

N-BOC-2S-Methyl Pyroglutamate as Potential Anxiolytic Agent: Integrated ADMET profiling, Molecular docking and *in vivo* Pharmacological evaluation

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ABSTRACT

Anxiety disorders are highly prevalent neuropsychiatric conditions such as medications like benzodiazepines, selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) are widely prescribed for their long-term use but it is limited due to delayed onset of action, adverse effects, tolerance, dependence and withdrawal symptoms. **Methods:** The current research used integrated *in vivo* and *in silico* methods to examine the anxiolytic potential and underlying mechanism of N-Boc-2S-methyl pyroglutamate using the elevated plus maze model. the chemical was procured according to conventional protocols and tested for anxiolytic action in wistar albino rats at given oral dosages of 25, 50 and 100 mg/kg using diazepam (1 mg/kg) as the reference standard. **Result:** The 100 mg/kg dose produced significant anxiolytic effects ($p < 0.0001$, $n = 6$) as evidenced by increased open-arm entries (14 ± 0.27) and time spent in open arms (163.35 ± 0.71), along with reduction in closed-arm entries (11.25 ± 0.02) and time in closed arms (136.82 ± 0.65) without signs of sedation or motor impairment. Molecular docking against the GABAA receptor (PDB ID: 8G5F) demonstrated strong binding affinity (-7.2 kcal/mol) and suggesting positive allosteric modulation. *In silico* ADMET analysis predicted high intestinal absorption (87.25%) and absence of significant toxicity with an estimated $LD_{50} > 1000$ mg/kg by using (Protox 3.0) software. **Discussion:** N-Boc 2S-methyl pyroglutamate showed dose-dependent anxiolytic activity in rats, peaking at 100 mg/kg in the elevated plus maze, comparable to standards. The five-membered lactam ring likely facilitates GABAA receptor binding, similar to diazepam proven by structural and docking evidence. **Conclusion:** In summary, the concordance between *in vivo* efficacy and *in silico* binding interactions indicates that N-Boc-2S-methyl pyroglutamate may be lead candidate for the development of safer anxiolytic agents and further pharmacological and mechanistic studies will validate their therapeutic potential.

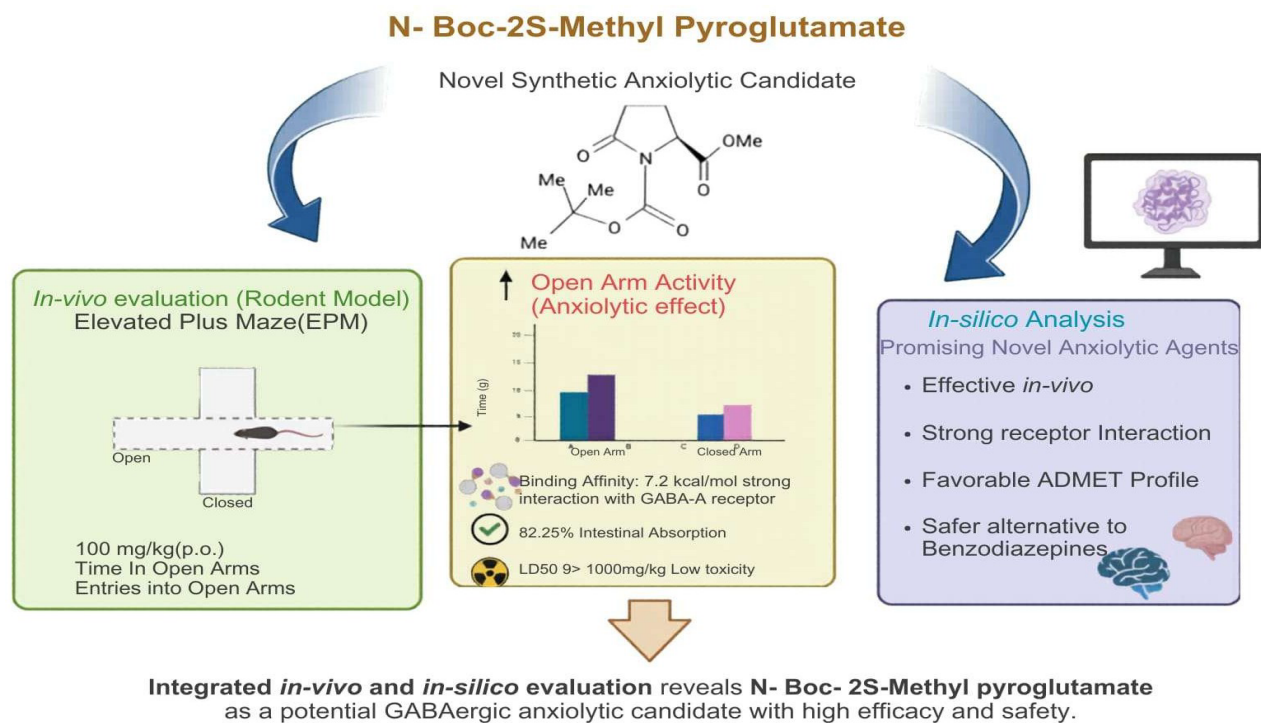
Keywords: Pyroglutamic acid, Anxiety disorders, GABAergic modulation, Elevated plus maze, Molecular docking, ADMET

