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ORIGINAL ARTICLE

The Contribution of Ionotropic GABAergic and N-methyl-D-Aspartic Acid Receptors in the Antidepressant-Like Effects of Hispidulin

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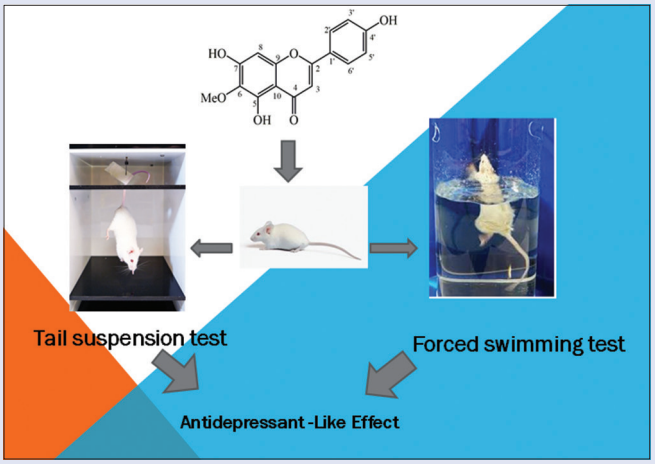
ABSTRACT

Background: *Salvia triloba*, commonly known as sage belongs to Lamiaceae family, is conventionally used as a brain-enhancing tonic. The purpose of this study was to evaluate hispidulin, a flavonoid isolated from *S. triloba* for its antidepressant-like effects and to identify its possible mechanisms of action. **Materials and Methods:** Mouse models of the forced swimming test (FST) and tail-suspension test (TST) were used to assess the antidepressant-like effects of hispidulin. **Results:** The results showed that hispidulin at the doses level of 1–10 mg/kg, intraperitoneal (ip) exerted significant antidepressant-like effects in both FST and TST. Pretreatment of animals with bicuculline (4 mg/kg, ip; a competitive γ-aminobutyric acid (GABA) antagonist) and N-methyl-D-aspartic acid (NMDA) (75 mg/kg, ip, glutamate receptor agonist) significantly blocked the reduction in immobility time of mice treated with hispidulin (3 mg/kg, ip) in FST. Furthermore, brain GABA levels were significantly decreased by coadministration of hispidulin with bicuculline, whereas glutamate levels were increased with combined administration of hispidulin and NMDA. Moreover, coadministration of sub-effective doses of hispidulin (0.5 mg/kg, ip) and ketamine (0.3 mg/kg, ip) or MK 801 (0.1 mg/kg, ip) also exerted significant antidepressant-like effects in FST. **Conclusion:** Taken together, these findings suggest that hispidulin possess significant antidepressant-like effects mediated most likely through GABAergic and glutamatergic mechanisms.

Key words: Antidepressant, forced swim test, GABAergic, glutamatergic, Hispidulin, tail-suspension test

SUMMARY

- Hipsidulin isolated from *Salvia triloba* exerted significant antidepressant-like effects in the tail-suspension and forced swimming tests. The antidepressant-like effect was antagonized by bicuculline (γ-aminobutyric acid A receptor antagonist) and N-methyl-D-aspartic acid (glutamate N-methyl-D-aspartic acid receptor agonist). Hispidulin also significantly increased brain γ-aminobutyric acid levels and decreased brain glutamate levels after the forced swimming test. Taking together these data suggest the involvement of GABAergic and glutamatergic mechanisms in the antidepressant-like effects of hispidulin.



Abbreviations used: DMSO: Dimethyl sulfoxide; FLX: Fluoxetine; FST: Forced swim test; GABA: γ-aminobutyric acid; 5HT2a: 5-hdroxytryptamine type 2a; 5-HTc: 5-hydroxytryptamine type 2c; IMP: Imipramine; MAO: Monoamine oxidase; MDD: Major depressive disorder; MK 801: Dizocilpine, NMDA: N-methyl-D-aspartic acid; OFT: Open field test; pCPA: Parachlorophenylalanine; TST: Tail-suspension test.

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INTRODUCTION

Major depressive disorder (MDD) is one of the most common psychiatric disorders and is a serious public health problem.[1] According to the WHO, the global prevalence of depression is 4.4% with approximately 322 million people living with depression.[2] The classical antidepressant drugs available for the treatment of clinical depression include monoamine oxidase inhibitors, tricyclic antidepressants, and selective serotonin reuptake inhibitor drugs. Although these drugs are effective in treating a measurable number of patients with MDD, almost 50% of patients do not respond to the first-line conventional antidepressant drugs. Furthermore, these drugs require 3–4 weeks before showing therapeutic effects.[3] Furthermore, these drugs associated with serious adverse effects such as sleepiness during the day, insomnia, restlessness, dry mouth, muscle spasms, nausea, constipation, profuse sweating, sexual disorders, weight gain, dizziness, and increased suicidal tendency over a prolonged period.[4,5] Thus, there is a dire need for the development of newer antidepressant agents acting through different mechanisms

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that may provide rapid onset of action for relieving depression symptomatology, devoid of the aforementioned adverse effects, and effective in nonresponsive patients to the conventional agents.

Diverse neuroscience research suggests that various neurotransmitter systems in addition to the monoamines are implicated in the pathophysiology of depression.[6] The glutamatergic system has been shown to be implicated in the pathophysiology of MDD and thus is a key target in mood disorders.[7] Recently, ionotropic N-methyl-D-aspartic acid (NMDA) receptor antagonist, ketamine was shown to produce rapid antidepressant effects in patients with MDD. This is in stark contrast to the prolonged delay in the onset of antidepressant effects of conventional antidepressant drugs. These rapid effects of ketamine provide an alternative to the monoaminergic deficiency hypothesis of depression. Recent evidence suggests that the drugs which target the monoamine pathways modulate the downstream signaling pathways responsible for their therapeutic efficacy, whereas ketamine may more directly target these pathways.[6] Dysfunction in the γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the mammalian central nervous system has also been implicated in psychiatric disorders, including anxiety and depression.[8] Reduced GABA level in the plasma,[9] cerebrospinal fluid,[10] and cortical tissues[11] have been shown to be associated with depression. Furthermore, GABA deficits were observed in the occipital cortex, anterior cingulate, and dorsomedial/dorsolateral prefrontal cortex of MDD patients using proton magnetic resonance spectroscopy.[12] Thus, the development of newer antidepressant agents targeting glutamate and GABA could potentially provide safe and rapid-acting alternative antidepressant agents to conventional drugs.

