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ORIGINAL ARTICLE **OPEN ACCESS**

Anxiolytic, *In-Silico* Docking and ADME Analysis of Some Active Compounds of *Catharanthus roseus*

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ABSTRACT
Anxiety as an emotion characterized by feelings of tension, worried thoughts and physical changes like increased blood pressure. The present study is focused on the evaluation of Leaves of Catharanthus roseus extract as anxiolytic using in-vivo animal models. In vivo evaluation of anxiolytic activity of the extract of Catharanthus roseus was carried out by using Elevated plus maze, Rotarod test and Marble burying test. In Elevated plus maze test decrease in the number of entries and time spent in the closed arms, in Rota rod Test decrease in fall of time and in Marble burying Test the decrease in number of marbles buried are an indicative of anxiolytic activity. The animals treated with the extract at 200 mg/kg, bd. wt and 400 mg/kg, bd. wt have shown significant($p<0.05$) anxiolytic response and this might be due to the presence of active constituents likequercetin, yohimbine, vindolinine, catharanthine, chlorgenic acid, vindoline, lurasidone, serpentinein the extract. Docking studies were performed for natural compounds present in the extract such as Quercetin, Yohimbine, Vindolinine, Catharanthine, Chlorgenic acid, Vindoline, Lurasidone, Serpentine and standard drug diazepam and fluoxetine against PDB ID:6VRH, 3HCD, 6HUP, 5WIU using ligand fit of maestro 9.1 (Schrodinger Software Inc.).The statistical verification of the model was evaluated with PROCHEK; a structure verification program relies on Ramachandran plot which determines the quality of the predicted structures. The results revealed thatQuercetin, Yohimbine, Vindolinine, Catharanthine, Chlorgenic acid, Vindoline, and standard drug diazepam and fluoxetine had shown highest glide scores which indicates a stronger receptor-ligand binding affinity among the various phytochemical constituents present in the extract. An in-silico study of these selected phytochemical constituents was also subjected to Swiss ADME, a web tool to evaluate their pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules to support drug discovery. The swiss ADME results revealed that the active constituents of the extract have shown zero violation of the Lipinski's rule indicating the probability of its higher oral bioavailability and TPSA score less than 140 clearly indicating better permeability into the tissues. From the above, the methanolic extract of leaves of Catharanthus roseus possesses significant anxiolytic activity.
Keywords: *Diazepam, Fluoxetine, Catharanthus roseus, Docking studies, Schrödinger software, Swiss ADME analysis*

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INTRODUCTION

Anxiety is an emotion that predates the evolution of man, children, adolescents and adults experience anxiety in different forms; while this is visible in some, it can be inferred in others from their physiological and psychological responses. Anxiety also varies in frequency and intensity in different persons, even in response to the same stimulus [1]. The American Psychological Association (APA) defines anxiety as "an emotion characterized by feelings of tension, worried thoughts and physical changes like increased blood pressure." The neural systems of the three major neurotransmitter systems serotonin, dopamine, and norepinephrine have been extensively studied in normal and pathological anxiety states. Increases in GABA neurotransmission mediate the anxiolytic effect of barbiturates and benzodiazepines. It is well known that dopamine D_2 blockade, the characteristic mechanism of antipsychotic medications, is also anxiolytic. 5-HT released from the nerve terminal binds to the postsynaptic 5-HT2C receptor subtype, which mediates anxiety. 5-HT1A is an auto- receptor on

the presynaptic neuron which, when stimulated, inhibits the release of 5-HT from the presynaptic neuron into the synapse [2]. Diazepam and Fluoxetine are standard drugs used in this study. *Catharanthus roseus* is a species of flowering plant in the dogbane family Apocynaceae. The different parts of plant are used as antihypertensive, in various types of lymphoma, leukemia, anti-diabetic, hypolipidemic, antioxidant, alzheimer's disease, antipyretic, antiulcer, astringent, diuretic, cough remedy, astringent, ease lung congestion, inflammation and anticancer in Indian herbal medicine [3]. Further, results also validate the *in-silico* analysis by molecular docking, Ramchandran plot and swiss ADME.

MATERIAL AND METHODS

Plant collection and drying

Leaves of *Catharanthus roseus* was collected from Medchal district, Telangana in the month of October and was identified and authenticated. The leaves were cleaned, dried under shade for about six days and coarsely powdered in a mixer grinder. The powdered material was stored or taken up for extraction process.

Preparation of methanolic extract of *Catharanthus roseus* leaf

The powdered material of leaves of *Catharanthus roseus* were extracted by soxhlation technique with methanol. Soxhlet extraction is the process of continuous extraction in which the same solvent can be circulated through the extractor for several times. This process involves extraction followed by evaporation of the solvent. The vapors of the solvent are taken to a condenser and the condensed liquid is returned to the drug for continuous extraction[4].