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GRAPHICAL ABSTRACT



INTRODUCTION

Anxiety disorders represent one of the most prevalent neuropsychiatric conditions worldwide, exerting a substantial burden on public health and quality of life. They are characterized by excessive fear, worry and behavioural disturbances often accompanied by somatic symptoms such as palpitations, tremors and sleep disturbances [1]. The long-term treatment of anxiety is difficult due to norepinephrine reuptake inhibitors (SNRIs), selective serotonin reuptake inhibitors (SSRIs) [2]. The conventional anxiolytics have undesirable side effects, delayed onset of action, tolerance, dependence and withdrawal liabilities [3]. Consequently, the search for safer and more effective anxiolytic agents with improved pharmacological profiles continues to be an area of active research [4]. The γ -aminobutyric acid type A (GABAA) receptor plays a central role in the regulation of neuronal excitability which is the primary molecular target for classical anxiolytic drugs like benzodiazepines. Positive allosteric modulation of the GABAA receptor modulate inhibitory neurotransmission leading to anxiolytic, sedative and anticonvulsant effects [5]. Non-selective interaction of anxiolytics with GABAA receptor subunits have resulted in undesirable effects such as sedation, muscle relaxation, cognitive impairment and dependence. Recent advances in neuropharmacology are emphasizing on the development of novel ligands with improved receptor selectivity with least undesirable effects and efficient safety profiles [6]. In this context, small molecules capable of modulating GABAergic neurotransmission without eliciting severe side effects are

considered. Pyroglutamic acid (5-oxoproline), a cyclic derivative of glutamic acid, is an endogenous molecule involved in the γ -glutamyl cycle that plays an important role in neurotransmitter metabolism and cognitive function [7]. Several studies have demonstrated that pyroglutamate derivatives have exhibited diverse pharmacological activities including Nootropic, neuroprotective, anticonvulsant and anxiolytic effects. Structural modification of the pyroglutamate scaffold may influence their interaction with central nervous system (CNS) targets, will have enhanced therapeutic potential than pyroglutamate [8]. Moreover, N-Boc (tert-butoxy carbonyl) protection and stereochemical substitution at 2s-position may improve molecular stability, lipophilicity and receptor binding affinity, which are key parameters for CNS drug development [9]. Molecular docking has emerged as a powerful *in silico* tool in modern drug discovery, enabling the prediction of ligand-receptor interactions, binding affinities and key amino acid residues involved in molecular recognition [10]. Docking studies reduce experimental cost and time while providing mechanistic insights into pharmacological activity at the molecular level [11]. *In-vivo* studies demonstrated statistically significant therapeutic effects, validating their drug development potential [12]. Integration of docking analysis with *in vivo* pharmacological evaluation provides a rational approach to validate biological activity and establish structure-activity relationships (SAR) for novel compounds. This combined strategy is particularly valuable for developing CNS-active agents targeting GABAA receptors [13].

Based on these considerations, the present study is designed to synthesize N-BOC 2S-methyl pyroglutamate

and validate *in silico* binding affinity and interaction pattern with the GABAA receptor and pharmacologically assess its anxiolytic potential using an established experimental animal model. Diazepam will be employed as the standard reference drug for comparative evaluation. The integrated approach combining synthetic chemistry, molecular docking and pharmacological screening will comprehensively evaluate the test compound's potential as a novel GABAA receptor modulator with anxiolytic properties.

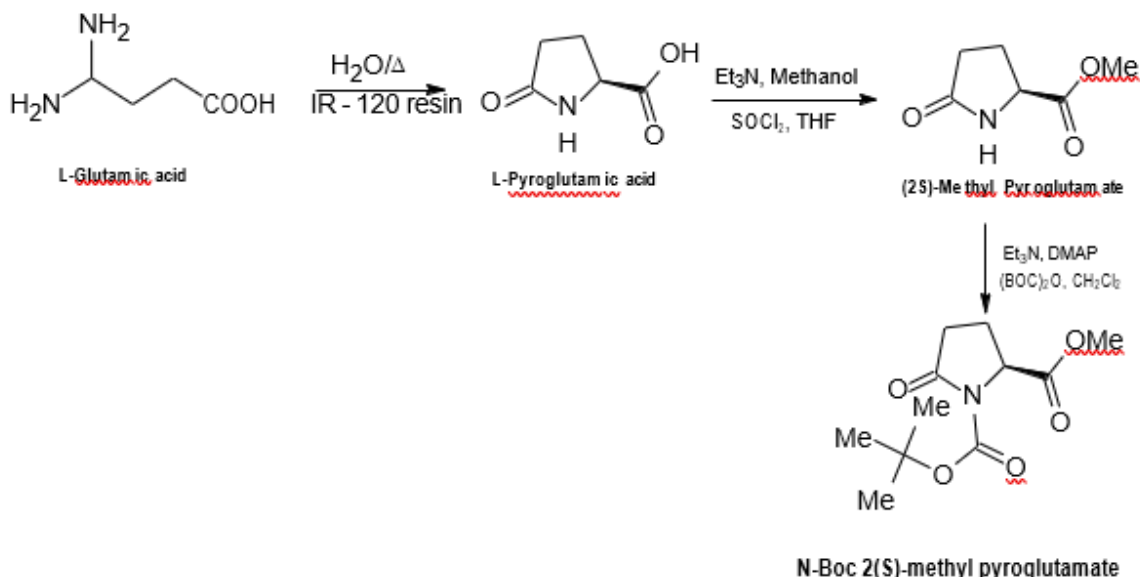
METHODS

2.1 Chemistry

Synthesis of N-Boc 2S-Methyl Pyroglutamate

0.01M (1.29gm) of L-pyroglutamic acid was dissolved in 20 ml methanol and thoroughly mixed with catalytic amount of concentrated sulfuric acid with constant

