride **(Group-XI):** Showed minimal portal inflammation and their lobular architecture was normal; **(D)** Liver section of the rat treated with 500 mg/kg of synthesized compound AB8 and intoxicated with carbon tetrachloride **(Group-X):** Showed minimal inflammation with moderate necrosis and portal triditis and their lobular architecture was normal.

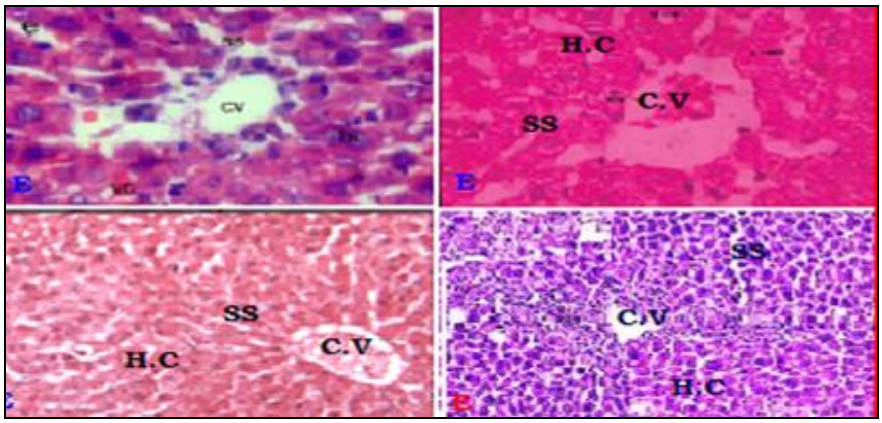


FIG. 26: STAINED WITH HEMATOXYLIN AND EOSIN: (E) Liver section of the rat treated with 250mg/kg of synthesized compound AB5, AB6, AB7 and AB8 (Group: VII, VIII, IX and X) and intoxicated with carbon tetrachloride

Histopathological scoring: [22] As prognostic indicator of chronic hepatitis, criteria have been evolved to classify chronic hepatitis by giving "hepatitis activity score" ranging from none to minimal/mild to moderate and severe describe by Knodell and Ishak based on the following features (**Table 3, 4**)

1. Necroinflammatory activity:

(i) Periportal necrosis *i.e.*: piece necrosis and / or bridging necrosis: Ranging from score 0 as "no necrosis" to score 4 as "multilobular necrosis".

(ii) Intralobular necrosis, focal or confluent: Ranging from score 0 as "none" to score 4 for "<10 focai" for focal necrosis and score 6 as "panacinar/ multiacinar" for confluent necrosis. (iii) Extent and depth portal inflammation: Ranging from grade 0 as "no inflammation" to grade 4 having "marked portal inflammation".

2. Stage of fibrosis: Extent and density of fibrosis: Ranging from score 0 as "no fibrosis to score 6 as "cirrhosis".

TABLE 3: FOR HEPATITIS ACTIVITY SCORE: NECRO INFLAMMATORY ACTIVITY

Group	Necrosis	Group	Necrosis
	Score 0 to Score 6		Score 0 to Score 6
I	0	III-XI	0
II	4	III	0
II	6	IV	0
II	4	V	0
II	4	VI	1
II	4	VII	1
II	5	VIII	1
II	5	IX	2
II	6	X	2
II	6	XI	1

TABLE 4: FOR HEPATITIS ACTIVITY SCORE: PORTAL INFLAMMATORY ACTIVITY

Group	Portal Inflammation Grade 0 to Grade 4	Group	Portal Inflammation Grade 0 to Grade 4
I	Grade 0	III-XI	Grade 0
II	Grade 4	III	Grade 0
II	Grade 3	IV	Grade 0
II	Grade 4	V	Grade 0
II	Grade 2	VI	Grade 1
II	Grade 4	VII	Grade 1
II	Grade 3	VIII	Grade 1
II	Grade 4	IX	Grade 2
II	Grade 4	X	Grade 2
II	Grade 3	XI	Grade 0

CONCLUSION: The above experimental data concluded that the synthesized compounds had the potential hepatocytes regenerator ability which was proved by Hepatitis Activity Score (HAC) shown *in vivo* and in silico molecular docking studies of synthesized compounds were revealed comparable binding energies and similar docking poses on target proteins such as 2V2T-NF-KB and known to be inhibitors of NF-KB.

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CONFLICT OF INTEREST: We declare that we have no conflict of interest.

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