Preliminary Phytochemical Screening

The extract was subjected to preliminary phytochemical screening to identify various phytoconstituents present in *Catharanthus roseus*[5].

Acute toxicity studies

Acute toxicity study was carried out in order to check the toxic effects for methanolic extract of *Catharanthus roseus* leaves as per Organization for Economic Cooperation and Development (OECD) that is up and down procedure (OECD guideline-425). The extract was administered to the respective groups orally and the animals were observed continuously for 24 h for behavioural, neurological and autonomic profiles and 72 h for any lethality. The animals were also further observed for toxic symptoms for 14 days[6].

Experimental protocol

Swiss albino mice (20 to 25 gm) were procured from Jeeva life sciences Uppal, Hyderabad. Present study was carried out in CPCSEA approved animal house of Gokaraju Rangaraju College of Pharmacy, Bachupally, Hyderabad, India. (Reg.No.1175/PO/ERe/S/08/CPCSEA). The care and maintenance of the animals were carried out as per the approved guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

In vivo methods for evaluation of anxiolytic activity

In vivo evaluation of anxiolytic activity of the methanolic extract of leaves of *Catharanthus roseus* was carried out in following models.

1. Elevated plus maze (EPM)
2. Rota rod test (RRT)
3. Marble Burying Test

Elevated plus maze(EPM)

EPM is extensively used method to examine anxiolytic effect in rodents. The plus maze apparatus is based on the innate aversion of rodents to open and high space. The apparatus has a central platform of 5 cm connected to two open arms (15 cm× 5 cm) and two closed arms (15 cm × 5 cm × 12 cm), bisecting each other. The maze was raised to a height of 25 cm from the ground.

Albino Mice of either sex weighing about 22-25 gm were selected for this study. Group I serves as control, Group II & III received MECR of 200 & 400 mg/kg for 1-5 days. Group IV received Diazepam at dose of 1 mg/kg for 1-5 days. Mice were placed at the centre of the maze facing an open arm. Number of entries into closed arm and time spent in closed arms was recorded for 5 mins. Entry into an arm is considered if the animal place it's all four paws in to the arm. After each test the maze was carefully cleaned up with a tissue paper [7].

Rota rod test

Mice of either sex weighing about 25-30 gm were selected for this study. The test consists of placing the mice upon a cylinder rotating at a speed of 12 rpm. At first, untreated animals were trained to walk on the cylinder on three consecutive sessions; the pharmacological treatments were tested by placing treated animals on the rota rod after the training sessions. Group I serves as control, Group II & III received MECR

of 200 & 400 mg/kg for 1-5 days. Group IV received Diazepam at dose of 1 mg/kg for 1-5 days. The number of falls and time of permanence were recorded during a period of 120 seconds were counted. The increase in the number of falls was indicative of coordination failures. After 60 minutes of administration of diazepam and MECR animals were tested for their motor coordination [8].

Marble burying test

The marble burying test was conducted as described [9]. 24 clear glass marbles (diameter,15 mm) were spaced 3 cm apart in 4 rows of six, on approx. 5 cm layer of saw dust bedding. Mice were introduced to these cages in which they had been previously habituated. Group I serves as control, Group II & III received MECR of 200 & 400 mg/kg for 1-5 days. Group IV received Fluoxetine at dose of 10 mg/kg*i.p*. After 30 min, the test was terminated, and the number of buried marbles was counted. The number of marbles buried by mice is noted[10].

Elevated plus maze test

Rota rod test

Marble burying test

Figure 1: *In vivo* methods for evaluation of anxiolytic activity

***In silico* modeling**

In silico analysis, molecular docking analysis is performed by Schrodinger software. Initially protein preparation is done by selecting PDB from RSCB site and active site is generated. With Chemsketch ligands are prepared and ligprep file is prepared in Schrodinger and final molecular docking is performed with visualization, Ramachandran plot is represented with Procheck tool and ADME studies are performed[11].

ADME Studies:

An *in-silico* study of Isolated compounds from *Catharantus roseus* was done for the prediction of ADME properties, Molecular weight, Total polar surface area (TPSA), iLOG P, number of rotatable bonds, number of hydrogen donor and acceptor atoms were calculated on the basis of Lipinski's rule of five [12]. In the present study, ADME was done by utilizing a web-based program (www.swissadme.ch). This software computes physicochemical descriptors as well as predicts pharmacokinetic properties and the drug-like nature of one or multiple small molecules (BBB, Cyp, Pgp). The compounds with positive values can cross readily in the BBB, while compounds with negative values are poorly distributed to the brain [13].

Statistical Analysis

Values are expressed as Mean ± SEM, (n=6). Statistical analysis was performed by using ANOVA followed by Dunnet's test by comparing with control and standard. Significant values were expressed as $p < 0.001$ and $p < 0.005$.

RESULTS

Methanolic extract of leaves of *Catharanthus roseus* was explored for its *in vivo* antianxiolytic activity using suitable rodent models. The results obtained in the study were included below.