stirring at room temperature. 2S-methyl pyroglutamate was obtained by refluxing the reaction mixture for 2 hrs. The contents were cooled at room temperature; and solvent was removed under reduced pressure using Soxhlet apparatus and an aqueous sodium bicarbonate solution was added to neutralize the crude product. L-pyroglutamate methyl ester was obtained by extracting with ethyl acetate, dried over anhydrous sodium sulphate. The methyl ester was then dissolved in 10 ml of chloroform, triethylamine (1.1 equiv.) and 4-dimethylaminopyridine (0.1 equiv.). Di-tert-butyl dicarbonate (Boc)₂O, 1.1 equiv.) was added gradually with continuous stirring. The progress of the reaction was monitored by thin-layer chromatography (TLC). The solvent was evaporated under reduced pressure to get N-Boc-2-S-methyl pyroglutamate [14,15] (Scheme:1). The product was further characterized by physical, chromatographic, and spectroscopic techniques.



Scheme: - 1. Synthetic route of N-Boc 2S-methyl pyroglutamate.

2.2 Anxiolytic Evaluation

2.2.1 Elevated Plus Maze Test: N-Boc-2S-methyl pyroglutamate was evaluated using the elevated plus maze model (EPM). Wistar albino rats weighing between 175 to 200 g were obtained from institutional animal facility. Rats were housed under controlled conditions (25 ± 2°C; 12-h light/dark cycle) with free access to standard food and water [16]. Elevated plus maze model is made up of two open arms (50 × 10 cm), two closed arms (50 × 10 ×

40 cm), and 25 cm of standard elevation. Animals were randomly assigned to five experimental groups (n=6) using a randomization procedure (Table-1). All doses were administered orally 60 min prior to behavioral assessment of experimental animals. Each wistar albino rat was placed in the center of the model facing toward an open arm. Behaviour of wistar albino rats in different groups were recorded after 5 min [17-19].

Table: - 1 Description of the five experimental groups for anxiolytic evaluation (n = 6)

Groups	
Control	Normal Saline
Standard	Diazepam (1 mg/kg, p.o.)
Test group-1	25 mg/kg
Test group-2	50 mg/kg
Test group-3	100 mg/kg

2.2.2 Molecular Docking

Molecular docking was performed by *in silico* evaluation studies using Python Prescription 0.8 (PyRx) to determine the binding interactions in allosteric active sites in GABAA receptors. [20-22]. Protein-ligand complexes were prepared using PyMol molecular graphics system (version 2.4.1) for three-dimensional visualization. The docking results and protein-ligand complexes were visualized in two dimension using Discovery Studio 2016 [23]. The three-dimensional crystal structure of the receptor was retrieved from the Protein Data Bank (PDB ID: 8G5F). Chemskech tool was used to prepare the structure of N-Boc 2S-methyl pyroglutamate and the standard medication, Diazepam. PyRx (version 0.8, Open Babel) was used to optimize the ligands and by Merck Molecular Force Field 94 (MMFF94) was applied to generate energetically stable conformations. For each ligand, approximately nine conformations were generated [24].

2.2.3 Evaluation of ADME properties

Pink region in Radar model delineated the acceptable range of physicochemical space for positive oral bioavailability of N-Boc 2S- methyl pyroglutamate. Different axes in spherical structure represented size, molecular weight, instauration, flexibility, polarity, topological polar surface area (TPSA), lipophilicity and solubility[25]. The specific pink region in Radar model found within the ideal range and different parameters like molecular weight ≤ 383.82 g/mol; proportion of carbons in the Sp3 hybridization in proportion: 0.50; flexibility: 7; TPSA: 93 Å; lipophilicity log P under 5; solubility: log S: -3.8019 were found to be within required range. According to the Veber rule, compound with 10-140 Å TPSA are better for oral bioavailability [26].

These parameters have also proved the design of molecule with improved ADMET properties [27]. as physicochemical characteristics and precise management of a compound is crucial in determining the therapeutic viability during key stages of drug development [28]. The test compound falls within the ideal bioavailability range (Table: 2) and adhere to both the Veber and Lipinski rules (Figure:1).

