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Bioactivity guided fractionation of *Bombax ceiba* L. root extract for antidepressant activity and elucidation of mechanism of action using *in silico* studies

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The glutamate system has received considerable attention in recent years as a target for development of novel therapeutics, including antidepressants. There are several lines of evidence showing the role of excitatory glutamatergic neurotransmission in depression and antidepressant activity of N-methyl D-aspartate (NMDA) antagonists like ketamine. The excitoxicity induced by glutamate receptor subtype NMDA is selectively and markedly inhibited by hesperidin. The hesperidin is one of the active constituents isolated from the roots of the cotton tree, *Bombax ceiba* L. (*Bombacaceae*). In this context, here, we studied the antidepressant activity of ethanolic extract of root of *B. ceiba* and its fractions *viz*., chloroform fraction CBCR (200 & 400 mg/kg); ethyl acetate fraction EABCR (200 & 400 mg/kg); n-butanol fraction (200 & 400 mg/kg) using two animal models of learned helplessness. To further elucidate the possible NMDA antagonist effect of hesperetin, an active metabolite of hesperedin, *in silico* docking studies were carried out using 5VIH protein of *Rattus norvegicus* (Brown rat) which consists of GluN1/GluN2A NMDA receptor agonist binding domains, using Inventus v 1.1. Results of *in silico* study revealed good binding affinity of hesperetin to 5VIH protein. The data indicated antidepressant like activity of the extract and its fractions. The effect was more promising with ethyl acetate and n-butanol fraction of the extract which could be attributed to the antagonistic effect of hesperetin to GluN1/GluN2A NMDA receptor, present in roots of *B. ceiba*.

Keywords: Cotton tree, Depression, Hesperidin, Hesperetin, Malabar Silk-cotton Tree, Shalmali, Shemal, Molecular Docking

Depression is a chronic mental disorder that causes changes in mood, thoughts, behaviour and physical health and leads to a decline in capacity to carry out even the simplest daily routine tasks[1]. Other than its chronic nature, symptoms associated with this mental disorder are often recurring and life threatening[2]. According to the World Health Organization (WHO) fact sheet 2021, depression is a common mental disorder and approximately more than 264 million people worldwide are said to suffer from this mental disorder[3].

The most important contributing factor for depression is exposure of day-to-day stressors in modern time. With the advancement of science, technology and economy, the nature of stress has changed markedly. Stressors interact to cause epigenetic changes altering gene expression, DNA structure and finally behaviour[4]. Moreover, stress induces activation of hypothalamic pituitary adrenal (HPA) axis, sympathetic hyperactivity along with brain processes of neuroplasticity and neurodegeneration[5]. Neurochemical, neurotransmitter and hormonal alterations after acute stress help body to face danger. However, a large number of evidences demonstrate that chronic stress is detrimental and can activate inflammatory pathways in brain and periphery[6]. Over-activated immune system, increased sympathetic nervous system activity, and reduced glucocorticoid responsiveness all culminates at mediating inflammatory responses during stress[7]. Moreover, there is a crosstalk between excessive activation of inflammatory pathways and alterations in glutamate metabolism, and both are involved in development of mood disorders[7]. It is further supported by the finding that chronic inflammation in physically ill patients often develops symptoms of depression[7]. HPA axis activation and hypercortisolemia is often seen in

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Abbreviations: EBCR, Ethanolic extract of *Bombax ceiba* root; CBCR, Chloroform extract of *Bombax ceiba* root; EABCR, Ethyl acetate fraction of *Bombax ceiba* root; NBBCR, n-butanol fraction of *Bombax ceiba* root; IMP, Imipramine hydrocholoride; VECH, Vehicle; FST, Forced swim test; TST, Tail suspension test; HPA, hypothalamic-pituitary adrenal; NMDA, N-methyl D-aspartate

depressed patients[8]. There are several lines of evidences showing antidepressant like actions of N-methyl D-aspartate (NMDA) antagonists[9]. Ketamine, an NMDA antagonist induces rapid antidepressant effects that are in contrast to the delayed onset of action of currently approved antidepressants[10-12].