Preparation of methanolic extract of leaves of *Catharanthus roseus*

The methanolic extract of leaves of *Catharanthus roseus* was prepared by soxhlation technique. The percentage yield of methanolic extract was calculated by using the following formula.

$$\% \text{ of yield obtained} = \frac{\text{Amount of extract obtained (gm)}}{\text{Total amount of powder used (gm)}} \times 100$$

% Yield of extract = 26.5/235 ×100 = 11.2% w/w

Preliminary phytochemical analysis

The preliminary phytochemical investigation of methanolic extract of leaves of *Catharanthus roseus* revealed the presence of bioactive compounds of which phenolic acids, flavonoids, alkaloids, steroids, terpenoids, carbohydrates and tannins were the most prominent.

Acute toxicity studies:

Methanolic extract of leaves of *Catharanthus roseus* was tested on Swiss albino mice up to a dose of 2000 mg/kg bd. wt. various morphological and behavioural characters were observed during the study. The animal did not exhibit any signs of toxicity or mortality bd. wt. All the animals were found to be safe even after 14 days of observation. Hence the extract was found to be safe up to 2000 mg/kg bd. wt.

***In vivo* anxiolytic activity models**

The methanolic extract of leaves of *Catharanthus roseus* was screened for its anxiolytic activity using the following models

Elevated plus maze

Elevated plus maze is ubiquitously used model to determine anxiolytic activity and constitutes a simple and routine rodent model for evaluation of behavioral exploratory activity in rodent models. The results are tabulated in Table 1.

Table 1: Effect of MECR on elevated plus maze

Groups	Treatment	No of entries into closed arms	Time spent in closed arms(sec)
I	Saline water	15.16+0.66	287+0.92
II	MECR (200 mg/kg, *p.o)*	12.66+0.60^{*,a}	266.5+0.96^{**,aa}
III	MECR (400 mg/kg, *p.o)*	8.66+0.45^{**,ns}	212.5+0.84^{**,aa}
IV	Diazepam (1 mg/kg, *i.p)*	7+0.33**	135+0.76**

Values were expressed as mean ± SEM (n=6). Statistical analysis was performed by using ANOVA followed by Dunnett's test. Results were compared with control group (** p<0.001, * p<0.005) and standard group (^{aa} p<0.001), ^{a} p<0.005).

Decrease in the number of entries and time spent in the closed arms is an indicative of anxiolytic activity. From the results it is clear that number of entries and time spent in the closed arms in control group was found to be more. But in groups treated with the MECR and standard (diazepam 1 mg/kg, *i.p*) the number of entries and time spent in closed arms was found to be decreased.

Rota rod test

The results of the Rota rod test are tabulated in the table 2. In rota rod test time of permanence were recorded to determine its anxiolytic effect. The time of permanence in control group was found to be more, but in the group treated with MECR and standard (diazepam 1 mg/kg, *i.p*), the time of permanence was found to be less.

Table 2: Effect of MECR on muscle rigidity using rota rod test

Groups	Treatment	Time of permanence (sec)
I	Saline water	289±0.91
II	MECR (200 mg/kg, *p.o)*	125±0.90 ^{*,a}
III	MECR (400 mg/kg, *p.o)*	90.83±1.38 ^{*,a}
IV	Diazepam (1 mg/kg, *i.p)*	63.16±0.72*

Values were expressed as mean ± SEM (n=6). Statistical analysis was performed by using ANOVA followed by Dunnett's test by comparing with control (*p<0.001) and standard (^{a} p<0.001), ns=non-significant.

Marble burying test

The results of marble burying test are tabulated in the table 3. In marble burying test, the number of marbles buried were recorded to determine its anxiolytic effect. From these results it is clear that number of marbles buried in control group were found to be more but, in the groups, treated with MECR and standard (fluoxetine 10 mg/kg, *i.p*), the number of marbles buried were found to be less.

Table 3:Effect of MECR on no. of marble buried in marble burying test.

Groups	Treatment	No of marbles buried
I	Saline water	20.66±0.30
II	MECR (200 mg/kg, *p.o)*	13.83±0.64^{*,a}
III	MECR (400 mg/kg, *p.o)*	10±0.52^{*,a}
IV	Fluoxetine (10 mg/kg, *i.p)*	4±0.52**

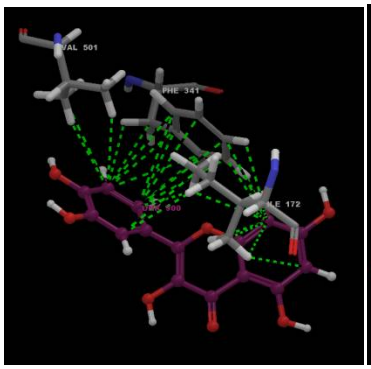
Values were expressed as mean ± SEM (n=6). Statistical analysis was performed by using ANOVA followed by Dunnett's test. Results were compared with control group (**p<0.001; * p<0.005) and standard group (^{a} p<0.01).

