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Molecular Docking, Synthesis and *In vivo* Assessment of New Phenothiazines as Inhibitors of Anxiety

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Heterocylics are the backbone of the medicinal chemistry. They played very imperative role in the discovery of new lead molecules. Phenothiazine is one of them and it has many biological activities as well. This article proposed the design and synthesis of new phenothiazine derivatives. This work includes the preparation of some new Schiff bases of phenothiazine. The synthetic scheme includes development of series of 1-(10H-phenothiazin-10-yl)-2-(4-((phenylimino)methyl) phenoxy) ethan-1-one (D1-D10). This work aims to design some new phenothiazine molecules. Phenothiazine is main heterocyclic moiety of this work to perform their computational study and to evaluate the prepared molecules as anxiolytic agents. All the derivatives was synthesized in the laboratory and characterized by applying different parameters. These parameters include melting point, solubility, Rf value, spectroscopic analysis like IR spectroscopy and 1H-NMR spectroscopy. Some software was used to dock the derivatives and to perform the computational studies. The synthesized compounds were screened for anti-anxiety activity and elevated plus maze method was incorporated to perform the activity. Statistical analysis of the prepared derivatives was done followed by one way ANOVA dunnett's test. Among them, some derivatives showed potent activity and some showed moderate activity against anxiety.

Keywords: Anti-anxiety activity; Autodock Vina; Chalcones; Chem Draw; Computational studies; Phenothiazine.

Carbocyclic compounds are organic cyclic compounds that have all of their carbon atoms arranged in rings. Heterocyclic compounds are those in which the ring structure contains at least one element other than carbon. In a heterocyclic ring, more than one heteroatom, which can be same or different from each other, either single bonded or double bonded such as oxygen, nitrogen, sulphur, phosphorous etc. Phenothiazine is the heterocyclic compound with one sulphide, one nitrogen, and four carbon atoms in a ring. Thiazines are employed as insecticides, tranquillizers, and dyes. There are many known 1,4-thiazine chemicals, the majority of which are phenothiazine derivatives[1].

The tricyclic fused ring thiazine molecules with the heteroatoms nitrogen and sulphur make up phenothiazine, a benzo derivative. Nitrogen in the phenothiazine nucleus should be regarded as non-aligned[2]. All phenothiazines are easily oxidised chemicals that are relatively cheap, widely available, well tolerated, and harmless. This is especially true when sunlight and moisture are

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present. Since they have a low oxidation potential and are easily capable of forming radical cations, phenothiazines are electron-donor chemicals. It can be discovered as the redox active component in donor acceptor systems as well as in ligands that are used to functionalize various surfaces[3].

Phenothiazine and several of its derivatives have been shown to exhibit a variety of biological actions in the past. Therefore, the value of phenothiazine chemicals as medications has long been understood[4]. The basic nitrogen of the ring, which gives electrons to the biological receptors through a charge transfer mechanism, is thought to be responsible for the phenothiazine's pharmacological effects[5]. An essential class of neuroleptics used to treat moderate to severe mental and emotional problems are phenothiazines[6]. Phenothiazine and its derivatives have several applications outside of biology; for example, complexes like methylene blue are well-known as dyes, bacterial stains, or redox indicators[7,8]. From the above view of modification in phenothiazine ring, this article forms new derivatives followed by structural substitution on nitrogen atom of the ring which is known to possess CNS activity to relieve mental disorders.

MATERIAL AND METHODS

Every chemical was purchased from Fine chemicals and CDH, they were of laboratory grade and utilized without purification. R_f value of every derivative was calculated to examine the progress of reaction while performing experiment. TLC slides was coated with silica gel then activated in hot air oven at 150°C for 30 minutes[9]. The mobile phase was n-hexane:ethyl acetate in ratio (1:2) was used as mobile phase and iodine glass chamber was used to visualize the spots. For checking melting point, an open capillary method was used and was unrectified. Determination of solubility of the derivatives was employed in various solvents like ethanol, benzene, ethyl methyl ketone, chloroform, ether, acetone and acetonitrile. Analytical studies like FT-IR spectroscopy and 1H-NMR spectroscopy was done at Central Instrumentation Facility lab, Punjab University, India. Bruker Advance Neo Spectrophotometer with 500 MHz frequency, DMSO as solvent and TMS as an internal standard was used for performing 1H-NMR study.