Recently, natural products have gained popularity for their usefulness in depression. Further studies on natural products and determining their mechanism of action along with a new combination of drug design and delivery techniques will provide an alternative to conventional antidepressant drugs. Various species of Salvia have been shown to possess antidepressant-like activities;[13,14] however, the phytoconstituents responsible for eliciting such effects remain unknown. Furthermore, recent evidence has also shown the involvement of GABAergic deficits and glutamate hyperactivity in the etiology of depression. We have previously shown that hispidulin, a flavonoid [Figure 1] isolated from *Salvia triloba* positively modulate $GABA_A$ receptors wherein it was found to potentiate GABA-induced current at recombinant α1 β2γ2 L expressed in *Xenopus* oocytes.[15] In the present study, we sought to investigate the antidepressant-like effects of hispidulin using classic mouse models of depression of the forced swimming test (FST) and tail-suspension test (TST) and attempted to elucidate the possible involvement of GABAergic and glutamatergic systems in its antidepressant effects.

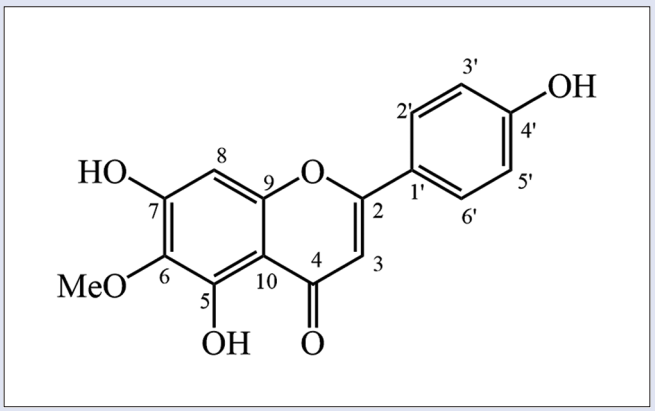

Figure 1: Chemical structure of hispidulin

MATERIALS AND METHODS

Drugs and chemicals

Hispidulin was isolated from *S. triloba*.[15] The chemicals and reagents used in this study were purchased from Sigma Aldrich Chemical Co. Ltd, MO, USA. These included dimethyl sulfoxide (DMSO), tween 20, normal saline, butanol, imipramine (IMP), ketanserin, WAY100635, ondansetron, parachlorophenylalanine (pCPA), SCH233390, haloperidol, ketamine, MK-801, NMDA, bicuculline, and prazosin.

Animals

Male Swiss albino mice weighing 20–25 g were purchased from the National Institute of Health, Pakistan. The animals were maintained in the animal facility of the University under standard laboratory conditions. All behavioral procedures were conducted in the morning between 9 am and 11 am. A total of 270 mice were used in the study. All procedures were approved by the Institutional Animal Ethical Committee of University of Malakand, (DAEC/PHARM/2016/15) and following the guidelines of the amended Animal Scientific Procedure Act UK, 1986.

Assessment of antidepressant activity

The tail-suspension test

The TST was carried out according to the method described by Steru *et al.*[16] with minor modifications we described previously.[17] The animals were subjected to a pretest training session 24 h before the actual test. After 24 h, the immobility time was recorded by an independent observer for a period of 5 min during the actual test time. Immobility was defined as the absence of all movements except for those necessary for respiration.

The forced swimming test

The FST was conducted according to the method described by Porsolt *et al.*[18] with minor modifications for mice described previously.[17] The animals were trained for swimming 24 h before the actual test using the same experimental conditions. After 24 h, mice were carefully placed in water for 6 min and the immobility time was recorded with a video camera for a period of the last 5 min when the initial struggling to escape the environment was over. The mouse was considered immobile when it remained motionless and maintained a hunched position with its head above the water surface. The various treatment groups (n = 6 mice/group) used in the study included the following.

1. Control group which was administered the same volume of the vehicle consisting of Tween-20, DMSO, and normal saline mixed in a ratio of 1, 4, and 95% respectively (intraperitoneal [ip])
2. IMP group; was administered IMP (30 mg/kg, ip)
3. Test compound groups were administered with hispidulin at the dose level of 1 mg/kg, 3 mg/kg, 10 mg/kg, and 30 mg/kg (ip)
4. Hispidulin (10 mg/kg; ip) + prazosin (1 mg/kg; ip)
5. Hispidulin (10 mg/kg; ip) + bicuculline (4 mg/kg; ip)
6. Hispidulin (10 mg/kg; ip) + haloperidol (0.2 mg/kg; ip)
7. Hispidulin (10 mg/kg; ip) + SCH23390 0.05 mg/kg; ip)
8. Hispidulin (10 mg/kg; ip) + ketanserin (1 mg/kg; ip)
9. Ketamine (0.5, 3,5, and 10 mg/kg; ip)
10. Hispidulin (1 mg/kg; ip) + ketamine (0.5 mg/kg; ip)
11. MK 801 (0.1, 0.5, and 1 mg/kg; ip)
12. Hispidulin (1 mg/kg; ip) + MK 801 (0.2 mg/kg; ip)
13. Hispidulin (3 mg/kg; ip) + NMDA (75 mg/kg; ip).