Although there are many antidepressants mainly targeting the monoaminergic system, the major challenge in the treatment of this disease is that a significant portion of the patients taking antidepressants fail to attain full remission besides the lag period in effect and adverse side effects. Between 20-30% patients develop resistant in treatment of depression in which the patients fail to respond to the available drugs or other therapeutic approaches[13]. Thus, there is a strong unmet need to identify novel drug targets in order to develop effective next generation of treatments acting beyond monoaminergic system. Accumulating evidence suggests that the NMDA receptor, a subtype of glutamate receptors, plays an important role in the neurobiology and treatment of this disease and can be targeted with potential drug candidates.

Bombax ceiba Linn. [Syn.*: Bombax malabaricum* DC.; *Salmalia malabarica* (DC.) Schott & Endl.; *Gossampinus malbarica* (DC) Merr.] commonly known as cotton tree or Malabar silk cotton tree, and locally shalmali, semal, simal and, shemal (Fam. *Bombaceacea*), is a tall deciduous tree distributed throughout Nepal, other part of tropical and subtropical Asia, Australia and Africa ascending the hills up to 1500 m height[14,15]. The roots of sapling up to about three-year-old are known as "semarkanda" and are used as a nerve tonic and as an astringent[16]. The plant posses stimulant, astringent, haemostatic, aphrodiasic, diuretic, emetic, demulcent, anti-inflammatory, antidiarrhoeal, cardiotonic, anti-dysenteric and antipyretic properties[17]. Hesperidin is one of the major constituents present in *B. ceiba* root[18]. The excitotoxicity induced by the glutamate receptor (NMDA subtype) ligand was markedly inhibited by hesperidin in one of the study[19]. Since NMDA activation has role in pathophysiology of depression, here, we tried to evaluate antidepressant effect of different extracts of *Bombax ceiba* and study the NMDA antagonist effect of hesperetin (an active metabolite of hesperidin), through *in silico* study.

Material and Methods

Plant material

Plant material was collected from local surrounding of Bauniya -6- Bauniya Kailali, Nepal; authenticated by, Dr IS Bisht, Principal Scientist, National Bureau of Plant Genetic Resources Regional Station, Niglat, Bhowali, District Nainital (Uttarakhand) (Specimen no. AO-01) and Senior Research officer Binod Kumar Basnet (183960) Department of Plant Resources, National Herbarium and Plant Laboratories, Godwari, Laiitpur, Nepal (Ref.No. 213-074-075).

Extraction

Roots of *B. ceiba* were shade dried and was coarsely powdered using a mixture grinder. The extraction of dried powdered material was carried out using ethanol (95%) as solvent in soxhlet apparatus. The ratio of solute (powdered plant material) to solvent (ethanol) used for extraction procedure was 1:4. The ethanolic extracted content was concentrated using Rota Vap and coded as EBCR.

The dried extract of *B. ceiba* root was further put into fractionation. The plant extract was dissolved in distilled water and partitioning was carried out using different solvents; chloroform, ethyl acetate and n-butanol with increasing polarity. The contents obtained after the partition were concentrated using Rota Vap and used for evaluation of activity. The chloroform fraction was coded as CBCR, ethyl acetate fraction was coded as EABCR and n-butanol fraction was coded as NBBCR.

Animals

Seven-week-old wistar albino rats procured from the animal house of Department of Pharmaceutical Sciences Bhimtal Campus, Uttarakhand, India were used in the study. The animals were housed in groups of 4 in home cage and had free access to standard animal feed and aqua water. They were maintained on 12 h of light/dark cycle and ambient temperature of 25±3°C. Animals were allowed to acclimatize to laboratory conditions 5 days before the experimentation. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC approval no: KUDOPS/58) and conducted in accordance with internationally accepted principles for laboratory animal use and care.

Experimental groups

All animals were randomly allocated to different groups each group containing 6 animals. Each animal model contains 10 groups, four groups for lower

dose (200 mg/kg), four for higher dose (400 mg/kg), one group for standard imipramine hydrochloride (15 mg/kg) and one for vehicle control. Group I received the vehicle (2% Tween 80 in distilled water) and served as negative control. Group II received the standard drug imipramine (15 mg/kg) and served as positive control. The test groups III-X, received two doses of ethanol extract and their solvent fractions, i.e. 200 and 400 mg/kg, respectively. The different doses of the extract, the solvent fractions and the standard drug were dissolved in 2% Tween 80 solution immediately prior to use and administered orally for 7 days. Doses of the extract were selected on the basis of the previous study[20].