Synthetic procedure

Synthetic scheme is represented in scheme I and their procedures is given below

Synthesis of compound (B)

(0.01 mol, 1.99 g) phenothiazine and (0.01 mol, 1.13 ml) chloroacetyl chloride were taken in a 250 ml RBF, along with 100 ml of dry acetonitrile and agitated until dissolved. After that, (0.02 mol, 2.76 g) K_2CO_3 was added in RBF and refluxed for six hrs. On the completion of six hours, The flask's content was filtered and solvent was evaporated under reduced pressure to obtain the 2-chloro-1-(10H-phenothiazin-10-yl)ethan-1-one (B). End product was recrystallized by ethanol[10-12].

Synthesis of compound (C)

(0.01 mol, 2.75 g) compound (B) and (0.01 mol, 1.36 g) 4-hydroxy benzaldehyde was dissolved in (100 ml) anhydrous acetonitrile were taken in a 250 ml RBF and (0.02 mol, 2.76 g) anhydrous K_2CO_3 was added to the round bottom flask, refluxed for seven hours. After reflux, the flask's content was cooled and filtered. Solvent was evaporated under reduced pressure to obtain the end product 2-(4-acetylphenoxy)-1-(10H-phenothiazin-10-yl)ethan-1-one (C). End product was recrystallized by ethanol[13-15].

Synthetic procedure for the preparation of (D1-D10):

In a 100 ml RBF, (0.01 mol, 3.61 g) 4-(2-oxo-2-(10*H*-phenothiazin-10-yl)ethoxy) benzaldehyde (C) was dissolved in 50 ml ethanol with a catalytic amount (0.5 ml) of glacial acetic acid and (0.01 mol) various substituted anilines (H, 3-Cl, 4-Cl, 3,4-Cl, 3-NO_2, 4-NO_2, 3,4-NO_2, 2,3-CH_3, 3-Cl-4-F, 4-Cl-2-NO_2) was added to this solution. This solution was refluxed for 3-5 hrs. After completion of reflux, the mixture was cooled and filtered to obtain the precipitate. Ethanol was used to recrystallize the compounds (D1-D10)[16-18].

Reagents & conditions

(i) Chloroacetyl chloride, Acetonitrile, Reflux 6 hrs. (ii) *p*-Hydroxy benzaldehyde, Acetonitrile, Reflux 7 hrs. (iii) Substituted aromatic amine, ethanol, glacial acetic acid, reflux 3-5 hrs.

Spectral data of the derivatives

1-(10H-phenothiazin-10-yl)-2-(4-((phenylimino) methyl)phenoxy)ethan-1-one (D1)

IR KBr (cm^{-1}): 3059 (CH, Ar, str), 2965 (CH, Ali, str), 1621 (C=O, str), 1361 (C=N, str), 1091 (C-O-C, str). 1H-NMR (500 MHz; DMSO)

ä: 8.66-7.77 (t, 4H, PTZ ring), 7.59-7.17 (d, 4H, PTZ ring), 6.99-6.72 (d, 8H, Ar-H), 2.51 (d, 1H, CH).

2-(4-(((3-chlorophenyl)imino)methyl)phenoxy)-1-(10H-phenothiazin-10-yl)ethan-1-one (D2)

IR KBr (cm^{-1}): 3026 (str, CH, Ar), 2971 (str, CH Ali), 1637 (str, C=O), 1309 (str, C=N), 1026 (str, C-O-C). ^{1}H-NMR (500 MHz; DMSO) ä: 7.76-7.15 (t, 4H, PTZ ring), 7.01-6.69 (d, 4H, PTZ ring), 6.59- 6.47 (d, 8H, Ar-H), 2.50 (d, 1H, CH).