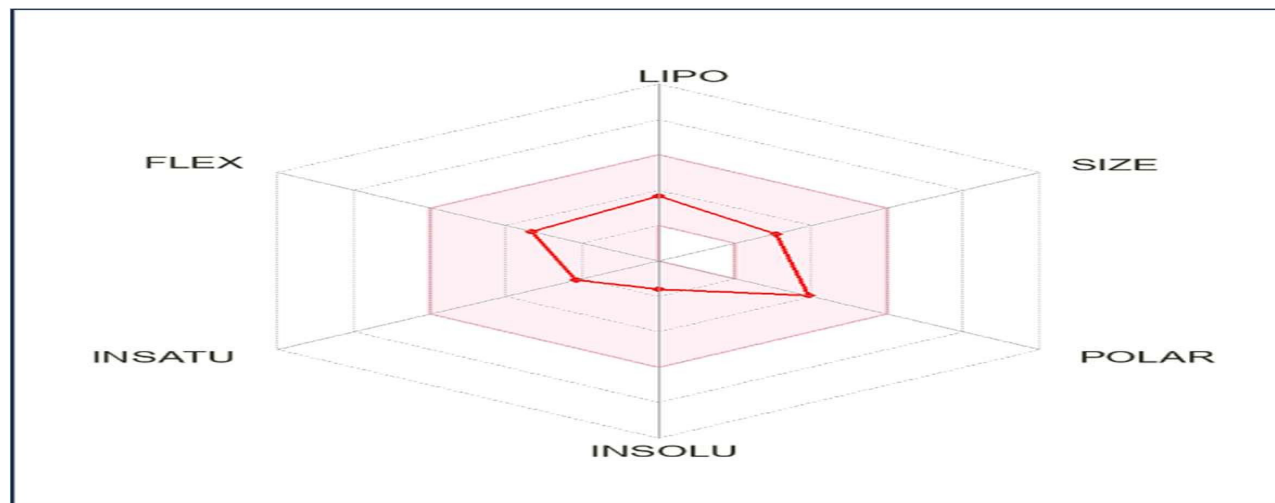


Figure: - 1 Bioavailability radar of N-Boc 2S-methyl pyroglutamate.

Table:2 Physicochemical properties, lipophilicity and water solubility of N-Boc 2S- methyl pyroglutamate.

Cpd.	Physicochemical Properties				Lipophilicity	Water solubility
	Molecular weight, g/mol	Fraction Csp3	Num. rotatable bonds	TPSA, Å ²	Log Po/w	LogS (ESOL)
1.	383.82 g/mol	0.50	7	93.14	3.18	-3.80

2.2.4 In Silico ADMET studies

The log volume of distribution values of test compound was -0.24 L/kg and intestinal absorption found to be 86.652%. CYP3A4, CYP2C9 and CYP2C19 have signified the metabolic enzymes which contributed to clinically low risk drug-drug interactions [29]. The test compound moderately reduced CYP3A4, CYP2C9, and CYP2C19 activity indicated that the metabolism of the substrate of these enzymes is not affected and unable to anticipate elevated plasma concentrations of co-administered medications. These suggested a low risk of

CYP-mediated drug-drug interactions and a favorable metabolic safety profile with no signs of cutaneous sensitivity. Excretion rate (log ml/min/kg) was found to be -1.206 (Table 3). The overall ADMET profile of compound was favorable and predicted by Swiss-ADME. However, there will be a possibility of drug-drug interaction due to anticipated suppression of CYP3A4, CYP2C9, and CYP2C19. These limitations of *in vivo* evaluation can be optimised by *in vitro* validation [30]. Protox software examined and estimated that oral acute toxicity was 2.735 mol/kg, which is class-4 and less hazardous at a dose of less than LD50 = <1000 mg/kg.

Table: - 3 Results of ADMET properties of test compound

Absorption	Distribution	Metabolism	Excretion (Log ml/min/Kg)	Toxicity
Intestinal absorption (% Absorbed)	VDss (Log L/kg)			Skin sensitization
86.652	-0.24	No inhibition of CYP3A4 substrate, CYP2C9, and CYP2C19	1.206	No

RESULTS

The anxiolytic evaluation studies of N-Boc 2S-methyl pyroglutamate were performed by elevated plus maze model by taking standard dose (1 mg/kg) of diazepam and reported to significantly increase time and the number of

entries into the open arms (figures 2 and 3) and closed arms (Figure 4 ,5). Various doses (25, 50 and 100 mg/kg) of test compound (N-Boc 2S-methyl pyroglutamate) were administered orally. The dose at which the test compound showed anxiolytic activity comparable to the standard drug was 100 mg/kg vs control $p < 0.0001$).

Table: 3. Readings of control, standard, test-1, test-2 and test-3 groups at different parameters (n=6) Data were expressed as Mean±SEM. Statistical analysis by oneway Anova followed by Tukey's post hoc tests. ****p<0.0001v/s controls

Group	Dose	No. of Entries		Time spent (sec)	
		Open arm	Close arm	Open arm	Close arm
Control Group	Saline	8.25±0.09	16.2±0.07	75.3±0.10	262.45±0.47
Standard Group	1 mg/kg	15.2±0.04	8.275±0.10	192.45±0.48	119.575±0.45
Test-1	25mg/kg	10.275±0.08	13.75±0.11	122.375±0.46	172.5±0.41
Test-2	50mg/kg	12.3±0.08	12.35±0.01	142.25±0.38	153.025±0.65
Test-3	100 mg/kg	14±0.27	11.25±0.02	163.35±0.71	136.825±0.65