Antidepressant activity

Forced swim test (*FST*)

The animals were treated daily for 1 week starting from day one, with the test drugs and standard drug. Wistar albino rats were forced to swim individually in a transparent glass container (20 cm in diameter and height of 50 cm) containing fresh water of 19 cm height, maintained at 25°C (±3°C). The activity was carried out in two session's i.e. The first was a pre-testing session in which all untreated rats were allowed to swim in the container for 15 min each without any behavioural recording. This was done in order to check the fitness level of each animal and to obtain a stable immobility time profile[21]. In the second session (24 h after the pre-test), the rats were allowed to swim for 5 min and the total duration of immobility was recorded, one hour after the dosing. The rats were judged to be immobile when they remained floating without struggling, and making movements necessary only to keep their heads above the water. On the contrary, swimming was defined active escape or struggling movements such as head dipping, paddling with all four legs, circling the tank, and clambering at the walls. Fresh water was replaced after each rat. Following the sessions, each rat was dried on a towel and placed in a heater before it was returned to its cage[22,23].

Tail suspension test (*TST*)

The TST is based on the fact that animals subjected to the short-term, inescapable stress are being suspended by their tail, develop an immobile posture. This protocol is conceptually related to the FST[24,25]. The animals were hanged at 50 cm above from ground using adhesive tape for period of 5 min and immobility time was noted. Adhesive tape was placed approximately 1cm from the tip of their tails. Treatment was given to animal for period of one week and on 7th day the immobility time was again measured after 60 min of drug administration. The rats were judged to be immobile when they hanged motionless, making those movements necessary for respiration only. The duration of immobility was recorded for 5 min from using a stopwatch[26,27].

Locomotor activity

In order to rule out any false positive results in TST and FST, changes in motor activity of animals treated with the extract, their fractions and standard were measured automatically using actophotometer (Medicraft photoactometer INCO, model No: 600M-4D, S.No: PA-0129, India). The locomotor activity was recorded individually for 10 min. The locomotor activity of the individual animal was recorded at day 0 (i.e. before administration of the test and standard). All the test drugs and standard drug were administered orally for seven days. At day 7, after 60 min of drug administration the final recording of locomotor activity was done. Apparatus was cleaned after each test session to prevent each rat from being influenced by the odours present in the urine and faeces of the previous rats[28].

Docking study

NovoDocker molecular docking, a computational procedure that predicts the non-covalent binding of macromolecules (receptor) and a small molecule (ligand), was used for docking studies. It is all-atom energy based Monte Carlo docking procedure[29]. The molecular docking of the active metabolite of hesperidin i.e hesperetin with the target protein (5VIH) was done using Inventus software. The various steps carried out in the docking of receptor with the target protein are as follows.

Selection of protein

The NMDA (ionotropic glutamate receptor) receptor plays an important role in depression. Stress cause excess release of the glutamate and activate NMDA receptor. Continuous activation of NMDA receptor leads to excitotoxicity leading to neuronal apoptosis or necrosis leading to depression[30]. In order to prevent neuronal apoptosis or necrosis it is necessary to prevent the binding of glutamate to NMDA receptors. On the basis of this, 5VIH was selected as the target protein which is a *Rattus norvegicus* (Brown rat) protein. The protein consists of GluN1/GluN2A NMDA receptor agonist binding domains with glycine and antagonist 4-flurophenyl-ACEPC ((S)-5-[(R)-2-amino-2-carboxyethyl]-4,5-

dihydro-1H-pyrazole-3-carboxylic acid). Thus selected protein 5VIH was downloaded from the Research Collaboratory for Structural Bioinformatics Protein Databank (RCSB PDB), and was converted into PDB format. The protein consisted of two chains A and B with agonist glycine bound to chain A and antagonist 4-flurophenyl-ACEPC bound to chain B[31]. The glycine and antagonist bound to protein were removed.

