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# Antidepressant Activity of *Enicostemma littorale* Blume in Shp2 (Protein Tyrosine Phosphatase)-inhibited Animal Model of Depression

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## ABSTRACT

**Background:** The objective of this study is to develop a new animal model based on signaling pathways to understand the pathophysiology, therapy of depression, and to investigate the antidepressant activity of *Enicostemma littorale* which is not yet established.

**Methods:** Animal models of depression were raised by physical methods and administration of methyl isobutyl ketone (100 mg/kg b.w., i.p.,) and a protein tyrosine phosphatase inhibitor, sodium orthovanadate (30 mg/kg b.w., i.p.,) to young Wistar rats. *E. littorale* aqueous extract (100 mg/kg b.w., oral) was administered. Forced swimming test (FST), biochemical, and histopathological parameters were performed with reference to fluoxetine (20 mg/kg b.w., oral) treatment.

**Results:** High-performance thin-layer chromatography confirmed the presence of swertiamarin, a unique glycoside present in the *Gentianaceae* family. FST indicated high rates of immobility in depressed groups and low rates in plant extract-administered group with reference to fluoxetine. Biochemical assays indicated significantly ($P < 0.05$) increased levels of total protein, superoxide dismutase, triglycerides, and total serum cholesterol, whereas significant reduction ($P < 0.05$) of glutathione peroxidase, catalase, and lipid peroxidation in plant extract-administered groups in comparison to the depressed groups. Histopathological analysis indicated disorganized neuronal architecture during depression whereas rejuvenation of neuronal patterns was observed during treatment with plant extract and fluoxetine.

**Conclusions:** This study shows that sodium orthovanadate induces depression in animals and also establishes the antidepressant activity of *E. littorale*.

**Keywords:** Depression, *Enicostemma littorale* Blume, sodium orthovanadate

## INTRODUCTION

Depression is a complex and heterogeneous disorder which involves several neurotransmitters and neurohormonal pathways that play crucial roles in the pathophysiology of depression whose mechanisms are not well understood.[1] The absence or low levels of brain-derived neurotrophic factor (BDNF) or altered serotonin signaling, stress pathways, and other genetic or epigenetic factors influence the sequential activation of BDNF-mitogen-activated protein kinase (MAPK) pathway thereby resulting in

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depression.[2,3] The knowledge about the downstream targets of the MAPK signaling and their interactions in the regulation of anxiety and depression in the brain is obscure[4] [Figure 1]. *Enicostemma littorale* Blume (Indian Gentian) possess antioxidant, hepatoprotective, antinociceptive, anti-inflammatory, and antilipidemic activities.[5,6]

## METHODS

### Chemicals

All the chemicals were of analytical grade purchased from Hi-Media Laboratories Pvt., Limited, (Mumbai, Maharashtra, India). Sodium orthovanadate was purchased from Sigma-Aldrich, (Coimbatore, Tamil Nadu, India). Methyl isobutyl ketone (MIBK) was purchased from Qualigens Pvt. Limited, (Mumbai, Maharashtra, India). The standard drug fluoxetine was purchased commercially as fluon capsules at PSG Healthcare Pharmacy, Coimbatore, India. Swertiamarin standard was purchased from Aktin Chemicals, China.

### Plant material and extraction

*E. littorale* plant on the whole was collected from the local herbal shops in Coimbatore district, Tamil Nadu, India, and was authenticated by the Botanical Survey