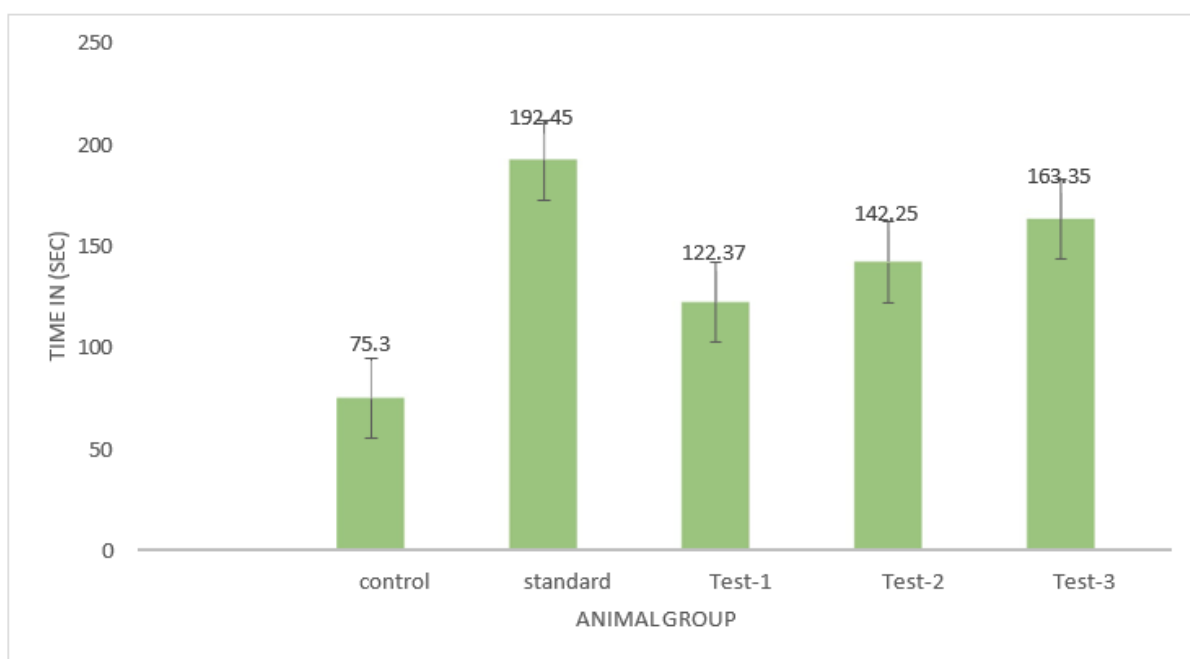


Figure:2. Average time spent in open arm by the wistar albino rats in different groups. Values were given as Mean $\pm$ SEM (n=6) $P < 0.0001$ v/s control was used to determine statistical significance.

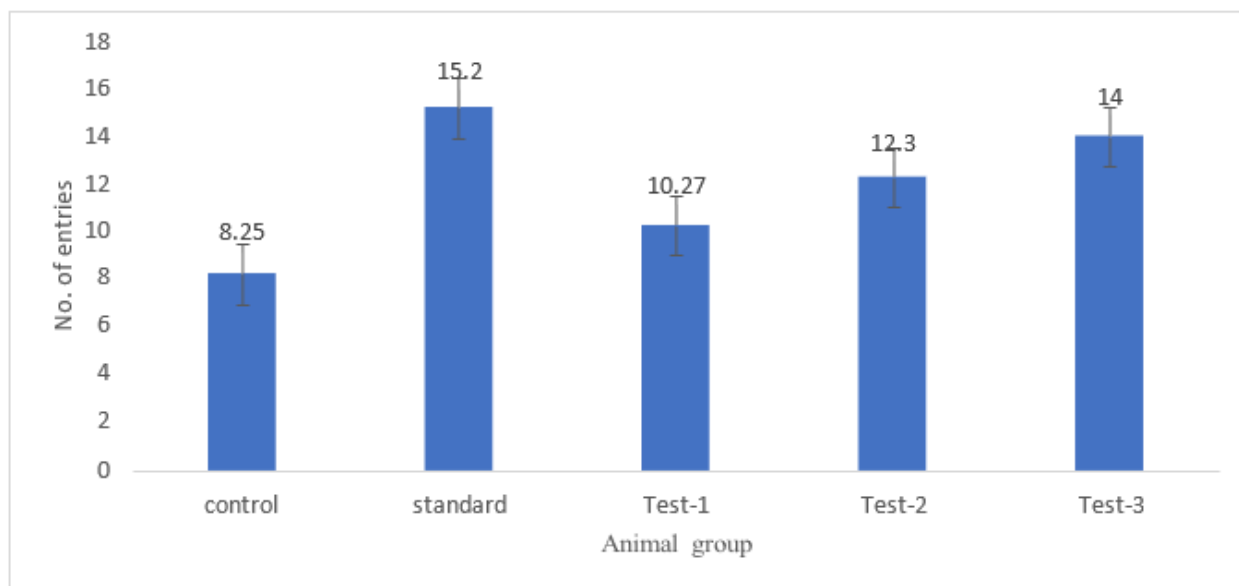


Figure: 3. Number of entries in open arm of control, standard, test-1, test-2 and test-3 animal groups by EPM. The values were expressed as Mean $\pm$ SEM (n=6) **** $P < 0.0001$.