Selection of the ligand

Hesperidin is reported to be present in root of *Bombax ceiba*[19] and its aglycone hesperetin has many beneficial therapeutic effects including potent antiinflammatory and neuroprotective effect[32]. The excitotoxicity induced by the glutamate receptor (NMDA subtype) ligand was markedly inhibited by hesperidin in one of the study[19]. Hesperidin is absorbed in the form of its aglycone, hesperetin, after removal of disaccharide by intestinal bacteria[33]. On the basis of above evidence the hesperetin was selected as a ligand. The hesperetin was downloaded and was converted into 3D form and finally hydrogen atoms were merged to the target receptor molecule.

Energy minimization and clash optimization

The crystalline protein 5VIH was stabilized by energy minimization. The protein attains the least energy level and become established. Many a times, energy minimization is unable to remove all stearic clashes from proteins structure which may create anomaly in outcome, during docking of protein and ligand/inhibitor. In order to prevent such anomaly, clash optimization of protein was done. This application based on monte carlo technique is used to remove clash from protein[29].

Molecular docking

In silico molecular docking is an automated and sophisticated computation structure based drug design method to study the interaction of ligand and a receptor. It determines how a ligand may bind to the active site cleft of a protein to produce energetically stable geometry of ligand receptor complex. Flexible ligands can rotate by its rotable bond in many different orientations and conformation in the active site, and then computes a score for each complex. The interaction energy is represented by dock score. This score is used to predict the binding affinity of a ligand towards receptor[34,35]. Some programs store the data for all of the tested orientation but most only keep a number of those with the best score[36]. The 4-flurophenyl-ACEPC is an antagonist binding with the NMDA 2A ionotropic glutamate receptor. Similarly, to find out the antagonistic effect of the hesperetin on 5VIH protein; the antagonist 4-flurophenyl-ACEPC was removed from its binding site (chain B) and hesperetin was docked instead.

Statistical analysis

The data obtained from the experiment was evaluated using Graph Pad Prism 5 using One-way Analysis of Variance (ANOVA) followed by Turkey's post-hoc analysis. All the results observed from the experiment were expressed as mean ± S.E.M and level of significance was set at P <0.05.

Results and Discussion

Locomotor activity

As shown in Fig. 1, administration of different doses (200 and 400 mg/kg) of ethanolic extract and its fraction showed no statistical difference in locomotor activity as compared to that of the respective control and standard both a day 0 and 7.

Forced swim test

The behavioural effects produced in the FST test owing to drug administration are presented in Fig. 2A. A significant reduction in immobility time was observed in the rats when treated continuously for one week with different doses of crude ethanolic extract, chloroform fraction, ethyl acetate fraction and n-butanol fraction of *B. ceiba* roots as compared to vehicle control. Both the doses of ethyl acetate

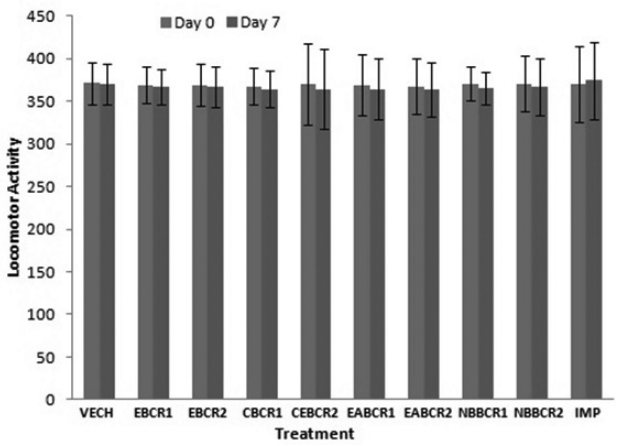

Fig. 1 — Effect of ethanolic extract and its fraction (chloroform, ethylacetate and n-butanol) on locomotor activity. [Each column represent the mean±SEM (n=6). The one-way ANOVA followed by Tukey's test was performed on raw data. Veh: Vehicle; EBCR: Ethanolic extract of root, CBCR: Chloroform fraction of root, EABCR: ethyl acetate fraction, NBBCR: n-butanol fraction; IMP: imipramine]

