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Synthesis, Characterization, Biological Evaluation and *In-Silico* Studies of Some Novel Isoxazole Derivatives

Durga Prasad*[1], Kalpana Devi[2], Rakesh Panda[3], Md. Salahuddin[4], Sakri Veerendra[1]

[1]*M. Pharm, Department of Pharmaceutical Chemistry, Al-Ameen College of Pharmacy, Bangalore, Karnataka, India*
[2]*Professor, Department of Pharmaceutical Chemistry, Al-Ameen College of Pharmacy, Bangalore, Karnataka, India*
[3] *Associate Professor, Department of Pharmaceutical Chemistry, Al-Ameen College of Pharmacy, Bangalore, Karnataka, India*
[4]*Prinipal and HOD, Department of Pharmaceutical Chemistry, Al-Ameen College of Pharmacy, Bangalore, Karnataka, India*
**Corresponding Author: Durga Prasad*

ABSTRACT: *In the present research work, an attempt has been made to synthesize some novel isoxazole derivatives with significant in-vitro anti-inflammatory, anti-oxidant, anti-microbial, anti-diabetic and in-vivo anti-depressant activity along with their in-silico studies. To obtain the target compounds, the synthetic pathway started from acetylation of indole when treated with acetic anhydride, followed by selective mono-deacetylation at N-1 position of indole to form an intermediate 3-Acetyl indole which is treated with substituted benzaldehydes and undergoes Claisen-Schmidt condensation to form α, β-Unsaturated ketones, the resultant intermediate is treated with hydroxylamine hydrochloride and undergo cyclisation to form isoxazoles. The synthesized compounds were screened for evaluating their In-vitro, In-vivo along with their In-silico studies where DPK-2 & DPK-3 had exhibited good anti-oxidant activity, compounds DPK-1 & DPK-2 had exhibited good anti-inflammatory activity, DPK-6 & DPK-7 exhibited very good ant-microbial activity, compounds DPK-2 & DPK-7 had exhibited good anti-diabetic activity and DPK-3 had exhibited very good anti-depressant activity. The Synthesized isoxazole derivatives possess significant in-vitro anti-inflammatory, anti-oxidant, anti-microbial, anti-diabetic and in-vivo anti-depressant activity which is backed up with their in-silico studies.*

KEYWORDS: *Isoxazoles, In-silico studies, In-vitro studies, In-vivo studies, Anti-Depressant.*

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I. INTRODUCTION

Heterocyclic compounds are essential structural moieties widely employed in medicinal chemistry as a valuable intermediate in organic synthesis. Among them, isoxazole is a five-membered aromatic ring containing adjacent oxygen and nitrogen atoms. Due to its structural stability and adaptability, the isoxazole moiety serves as a versatile scaffold in the design and synthesis of numerous biologically active molecules, including pharmaceutical and agrochemical agents.

Isoxazole is heterocyclic compound with a five-membered aromatic ring containing adjacent oxygen and nitrogen atom at 1 and 2 positions. Many biologically active compounds possess these heterocyclic compounds **[1-3]**. Derivatives containing isoxazole/isooxazoline fragments possess biological activities such as anticancer **[4-5]**, anti-inflammatory **[6-7]**, antibacterial **[8-9]**, anti-Alzheimer's disease **[10-11]**, antioxidant **[12-13]**, insecticidal **[14]**, antifungal **[15-16]** and antidiabetic **[17-18]**. They also possess significant role as an immunomodulator in treatment of auto-immune conditions like arthritis **[19]** and as an important anti-TB pharmacophore as reported in literatures **[20-23]**.

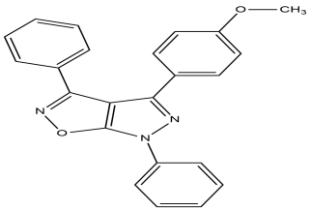
Among five membered nitrogen-containing heterocyclic compounds, indole containing scaffolds gained remarkable attention for its vast role in process of drug discovery as they encompass numerous therapeutic and pharmaceutical properties. The present research involves the synthesis, purification, characterization, docking and biological evaluation of some novel Isoxazole derivatives from indole via Claisen- Schmidt condensation reaction. The synthesized compounds will be screened for their most appropriate biological activity.

Objectives:

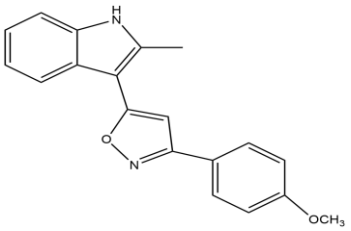
1. To synthesize series of novel isoxazole derivatives.
2. To characterize the synthesized isoxazole derivatives using spectral analysis like IR, NMR and Mass spectra.
3. To assess the drug likeness of derivatives using Swiss ADME and molinspiration web servers.
4. To conduct online toxicity predictions using PyRx software.
5. To evaluate *in-vitro* anti-inflammatory, anti-oxidant, anti-microbial and anti-diabetic activities of synthesized isoxazole derivatives.
6. To evaluate *in-vivo* anti-depressant activity of synthesized isoxazole derivatives.

II. REVIEW OF LITERATURE

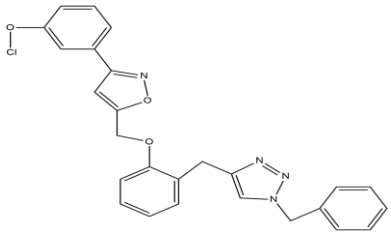
1. Reheim M A M A and co-workers synthesized a series of novel fused isoxazole derivatives. In particular, pyrazolo[4,3-d] isoxazole for their potential anti-microbial and anti-oxidant activities. The structures of the target compounds were supported by the results of 1 H-NMR, IR and mass spectroscopy. The antioxidant potential of these compounds was assessed using DPPH and ABTS assays showing impressive concentration dependent activity **[24]**.



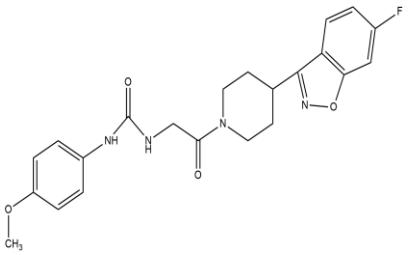
2. Bhatia R and co-workers carried out design and synthesis of a series of 15 indole-3-subtituted isoxazoles having potential anti-inflammatory as well as analgesic properties. Most of the compounds exhibited comparable *in-vivo* anti-inflammatory and analgesic activity in rats to the reference drugs. Some compounds were found to show most significant anti-inflammatory activity and reduced the paw edema which were comparable to reference drug indomethacin. The COX inhibition studies revealed that these compounds have 2–3 folds selectivity for COX-2 with moderate inhibition activity **[25]**.



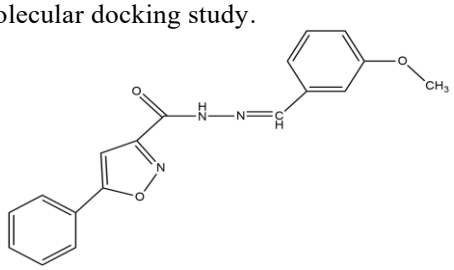
3. Bouzammit R and co-workers designed and synthesized novel isoxazole–triazole conjugates. The structures of the target conjugates and intermediate products were characterized by standard spectroscopic techniques (1H NMR and 13C NMR) and confirmed by mass spectrometry (MS). The all-synthesized compounds were screened for their antibacterial activity against three ATCC reference strains, namely Staphylococcus aureus ATCC 25923, Staphylococcus aureus ATCC BAA-44, and Escherichia coli ATCC 25922. The findings indicate that conjugate exhibits a stronger antibacterial response against the tested Escherichia coli ATCC 25922 strains compared to the standard antibiotics **[26]**.



4. Shantharam C S and co-workers carried out Synthesis of a new series of urea/thiourea derivatives of Gly/Pro conjugated benzisoxazole whose Structures were characterized by physical and spectroscopical data and has been screened for their in vitro antiglycation activity. Several compounds showed promising activity with compared to standard rutin. Further, it was found that compounds containing methoxy and bromine substituents have exerted highly potent activity **[27]**.



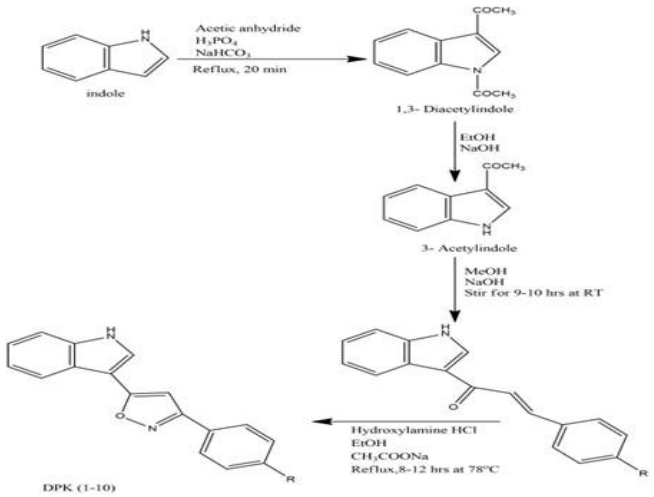
5. Agrawal N and co-workers designed and synthesized a series of phenyl isoxazole carbohydrazides, which was screened for both MAO-A and MAO-B inhibition using Amplex Red assays. None of the compounds inhibited the MAO-A activity while most of them significantly inhibited MAO-B in the micromolar to nanomolar range. Enzyme kinetic studies revealed the reversible and competitive nature of compound towards MAO-B inhibition. The results of the enzyme inhibition assay were aligned with molecular docking study, with molecular docking study.