2-(4-(((4-chlorophenyl)imino)methyl)phenoxy)-1-(10H-phenothiazin-10-yl)ethan-1-one (D3)

IR KBr (cm^{-1}): 3055 (CH, Ar, str), 2964 (CH, Ali, str), 1621 (C=O, str), 1386 (C=N, str), 1021 (C-O-C, str). 1H-NMR (500 MHz; DMSO) ä: 7.59-7.40 (t, 4H, PTZ ring), 7.37-7.14 (d, 4H, PTZ ring), 6.97- 5.96 (d, 8H, Ar-H) 3.53 (s, 2H, CH2), 2.45 (d, 1H, CH).

2-(4-(((3,4-dichlorophenyl)imino)methyl)phenoxy)-1-(10H-phenothiazin-10-yl)ethan-1-one (D4)

IR KBr (cm^{-1}): 3060 (CH, Ar, str), 2923 (CH, Ali, str), 1623 (C=O, str), 1364 (C=N, str), 1013 (C-O-C, str). 1H-NMR (500 MHz; DMSO) ä: 7.70-7.39 (t, 4H, PTZ ring), 7.36- 7.18 (d, 4H, PTZ ring), 7.15-6.76 (d, 7H, Ar-H), 3.10 (s, 2H, CH2), 2.33 (d, 1H, CH).

2-(4-(((3-nitrophenyl)imino)methyl)phenoxy)-1-(10H-phenothiazin-10-yl)ethan-1-one (D5)

IR KBr (cm^{-1}): 3056 (CH, Ar, str), 2962 (CH, Ali, str), 1638 (C=O, str), 1308 (C=N, str), 1032 (C-O-C, str). 1H-NMR (500 MHz; DMSO) ä: 8.68-7.65 (t, 4H, PTZ ring), 6.99-6.76 (d, 4H, PTZ ring), 6.73- 6.04 (d, 8H, Ar-H), 3.39 (s, 2H, CH2), 2.38 (d, 1H, CH).

2-(4-(((4-nitrophenyl)imino)methyl)phenoxy)-1-(10H-phenothiazin-10-yl)ethan-1-one (D6)

IR KBr (cm^{-1}): 3026 (CH, Ar, str), 2971 (CH, Ali, str), 1637 (C=O, str), 1309 (C=N, str), 1026 (C-O-C, str). 1H-NMR (500 MHz; DMSO) ä: 7.57-7.19 (t, 4H, PTZ ring), 6.93-6.86 (d, 4H, PTZ ring), 6.80- 6.60 (d, 8H, Ar-H), 3.81 (s, 2H, CH2), 2.56 (d, H, CH).

2-(4-(((3,4-dinitrophenyl)imino)methyl)phenoxy)-1-(10H-phenothiazin-10-yl)ethan-1-one (D7)

IR KBr (cm^{-1}): 3056 (CH, Ar, str), 2962 (CH, Ali, str), 1618 (C=O, str), 1308 (C=N, str), 1032 (C-O-C, str).^{1}H-NMR (500 MHz; DMSO) ä: 8.68-7.03 (t, 4H, PTZ ring), 6.90- 6.75 (d, 4H, PTZ ring), 6.66- 6.48 (d, 8H, Ar-H), 3.59 (s, 2H, CH_2), 2.42 (d, 1H, CH).

2-(4-(((3-chloro-4-fluorophenyl)imino)methyl)phenoxy)-1-(10H-phenothiazin-10-yl)ethan-1-one (D8)

IR KBr (cm^{-1}): 3060 (CH, Ar, str), 2923 (CH, Ali, str), 1623 (C=O, str), 1364 (C=N, str), 1013 (C-O-C, str). ^{1}H-NMR (500 MHz; DMSO) ä: 8.68-7.22 (t, 4H, PTZ ring), 7.04-6.91 (d, 4H, PTZ ring), 6.78- 6.70 (d, 8H, Aromatic H), 2.52 (d, 1H, C-H).