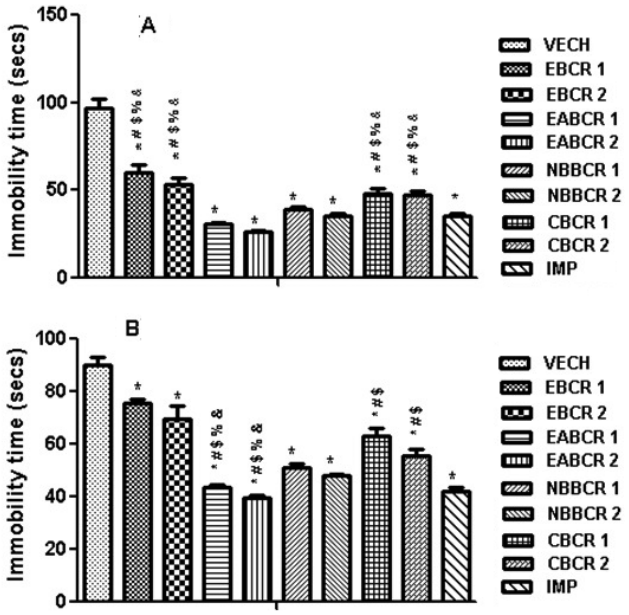

Fig. 2 — Effect of ethanolic extract and its fraction (chloroform, ethylacetate and n-butanol) on (A) Forced swim test; and (B) Tail suspension test. [Each column represent the mean±SEM (n=6). The one-way ANOVA followed by Tukey's test was performed on raw data. $^{*}P$ <0.05 *vs*. control, $^{\#}P$ <0.05 *vs*. EABCR 1, $^{\$}P$ <0.05 *vs*. EABCR 2, $^{\%}P$ <0.05 *vs*. NBBCR 1, $^{\&}P$ <0.05 *vs*. NBBCR 2. Veh: Vehicle; EBCR: Ethanolic extract of root, CBCR: Chloroform fraction of root, EABCR: Ethyl acetate fraction, NBBCR: n-Butanol fraction; IMP: Imipramine]

and n-butanol fraction produced most significant antidepressant like action than ethanol extract and choloroform fraction. Standard drug treatment with imipramine also resulted in significant decrease in immobility period as compared to vehicle treated group and the effect is comparable to ethyl acetate and n-butanol fraction.

Tail suspension test

Both 200 and 400 mg/kg doses of crude ethanolic extract, chloroform fraction, ethyl acetate fraction and n-butanol fraction of *B. ceiba* roots significantly decreased immobility period in TST as compared to vehicle control on day 7. Both ethyl acetate and n-butanol fraction were found to be more effective than ethanol extract and choloroform fraction. A significant reduction in immobility time was also observed in the rats treated with standard drug impiramine as compared to vehicle control (P <0.05). The effects produced by ethyl acetate and n-butanol fraction were found to be comparable with impiramine Fig. 2B.

Fig. 3 — Interaction between 5VIH (NMDA recptor) and hesperitin

Table 1 — The docking scores of different poses of Hesperetin

Poses number	Bio-affinity
First	–5.46 kcal/mol
Second	–6.47 kcal/mol
Third	–5.07 kcal/mol
Fourth	–6.46 kcal/mol
Fifth	–6.35 kcal/mol
Sixth	–6.35 kcal/mol
Seventh	–6.00 kcal/mol
Eight	–6.44 kcal/mol

Docking result

After docking of the ligand (hesperetin) with protein, eight possible poses were obtained (Fig. 3). Then binding affinity of each pose was calculated. Among eight poses the bio-affinity of all was found above –5 and the second pose was found as best binding pose with highest binding affinity (–6.47 kcal/mol) (Table 1).

Depression is a most common life threatening illness that takes away person's ability to carry out their daily activity. There are lots of drugs in market that act through various mechanisms which involve serotonergic, noradrenergic and dopaminergic system. However, heterogeneous clinical response to anti-depressants and susceptibility to adverse effect are major clinical problems. Therefore there is an urgent need for effective antidepressant drugs in the market for complete remission. In the present study, we demonstrated significant antidepressant effect of *B. ceiba* root extract in two animal models of depression *viz*., FST and TST. The immobility time recorded in these models reflect despair behavior or failure to adapt to stress[37,38]. Antidepressant can be distinguished from stimulant because stimulant causes marked motor stimulation but antidepressant does not[37].