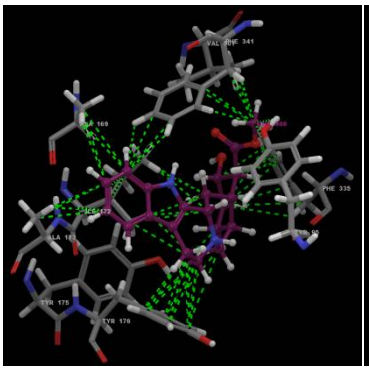
***In silico* modeling**
Molecular docking

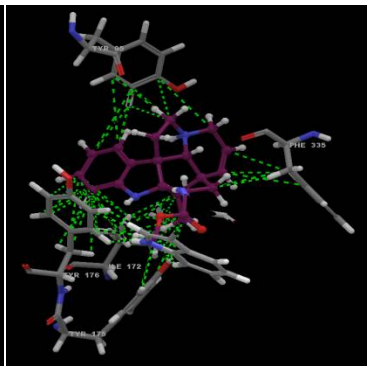
Table 4: Glide score of protein 6VRH, 3HCD, 6HUP and 5WIU

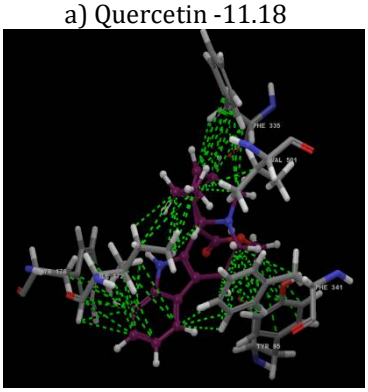
Compounds	6VRH	3HCD	6HUP	5WIU
Quercetin	-11.18	-11.04	-6.27	-9.26
Yohimbine	-10.24	-12.69	-6.34	-9.29
Vindolinine	-9.77	-1.83	-5.59	-8.33
Catharanthine	-9.50	-3.82	-5.88	-6.13
Chlorgenic acid	-8.03	-8.82	-7.67	-9.88
Vindoline	-6.30	--	-4.20	-6.26
Lurasidone	-3.57	--	-5.06	-7.67
Serpentine	-7.83	--	-4.92	-6.74
Gallic acid	-7.34	-7.94	-5.61	-7.37
Fluoxetine	-9.44	-8.03	-4.97	-8.96
Diazepam	-7.42	-6.99	-5.17	-6.56