III. MATERIALS AND METHODS

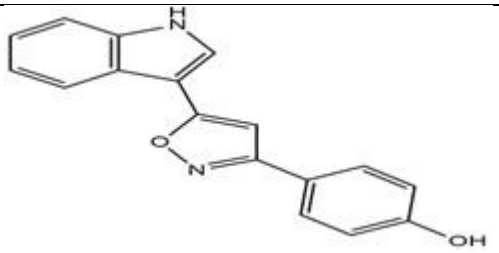
1. Synthetic Methodology:

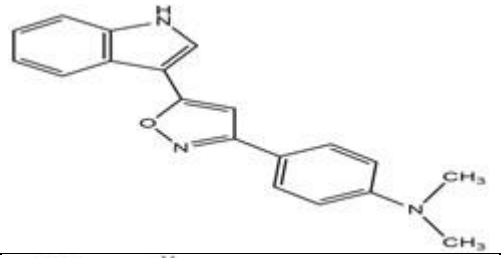
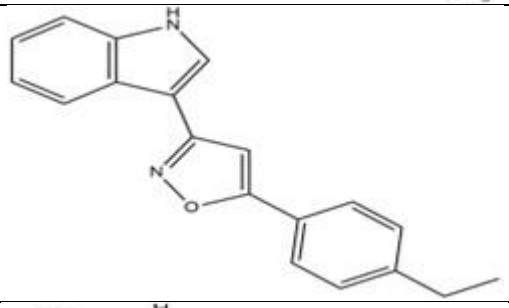
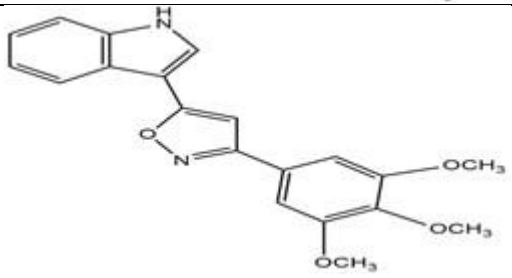
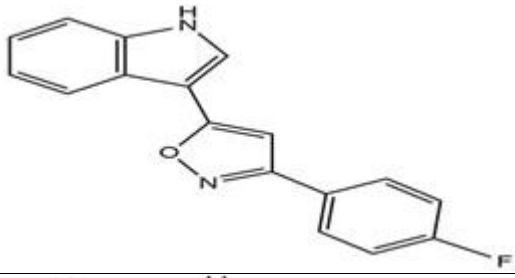
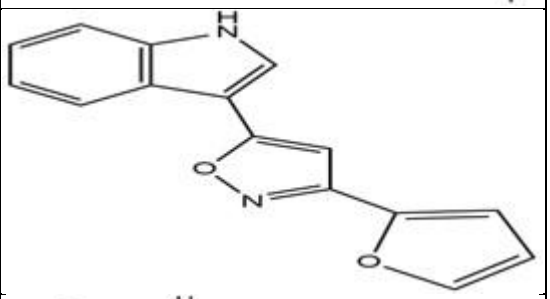
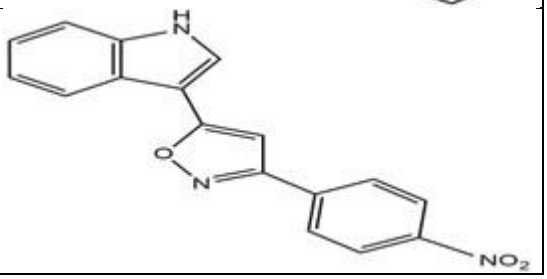
1.1. Synthetic scheme:

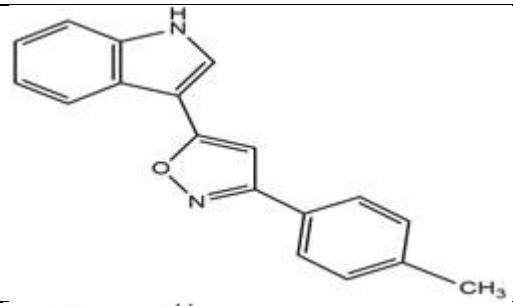
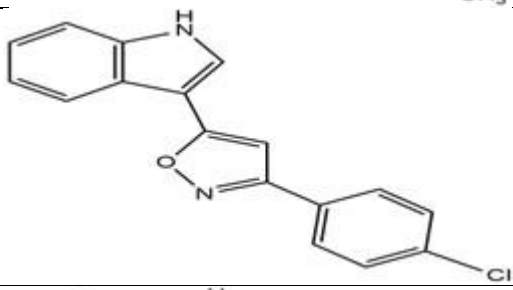
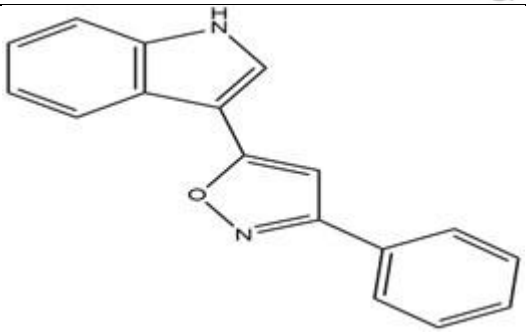


1.2. Structure of synthesized isoxazole derivatives:

Table I: List of isoxazole derivatives.

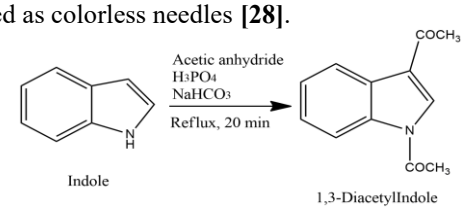
Compound code	IUPAC Name	Structure
DPK-1	3-(1H-indol-3-yl)-5-(4-hydroxyphenyl) isoxazole	

DPK-2	5-(4-(dimethyl amino) phenyl)-3-(1H-indol-3-yl) isoxazole	
DPK-3	3-(1H-indol-3-yl)-5-(4-ethylphenyl) isoxazole	
DPK-4	5-(3,4,5-trimethoxyphenyl)-3-(1H-indol-3-yl) isoxazole	
DPK-5	3-(1H-indol-3-yl)-5-(4-fluorophenyl) isoxazole	
DPK-6	3-(furan-2-yl)-5-(1H-indol-3-yl) isoxazole	
DPK-7	3-(1H-indol-3-yl)-5-(4-nitrophenyl) isoxazole	

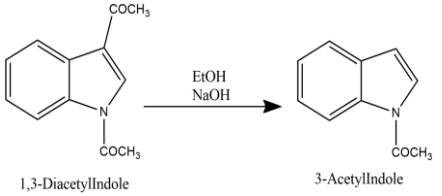
DPK-8	3-(1H-indol-3-yl)-5-(4-methylphenyl) isoxazole	
DPK-9	5-(1H-indol-3-yl)-5-(4-chlorophenyl) isoxazole	
DPK-10	3-(1H-indol-3-yl)-5-phenyl isoxazole	

1.3. Steps Involved in Synthesis of Isoxazole Derivatives:

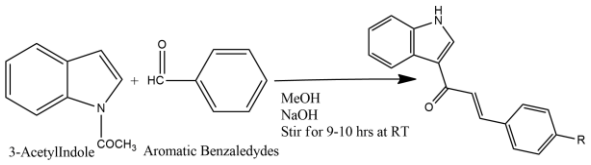
Step 1: Synthesis of 1,3- Diacetyl indole: To 1 gm of indole in RBF,10 ml of acetic anhydride and about 25 drops of 85 % phosphoric acid has been added drop wise. Then, the calcium guard was attached to reflux condenser and the flask was placed on steam bath and refluxed for 20 min. The reaction was cooled to r.t and poured on to crushed ice. The acetic anhydride and excess of phosphoric acid was neutralized by slow addition of sodium bicarbonate (10gm). Then, the mixture was allowed to stand in ice bath for about 10min for the product to precipitate followed by filtration under vaccum. The product was re-crystallized from ethanol. As a result, 1,3-Diacetylindole (55%) was obtained as colorless needles **[28]**.



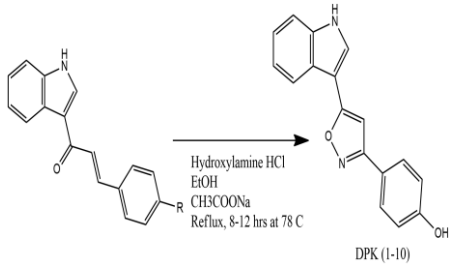
Step 2: Synthesis of 3-Acetylindole: 1,3- Diacetyl indole (1 g) was suspended in ethanol (5 ml) and sodium hydroxide (10 ml) of 2 N was added. The mixture was stirred and warmed until the diacetyl indole had dissolved, the product after being precipitated by dilution with water, was collected and crystallized from ethanol (0.69 g, 87 %) was obtained.



Step 3: Synthesis of Chalcone Derivatives (AIC): To a solution of 3-acetylindole(2gm) (0.01mol) in methanol (50ml), Aromatic aldehyde (2ml) (0.01 mol) was added in the presence of 10% sodium hydroxide (5ml). The reaction mixture was stirred for 9-10 h at room temperature and then the mixture was poured into ice water. The compound obtained was filtered, washed with water and recrystallized using methanol **[29]**.



Step 4: Synthesis of isoxazole derivatives (DPK): To chalcone (0.01 mol,1equiv) in a round-bottom flask, hydroxylamine hydrochloride (0.012,1.2equiv) and sodium acetate (1.2equiv) were added to the flask. Then, ethanol was added as the solvent. The flask was attached to a reflux condenser for the mixture to reflux with heat for 8 hours at 78 °C while stirring continuously. After completion, reaction mixture was cooled to room temperature and poured into ice-cold water (100–200 mL) to precipitate the product. The mixture was allowed to stand for 10–15 min, followed by vacuum filtration to precipitate the solid. Then, washed with cold water to remove residual salts and impurities. The crude product was obtained by recrystallisation from ethanol for better purity.



2. Docking Methodology:

In-silico docking techniques are becoming increasingly significant in the drug discovery process due to their cost-effective identification of potential drug candidates. Docking techniques predict the native position, confirmation (native pose or native binding mode) and orientation of a ligand within the binding side of a target protein. Docking software like PyRx 4.0 uses the Lamarckian genetic algorithm, Monte Carlo simulated annealing and genetic and evolutionary methods to predict ligand flexibility while keeping the target protein receptor rigid. The scoring functions of ligands are based on the AMBER force field, including hydrogen bonding, electrostatic interactions, Vander Waals forces, desolvation terms and conformational entropy. PyRx 4.0 can predict receptor flexibility by allowing the side chains to move freely **[30,31]**.