The results obtained from present study shows that the ethanolic extract and its fraction posses

antidepressant like effect evident by significant reduction in the immobility time in FST and TST. Neither ethanolic extract nor its fractions altered motor activity, thus showing that increased motor activity is not associated with antidepressant effect. Likewise positive control did not alter locomotor activity. In both the models of depression, the activity of the ethyl acetate fraction (EABCR) and n-butanol fraction were found to be superior to that of the chloroform fraction (CBCR)

Hesperedin principally an antioxidant and anti-inflammatory bioflavonoid is present in the root of *B. ceiba*[9]. It is absorbed in the form of its aglycone, hesperetin, after removal of disaccharide by intestinal bacteria and is metabolized in the intestinal epithelium and liver[33]. Glucuronides and sulfoglucouronides of the hesperetin are the major conjugates derived from hesperidin in rat plasma, and they reach a peak level between 4 and 6 h[39]. Hesperedin has many therapeutic effect including hypolipedemic, antimicrobial, anticancer, antiulcer, antihypertensive, anticarcinogenic effect and similarly its aglycone, hesperetin has many benificial effect such as antioxidant, antiinflamatory, neuroprotetive, antiviral and cholestrol lowering effect[19,40-43]. The excitoxicity induced by the glutamate receptor subtype NMDA is selectively and markedly inhibited by hesperidin[19].

A functional NMDA-glutamatergic receptor comprises of heterotetramer complex with at least two obligatory GluN1 subunits and two regionally localized variables GluN2(A-D) subunits. The GluN1/GluN2B TM segments are considered to be the part of the receptor that forms the binding pockets for uncompetitive NMDA receptor antagonists[44]. The activation of the NMDA receptor requires two agonists, glycine and two molecules of agonist glutamate[45]. The glycine and glutamate bind to the respective region on GluN1 and GluN2 subunit, respectively before the channel gets activated. Binding of the glycine and glutamate on NMDA receptor selectively opens cationic ion channels and significant amount of Ca^{2+} enters inside neuron. Continuous activation of large numbers of NMDA receptors leads to increases in intracellular calcium loads and catabolic enzyme (DNAses, proteases, phosphatases and phospholipases) activities triggering a cascade of events finally leading to apoptosis or necrosis[30]. Stress causes excess release of glutamate in experimental animal models and thus glutamate excitotoxicity. Antidepressants were found to inhibit stress induced presynaptic release of glutamate in these models. Several studies report elevated concentrations of glutamate in plasma and increased concentration of glutamine in the cerebrospinal fluid of depressive patients[46].

On the basis of docking result obtained from the CADD, the binding affinity of the hesperetin to protein 5VIH (Crystal structure of GluN1/GluN2A NMDA receptor agonist binding domains with glycine and antagonist, 4-fluorophenyl-ACEPC) was found to be higher than that of −5 kcal/mol. It means that the molecule has good binding affinity with GluN1/GluN2A NMDA receptor and shows antagonistic effect. As a result, inhibition of the NMDA receptor by hesperetin might have prevented the excitotoxicity of neuronal cell thus exhibiting antidepressant like effect. In order to find out whether the hesperetin act on GluN1/GluN2A NMDA receptor inside our body it is necessary to carry out further *in vitro* and *in vivo* studies of the hesperetin.

Conclusion

The preliminary investigation on the ethyl acetate and n-Butanol fraction of *Bombax ceiba* root extract showed highest antidepressant activity in both the animal models without having any effect on locomotor activity. The effect was found to be comparable with the standard drug imipramine, thus suggesting *B. ceiba* as a potential nutraceutical for antidepressant effect. The *in silico* docking study revealed NMDA antagonistic activity of hesperetin which is an active metabolite of hesperdin. Further, phytochemical studies could be extended in future to quantify the amount of hesperidin in each fraction along with mechanistic studies to elucidate the role of gluatmate system and to correlate *in silico* results well with *in vivo* results.

Conflict of Interest

Authors declare no competing interests.

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