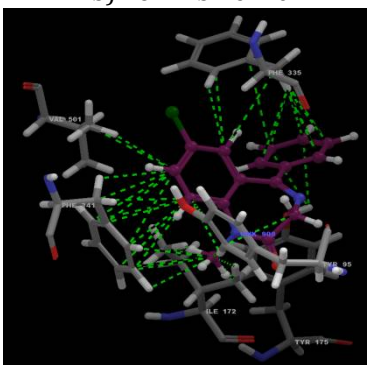
6VRH

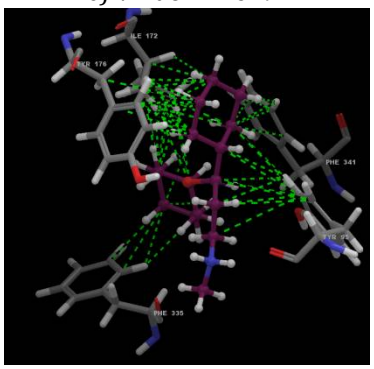

a) Quercetin -11.18


b) Yohimbine -10.24

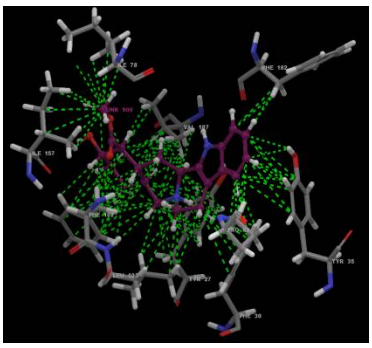

c) Vindolinine -9.77

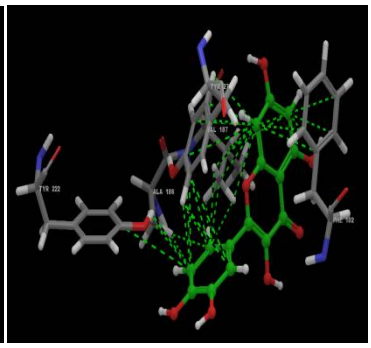

d) Catharanthine -9.50

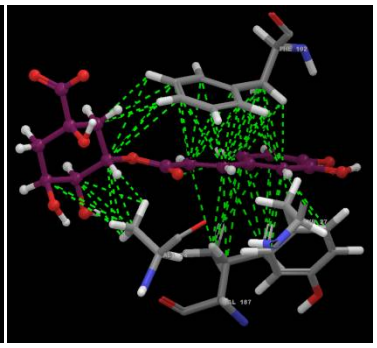

e) Diazepam -7.42

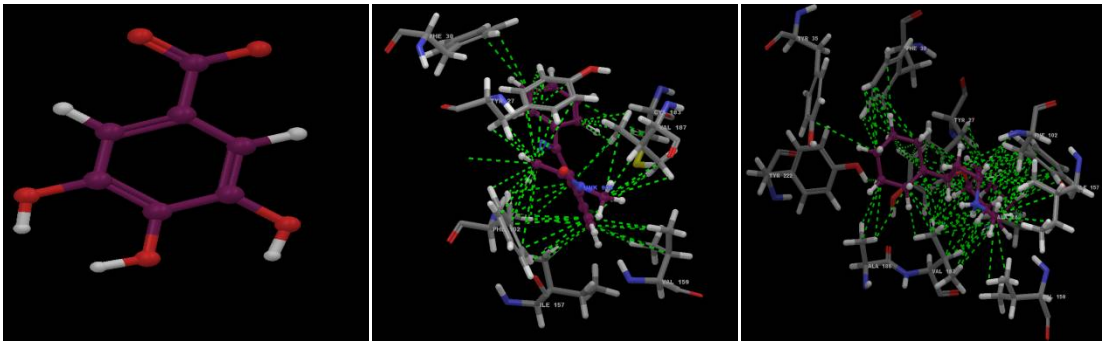

f)Fluoxetine -9.44

3HCD


a) Yohimbine -12.69

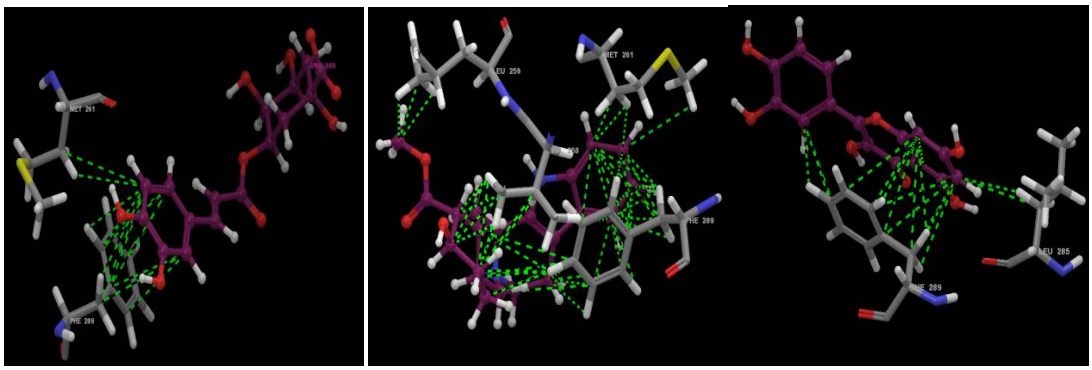

b) Quercetin -11.04


c) Chlorgenic acid -8.82

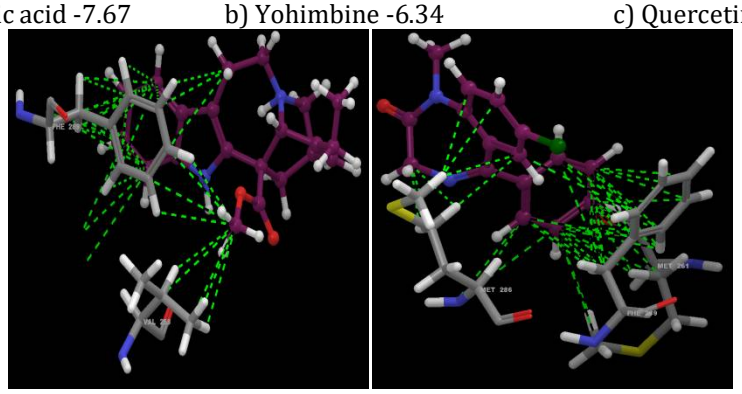


d) Gallic acid -7.94 e) Diazepam -6.99 f)Fluoxetine -8.03