2-(4-(((4-chloro-2-nitrophenyl)imino)methyl)phenoxy)-1-(10H-phenothiazin-10-yl)ethan-1-one (D9)

IR KBr (cm^{-1}): 3070 (CH, Ar, str), 2912 (CH, Ali, str), 1621 (C=O, str), 1383 (C=N, str), 1018 (C-O-C, str). ^{1}H-NMR (500 MHz; DMSO) ä: 7.70-7.41 (t, 4H, PTZ ring), 7.39- 7.01 (d, 4H, PTZ ring), 6.98-6.76 (d, 7H, Ar-H), 3.10 (s, 2H, CH_2), 2.51 (d, 1H, CH).

2-(4-(((2,3-dimethylphenyl)imino)methyl)phenoxy)-1-(10H-phenothiazin-10-yl)ethan-1-one (D10)

IR KBr (cm^{-1}): 3059 (CH, Ar, str), 2965 (CH, Ali, str), 1621 (C=O, str), 1361 (C=N, str), 1013 (C-O-C, str). ^{1}H-NMR (500 MHz; DMSO) ä: 8.64-7.05 (t, 4H, PTZ ring), 6.99-6.80 (d, 4H, PTZ ring), 6.76- 6.50 (d, 7H, Ar-H), 3.88 (s, 2H, CH_2), 2.15 (d, 1H, CH).

Docking study

Docking study was executed by Autodock Vina v.1.2 docking software[19,20] on Samson[21] platform by OneÅ, 2022, for purpose of visualising & calculating protein-receptor interfaces. The MOE Site Finder tool[22], that employs a linear method to determine possible binding sites in a protein beginning from its tridimensional structure was used to predict the receptor site. Rather than energy models, this method is based on alpha spheres, which are a generalisation of convex hulls[23]. The protein structure was generated using the default parameters of MOE Quick Prep. Prior to the experimentation, all the receptors were transformed to the *mol2 structural layout using Chem3D 16.0. All the receptors were pre-set to reduce the predetermined thousand stepladders with N= 1000, M= 25 and Et = 0.05 kcal/mol before conducting a the docking experiment in Autodock Vina v.1.2.0. The variables N, M, and

Et represent the maximum number of minimisation steps, consecutive minimisation steps, and energy difference between stepladders that is less than the threshold. The structure of GABA-A receptor (human synaptic PDB ID: 6D6U)[24] was obtained and used in order to perform docking simulations. A docking research has previously made advantage of this structure[25]. The size and centre coordinates of the search domain box around the active binding site predicted by MOE were 147.0 x 141.0 x 138.7 and 73.5 x 34.7 x 66.9, respectively. Angstrom units were used for all coordinate systems. There were 10 binding modes, 32 exhaustiveness, and a maximum energy difference of 3 kcal/mol. The docking experiment was carried out by OneAngstrom 2022 using Autodock Vina 1.2. on the Samson platform, and the results were recorded for a subsequent computational analysis.

Physicochemical Properties

The derivatives were synthesized in the way that they have ability to cross blood brain barrier (BBB) and for this ability it is necessary to acquaint with lipophilicity of the derivatives for the transportation of the drug to brain. The Log BBB value has been determined to know the ability of a drug to reach to central nervous system. Additionally**,** Chem Draw ultra 16.0 and ORSIS property explorer was used to compute other physicochemical parameters of derivatives as well as the standard medicine Diazepam. These parameters include Log P, molecular weight, molar refractivity, molecular topological index, Ovality, no. of rotatable bonds, and topological surface area.