Mechanism (s) of action

Involvement of GABAergic mechanism

Mice were pretreated with a completive GABA antagonist, bicuculline (4 mg/kg) 30 min before treating with hispidulin (10 mg/kg). After

40 min of hispidulin treatment, the immobility time of mice was recorded in FST.

Involvement of glutamatergic mechanism

The role of glutamatergic mechanism was evaluated by treating mice with a subeffective dose of ketamine (0.5 mg/kg), or MK 801 (0.2 mg/kg) 30 min before administration of hispidulin (1 mg/kg), IMP or saline. After 40 min, the mice were assessed for immobility time in FST. Furthermore, mice were also pretreated with NMDA (75 mg/kg) 30 min before administering with hispidulin (3 mg/kg). Immobility was recorded in FST 30 min after hispidulin injection.

Evaluation of adrenergic, dopaminergic, and serotonergic mechanisms

For assessing the involvement of adrenergic or dopaminergic mechanisms, mice were pretreated with either prazosin (1 mg/kg) (α1-adrenergic receptor antagonist) or haloperidol (0.2 mg/kg) or SCH 23390 (0.05 mg/kg), respectively, 30 min before treatment with vehicle or hispidulin (10 mg/kg). Similarly, the contribution of the serotonergic system was evaluated by pretreatment with pCPA. pCPA acts by reducing the concentration of serotonin in the brain by inhibiting its biosynthesis. Mice were injected with either vehicle or pCPA at the dose of 300 mg/kg once daily for three consecutive days. On the 4th day, mice received hispidulin (10 mg/kg) fluoxetine (20 mg/kg) or saline. In all cases, the immobility time was determined after 40 min using FST.

Neurochemical analysis

Determination of γ-aminobutyric acid and glutamate level in brain tissue

Mice (n = 6) were decapitated by guillotine after behavioral testing in FST and brains were collected. The brains were homogenized using 5 ml of 0.01 M ice-cold hydrochloric acid with an UltraTurax homogenizer (IKA, Wilmington, NC, USA). The homogenate was transferred to ice cold absolute alcohol (10 ml) and kept at 0°C for 1 h. After 1 h, the homogenate was centrifuged at 10,000 rpm for 20 min (Beckman Coulter, USA). The supernatant was collected and washed with 70% ethanol (5 mL). This process was done three times, and the supernatants were combined. The content was dried by evaporation on a water bath at 75°C under a stream of air. Water (1 mL) and Chloroform (3 mL) were added to the dry mass and was centrifuged at 2000 g for 10 min (Beckman J2-21, rotor JA 20, Fullerton, CA, USA). The supernatant which contained GABA or glutamate was collected, and a 10 μL spot was applied on Whatman paper (No. 41). Standard solutions of GABA and glutamate at a concentration of 2 mM are also spotted using a micropipette. The spots are dried with a hairdryer. The chamber was saturated for 45 min with the mobile phase consisting of water: n-butanol and acetic acid (60 ml: 50 ml: 12 mL). Ascending technique was used to develop paper chromatogram. The paper was then dried with the hot air stream and was sprayed with 0.5% ninhydrin solution in 90% ethanol. The paper was allowed to dry for 1 h at 90°C. A blue color spot was developed. The portions carrying GABA and glutamate corresponding with the standards are cut and eluted with 0.005% $CuSo_4$ in 75% ethanol. Their absorbance was measured against a blank at 515 nm.[19] The levels of GABA and glutamate were determined using the following formula:

$$A = \frac{\text{Unknown OD}}{\text{Standard OD}} \times \frac{\text{Standard in mg}}{\text{Volume spotted } (\mu l)} \times \frac{100}{X}$$

Where,

A = Amino acid content in μmole/wet weight issue

X = Weight of the tissue in grams

Statistical analysis

Data were analyzed with GraphPad Prism (version 5.0) and are indicated as mean ± standard error of the mean. Various treatment groups were compared using one–way analysis of variance. *Post hoc* comparison was made by applying Tukey's test $P < 0.05$ was considered for data significance.

RESULTS

Evaluation of antidepressant-like activity in TST and FST

Figure 2 indicates the effects of IMP and hispidulin treatment in [Figure 2a] TST and [Figure 2b] FST. The result showed that hispidulin at the doses of 1–10 mg/kg, exerted significant antidepressant-like effects as indicated by a significant reduction in the immobility time of mice in TST (F (5,30) =7.21*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, respectively, $n = 8$) and FST (F [5,30] =9.5*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, respectively, $n = 8$). Hispidulin (0.5 mg/kg) did not significantly decrease immobility in either TST or FST ($P > 0.05$). IMP (30 mg/kg) also caused significant decreases in immobility time in both TST (F [5,30] =7.21***$P < 0.001$) and FST (F [5,30] =9.5***$P < 0.001$). The antidepressant-like effect of hispidulin at (10 mg/kg) was similar to IMP (30 mg/kg).

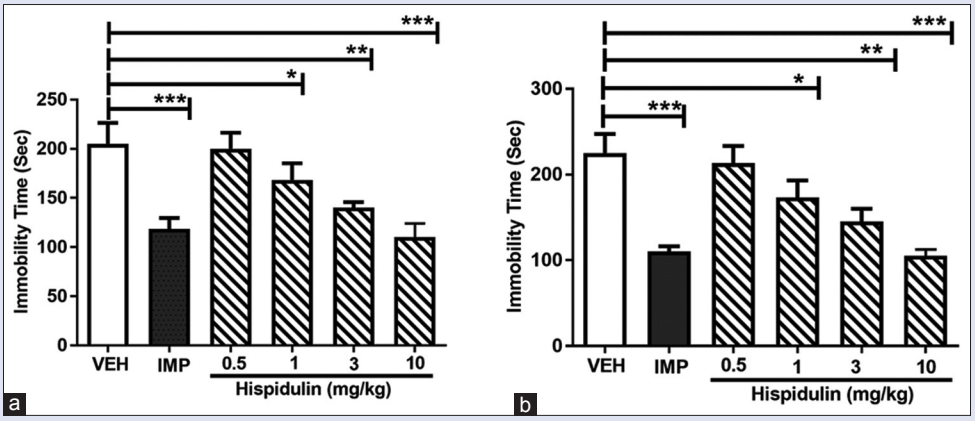

Figure 2: The antidepressant-like effects of hispidulin at various doses (0.5, 1, 3 and 10 mg/kg in tail suspension test (a) and forced swimming test (b). The values represent mean + standard error of the mean (n = 8 in each group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, One-way analysis of variance, followed by Tukey's *post hoc* test