Proteins involved-

1. The *in-silico* anti-depressant activity of the synthesized compounds was screened using Crystal structure of the chimeric protein of 5-HT1B-BRIL with PDB ID: **4IAR** in complex with ergotamine.
2. The *in-silico* antioxidant activity of the synthesized compounds was screened with PDB ID: **6HN3** using wildtype form (apo) of human GPX4 with Se-Cys46.
3. The *in-silico* anti-inflammatory activity of the synthesized compounds was screened using crystal structure of lectin-like Ox-LDL receptor 1 with PDB ID: **6TLA.**
4. The *in-silico* anti-microbial activity of the synthesized compounds was screened using x-ray structure of human sigma alcohol dehydrogenase with PDB ID: **1AGN.**
5. The *in-silico* antidiabetic activity of the synthesized compounds was screened using Crystal Structure of Human Dipeptidyl Peptidase IV (DPPIV) with PDB ID: **2OLE** complexed with Cyclic Hydrazine Derivatives.

3. In-Silico Physicochemical Properties and Toxicity Studies:

Swiss-ADME is an online tool used to determine pharmacokinetic descriptors, predict ADME parameters, pharmacokinetic properties, drug-likeness, and medicinal chemistry friendliness of ligand molecules to aid the drug discovery process. Molinspiration is another web-based tool used to calculate molecular properties. The SMILES notation of the sketched structures was generated using ChemSketch software and copied. The SMILES notations of each ligand were then pasted into the online tools Swiss ADME and Molinspiration, and the run button was clicked to obtain the desired properties of the ligands **[30]**.

Protox-3 is a free online tool used to predict the toxicity by helping to minimize animal testing and save animal lives. A unique feature of the protocols-3 web server is its ability to predict oral toxicity (acute rodent toxicity), organ toxicity(hepatotoxicity), toxicological endpoints (such as mutagenicity, carcinogenicity, cytotoxicity and immunotoxicity. The SMILES notations of each ligand were then pasted into protox-3 web tool, and the run button was clicked to obtain the toxicity profile of the ligands.

4. Biological Activity:

4.1. In-vitro antioxidant activity (DPPH Free Radical Scavenging Assay):

DPPH is a stable free radical method characterized by the delocalization of the spare electron over the molecule. The delocalization also gives rise to the deep violet colour showing absorbance around 516nm.

A stock solution of DPPH (molecular weight=394.33g/mol) was prepared by dissolving 0.39mg of DPPH in 100mL of methanol(1mM). The solution was kept under dark condition for 90mins for stabilization (DPPH slowly deteriorates over a period after 120mins).

Different concentrations of synthesized compounds of 50, 100, 150, 200, 250 and 500µg/ml were prepared by diluting 0.5ml, 1.0ml, 1.5ml, 2ml and 5ml of stock solution in 10ml volumetric flask by using methanol solution prepared. In the same manner, Standard solution using ascorbic acid were prepared for concentrations ranging from 50µg/ml to 500µg/ml. The radical scavenging ability of synthesized compounds and the ascorbic acid (standard) was tested based on the radical scavenging effect on DPPH free radical. In clean and labelled test tubes, 4.9mL of DPPH solution was mixed with 0.1mL of different concentration of compounds and standard. The tubes were incubated at room temperature in dark for 30 minutes and the optical density was measured against blank solution(methanol) at 516 nm using UV-Visible Spectrophotometer The anti-oxidant activity was calculated using the formula **[32]**:

% Inhibition = [(Absorbance of control - Absorbance of sample) / Absorbance of control)] x 100

4.2. In-vitro anti-inflammatory activity (Bovine Serum Albumin denaturation assay):

In this method, Denaturation of proteins which is a cause of inflammation, was assessed.

A stock solution of 1% w/v of BSA was prepared in phosphate buffer saline and pH was adjusted 7.4 by using glacial acetic acid, DMF (Dimethyl formamide) and Phosphate Buffer (0.2M, Ph 7.4).

Different concentration of synthesized compounds of 50,100, 200 and 400µg/ml were prepared by using methanol as a solvent. Diclofenac sodium was used as a standard. 0.1mL of each sample was transferred to a volumetric flask (10mL) using a 1mL of micro pipette; 4.9 mL of 2% BSA was added to all the above flasks and incubated at 27± 1°C for 15 minutes. Denaturation was induced by keeping the reaction mixture at 80 ±1°C in a water bath for 10 minutes. The control consists of 4.9 ML of 2% BSA solution with 0.1mL methanol. After cooling the turbidity was measured at 660 nm using UV-visible Spectrophotometer. The control represents 100% protein denaturation. The percentage inhibition of BSA denaturation was calculated using the formula **[33]**:

% Inhibition = [(Absorbance of control - Absorbance of sample) / Absorbance of control)] x 100

4.3. In-vitro anti-microbial activity (Agar Well Diffusion Method):

Agar well diffusion method is widely used to evaluate the antimicrobial activity of drug molecules where the agar plate surface is inoculated by spreading a volume of the microbial inoculum over it and diameter of zone formed by the introduction of drug in the wells measured for its zone of inhibition by screening against standard culture of microbes.

In this method, the culture media was prepared by pouring sterilized nutrient agar media such as Mueller-Hinton agar into petri dishes and allowing them to solidify after cooling. These plates are then inoculated with a standard microbial sub-culture containing gram +ve *staphylococcus aureus (ATCC 25923)* and gram -ve *Escherichia coli (ATCC 25922)* bacteria by ensuring even distribution of sub-cultures throughout media by swabbing with an inoculating loop. Then the wells of diameter 6 to 8 mm were made using sterile cork borer upon solidified agar media and into each well a volume of 0.1-0.2ml of test compounds having concentration of 1mg/ml were added and compared against standard solution of streptomycin for zone of inhibition **[34,35]**.

4.4. In-vitro anti-diabetic activity (α-amylase inhibitory assay):

The α-amylase inhibition assay utilized the 3,5-dinitrosalicylic acid (DNSA) method. Stock solutions of test compounds at various concentration of 1mg/ml was prepared using methanol. From this the stock solution, different concentration of synthesized compounds of 100, 200 and 500µg/ml were prepared. Initially, 250µL of prepared sample solution is mixed with freshly prepared 0.02 M sodium phosphate buffer at pH 6.9 and 250µL of prepared α-amylase of concentration 0.5 mg/ml was added and the mixture was pre-incubated at room temperature for 10 minutes. Then, 250µL of 1% starch solution prepared using 0.02 M sodium phosphate buffer at pH 6.9 was added to the mix and further incubated at 25^0C for 10 minutes. To halt the reaction, 500 µL of DNSA (Dinitro salicylic acid) was added and the tubes were boiled in a water bath for 5 minutes until a yellowish-orange colour is developed and the tubes were left to cool under room temperature. The reaction mix was made to dilute by adding 5 ml of distilled water and measured for absorbance at 540 nm using UV-visible Spectrophotometer. The percentage α-amylase inhibition was calculated using the formula **[36]**:

% Inhibition = [(Absorbance of control - Absorbance of sample) / Absorbance of control)] x 100

4.5. In-vivo anti-depressant activity (Forced Swim Test):

This method was used to evaluate *in-vivo* anti-depressant activity via forced swim test using Swiss albino mice weighing about 20-25gm. The animals were classified into group of six (n=6) where each group involves six animals. The first group was treated with control prepared using 2% tween 80 suspension, the second group was treated with a negative control diazepam of dose 2mg/kg, the third group was treated with diazepam to induce depression along with standard drug imipramine of dose 10mg/kg, then the subsequent three groups were treated with diazepam to induce depression along with three different test compounds having dose of 10mg/kg. Treatment was administered using intraperitoneal route. The test was carried for 7 days, where pre-test was carried out for 6 days to train the mice to swim in plexiglass cylinder (10x25 cm) filled with water of 10 cm depth while maintaining temperature at 25^{o}C. On 7th day, the test was carried out by placing the mice of each group in the cylinder to assess immobility time. The test duration lasts for 6 minutes where the last 4 minutes were considered to assess immobility time. The duration of immobility signifies the depressant activity among the mice **[37]**.

IV.RESULTS

1. Physical properties and spectral data of the synthesized compounds:

1.1. 3-Acetyilndole:

1. Physical properties: Molecular formula: $C_{12}H_{11}NO_2$, Molecular weight: 201.22, Colour: Brown, Melting point (^{o}C):151,
% Yield:60
2. FT-IR Spectra(cm^{-1}): 3270(N-H str), 3053(Ar C-H str), 1647(C=O str), 1480(C-N str), 1449(C-C str).

1.2. Physical properties and spectral data of (E)-3-(1H-Indol-3-yl)-1-(4-substituted) prop-2-en-1-one (AIC):

1.2.1 (E)-3-(1H-Indol-3-yl)-1-(4-ethylphenyl) prop-2-en-1-one (AIC-3):

1. Physical properties: Molecular formula: $C_{19}H_{17}NO$, Molecular weight: 275.35, Colour: Yellow, Melting point (^{o}C):140,
% Yield:75
2. FT-IR Spectra(cm^{-1}): 3403(NH-str), 1592(C=O str),1524 (C=C str).

1.2.2.(E)-3-(1H-Indol-3-yl)-1-(4-fluorophenyl) prop-2-en-1-one (AIC-5):

1. Physical properties: Molecular formula: $C_{17}H_{12}NO$, Molecular weight: 273.35, Colour: Pale Yellow, Melting point (^{o}C):220,
% Yield:60
2. FT-IR Spectra(cm^{-1}): 3401(NH-str), 1592(C=O str),1515 (C=C str), 1309(C-F str).

1.2.3. (E)-3-(1H-indol-3-yl)-1-(furan-2-yl) prop-2-en-1-one (AIC-6):

1. Physical properties: Molecular formula: $C_{16}H_{11}NO_2$, Molecular weight: 249.35, Colour: Yellow, Melting point (^{o}C):190,
% Yield:70
2. FT-IR Spectra(cm^{-1}):3396(NH-str), 1608(C=O str),1589 (C=C str).