Biological evaluation

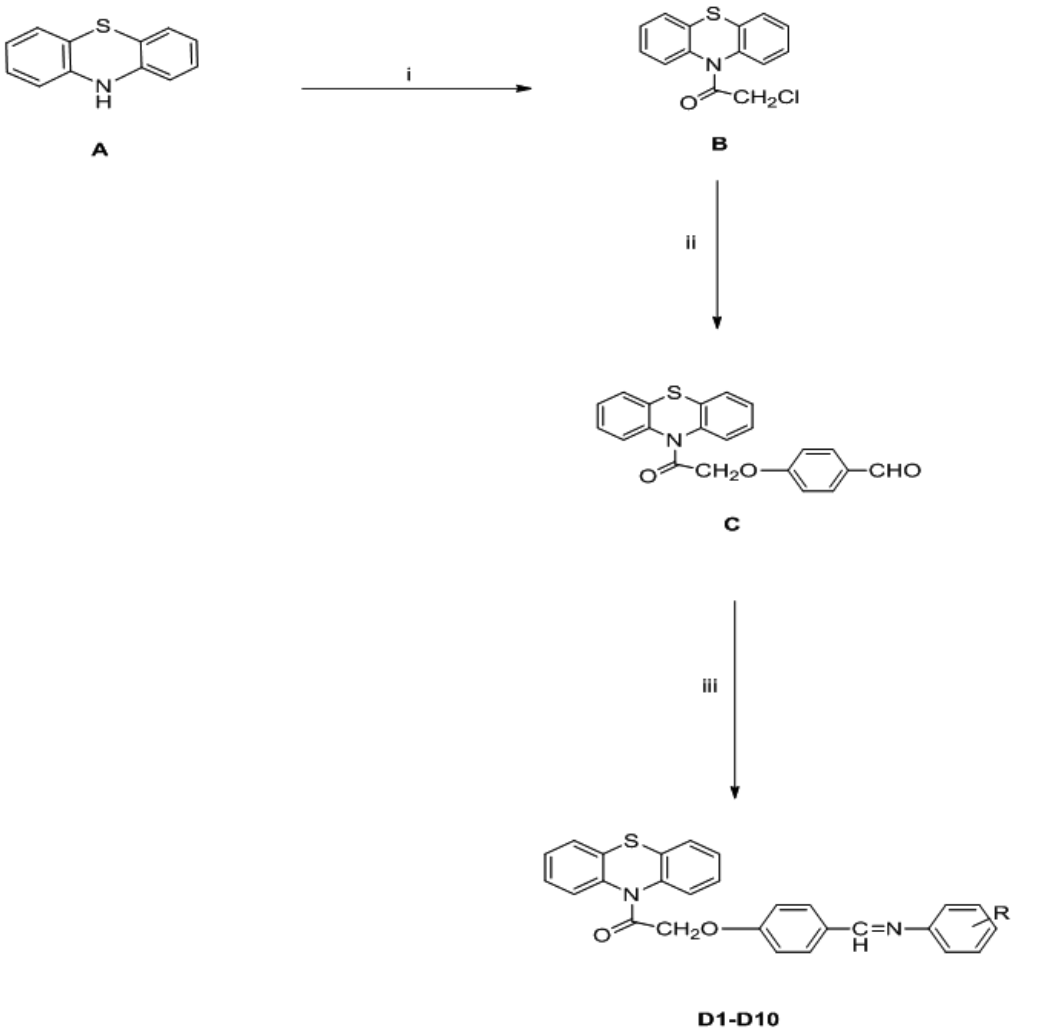
Swiss *albino* mice (either sex, 20-25 g) were taken from the IFTM University's animal facility in Moradabad, India. They were housed in groups in polypropylene cages. Wood shavings were utilized as bedding, and the temperature and lighting were controlled to be at 25±2°C. The rats had access to food and water without restriction. The experimental animal protocols received the necessary approval from the institutional animal ethical committee. All compounds with a 5 mg/kg concentration were prepared in a 1% tween 80 suspension and given intraperitoneally on the test day with the dose of 0.4 ml of animal's bodyweight. The control group received 1% tween 80 suspension agent mixed with normal saline[26-28]. As a conventional anxiolytic, diazepam (2 mg/kg, i.p.) was utilized. The apparatus was in plus shape, consisting of two open and two closed arms (each arm was in 50cm length x 10cm width) with a forty cm high wall. The wooden construction raised to a height of 50 cm above the ground with the help of a single central support. The animal was standing in the centre platform of the maze, facing an open arm[9]. Every participant was removed from the maze with great care, and a standard 5-minute test period was employed. For both closed and open arms, the frequency and length of arm visits were noted individually. (Open arm entries + closed arm entries / total time spent) x 100 was the formula used to calculate the percentage of each mouse's entries made with open arms.