6HUP

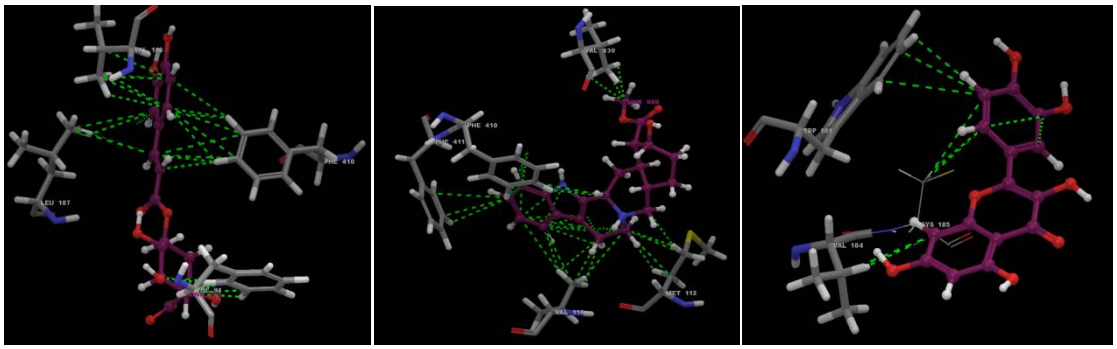


a) Chlorgenic acid -7.67 b) Yohimbine -6.34 c) Quercetin -6.27

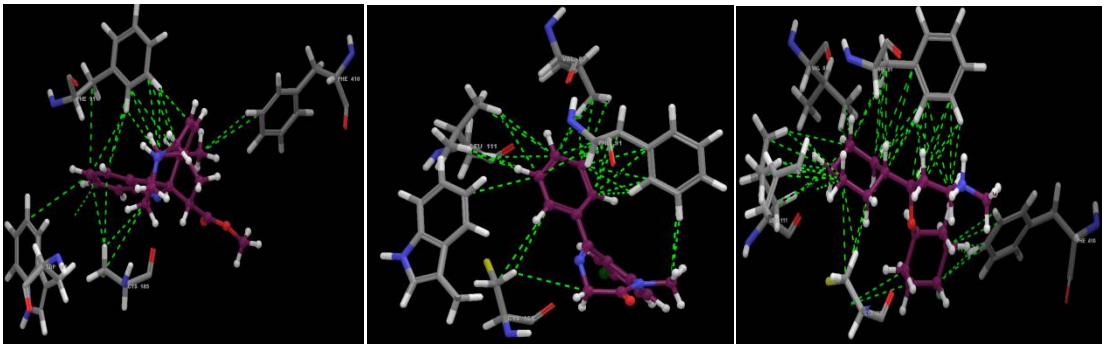


d) Catharanthine -5.88 e) Diazepam -5.17 f)Fluoxetine -4.97

5WIU



a) Chlorgenic acid -9.88 b) Yohimbine -9.29 c) Quercetin -9.26



d) Vindolinine -8.33 e) Diazepam -6.56 f)Fluoxetine -8.96

Figure 2: Docking visualization of protein a) 6VRH b) 3HCD c) 6HUP d) 5WIU

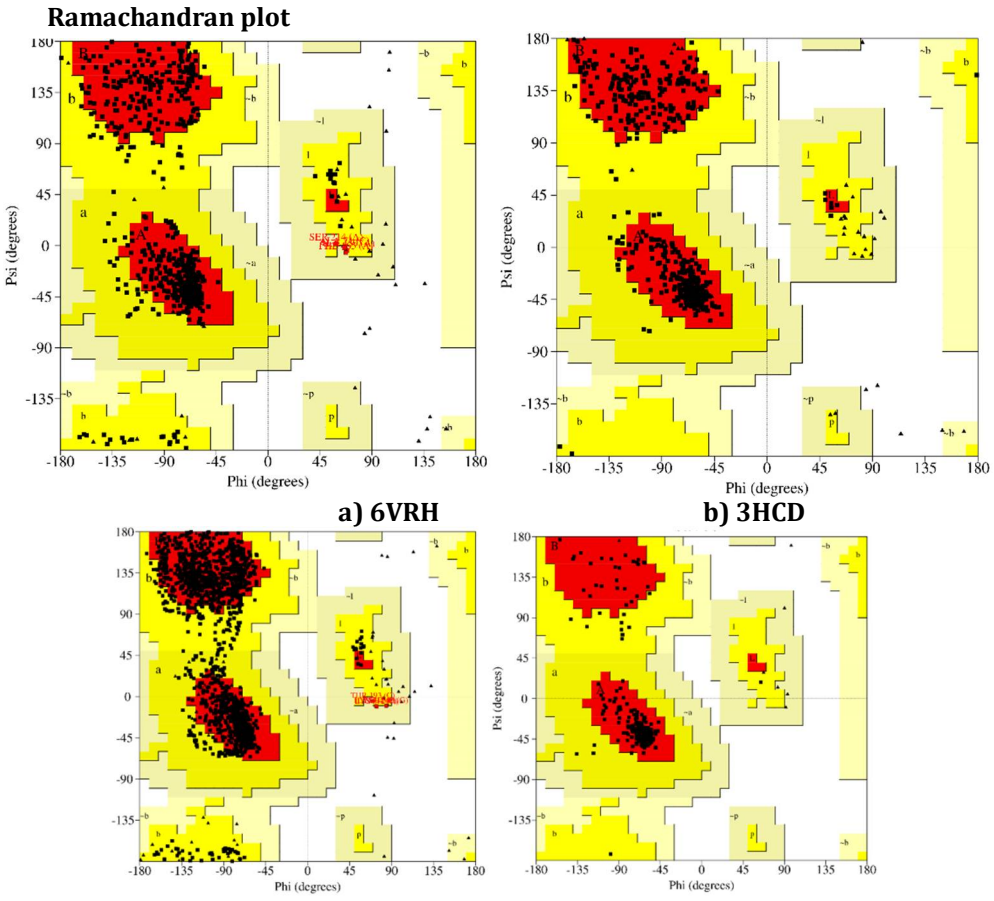


Figure 3: Ramachandran plot of protein a) 6VRH b) 3HCD c) 6HUP d) 5WIU by Procheck