1.3. Physical properties and spectral data of 3-(1H-indol-3-yl)-5-(4-ethylphenyl) isoxazole (DPK):

1.3.1. 3-(1H-indol-3-yl)-5-(4-hydroxyphenyl) isoxazole (DPK-1):

1. Physical properties: Molecular formula: $C_{17}H_{12}N_2O_2$, Molecular weight: 276.35, Colour: Yellow, Melting point (^{o}C):190,
% Yield:80
2. FT-IR Spectra(cm^{-1}): 3361 (NH-str), 1683 (Ar C=C str), 1524 (C=N str), 1242 (C-O str).

1.3.2. 5-(4-(dimethyl amino) phenyl)-3-(1H-indol-3-yl) isoxazole (DPK-2):

1. Physical properties: Molecular formula: $C_{19}H_{17}N_3O$, Molecular weight: 303.35, Colour: Yellow, Melting point (^{o}C):190,
% Yield:70
2. FT-IR Spectra(cm^{-1}): 3242(NH str), 2916(Ar CH str), 1602 (Ar C=C), 1524 (C=N str), 1361 (C-N str).

1.3.3. 3-(1H-indol-3-yl)-5-(4-ethylphenyl) isoxazole (DPK-3):

1. Physical properties: Molecular formula: $C_{19}H_{16}N_2O$, Molecular weight: 276.00, Colour: Yellow, Melting point (^{o}C):138,
% Yield:60
2. FT-IR Spectra(cm^{-1}): 3373 (NH-Str), 2161 (C=C str), 1482 (C=N str), 1 (Ar C=C), 1219 (C-O str), 1096 (C-N str).

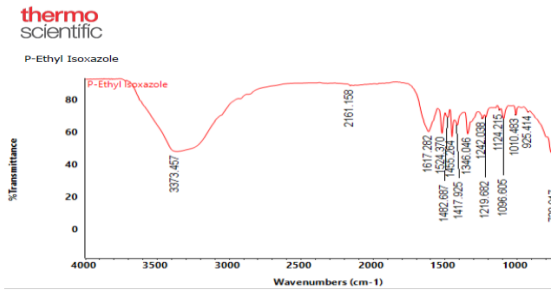


Figure 1 : IR spectrum of compound 3-(1H-indol-3-yl)-5-(4-ethylphenyl) isoxazole (DPK-3)

3. ^{1}H-NMR spectra: δ:H_1(6.78), $H_{2\text{-}5}$(7.17-7.26), H_6(7.51), H_7(10.7), H_8(7.07), $H_{9\text{-}10}$(6.86-6.97), H_{11}(7.35).

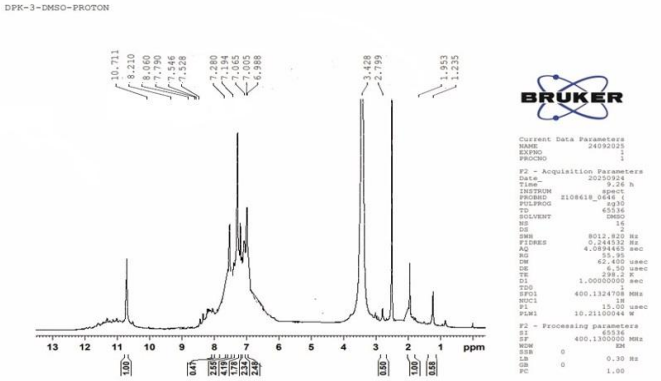


Figure 2:^{1}H-NMR spectrum of compound 3-(1H-indol-3-yl)-5-(4-ethylphenyl) isoxazole

4. ^{13}C-NMR spectra: δ:C_1(111.7), $C_{2\text{-}3}$(150.3-151.2), C_4(118.3), C_5(131.1), C_6(129.6), C_7(140.5), C_8(127.1), C_9(131.1), C_{10}(32.9), C_{11}(19.0), C_{12}(115.2), C_{13}(126.7), C_{14}(126.5), C_{15}(136.8), $C_{16\text{-}19}$(116.6-121.0)

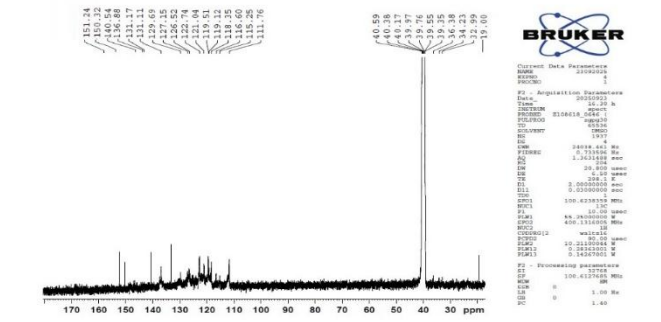


Figure 3:^{13}C-NMR spectrum of compound 3-(1H-indol-3-yl)-5-(4-ethylphenyl) isoxazole

5. Mass Spectra: (m/z): 289.16 [M+1]

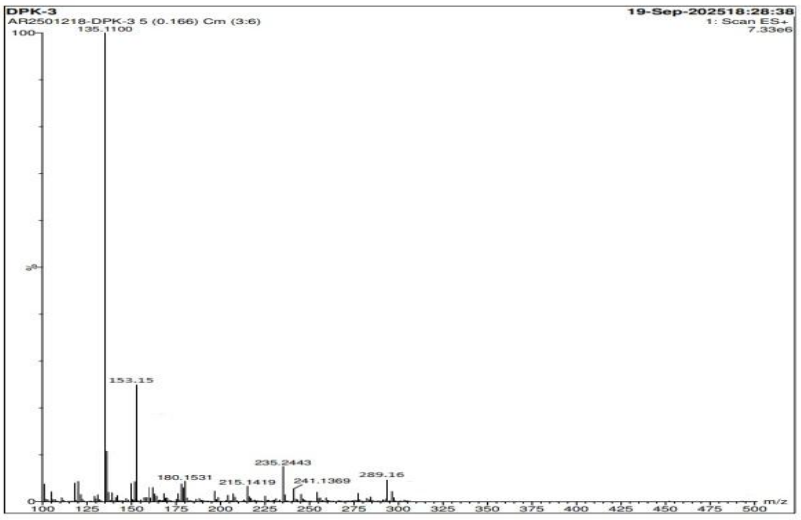


Figure 4: Mass spectra of compound 3-(1H-indol-3-yl)-5-(4-ethylphenyl) isoxazole

1.3.4. 5-(3,4,5-trimethoxyphenyl)-3-(1H-indol-3-yl) isoxazole (DPK-4):

1. Physical properties: Molecular formula: $C_{20}H_{18}N_2O_4$, Molecular weight: 350.35, Colour: Brown, Melting point (°C):180,
   % Yield:60
2. FT-IR Spectra(cm^{-1}): 3362 (NH str), 2932 (Ar C-H str), 1617 (Ar C=C), 1505 (C=N), 1235,1150,1099 (C-O str).

1.3.5. 3-(1H-indol-3-yl)-5-(4-fluorophenyl) isoxazole (DPK-5):

1. Physical properties: Molecular formula: $C_{17}H_{11}FN_2O$, Molecular weight: 278.00, Colour: Light brown, Melting point (°C):195, % Yield:75
2. FT-IR Spectra(cm^{-1}): 3399 (NH-str), 3055 (CH str), 1619 (Ar C=C str), 1482 (C=N str), 1220 (C-F str).

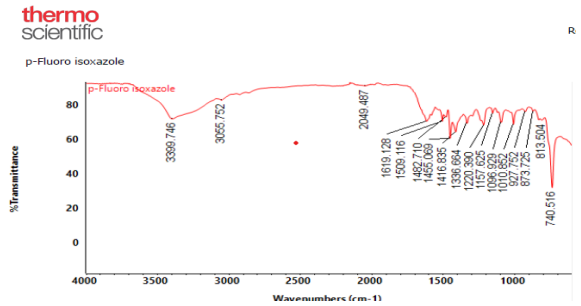


Figure 5 : IR spectrum of compound 3-(1H-indol-3-yl)-5-(4-fluorophenyl) isoxazole

3. ^{1}H-NMR spectra: δ:H_1(6.78), $H_{2\text{-}5}$(7.17-7.26), H_6(7.51), H_7(10.7), H_8(7.07), $H_{9\text{-}10}$(6.86-6.97), H_{11}(7.35)

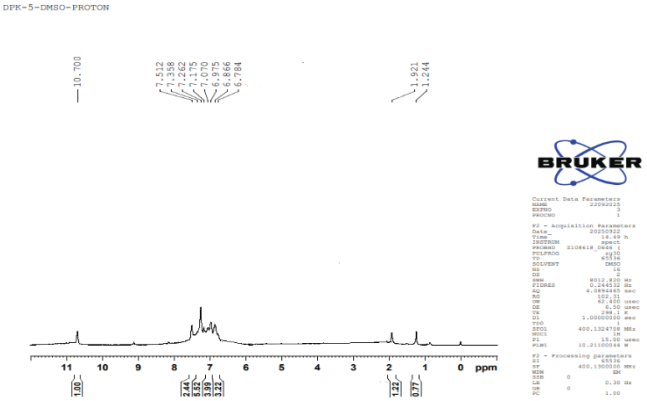


Figure 6 : ^{1}H-NMR spectrum of compound 3-(1H-indol-3-yl)-5-(4-fluorophenyl) isoxazole

4. ^{13}C-NMR spectra: δ:C_1(79.4), C_2 (166.2), C_3 (146.6), $C_{4\text{-}5}$ (120.9-122.6), C_6 (116.5), C_7 (136.8) (Ar-F), C_8 (118.3), C_9 (127.04), C_{10} (111.7), C_{11} (129.6), C_{12} (126.4), C_{13} (131.1), $C_{14\text{-}16}$ (115.1-119.5), C_{17}(127.14)