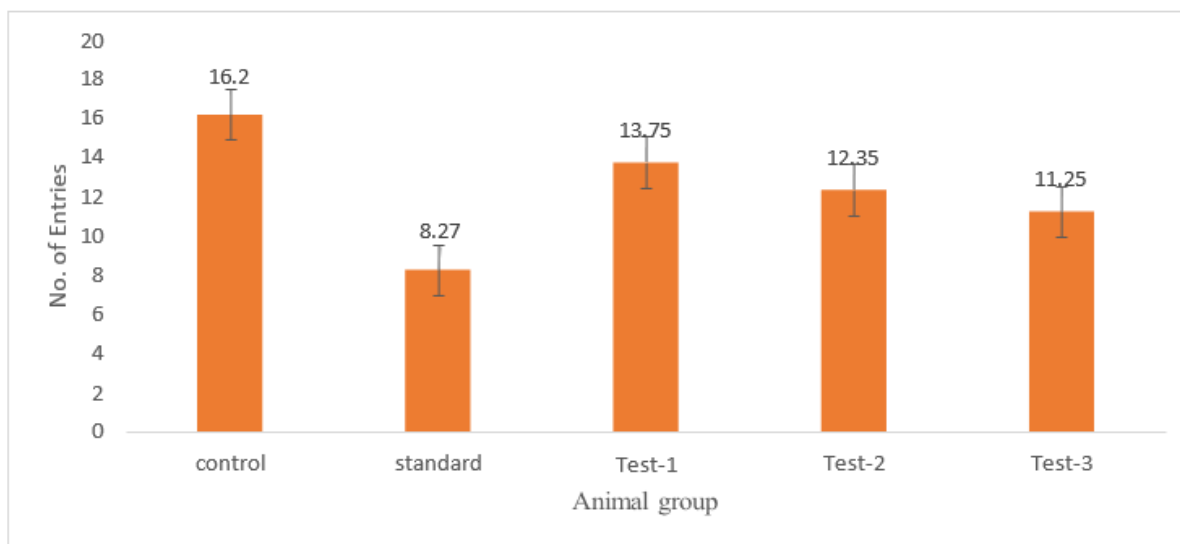


Figure: 4. Number of entries in closed arm by EPM in control, standard, test-1, test-2 and test-3 animal groups. Values were expressed as Mean $\pm$ SEM (n=6) ****P<0.0001.

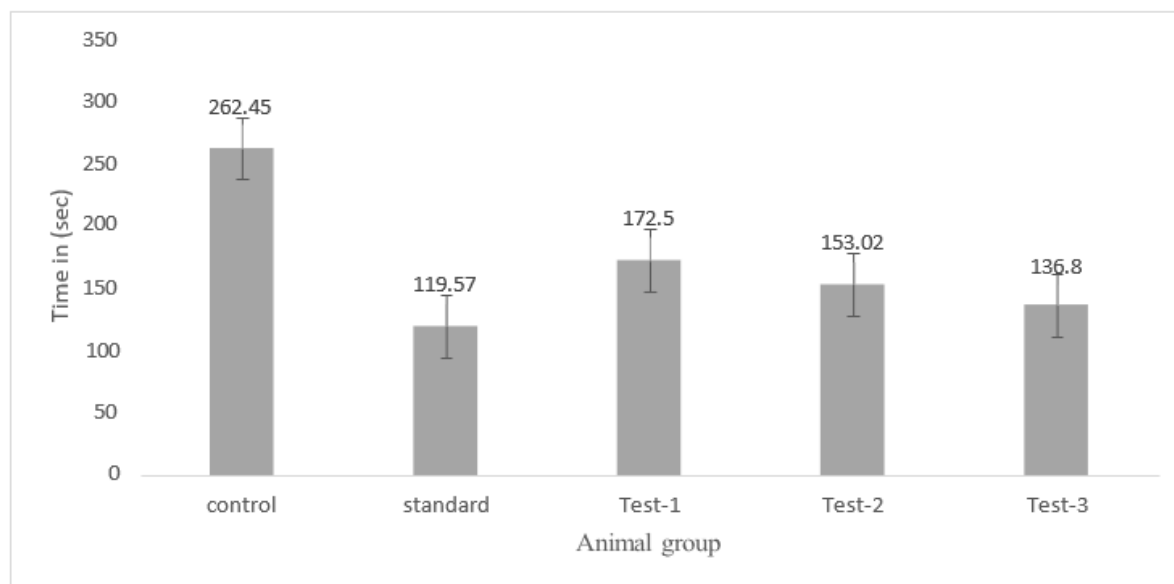


Figure: 5. Average time spent in close arms by different animal groups. Values were expressed as Mean $\pm$ SEM (n=6) ****P<0.0001.

3.2 Docking analysis of N-Boc 2S-methyl pyroglutamate and Diazepam

The docking results with GABAA complex receptor has provided useful information about the type of binding in test molecule were found to be compatible with pharmacological findings. The docking results showed that the test compound was best accommodated in active site residues of the target enzymes (ILE 265, TYR B:328,

LEU 321, TRP 272, GLN 268, VAL 269, LEU 325). The structure of test compound (docking score = -7.2, Table 2) and its greater negative binding affinity to the receptor supposed to be responsible for anxiolytic action. A significant π -sigma interaction was observed between the ligand and TRP (C:272) indicated the strong hydrophobic and aromatic contributions to ligand stabilization within the receptor cavity [31]. Such interactions are known to play a critical role in enhancing binding affinity and

maintaining conformational stability of ligand-receptor complexes. The ligand has established several Vander Waals interactions with surrounding residues including TRP C:272 involved in π -alkyl interactions and contribute to ligand stabilization within the hydrophobic pocket. VAL C:269 and ILE C:265 forming alkyl interactions may also enhanced the binding affinity. LEU B:321 and LEU B:325 supposed to anchor the hydrophobic nature of the ligand. TYR B:323 participating in van der Waals interactions and GLN C:268-contributed polar proximity aiding correct ligand orientation (figure-6). These interactions have contributed to the proper orientation of

the ligand and reinforce the stability of the docking complex of test compound [32]. The predominance of hydrophobic residues within the binding pocket suggested that the hydrophobic forces are a major driving factor for ligand binding. The heterocyclic core and carbonyl functionalities of the ligand were oriented toward the central region of the active site which facilitate the close contact with surrounding residues and contributing to enhanced binding stability. The bulky tert-butoxycarbonyl (BOC) group occupied a hydrophobic sub-pocket which further support the ligand anchoring through hydrophobic interactions [33].