Involvement of GABAergic mechanism in the antidepressant-like effects of hispidulin

The results depicted in Figure 3 shows that pretreatment of mice with $GABA_A$ antagonist bicuculline (4 mg/kg) decreased the antidepressant-like effect of hispidulin. Co-administration of bicuculline with hispidulin (3 mg/kg) blocked the reduction in immobility time observed with hispidulin (3 mg/kg) alone ($^{\#\#}P < 0.01$, $n = 8$). There was no significant difference in the immobility time of vehicle control and hispidulin, indicating the possible role of GABAergic mechanism in the antidepressant effect exerted by hispidulin.

Contribution of glutamatergic N-methyl-D-aspartic acid receptors in the antidepressant-like effects of hispidulin

Figure 4a and b shows the antidepressant-like effect of ketamine and MK 801. The results indicate that ketamine at the dose level of 1, 3, and 10 mg/kg, significantly decreased the immobility time of mice ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; $n = 8$) in FST. Similar results were obtained with MK 801 which also caused a profound decrease in the immobility of mice at the doses of 0.5, 1, and 3 mg/kg ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; $n = 8$). Ketamine and MK801 were found to be ineffective at the doses of at 0.5 and 0.1 mg/kg, respectively. However, treatment of mice with (sub-effective doses of either ketamine (0.5 mg/kg) or MK 801 (0.1 mg/kg) and hispidulin (0.5 mg/kg) caused a significant reduction in the immobility time in FST ($^{*}P < 0.05$) [Figure 4c and d]. Under similar conditions, IMP, the reference drug also significantly decreased the immobility time of mice ($^{***}P < 0.001$). These results indicate the possible partial involvement of the glutamatergic system in the antidepressant-like effects of hispidulin.

The involvement of NMDA receptors was further studied by co-treatment of mice with a sub-effective dose (75 mg/kg) of NMDA (NMDA receptor agonist) and hispidulin (3 mg/kg) in FST [Figure 5]. The results showed that pretreatment of mice with a sub-effective dose of NMDA significantly reversed the reduction in immobility time caused by hispidulin, thus reducing its antidepressant-like effect ($^{**}P < 0.01$; $n = 8$).

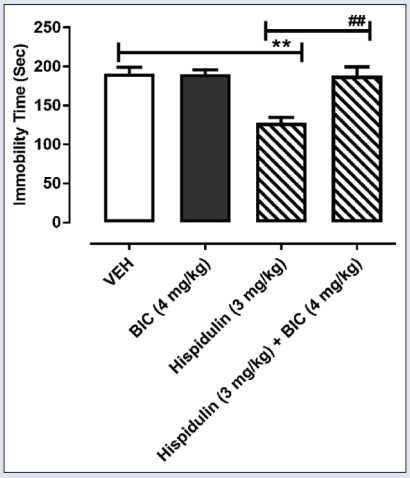

Figure 3: The effects of pretreatment with bicuculline (4 mg/kg, intraperitoneal) on the antidepressant-like effects exerted by hispidulin (3 mg/kg, intraperitoneal) in the forced swimming test. The values represent mean + standard error of the mean ($n = 8$ in each group). $^{**}P < 0.01$ compared to the vehicle while $^{\#}P < 0.05$ compared to hispidulin alone, one-way analysis of variance, followed by Tukey's *post hoc* test

These results further confirm that NMDA receptors are implicated in the antidepressant effect of hispidulin.

Evaluation of hyperactivity in open field test

Figure 6 shows the effects of hispidulin on the hyperactivity in the open field test (OFT). Hispidulin at the doses of 1, 3, and 10 mg/kg, did not cause any significant increase in the rearing frequency (number of times the animals and stands on the rear paws) [Figure 6a] and ambulation frequency (number of squares crossed) [Figure 6b] as compared to the vehicle ($P > 0.05$) indicating no significant effects on the motor activity of mice. Similar effects were observed with IMP (30 mg/kg).

Estimation of γ-aminobutyric acid and glutamate levels in brain tissue after the forced swimming test

Figure 7 depicts the results of various drug treatments on GABA and glutamate level in brain tissues following FST. Hispidulin at the doses of 1–10 mg/kg significantly increased the level of GABA in brain tissues ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; $n = 8$) and pretreatment of animals with bicuculline (4 mg/kg) significantly decreased the brain GABA levels ($^{\#}P < 0.05$) compared to hispidulin treatment alone, indicating GABAergic mechanism. Hispidulin (1–10 mg/kg) also significantly decreased the level of glutamate in brain tissue ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; $n = 6$). In contrast, pretreatment of animals with NMDA (75 mg/kg) significantly reversed the decreases in glutamate level observed with hispidulin (3 mg/kg) ($^{\#\#}P < 0.01$) indicating the involvement of glutamatergic mechanisms. IMP (30 mg/kg), the reference tricyclic antidepressant drug also did not cause any increase in the levels of GABA or a decrease in glutamate level ($P > 0.05$).

Involvement of dopaminergic and adrenergic mechanisms in the antidepressant effects of hispidulin

Figure 8 depicts the results of co-administration of various antagonists of the dopaminergic and adrenergic system with hispidulin. The results showed that co-administration of SCH23390 (0.05 mg/kg) [Figure 8a] or haloperidol (0.2 mg/kg) [Figure 8b] with hispidulin did not cause a significant increase in the immobility of mice in FST ($P > 0.05$). The results obtained with prazosin (1 mg/kg) were similar where pretreatment of mice with the antagonist did not reverse the antidepressant-like effects of hispidulin [Figure 8c]. These results suggest that neither dopaminergic noradrenergic mechanisms are implicated in the antidepressant-like effects of hispidulin ($P > 0.05$).