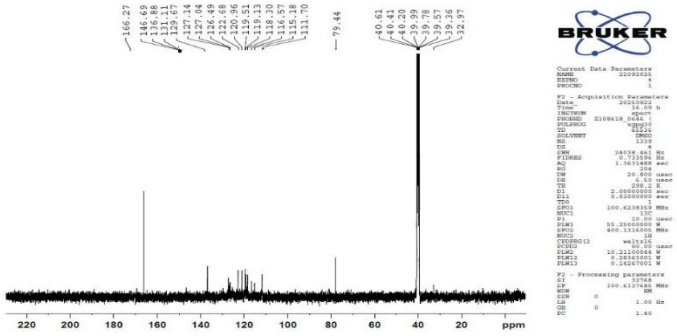


Figure 7 : ^{13}C-NMR spectrum of compound 3-(1H-indol-3-yl)-5-(4-fluorophenyl) isoxazole

5. Mass spectra: (m/z): 277.28 [M+1]

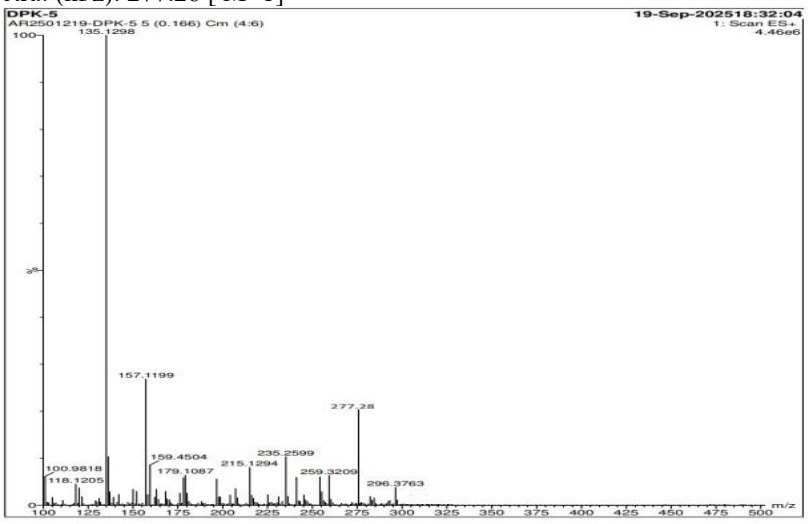


Figure 8 : Mass spectra of compound 3-(1H-indol-3-yl)-5-(4-fluorophenyl) isoxazole

1.3.6. 3-(furan-2-yl)-5-(1H-indol-3-yl) isoxazole (DPK-6):

1. Physical properties: Molecular formula: $C_{15}H_{10}N_2O_2$, Molecular weight: 250.25, Colour: Brown, Melting point (°C):176,
   % Yield:75
2. FT-IR Spectra(cm^{-1}): 3400 (NH-str), 3055 (CH str), 1617(Ar C=C str),1493 (C=N str), 1455 (C=C), 1124 (C-O str).

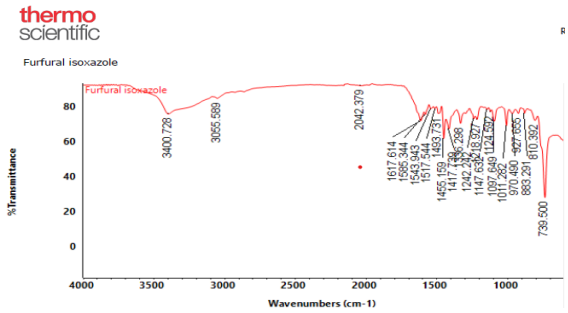


Figure 9 : IR spectrum of compound 3-(furan-2-yl)-5-(1H-indol-3-yl) isoxazole

3. ^{1}H-NMR: δ:H_1(6.78), $H_{2\text{-}5}$(7.17-7.26), H_6(7.51), H_7(10.7), H_8(7.07), $H_{9\text{-}10}$(6.86-6.97), H_{11}(7.35)

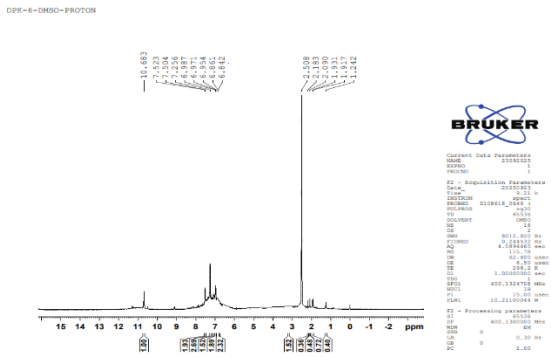


Figure 10: ^{1}H-NMR spectrum of compound 3-(furan-2-yl)-5-(1H-indol-3-yl) isoxazole

4. ^{13}C-NMR spectra: δ:C_1 (79.7), C_2 (146.6), C_3 (129.6), C_4 (136.9), C_5 (127.1), $C_{6\text{-}7}$ (115.2-116.21), $C_{8\text{-}10}$ (111.7-119.5), C_{11} (126.5), C_{12} (118.3), C_{13} (125.1), $C_{14\text{-}15}$ (120.9-122.6)

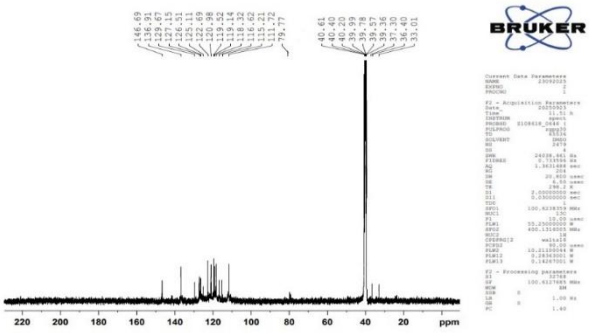


Figure 11: ^{13}C-NMR spectrum of compound 3-(furan-2-yl)-5-(1H-indol-3-yl) isoxazole

5. Mass spectra: (m/z): 250.56 M^+

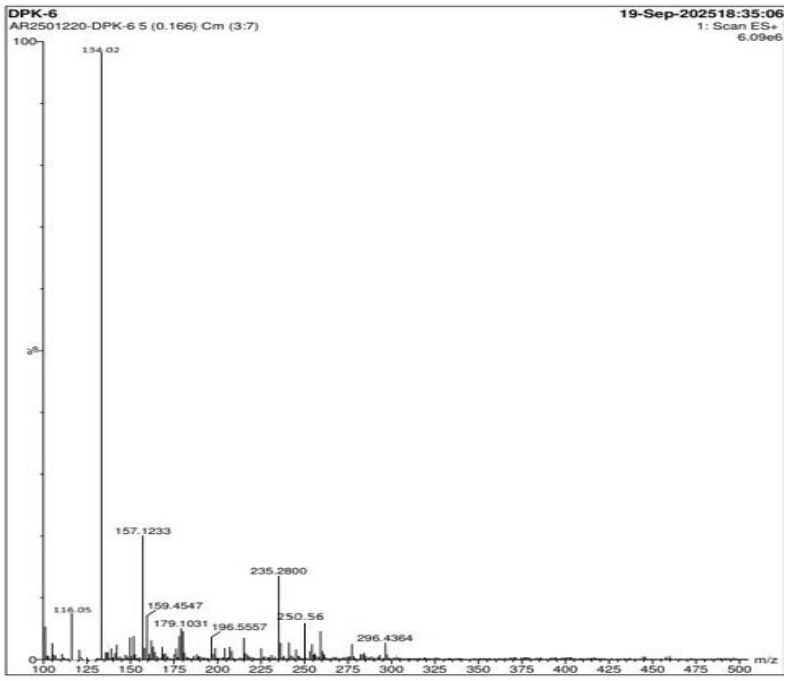


Figure 12: Mass spectra of compound 3-(furan-2-yl)-5-(1H-indol-3-yl) isoxazole

1.3.7. 3-(1H-indol-3-yl)-5-(4-nitrophenyl) isoxazole (DPK-7):

1. Physical properties: Molecular formula: $C_{17}H_{11}N_3O_2$, Molecular weight: 305.35, Colour: Brown, Melting point (°C):207,

% Yield:70

2. FT-IR Spectra(cm^{-1}): 3399 (NH str), 3055 (Ar C-H str), 1601 (Ar C=C), 1494 (C=N), 1515(NO_2 str).

1.3.8. 3-(1H-indol-3-yl)-5-(4-methylphenyl) isoxazole (DPK-8):

1. Physical properties: Molecular formula: $C_{18}H_{14}N_2O$, Molecular weight: 274.35, Colour: Brown, Melting point (°C):171,

% Yield:80

2. FT-IR Spectra(cm^{-1}): 3395 (NH str), 1617 (Isoxazole C=C str),1547 (Ar C=C), 1481 (C=N), 1416 (methyl C-H str).

1.3.9. 3-(1H-indol-3-yl)-5-(4-chlorophenyl) isoxazole (DPK-9):

1. Physical properties: Molecular formula: $C_{17}H_{11}ClN_2O$, Molecular weight: 294.35, Colour: Yellow, Melting point (°C):145,

% Yield:50

2. FT-IR Spectra(cm^{-1}): 3404 (NH str), 3055 (CH str),1619 (Isoxazole C=C str), 1489 (Ar C=C), 1455 (C=N), 823 (C-Cl str).

1.3.10. 3-(1H-indol-3-yl)-5-phenyl isoxazole (DPK-10):

1. Physical properties: Molecular formula: $C_{17}H_{12}N_2O$, Molecular weight: 260.35, Colour: Dark Brown, Melting point (°C):141,

% Yield:70

2. FT-IR Spectra(cm^{-1}): 3258 (NH str), 3056,2925 (CH str), 1601 (Isoxazole C=C str), 1491 (Ar C=C), 1450 (C=N)

2. *In-Silico* activity for the synthesized compounds:

2.1. *In-silico* anti-depressant activity:

In-silico anti-depressant activity of the synthesized isoxazole derivatives were screened using the target protein with **PDB ID:4IAR**. The compounds **DPK-8 and DPK-2** exhibited binding affinity with a value of **-10.8 and -10.0** kcal/mol respectively.