Table 5: Ramachandran plot statistics by Procheck

Protein	Favored regions (%)	Additional allowed regions (%)	Generously allowed regions (%)	Disallowed regions (%)
6VRH	85.2	14.2	0.6	0.0
3HCD	92.6	7.4	0.0	0.0
6HUP	85.5	14.5	0.0	0.0
5WIU	95.5	4.5	0.0	0.0

Table 6: Swiss ADME properties of Active Compounds of *Catharanthus roseus*

	Pub chem CID	Mol wt g/mol	TPSA Å²	iLOGP	H bond acceptors	H bond donors	Lipinski violations
Quercetin	5280343	302.24	131.36	1.63	7	5	0
Yohimbine	8969	354.44	65.56	2.79	4	2	0
Vindolinine	24148538	336.43	41.57	3.1	3	1	0
Catharanthine	5458190	336.43	45.33	3.42	3	1	0
Chlorgenic acid	1794427	354.31	164.75	0.96	9	6	1
Fluoxetine	3386	309.33	21.26	3.46	5	1	0
Diazepam	3016	284.74	32.67	2.68	2	0	0

Swiss ADME

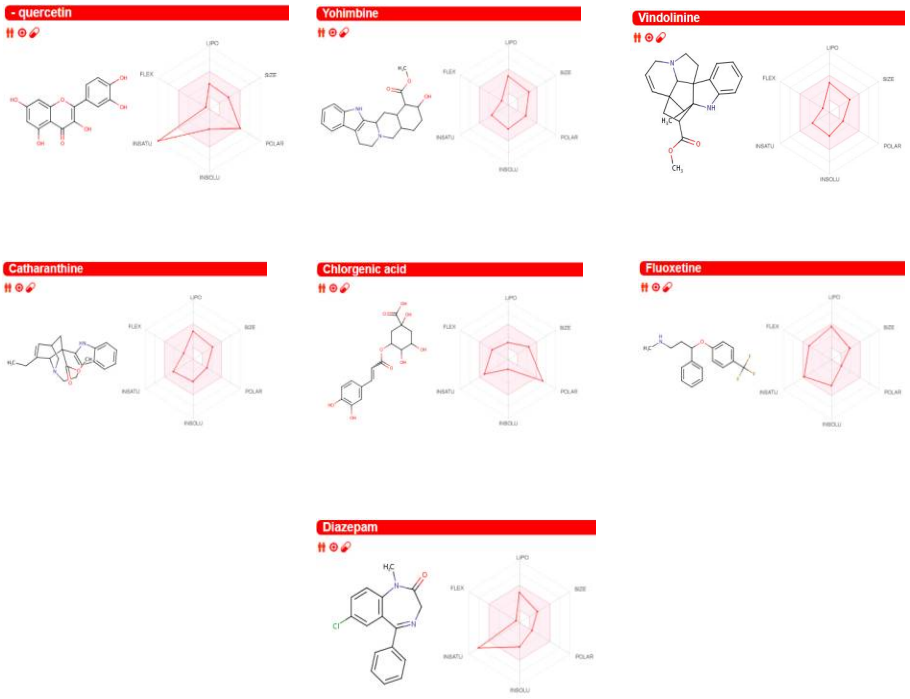


Fig 4: Swiss ADME properties of Active Compounds of *Catharanthus roseus*

DISCUSSION

Anxiolytic activity:

Methanolic extract of leaves of *Catharanthus roseus* was explored for its *in vivo* anxiolytic activity. The preliminary phytochemical investigation of methanolic extract of leaves of *Catharanthus roseus* revealed the presence of bioactive compounds of which phenolic acids, flavonoids, and alkaloids were the most prominent. Elevated plus maze testis based on the natural aversion of rodents to open spaces, and uses conflict between exploration and this aversion. It includes elements of neophobia exploration, avoidance conflict, thus it is often called as an unconditioned spontaneous behavioral conflict model. MECR showed its anxiolytic activity by decreasing the closed arm entries and time spent in closed arms. Rotarod test is based on the measuring parameters like endurance, grip strength and motor coordination of subjects and can be used for evaluating the motor coordination in rodent models. MECR also reduced the motor coordination and gripping strength. Marble burying testis a useful model of OCD. The novel nature of marble induces the anxiety in the mice and the natural response of a mouse to dig relates to the marble burying behavior [14]. MECR prominently decreased the number of marbles buried by the Swiss albino mice in Marble burying test.