Involvement of serotonergic mechanisms in the antidepressant effects of hispidulin

Figure 9 shows the effect of serotonin synthesis inhibitor, pCPA [Figure 9a], or 5-HT receptor antagonists, WAY100635 [Figure 9b] ketanserin [Figure 9c] and ondansetron on the antidepressant-like effects of hispidulin. The results showed that pretreatment of mice with pCPA (100 mg/kg) did not significantly decrease the antidepressant-like effect of hispidulin (3 mg/kg) as evidenced by no increase in the immobility time of mice ($P > 0.05$). Similar results were observed with receptor antagonists, where pretreatment of mice with 5-HT 1a receptor antagonist, WAY 100635 (0.1 mg/kg), 5-hdroxytryptamine type 2a (5HT2a) antagonist, ketanserin (1 mg/kg) and 5-hydroxytryptamine type 2c (5-HTc) receptor antagonist, ondansetron (1 mg/kg) did not cause any statistically significant increase in the reduction of immobility time observed with hispidulin at the dose of 3 mg/kg ($P > 0.05$).

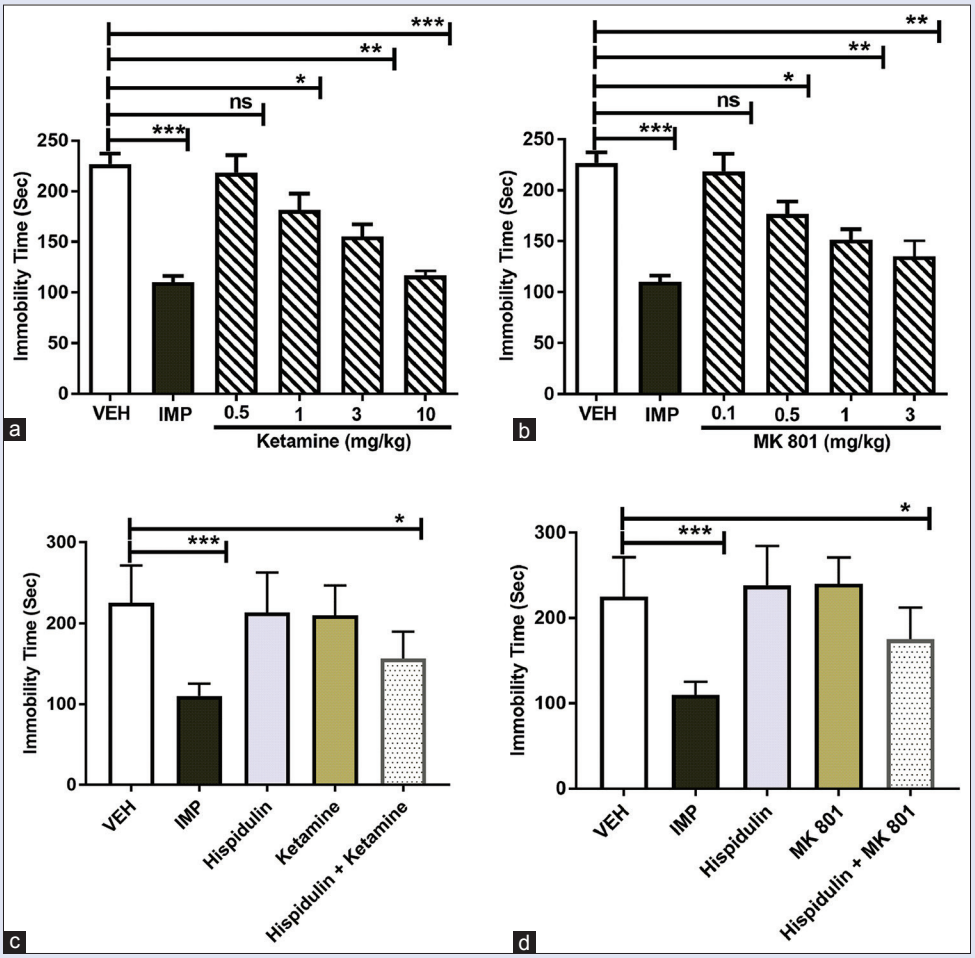

Figure 4: The antidepressant-like effects of different doses of ketamine (a), MK 801 (b) and antidepressant-like effects of coadministration of sub-effective doses of hispidulin (0.5 mg/kg, intraperitoneal) and ketamine (0.3 mg/kg, intraperitoneal) (c) and hispidulin (0.5 mg/kg, intraperitoneal) and MK801 (0.1 mg/kg, intraperitoneal) (d) in the forced swimming test. The values represent mean + standard error of the mean (n = 8 in each group). *P < 0.05, **P < 0.01, ***P < 0.001 compared to the vehicle, one-way analysis of variance, followed by Tukey's *post hoc* test

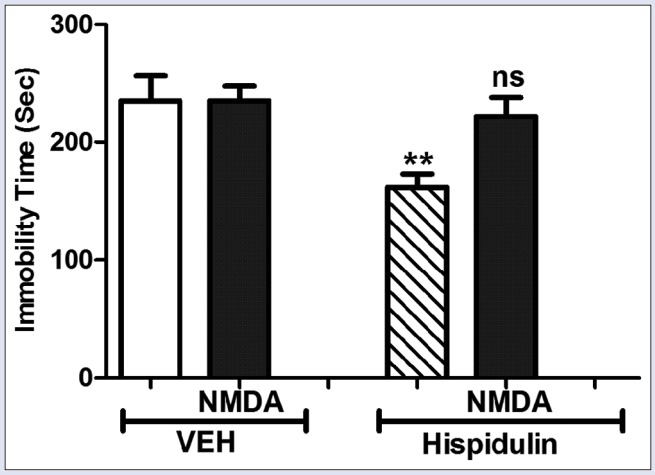

Figure 5: The effects of pretreatment with N-methyl-D-aspartic acid (75 mg/kg, intraperitoneal) on the antidepressant-like effects exerted by hispidulin (3 mg/kg, intraperitoneal) in the forced swimming test. The values represent mean + standard error of the mean (n = 8 in each group). **P < 0.01 compared to the vehicle, one way analysis of variance, followed by Tukey's *post hoc* test