Table 2: Binding energy of the compounds with target protein **PDB ID: 4IAR**

Ligand	Binding Affinity
DPK-1	-9.9
DPK-2	-10.0
DPK-3	-9.8
DPK-4	-9.1
DPK-5	-9.6
DPK-6	-9.4
DPK-7	-9.5
DPK-8	-10.8
DPK-9	-9.8
DPK-10	-9.7
Imipramine	-8.2

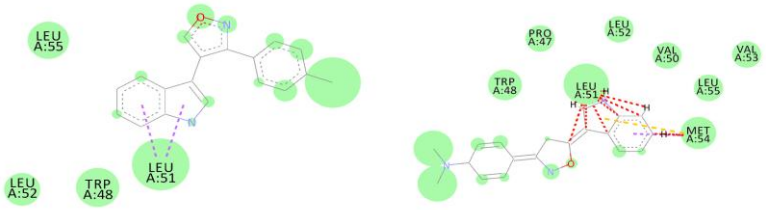


Figure 13: Docking interaction of **DPK-8** and **DPK-2** with **PDB ID-4IAR.**

2.2. *In-silico* anti-inflammatory activity:

In-silico anti-inflammatory activity of the synthesized isoxazole derivatives were screened using the target protein with **PDB ID:6TLA**. The compounds **DPK-5 and DPK-8** exhibited binding affinity with a value of **-7.8 and -7.9** kcal/mol respectively.

Table 3: Binding energy of the compounds with target protein **PDB ID: 6TLA**

Ligand	Binding Affinity
DPK-1	-7.4
DPK-2	-7.4
DPK-3	-7.6
DPK-4	-7.0
DPK-5	-7.8
DPK-6	-6.8
DPK-7	-7.6
DPK-8	-7.5
DPK-9	-7.9
DPK-10	-7.6
Diclofenac sodium	-6.4

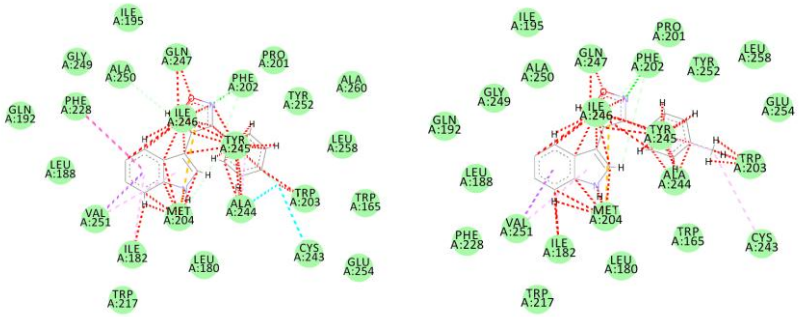


Figure 14: Docking interaction of **DPK-5** and **DPK-8** with **PDB ID-6TLA.**

2.3. *In-silico* anti-microbial activity:

In-silico anti-microbial activity of the synthesized isoxazole derivatives were screened using the target protein with **PDB ID:1AGN**. The compounds **DPK-1 and DPK-2** exhibited binding affinity with a value of **-9.6 and -9.6** kcal/mol respectively.

Table 4: Binding energy of the compounds with target protein **PDB ID: 1AGN**

Ligand	Binding Affinity
DPK-1	-9.6
DPK-2	-9.6
DPK-3	-8.6
DPK-4	-7.8
DPK-5	-8.2
DPK-6	-7.9
DPK-7	-8.4
DPK-8	-9.3
DPK-9	-8.2
DPK-10	-9.5
Streptomycin	-7.0

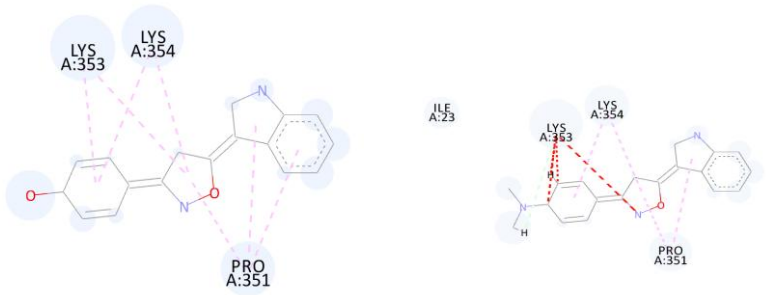


Figure 15: Docking interaction of **DPK-1** and **DPK-2** with **PDB ID-1AGN.**

2.4. *In-silico* anti-oxidant activity:

In-silico anti-oxidant activity of the synthesized isoxazole derivatives were screened using the target protein with **PDB ID:6HN3**. The compounds **DPK-8 and DPK-10** exhibited binding affinity with a value of **-7.5 and -7.4** kcal/mol respectively.

Table 5: Binding energy of the compounds with target protein **PDB ID: 6HN3**.

Ligand	Binding Affinity
DPK-1	-7.3
DPK-2	-7.3
DPK-3	-7.0
DPK-4	-6.4
DPK-5	-6.9

DPK-6	-6.8
DPK-7	-6.6
DPK-8	-7.5
DPK-9	-6.6
DPK-10	-7.4
Ascorbic acid	-6.5

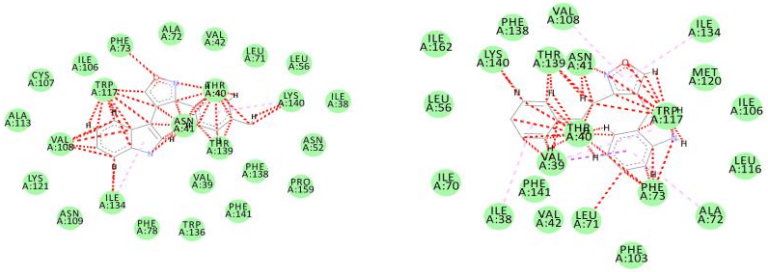


Figure 16: Docking interaction of **DPK-8** and **DPK-10** with **PDB ID-6HN3.**

2.5. *In-silico* anti-diabetic activity:

In-silico anti-diabetic activity of the synthesized isoxazole derivatives were screened using the target protein with **PDB ID:2OLE**. The compounds **DPK-1 and DPK-8** exhibited binding affinity with a value of **-9.7 and -8.8** kcal/mol respectively.

Table 6: Binding energy of the compounds with target protein **PDB ID: 2OLE**.

Ligand	**Binding Affinity**
DPK-1	-9.7
DPK-2	-7.9
DPK-3	-8.6
DPK-4	-8.0
DPK-5	-8.8
DPK-6	-8.1
DPK-7	-8.4
DPK-8	-8.8
DPK-9	-8.8
DPK-10	-8.7
Acarbose	-8.4

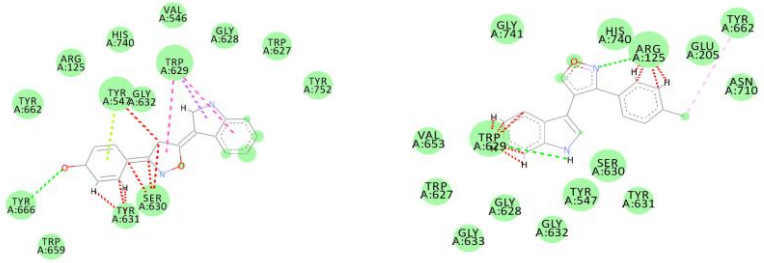


Figure 17: Docking interaction of **DPK-1** and **DPK-8** with **PDB ID-2OLE.**

3. *In-Silico* Physicochemical Properties:

All the designed and synthesized compounds exhibited a good lipophilic profile and the bioactivity score of the compounds exhibited good ion channel modulation and nuclear receptor ligand effect.

3.1. *In-silico* Physicochemical Studies of compounds obtained from Molinspiration:

Table 7: *In-silico* Physicochemical Studies of compounds obtained from Molinspiration.

Compound code	miLogP	TPSA	n- atoms	MW (g/mol)	nO N	n OH NH	n-violations	n rotb	volume
DPK-1	3.69	62.05	21	276.29	4	2	1	2	241.27
DPK -2	4.27	45.06	23	303.35	4	1	0	3	279.16
DPK -3	5.08	41.82	22	288.34	3	1	1	3	266.61
DPK -4	3.79	69.51	26	350.37	6	1	0	5	309.89
DPK -5	4.33	41.82	21	278.28	3	1	0	2	238.18
DPK -6	3.31	54.96	19	250.26	4	1	0	2	224.8
DPK -7	4.12	87.65	23	305.29	6	1	0	3	256.5
DPK -8	4.61	41.82	21	274.32	3	1	0	2	249.81
DPK -9	4.84	41.82	21	294.74	3	1	0	2	246.78
DPK -10	4.16	41.82	20	260.31	3	1	0	2	233.25

3.2. *In-silico* Physicochemical Studies of compounds obtained from Swiss ADME:

Table 8: *In-silico* Physicochemical Studies of compounds obtained from Swiss ADME

Compound code	DPK -1	DPK -2	DPK -3	DPK -4	DPK -5	DPK -6	DPK -7	DPK -8	DPK -9	DPK -10
No. of heavy atoms	21	23	22	26	21	19	23	21	21	20
No. of aromatic heavy atoms	20	20	20	20	20	19	20	20	20	20
No. of rot bonds	2	3	3	5	2	2	3	2	2	2
No. of H-bond acceptors	3	4	2	5	3	3	4	2	2	2
No, of H-bond donors	2	3	1	1	1	1	1	1	1	1

Molar refractivity	81.25	89.61	89.00	98.71	79.19	71.50	88.05	84.20	84.24	79.23
Total polar surface area (A°)	62.05	45.06	41.82	69.51	41.82	54.96	87.65	41.82	41.82	41.82
Log $P_{O/w}$ (iLog P)	2.15	1.92	3.00	3.30	2.65	2.48	2.24	2.79	2.81	2.54
Water solubility	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
BBB permeant	Yes	No	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
GI absorption	High	High	High	High	High	High	High	High	High	High
Drug-likeliness (violation)	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation
Lead-likeliness	Yes	Yes	No: 1 violation: XLOGP3>3,5	No: 2 violations: MW>350 XLOGP3>3,5	No; 1 violation: XLOGP3>3.5	Yes	No; 1 violation: XLOGP3>3.5	No; 1 violation: XLOGP3>3.5	No; 1 violation: XLOGP3>3.5	No; 1 violation: XLOGP3>3.5