RESULTS AND DISCUSSION

From the prospective of designing new bioactive heterocyclic compounds to treat anxiety, this research article summarizes the formation of ten new phenothiazine analogues (Scheme-I) which have been characterized by performing spectroscopic methods. The compound (C) was synthesized by treating compound (B) with 4-hydroxy benzaldehyde, under taking acetonitrile as solvent and anhydrous potassium carbonate as base which was then treated with substituted anilines to form a series of 1-(10H-phenothiazin-10-yl)-2-(4-((phenylimino)methyl)phenoxy)ethan-1-one (D1-D10)

All the newly synthesized analogues were characterized by IR and 1H-NMR spectroscopy. The spectrograph of the analogues gave sufficient results to interpret the data at significant range for the identification of functional groups. The analytical statistics interrelated with the structure of derivatives. The frequency of FT-IR bands provided the presence of functional groups. The frequency of aromatic C-H stretch was detected at the expanse of 3070-3026cm^{-1}. The aliphatic CH stretching was detected at the expanse 2971-2912cm^{-1}. The absorption band for C=N stretching was detected at the expanse of 1386-1308cm^{-1}. The C=O stretching was detected at the expanse of 1638-1621cm^{-1}. The C-O-C stretching was observed at the expanse of 1091-1013cm^{-1}. Aromatic protons were identified by illustrating the NMR spectra of the derivatives. The aromatic protons of all the derivatives (D1-D10) showed

a chemical shift at expanse of 8.68-7.03 ppm in the form of triplet phenothiazine ring. Doublet of phenothiazine ring's proton was detected at 7.39-6.75. Doublets of aromatic protons were detected in the range 7.15- 5.96. A singlet of CH_2 was observed at ä 3.10- 3.88 and doublet was observed at 2.56-2.15 corresponding to CH. PDB ID: 6D6U which is a human synaptic GABA-A receptor was downloaded from protein data bank and employed to dock the derivatives. Autodock vina 1.2.0 was used as docking software and the docking score of the derivatives showed their potency to bind with receptor, the higher the negative value, greater will be the potency to bind. Therefore, among the entire designed derivatives compound D4, D5 and D7 exhibited best docking i.e. -9.9, -9.9 and -10.2 respectively. This docking score revealed that these three derivatives have excellent binding affinity with receptor. A set of physicochemical parameters and docking score of the derivatives was shown in Table 1. Log P value of the derivatives has drawn within the range of 5.70-6.51, these value showed



Scheme I. Synthesis of Phenothiazine Derivatives

Table 1. Physicochemical Properties And Docking Score of the Derivatives

No.	Comp. Code	MW	Log P	MR	ASAÅ^2	TPSAÅ^2	MTI	WI	Ov	HBA	HBD	nRB	Docking score	Log BBB
1	D1	436.12	5.91	12.99	663.94	41.9	25939	3396	1.60	3	0	6	-9.0	0.118
2	D2	470.08	6.53	13.48	668.50	41.9	27499	3726	1.62	3	0	6	-9.5	0.103
3	D3	470.97	6.54	13.48	696.09	41.9	27621	3752	1.61	3	0	6	-9.6	0.103
4	D4	505.41	7.16	13.97	716.33	41.9	29187	4085	1.62	3	0	6	-9.9	0.099
5	D5	481.11	5.81	13.60	708.31	93.71	32296	4454	1.62	4	0	7	-9.9	0.063
6	D6	481.52	5.81	13.60	709.06	93.71	32766	4532	1.63	5	0	7	-9.6	0.071
7	D7	526.52	5.70	14.21	730.16	145.52	39295	5629	1.63	5	0	8	-10.2	0.019
8	D8	488.07	6.70	13.49	700.97	41.9	29187	4085	1.61	4	0	6	-9.6	0.104
9	D9	515.96	6.43	14.09	712.75	93.71	33553	4746	1.62	4	0	7	-9.4	0.002
10	D10	464.15	6.57	13.91	708.11	41.95	30869	4033	1.61	3	0	6	-9.3	0.091
11	Diazepam	284.74	2.84	80.88	475.24	32.67	5393	726	1.421	2	0	1	-5.4	0.125