DISCUSSION

In the current study, hispidulin isolated from *S. triloba* L produced antidepressant-like effects in mouse models of FST and TST. The study provides convincing evidence that the antidepressant-like effects of hispidulin are mediated through GABAergic and glutamatergic mechanisms.

The FST and TST are the validated and most widely used screening tests for assessment of the antidepressant properties of pharmacological substances and are sensitive to all major classes of antidepressant drugs.[15,20] The principle of these tests is the observation that rodents after an initial period of struggling to escape the stressful situation in the inescapable environment become immobile, reflecting a condition similar to human depression. If the animals are pretreated with antidepressant substances, they continue their struggle for a longer period to escape the stressful environment than after vehicle/control treatment. Both models have been validated and have shown excellent correlation between clinical potency and effectiveness of antidepressant agents.[21] In the present study, hispidulin at the doses of 1–10 mg/kg significantly reduced the immobility time of mice in FST and TST, indicating antidepressant-like activity.

Certain drugs such as psychoactive stimulants produce false-positive results in both TST and FST, as they increase locomotor activity and

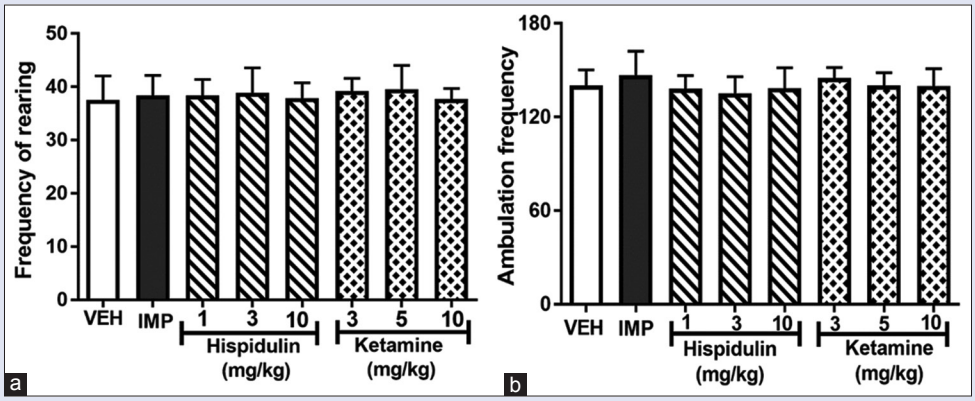

Figure 6: Effects of hispidulin (1–3 mg/kg, intraperitoneal), ketamine (3–10 mg/kg, intraperitoneal), imipramine (30 mg/kg, intraperitoneal) or vehicle on the locomotor activity (a) frequency of rearing, (b) ambulation of frequency; of mice in open field test. Data were expressed as the mean ± standard error of the mean of 6 animals

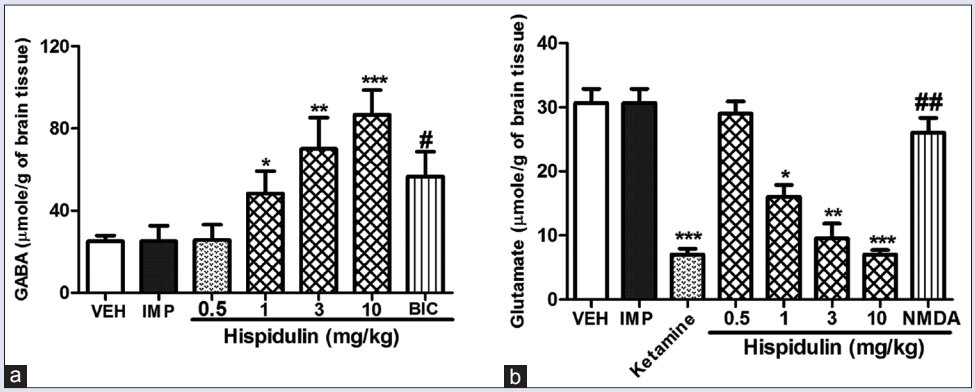

Figure 7: Effect of various treatment groups on γ-aminobutyric acid (a) and glutamate (b) levels in the brain of mice. Data were expressed as the mean ± standard error of the mean of 6 animals. *P < 0.05, **P < 0.01, ***P < 0.01 as compared to vehicle control, #P < 0.05, ##P < 0.01 as compared to hispidulin alone; 7one-way analysis of variance followed by Tukey's *post hoc* test

thus decrease the immobility time of mice.[22] The antidepressant-like effect of hispidulin (1–10 mg/kg) does not appear to be due to hyperactivity as it failed to increase the locomotor activity in OFT. Similar results were also shown by ketamine (3–10 mg/kg) which did not cause an increase in the locomotor activity in OFT. Flavonoids such as rutin, quercetin, and hesperidin have been reported to possess antidepressant-like effects.[23] However, to the best of our knowledge, this is the first study reporting the antidepressant-like effects of hispidulin.