3.3. *In-silico* Toxicity Studies:

In-silico toxicity studies were carried out for all the synthesized isoxazole derivatives

Table 9: *In-silico* toxicity Studies of compounds obtained from protox 3.0

Compound code	Predicted LD50 (mg/kg)	Hepatotoxicity	Neuro toxicity	Nephrotoxicity	Respiratory Toxicity	Cardio toxicity
DPK -1	1000	Active	Inactive	Inactive	Inactive	Inactive
DPK -2	612	Active	Active	Inactive	Active	Inactive
DPK -3	612	Active	Inactive	Inactive	Inactive	Inactive
DPK -4	1000	Active	Inactive	Inactive	Active	Inactive
DPK -5	612	Active	Active	Inactive	Inactive	Inactive
DPK -6	565	Active	Active	Inactive	Inactive	Inactive
DPK -7	612	Active	Inactive	Inactive	Inactive	Inactive
DPK -8	612	Active	Inactive	Inactive	Inactive	Inactive
DPK -9	500	Active	Active	Inactive	Inactive	Inactive
DPK -10	612	Active	Inactive	Inactive	Inactive	Inactive

4. Biological Activity:

4.1. *In-vitro* antioxidant activity (DPPH free radical scavenging assay):

All the synthesized compounds were screened for *in-vitro* antioxidant activity by DPPH free radical scavenging assay at concentration of 50µg/ml, 100µg/ml, 200µg/ml, 400 µg/ml and 600µg/ml. Ascorbic acid was used as a standard. DPPH is a stable free radical imparts purple colour solution with methanol at room temperature. The *in-vitro* antioxidant activity of the synthesized compounds was determined by measuring the degree of coloration which indicates the free radical scavenging potential of the compounds. Compounds **DPK-2** and **DPK-3** displayed the most potent antioxidant activity. The calculation of results is based on UV absorbance values which determines the colour intensity of the compounds.

Table 10: Percentage inhibition of the synthesized compounds for *in-vitro* antioxidant activity.

Compound code	% Inhibition					IC_{50}
	50µg	100µg	200µg	400µg	600µg	
DPK-1	4.65	16.27	9.30	23.25	52.55	521.76
DPK-2	2.32	18.60	46.51	46.51	69.76	389.59
DPK-3	20.90	34.88	58.13	62.79	72.09	313.63
DPK-4	16.27	25.58	27.90	44.18	51.16	533.14
DPK-5	4.65	27.90	34.68	46.51	62.79	453.24
DPK-6	18.60	28.90	32.55	46.51	48.51	579.07

DPK-7	18.60	23.25	48.85	39.53	51.16	531.94
DPK-8	2.32	11.62	11.62	34.88	51.16	537.96
DPK-9	13.95	30.23	48.83	58.13	60.46	427.38
DPK-10	2.32	23.25	44.18	51.16	53.48	513.56
Ascorbic acid	24.60	31.25	45.76	52.35	56.67	438.72

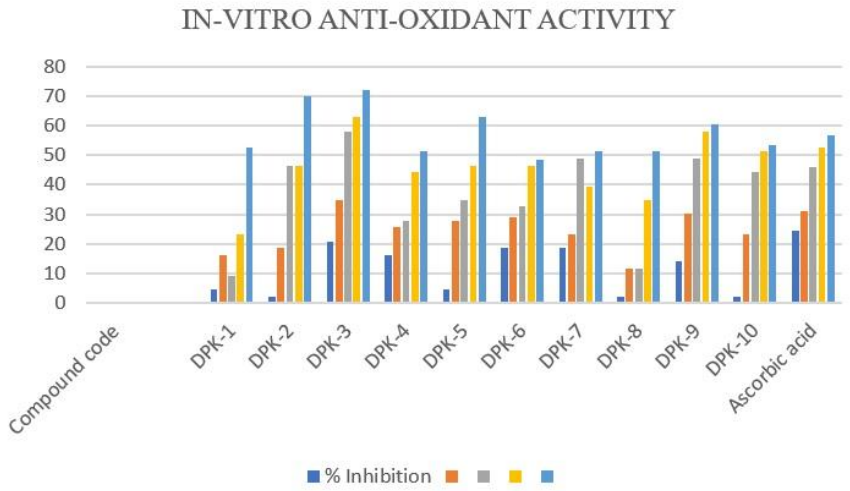


Figure 18: Graphical representation of *in-vitro* antioxidant activity by using DPPH free radical scavenging assay

Figure 19: *In-vitro* antioxidant activity by using DPPH free radical scavenging assay.

4.2. *In-vitro* anti-inflammatory activity (Bovine Serum Albumin denaturation assay):

Among all the synthesized compounds screened for *in-vitro* anti-inflammatory activity by bovine serum albumin denaturation assay at concentration of 50 µg/ml, 100µg/ml, 200µg/ml and 400µg/ml. Standard drug used for the activity was diclofenac sodium. The *in-vitro* anti- inflammatory activity of the synthesized compounds was determined by measuring the UV absorbance values of the denatured compounds. Compounds **DPK-1** and **DPK-2** displayed the most potent anti-inflammatory activity.

Table 11: Percentage inhibition of the synthesized compounds for *in-vitro* anti-inflammatory activity.

Compound code	**% Inhibition**				**IC_{50}**
	50µg	**100µg**	**200µg**	**400µg**	
DPK-1	6	14	29	51	343.33
DPK-2	5	17	33	54	322.44
DPK-3	9	17	25	46	389.18
DPK-4	12	21	34	46	392.64
DPK-5	5	14	25	45	395.00

DPK-6	8	19	29	48	368.75
DPK-7	4	13	31	49	358.88
DPK-8	6	18	24	47	376.82
DPK-9	9	15	30	48	369.23
DPK-10	11	19	23	48	370.27
Diclofenac Sodium	5	16	32	48	367.44

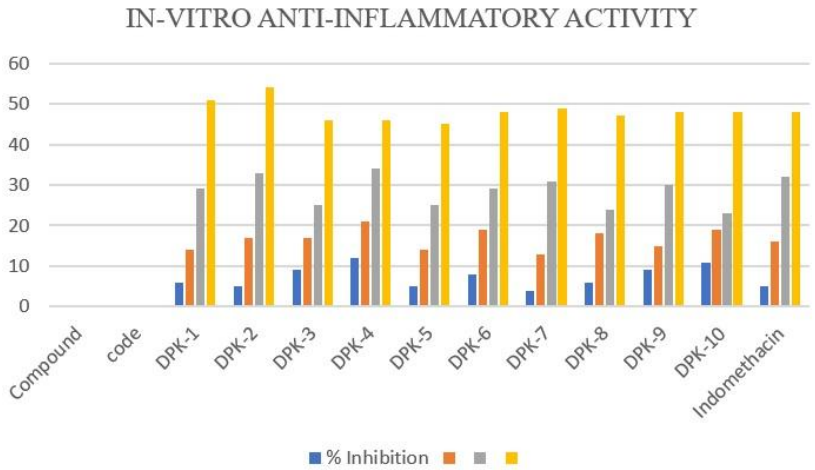


Figure 20: Graphical representation of *in-vitro* anti-inflammatory activity by using Bovine serum albumin assay

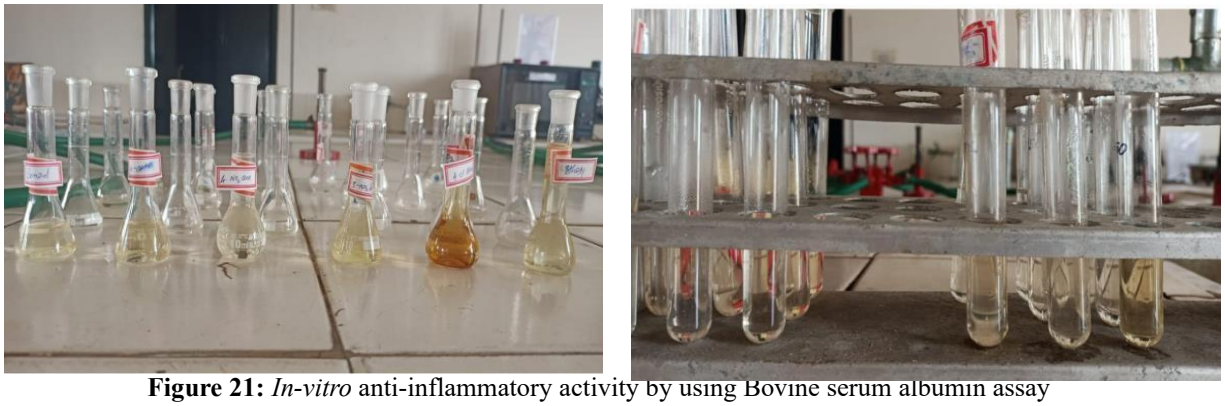


Figure 21: *In-vitro* anti-inflammatory activity by using Bovine serum albumin assay

4.3. *In-vitro* anti-microbial activity (Agar Well Diffusion Method):

The compounds were screened for antimicrobial activity against different bacteria strains at concentration of 1mg/ml for gram negative *Escherichia Coli* bacteria and gram-positive staphylococcus aureus bacteria. Compounds **DPK-6** and **DPK-7** displayed the most potent anti-microbial activity.

Table 12: Zone of inhibition of the synthesized compounds for *in-vitro* anti-microbial activity.