MW- Molecular weight; TPSA- Topological Surface Area; HBD- Hydrogen Bond Donor; Log PMTI- Molecular topological index; HBA- Hydrogen Bond Acceptor; MR- Molecular refractivity; WI- Wiener Index; nRB- No. of Rotatable Bonds; ASA- Accessible Surface Area; Ov- Ovality; BBB- Blood Brain Barrier

Table 2. Calculation of Anti-Anxiety Evaluation

Cpd. Code	Time Spent (open arm)	No. of Entry (open arm)	% No. of entrances (open arm)
D1	55.74±0.12	5.16± 0.12	49.89
D2	41.51±1.67	7.13±0.67	41.49
D3	61.39±2.01	2.60±0.37	50.89
D4	59.26±0.56*	8.61±1.31*	60.15
D5	70.56±2.89*	7.33±0.85*	63.18
D6	54.17±1.75	6.11±0.98	49.46
D7	60.53±1.56*	4.19±0.38*	55.49
D8	59.23±2.23	7.11±0.87	50.44
D9	54.12±0.85	9.77±1.55	43.28
D10	54.32±2.00	4.13±0.73	51.16
Diazepam	91.87±3.54	10.97±0.67	65.46
Vehicle	41.15±4.22	3.24±0.81	21.24

Mean ± SEM (n=6), One way ANOVA followed by Dunnett's test,
At significant level *P<0.05 compared with vehicle

the partition coefficient of the derivatives. Number of rotatable bonds of the derivatives was 6-8 in which six compounds (D1, D2, D3, D4, D8 and D10) possess 6 rotatable bonds, compounds D5, D6 and D9 contains 7 rotatable bonds and only compound D7 contains 8 rotatable bonds. All the prepared derivatives, was subjected for anti-anxiety activity investigation, Diazepam was selected as reference drug and elevated plus maze (EPM) model was employed for computing the activity results (Table 2). From the results acquired through EPM model, it has been found that compounds D4, D5 and D7 showed maximum potency when compared to the reference drug Diazepam.

CONCLUSION

This work completes the development and carrying out the synthetic procedures to prepare the newly designed phenothiazines. Chem draw ultra 16 was used to calculate the physicochemical properties (molecular weight, topological surface area, hydrogen bond donor, log p, molecular topological index, hydrogen bond acceptor, molecular refractivity, wiener index, no. of rotatable bonds, accessible surface area, ovality) of all the derivatives as well as standard drug diazepam. The crystal structure of human synaptic GABA-A receptor with PDB ID: 6D6U was obtained from the protein data bank and applied to execute docking. Docking study was done by using Autodock Vina and docking score revealed that the derivatives have good potentiality against anxiety. After performing computational study, research work was preceded for the evaluation of anti-anxiety activity and EPM approach was used. In comparison to diazepam, the results showed that three analogues 3,4-Cl (D4), 3-NO_2 (D5) and 3,4- NO_2 (D7) were most active against anxiety. Diazepam was used as a reference medication for the assessment of anti-anxiety activity.

In concluding remark, we have successfully designed and executed the synthetic scheme to prepare phenothiazine derivatives and found them active as anti-anxiety agents. However there is always a possibility of a drug to be explored for other biological activities. For future prospective, the reaction scheme could be good lead molecules and to be explore their other pharmacological activities like anti-psychotic activity, anti-depressant activity, anti-convulsant activity, anti-parkinson activity and various neuro-pharmacological activities.

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Conflict of interest

The authors do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

The study protocol was approved by Animal Ethical Committee, Committee for Control and Supervision of Experiments on Animals (CPCSEA), Government of India, New Delhi (Reg. No. 837/PO/RE/S/04/CPCSEA).

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Authors' Contribution

Pooja Saini: designing the scheme, synthetic work, paper writing, performing activity; Sushil Kumar: designing the scheme, interpretation of analytical data, paper editing; Swatantr Bahadur Singh: synthetic work, performing activity, paper writing.

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