Growing evidence from research conducted in the past decade has led researchers to look beyond the monoaminergic synapse for alternative targets to antidepressant drugs. GABA is the most important inhibitory neurotransmitter in the mammalian central nervous system and is responsible for the overall balance between excitation and inhibition among neurons.[24] Recently, many studies have shown a positive correlation between GABA dysfunction and various mental disorders, including anxiety, depression, and bipolar disorders.[25,26] In the clinical setting, drugs, such as lithium, carbamazepine, and valproate, have been shown to improve depressive symptoms possibly through reducing the metabolism of GABA and thus improving GABA content in the brain.[27] Furthermore, drugs possessing preferential selectivity for GABA α2/α3 subtypes of $GABA_A$ receptors have been proposed to be therapeutic targets for antidepressant agents. Clinical evidence shows that eszopiclone, an anxiolytic/hypnotic acting preferentially on α2/α3 $GABA_A$ receptors was found to possess enhanced antidepressant effect when coadministered with an antidepressant drug.[28] In the current study, coadministration of bicuculline (4 mg/kg) partially reversed the reduction in immobility time in mice induced by hispidulin (3 mg/kg). This reduction in immobility time of hispidulin by bicuculline was statistically significant compared to treatment with hispidulin alone ($^{\#}P < 0.05$) indicating the involvement of GABAergic mechanisms. These observations also suggest the contribution of other mechanisms in the antidepressant-like effects of hispidulin.

Glutamate is the principal excitatory neurotransmitter in the brain and is present in almost 60% of synapsis in the CNS.[29] The extracellular level of glutamate is essential for maintaining the normal physiological process and postmortem studies have indicated abnormal glutamate transmission in depression.[30] Several studies have implicated NMDA type of glutamate receptors in the pathophysiology and mechanisms of various antidepressant agents.[22] Thus, NMDA receptor antagonists, including memantine,[31] ketamine,[32] 1-aminocyclopropanecarboxylicacid, 2-amino-7-phosphonoheptanoic acid, and Dizocilpine (MK 801).[33] In this study also, ketamine and MK-801 exerted significant antidepressant-like effects. Furthermore, coadministration of NMDA (75 mg/kg), an agonist at NMDA glutamatergic receptors, caused a significant decrease in the antidepressant-like effect of hispidulin (3 mg/kg) compared to hispidulin alone ($^{\#}P < 0.05$), indicating the involvement of glutamatergic mechanisms in the antidepressant-like effects of hispidulin. Similar to the effects observed with bicuculline,

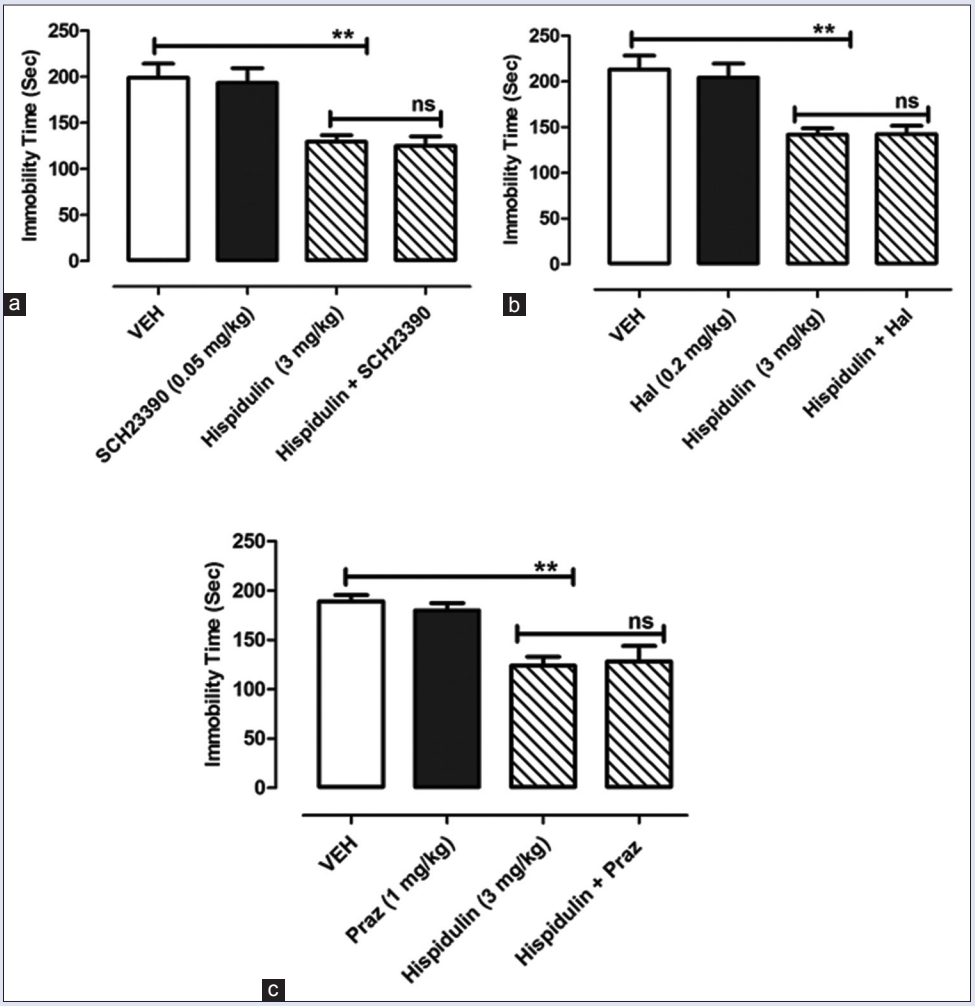

Figure 8: Effect of pretreatment of mice with SCH233390 (0.05 mg/kg, intraperitoneal a dopaminergic D1 receptor antagonist) (a), haloperidol (0.2 mg/kg, intraperitoneal, a dopaminergic D2 receptor antagonist) (b) and prazosin (1 mg/kg, intraperitoneal α1-adrenergic antagonist) (c) on the anti-immobility effect of hispidulin in the forced swimming test. Data are presented as the mean ± standard error of the mean ** $P < 0.01$, in comparison to the vehicle-treated group, ns (not significant) compared with hispidulin (3 mg/kg) treated group

NMDA did not completely block the antidepressant-like effects of hispidulin confirming that both GABAergic and glutamatergic neurotransmission may contribute to the antidepressant-like effects of hispidulin. Many studies have shown that concurrent treatment with antidepressant drugs might produce better and faster therapeutic response compared to single agents.[34,35] In this study, we found that significant reduction in immobility time of mice simultaneously administered with sub-effective doses of hispidulin (0.5 mg/kg) and ketamine (0.5 mg/kg) or MK 801 (0.1 mg/kg) in FST (*$P < 0.05$) indicating synergistic effect. When administered alone, neither of these agents produced a significant antidepressant-like effect.