Compounds Code (1mg/ml)	**Zone of inhibition (mm)**	
	Gram + ve	**Gram - ve**
	Staphylococcus aureus	***Escherichia Coli***
DPK-1	10.0	5.0
DPK-2	11.0	8.0
DPK-3	15.0	0
DPK-4	17.0	6.0

DPK-5	8.0	0
DPK-6	16.0	12.0
DPK-7	21.0	10.0
DPK-8	11.0	8.0
DPK-9	9.0	7.0
DPK-10	9.0	9.0
Streptomycin	24.0	21.0

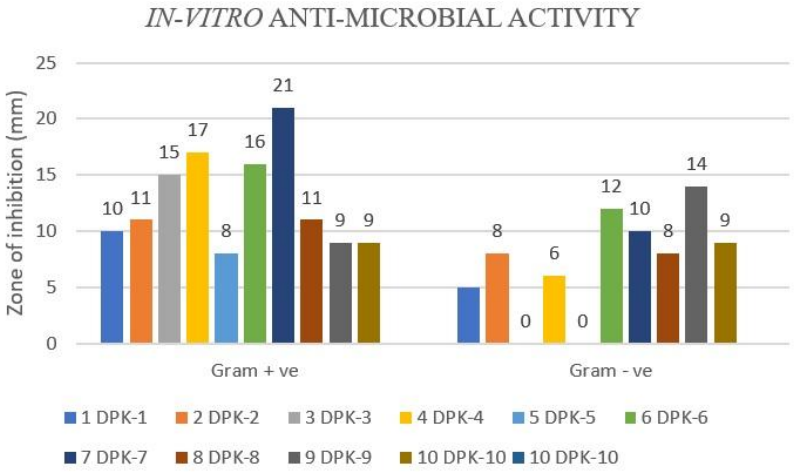


Figure 22: Graphical representation of *in-vitro* antimicrobial activity by using Agar well diffusion method

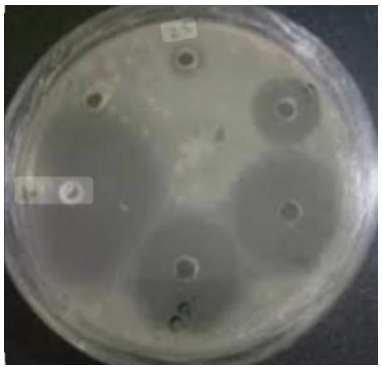


Figure 23: *In-vitro* antimicrobial activity by using Agar well diffusion method

4.4. *In-vitro* anti-diabetic activity (Alpha amylase inhibitory assay):

All the synthesized compounds were screened for *in-vitro* antidiabetic activity by using α- amylase inhibitory assay at concentration of 100µg/ml, 200µg/ml and 500µg/ml. Acarbose was used as a standard. The *in-vitro* antidiabetic activity of the synthesized compounds was determined by measuring percentage inhibition. Compounds **DPK -2** and **DPK -7** displayed the most potent anti-diabetic activity.

Table 13: Percentage inhibition of the synthesized compounds for *in-vitro* anti-diabetic activity.

Compound code	% Inhibitory Concentration			IC_{50}
	100µg/ml	200µg/ml	500µg/ml	
DPK-1	22.15	53.25	87.52	478.01
DPK -2	28.15	49.15	96.65	421.02
DPK -3	25.65	54.25	86.13	493.38
DPK -4	21.50	63.15	85.40	492.95
DPK -5	30.25	54.50	89.25	474.57
DPK -6	27.85	45.61	86.71	487.70
DPK -7	18.95	61.50	87.92	464.77
DPK -8	23.25	59.60	86.50	486.95
DPK -9	20.20	47.25	85.50	490.35
DPK -10	23.26	66.30	89.20	467.03
Acarbose	39.00	54.50	93.50	449.54

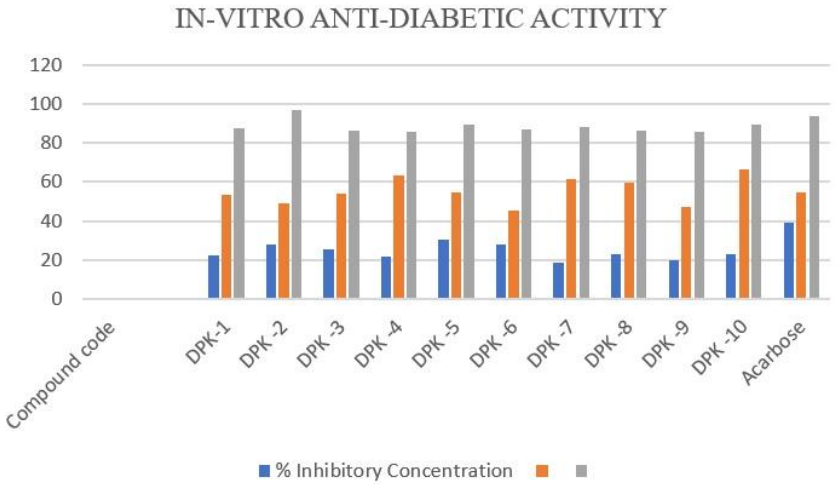


Figure 24: Graphical representation of *in-vitro* diabetic activity by using Alpha amylase inhibitory assay

Figure 25: *In-vitro* diabetic activity by using Alpha amylase inhibitory assay

4.6. *In-vivo* anti-depressant activity (Forced Swim Test):

The synthesized compounds were screened for *in-vivo* anti-depressant activity by Forced Swim Test. The standard drug used for the activity was Imipramine. The *in-vivo* anti-depressant activity of the synthesized compounds was determined by using immobility time(sec). Compound **DPK-3** displayed the potent anti-depressant activity.

Table 14: *In-vivo* anti-depressant activity of synthesized compounds by Forced swim test in mice.

Groups	Dose (mg/kg)	Immobility time (s) (mean ± SEM)	Change from control (%)
Control (2% tween 80)	-----	233.83±0.60	-----
Positive control- Diazepam	2	190.83±1.42	18.44
Standard- Imipramine	10	225.00±1.75	3.79
DPK-3	10	217.50±1.47	7.04
DPK-5	10	210.16±1.83	10.17
DPK-6	10	203.66±4.19	12.95

Values represent the mean ±SEM (n=6), *Significantly compared to control (Dunnet's test; *p<0.05, **p<0.01) by using one way Analysis of Variance (ANOVA) followed by multiple comparison Dunnett's test.

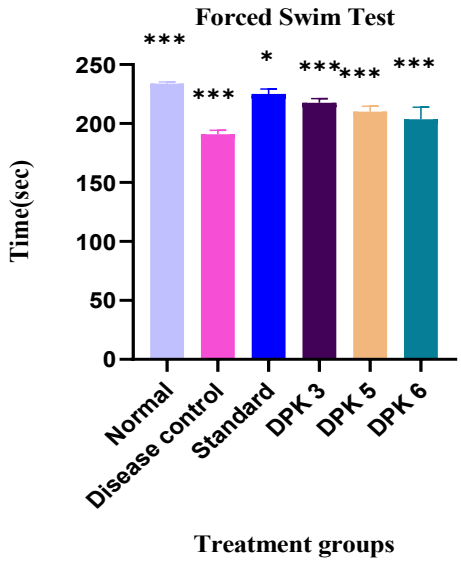


Figure 26: Graphical representation of compounds for *in-vivo* anti-depressant activity by Forced swim test

V.CONCLUSION:

The research paper aims to synthesize, characterize, conduct *in-silico* studies, and evaluate some biological activities of some novel isoxazole derivatives. The synthetic pathway started from the formation of 1,3-diacetylindole intermediate when indole reacts with acetic anhydride in presence of phosphoric acid and sodium bicarbonate followed by deacetylation in presence of sodium hydroxide and ethanol leading to formation of 3-acetylindole. Further, the resultant compound underwent Claisen-Schmidt condensation reaction with substituted benzaldehydes in presence of methanol and sodium hydroxide to give substituted indole-chalcones. Later, the

intermediates were treated with hydroxylamine hydrochloride in presence of ethanol and sodium acetate to form various isoxazole derivatives.

All final compounds were elucidated and confirmed through spectral analysis using IR, ^{1}H- NMR, ^{13}C-NMR, and MS spectroscopy. The identification of specific functional groups, such as fluoro, nitro, methoxy, ethoxy, methyl, and hydroxy groups in the isoxazole derivatives and intermediate compounds, was done using IR.

All synthesized compounds were screened for in-silico studies utilizing various online tools, including Swiss ADME, molinspiration, and ProTox-3. Most of the compounds complied to Lipinski's rule of five.

Molecular docking was conducted using PyRx 4.0 to determine the binding affinity (kcal/mol) of the compounds for antioxidant (PDB ID:6HN3), anti-inflammatory (PDB ID:6TLA), anti-microbial (PDB ID:1AGN), antidiabetic (PDB ID:2OLE), and antidepressant activity (PDB ID:4IAR). Almost all the compounds showed favorable binding affinities compared to the reference compounds.

All the synthesized compounds were subjected to *in-vitro* anti-oxidant, anti-inflammatory, anti-microbial, anti-diabetic and *in-vivo* anti-depressant activity demonstrating good to moderate efficacy. For *in-vitro* anti-oxidant activity (DPPH Radical scavenging assay), DPK-2 and DPK-3 exhibited the highest percentage of inhibition compared to standard drug ascorbic acid. For *in-vitro* anti-inflammatory activity (Bovine serum albumin assay), DPK-1 and DPK-2 exhibited the highest percentage of inhibition compared to standard drug indomethacin. For *in-vitro* anti-microbial activity (Agar well diffusion method), DPK-6 and DPK-7 exhibited potent zone of inhibition compared to standard drug streptomycin. For *in-vitro* anti-diabetic activity (Alpha amylase inhibitory assay), DPK-2 and DPK-7 better activity compared to standard drug acarbose. For *in-vivo* anti-depressant activity (Forced swim test), DPK-3 exhibited significant activity compared to standard drug imipramine. To conclude, the presence of p-nitro, p- dimethyl amino and p-hydroxy groups as substituents likely enhanced the overall pharmacological efficiency, suggesting the electron withdrawing groups may have a synergistic effect. Additionally, the fusion of isoxazole with indole ring may also have contributed to overall biological efficacy.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article. The research was carried out using the institutional facilities of Al-Ameen College of Pharmacy, Bengaluru, India.

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