Table: - 4. Docking score of test compound and standard drug

Test Sample	Docking Score
Test Compound (N-Boc 2S-methyl pyroglutamate)	-7.2 kcal/mol
Standard drug (Diazepam)	-8.8 kcal/mol

Several non-covalent interactions, including hydrophobic contacts and aromatic interactions, are evident in the three-dimensional pose and play a crucial role in stabilizing the ligand-receptor complex (figure:6). Spatial proximity of aromatic residues to the ligand framework suggested the presence of π -related interactions, which are known to significantly enhance the binding affinity and receptor modulation. Additionally, the absence of steric clashes or unfavorable contacts confirmed the suitability of the ligand conformation within the receptor

active site [34]. The functional groups of the ligand, particularly the carbonyl and heterocyclic moieties were oriented toward the active site in a manner that minimizes steric clashes and promotes effective molecular recognition. No unfavorable interactions were detected, further supporting the suitability of the synthesized compound for receptor binding. This compound displayed good docking results which correlate with the biological results [35].

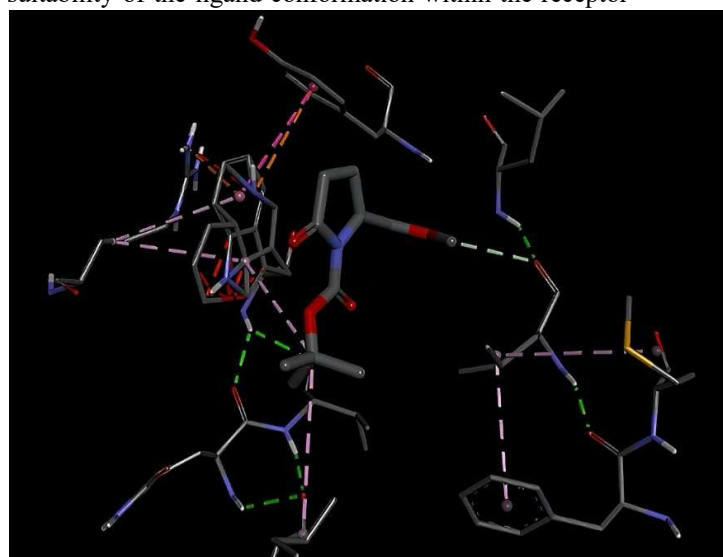


Figure: 6. The 3D and 2D docking conformation binding interactions of N-Boc 2S-Methyl Pyroglutamate against active site GABAA receptor (PDB ID: 8G5F). Ribbon and Line Model showed the binding pocket structure of Protein GABAA complexed with test derivative.

The docking results revealed that the standard drug and test compound have comparable accommodated ligand interactions with the target enzyme. Docking score of

standard drug was -8.8kcal/mol, and docking score of test compound was -7.2kcal/mol. The residue range score ILE 37, TYR 244, LEU 251, SER 17, GLY 14, GLU 34, PHE

247 signifies their affinity to active site of target enzyme (Table:2). The ligand is stably accommodated in the binding pocket through a combination of hydrogen bonding, π - π stacking, π -alkyl and van der Waals interactions, indicating favorable molecular recognition [36-38]. In the 3D interaction diagram, ligand showed prominent π - π stacked interactions with aromatic residues (notably Phe and Tyr), which contributed significantly to binding stabilization [38]. π -alkyl interactions with hydrophobic residues such as Leu and Ile further enhanced the hydrophobic complementarity. Conventional hydrogen bonds formed with polar residues (e.g., Glu, Ser, or Cys) help anchor the ligand within the

pocket while surrounding residues contribute additional stabilization via van der Waals forces [39-40]. These findings were supported by the 2D interaction map, which highlighted the important interacting residues such as LEU A:13, ILE C:37, TYR C:244, PHE C:247, PHE C:280, LEU C:248, LEU C:251, GLU C:34, SER A:17, GLY A:14, and CYS C:41. Hydrophobic

and van der Waals interactions were indicated by proximity-based contacts, while π - π stacking and π -alkyl interactions are represented by dashed lines (figure-7). all these results validated the anxiolytic impact of test compound by their interaction and docking score which were also found to be comparable to standard drug.