The anxiolytic effect of quercetin has been shown to be due to its anti-inflammatory effects and appropriate regulation of BDNF and iNOS expression. Thus, quercetin provides the potential as a therapeutic agent to inhibit anxiety-like symptoms in neuropsychiatric diseases, such as anxiety. Quercetin inhibits ROS-producing enzymes and is known to prevent neuronal damage induced by oxidative stress [15,16]. Also, it has been found to exert anxiolytic properties and can improve cognitive functions in neurobehavioral disorders [17]. It has been clearly showed that the anti-anxiety effect of chlorogenic acid was blocked by flumazenil suggesting that this polyphenol reduces anxiety by activating

the benzodiazepine receptor [18]. The structural similarity of indole alkaloids (exogenous agonists) to endogenous neurotransmitters like serotonin has led investigators to predict the potential neurological activity of these molecules [19].

Molecular docking

Molecular docking continues to holds great promise in the field of computer-based drug design which screens small molecules by orienting and scoring them in the binding site of a protein. The docking analysis of isolated compounds from methanolic extract of *Catharanthus roseus*and standard drugs like diazepam and Fluoxetine were carried out using Schrödinger software.The various constituents identified in the plant extract areQuercetin, Yohimbine, Vindolinine, Catharanthine, Chlorgenic acid, Vindoline, Lurasidone, Serpentine and standard drug diazepam and fluoxetine were subjected to docking against PDB ID:6VRH, 3HCD, 6HUP, 5WIU**.**

The highest glide scores were observed with Quercetin, Yohimbine, Vindolinine, Catharanthine, Chlorgenic acid, Vindoline, Lurasidone, Serpentine and standard drug diazepam and fluoxetine with almost all the selected proteins with PDB ID:6VRH, 3HCD, 6HUP, 5WIU.The glide scores of the quercetin, yohimbine, vindolinine, chlorogenic acid, were found to be more than the glide score of standard drug diazepam and fluoxetin stating that the compounds might have shown greater affinity to bind to the proteins. These results clearly indicate that the chemical constituents mentioned above might have shown similar mechanism to that of the standard drug diazepam and fluoxetine in reducing anxiety. The proteins identified namely PDB ID:6VRH, 3HCD, 6HUP, 5WIUare modeled and the qualities of the 3D model were evaluated using the PROCHECK program and assessed using the Ramachandran plot. It is evident from the Ramachandran plot that predicted models have most favorable regions, additionally allowed regions, generally allowed regions and disallowed regions. Such a percentage distribution of the protein residues determined by Ramachandran plot shows that the predicted models are of good quality. According to Ramachandran plot a good quality model would be expected to have over 90 % in the most favoured region. Proteins like6VRH, 3HCD, 6HUP, 5WIU showed 90% favoured a region which clearly indicates that the selected models in the present study are of good quality[13].

Swiss ADME

Lipinski's rule of five is to evaluate drug-likeness or determine if a chemical compound with a certain pharmacological or biological activity has chemical properties and physical properties that would make it an orally active drug in humans. In the present study, all the selected active constituents like quercetin, yohimbine, vindolinine, catharanthine, and standard drug diazepam and fluoxetine has zero violations except chlorgenic acid which has one violation out of five. Any compound with zero violation clearly indicates the probability of its higher oral bioavailability.

Topological polar surface area (TPSA) allows the prediction of transport properties of drug candidates in the intestines and blood-brain barrier. The TPSA score in all the selected active constituents of the extract except chlorgenic acid and standard drugs diazepam and fluoxetine was found to be less than 140 which clearly indicated better permeability into the tissues. Swiss ADME web tool enables the computation of key physicochemical, pharmacokinetic, drug-like and related parameters for one or multiple molecules. Number of H-bond acceptors should be in a range of 0-10 and number of H-bond donors should be 0-5. All the selected active constituents in the present study were found to be within the range. A negative value for iLogP means the compound has a higher affinity for the aqueous phase (it is more hydrophilic); when iLogP equals 0 the compound is equally partitioned between the lipid and aqueous phases; a positive value for iLogP denotes a higher concentration in the lipid phase (i.e., the compound is more lipophilic). In the present study all the active constituents have shown a positive iLogp value clearly indicating a higher concentration in the lipid phase [11] (Table 6, Fig 4)..

CONCLUSION

Anxiolytic activity of MECR, high-throughput screening using Swiss ADME followed by molecular docking using Schrodinger has proved to be useful in finding some possible lead compounds. The docking score determined in this study can be correlated with biological activities. The detailed analysis of the selected PDB ID: 6VRH, 3HCD, 6HUP, 5WIU against ligands may improve our knowledge in understanding the binding interactions in detail.

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CONFLICT OF INTEREST

All authors have no conflicts of interest to declare.

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