Other biogenic amines, including noradrenaline, dopamine serotonin have also been implicated in depression and play a pivotal role in the pathophysiology of depressive disorders. However, our results showed that pretreatment of animals with prazosin (α1-adrenergic antagonist), SCH23390 (D1 dopamine antagonist), haloperidol (D2 dopamine receptor antagonist), or prazosin (α1-adrenergic receptor antagonist), or WAY 100635 (5-HT 1a receptor antagonist), ketanserin (5HT2a antagonist) and ondansetron (5-HTc receptor antagonist) did not block the decrease in immobility time of hispidulin indicating that these neurotransmitter mechanisms are not required for the antidepressant-like effect of hispidulin.

In neurochemical assays, hispidulin increased GABA level in brain tissues following FST indicating the role of GABA in the antidepressant-like effects of hispidulin. Similarly, hispidulin also caused a significant decrease in brain glutamate levels after FTS. In contrast, the glutamate receptor agonist, NMDA reversed the reduction in glutamate level caused by hispidulin indicating glutamatergic mechanisms also play a role in the antidepressant-like effects of hispidulin. Hispidulin may enhance GABA levels and decrease glutamate levels after FTS through different mechanisms which may include changes in GABA and glutamate reuptake, decreases or increases in their release and/or modulation at the receptor level.[22]

CONCLUSION

Hispidulin demonstrated antidepressant effects in FST and TST most likely mediated in part through GABAergic and glutamatergic mechanisms. The findings of this study suggest that hispidulin may serve a potential agent for drug development acting through GABA and/or glutamate mechanisms against depression.

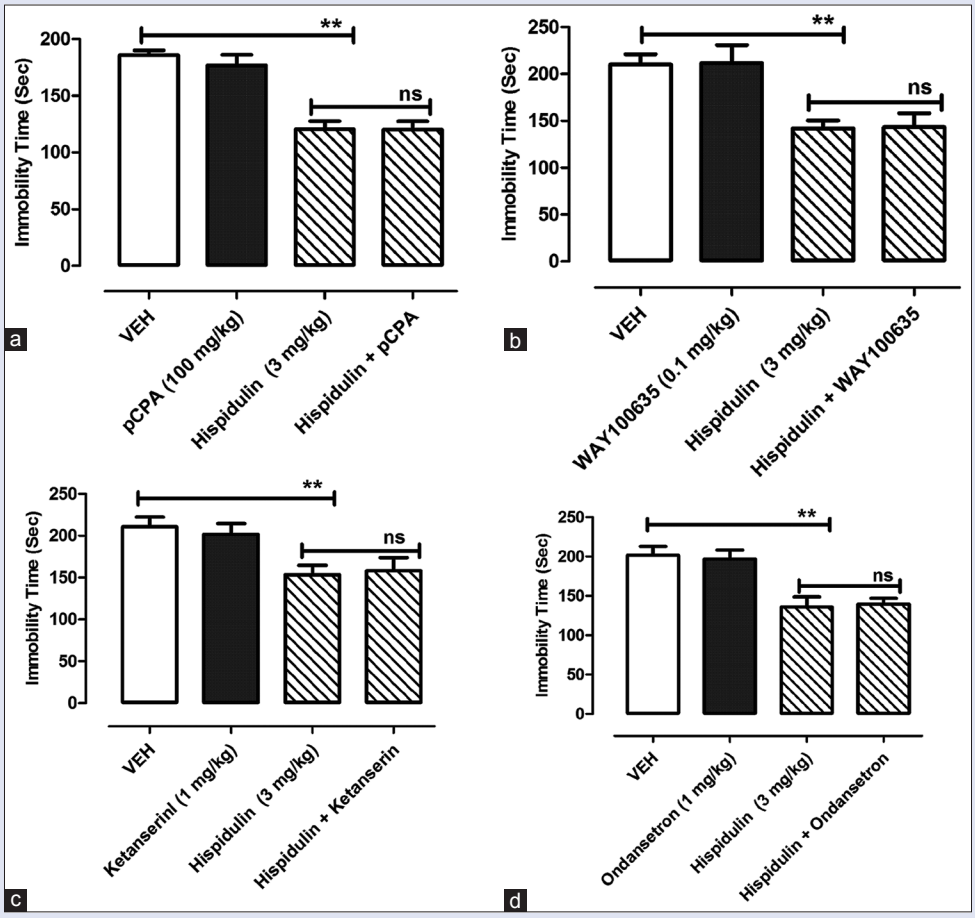

Figure 9: Effect of coadministration with parachlorophenylalanine (100 mg/kg, intraperitoneal) (a), WAY100635 (0.1 mg/kg, intraperitoneal) (b), ketanserin (1 mg/kg, intraperitoneal) (c), ondansetron (1 mg/kg, intraperitoneal) (d) on the antidepressant-like effect induced by hispidulin (3 mg/kg, intraperitoneal) in the forced swimming test. The values represent mean ± standard error of the mean ($n = 6$ in each group). Data were analyzed using one-way analysis of variance followed by Tukey's *post hoc* test, $^{**}P < 0.001$, compared with the vehicle control group, ns (not significant) compared with hispidulin (3 mg/kg) treated group

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Conflicts of interest

There are no conflicts of interest.

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