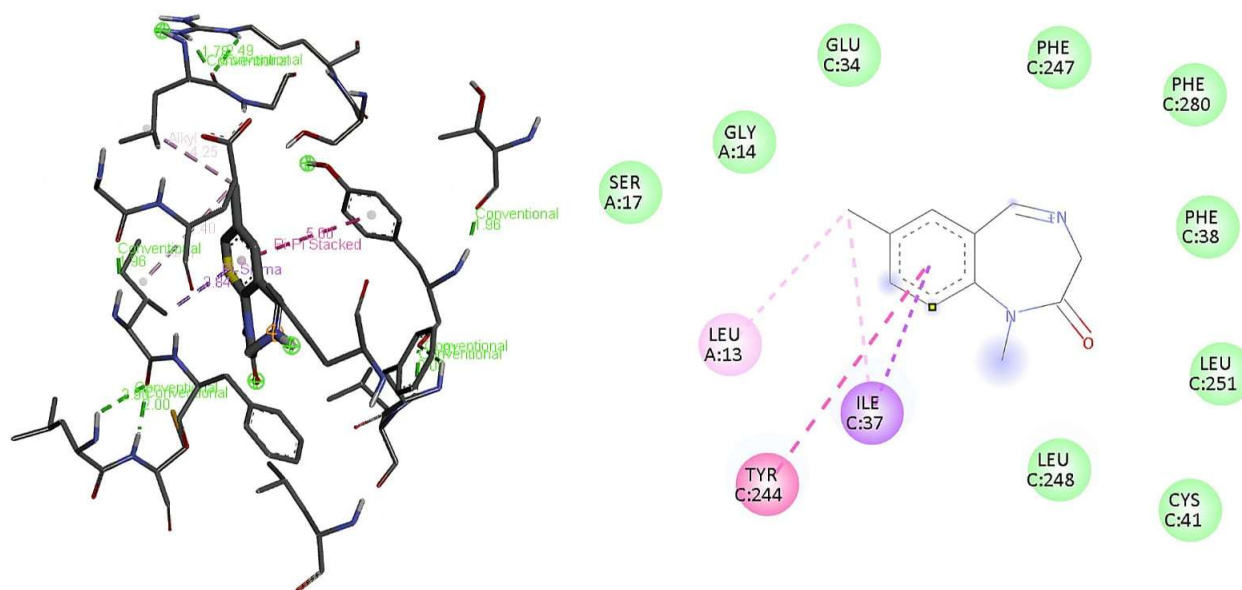


Figure: -7. The 3D and 2D docking conformation of Diazepam binding interactions with the GABAA receptor active site (PDB ID: 8G5F). Models of Ribbons and Lines displayed the binding pocket structure of Protein GABAA complex protein with diazepam.

3.3 In Silico ADME prdiction

The Swiss ADME tool, which forecasted the physically and pharmaceutically relevant components of test derivative was used to calculate absorption, distribution, metabolism and excretion. The ADME profile of test derivative was evaluated using pkCSM to predict the pharmacokinetics. The accessible web server provided platform for rapid evaluation of pharmacokinetic parameters, to predict the toxicity features and other ADMET-associated qualities of novel compounds [41-45].

DISCUSSION

Benzodiazepines are widely used to treat various anxiety disorders, but due to their undesirable side effects, a wide range of target moieties are continuously developed worldwide by structural modification, extraction and

synthetically synthesizing novel molecules with efficient anxiolytic activity and least undesirable effects[46]. In present investigation, N-Boc-2S-methyl pyroglutamate was synthesized, validated by statistical tools for efficacy and then evaluated for possible anxiolytic activity using *in vivo* elevated plus maze model. Pyroglutamate (pGlu) was synthesized non-enzymatically from glutamate, glutamine and gamma-glutamylated peptides, or enzymatically by gamma-glutamylcyclotransferase acting on L-amino acids. It is marketed over-the-counter as a "smart drug" to enhance cerebral blood flow. N-Boc-2S-methyl pyroglutamate was synthesized and evaluated for anxiolytic activity at doses of 25 mg/kg, 50 mg/kg and 100 mg/kg in the elevated plus maze model. It significantly increased the time spent and number of entries in the open arms while decreasing those in the closed arms[47-49]. The most significant effects were

observed at 100 mg/kg, comparable to the standard drug, moderate anxiolytic effect was observed at 25 mg/kg and 50 mg/kg. The anxiolytic effects of N-Boc-2S-methyl pyroglutamate may be linked to its five-membered cyclic lactam ring (2-pyrrolidone). The conformational rigidity and optimal ring size enable favorable interactions within the GABAA receptor binding pocket[51-52]. The mechanism of action of test derivative resembled with standard drug, which may indicate their capability to modulates the gamma-aminobutyric acid GABAA receptor complex. Moreover, lactam ring of N-Boc 2S-Methyl Pyroglutamate supposed to improved the receptor selectivity and binding stability to neuroactive amino acid derivatives. Ligand-receptor compatibility, typically observed in GABAergic modulators associated with anxiolytic effects was confirmed by molecular docking results and *in vivo* pharmacological activity.

CONCLUSION

This investigation systematically assessed the anxiolytic efficacy of N-Boc-2S-methyl pyroglutamate through integrated *in silico* molecular docking studies and *in vivo* behavioral paradigms. These findings provide preliminary validation of its anxiolytic potential. confirmatory receptor-binding kinetics, targeted mechanistic studies and comprehensive preclinical profile remain essential. This work established a promising scaffold for derivative synthesis, facilitating the rational design of novel GABA-modulating agents for anxiety disorder.

Ethics approval and Consent to participate

This study did not involve human participants. All animal experiments were conducted in compliance with CPCSEA guidelines and were approved by the Institutional Animal Ethics Committee (I.A.E.C) of M.J.P Rohilkhand University (Approval No.: MJPRU/PY/IAEC/25/20). Proper care was taken to minimize animal suffering during evaluation studies.

Conflict of Interest

The authors declare that they have no conflict of interest.

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