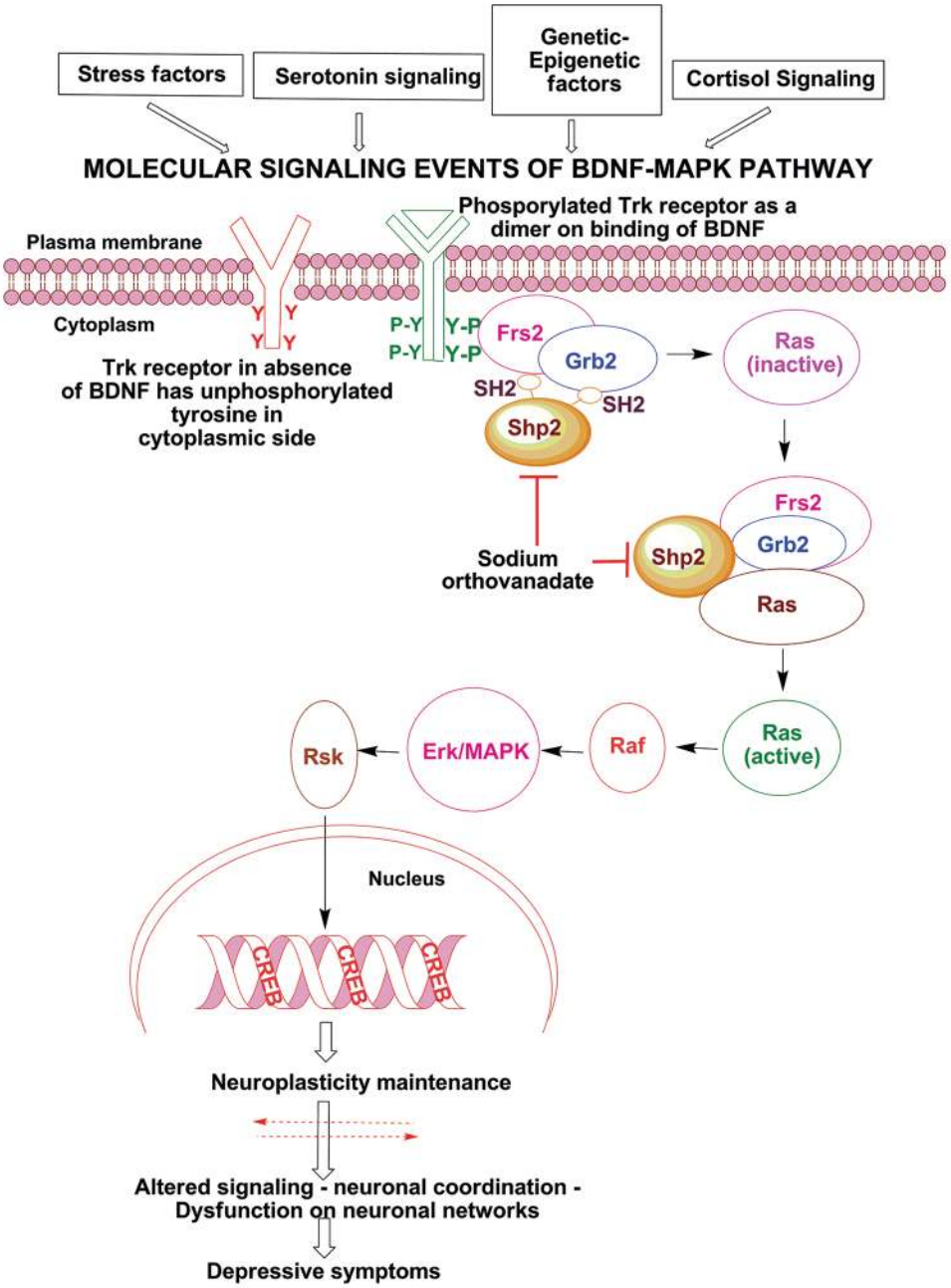


**Figure 1: Molecular signaling events of brain-derived neurotrophic factor-mitogen-activated protein kinase pathway and depression**

of India, Southern Regional Centre, Coimbatore (No. BSI/SRC/5/23/2010-11/Tech-2051). The vegetative and reproductive parts of *E. littorale* (1000 g) were shade dried, powdered in a mixer grinder, and stored in airtight containers. The dried plant powder was mixed with various solvents (1:3 ratio), namely water, ethanol, acetone, chloroform, and petroleum ether and filtered, and the phytochemical analysis[7,8] was performed to identify the efficient solvent for the study. The aqueous extract which showed the presence of more phytochemicals was prepared in large scale.[5]

## High-performance thin-layer chromatography of *Enicostemma littorale*

Two microliter plant extract and standard solutions were loaded as 5 mm band length in a 3×10 silica gel 60$F_{254}$ TLC plate using Hamilton syringe and CAMAG Linomat 5 instrument. TLC was performed using the mobile phase consisting of ethyl acetate, methanol, and water in the ratio 7.7:1.2:0.5, respectively. The plate was taken out and developed when the mobile phase was at 90 mm of height, and the developed plate was photo-documented using CAMAG Reprostar 3 in which the images were exposed and captured at visible light, ultraviolet (UV) 254 nm, and UV 366 nm. Then, the plate was scanned at UV 254 nm using CAMAG TLC Scanner 3 and then was derivatized using *p*-anisaldehyde-sulfuric acid and dried at 100°C in a hot air oven and photo-documented in visible light and UV (366 nm).

## Experimental animals

Young male Albino rats of Wistar strain (30 ± 20 g) were procured from Chennai, Tamil Nadu, India. The ethical clearance for handling of these experimental animals was obtained from the Institutional Animal Ethics Committee (IAEC) of PSG Institute of Medical Sciences and Research, Coimbatore, that acts under the guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India (CPCSEA/No: 158/1999/CPCSEA). The animals were acclimatized under standard laboratory conditions for 3 days with controlled temperature (29° ± 5°C), humidity (55% ± 5%), and 12 h light/dark cycles and maintained under the same conditions throughout the experimental period.

## Experiment groups

The experimental animals were divided into seven groups with three rats each including the normal healthy animals as Group I. Rats that were depressed using MIBK (100 mg/kg b.w., i.p., 5 days) and physical methods served as untreated depression control (Group II). The MIBK-induced depressed rates were treated with the standard drug, fluoxetine (20 mg/kg b.w., oral, 7 days) (Group III). The MIBK-induced depressed rats were treated with the aqueous extract of *E. littorale* (100 mg/kg b.w., oral, 7 days) (Group IV). Simultaneously, a group of animals were administered with sodium orthovanadate (protein tyrosine phosphatase [PTP] inhibitor ($LD_{50}$ is 330mg/kg b.w., rat) (30 mg/kg b.w., i.p., 5 days)[9] and subjected to physical methods for developing depression (Group V). The PTP inhibitor-induced depressed rats were treated with fluoxetine (20 mg/kg b.w., oral, 7 days) (Group VI). The PTP inhibitor-induced depressive rats were treated with aqueous extract of *E. littorale* (100 mg/kg b.w., oral, 7 days) (Group VII). Previous studies[10-12] on medicinal activities of *E. littorale* indicate various dosages of the plant extracts based on which the dosage was fixed in this study.

## Induction of depression

Depression was induced by physical and chemical methods in the young albino Wistar rats. Animals were starved for 24 h due to food deprivation and were injected with MIBK[13] (100 mg/kg, i.p.) with a 1:20 dilution to induce depression. Sodium orthovanadate (PTP inhibitor) (30 mg/kg, i.p.) was prepared in saline. After such chemical treatments, the rats were subjected to food deprivation for 24 h and were kept in a rotatory shaker (300 rpm for 10 min) which can further increase the susceptibility to depression. The status of depression was diagnosed by the forced swimming test (FST) on the 7$^{th}$ day after induction of depression and followed by treatments on the 3$^{rd}$ and 7$^{th}$.[14] FST was performed thrice for each test animal in each group and their mean was calculated for data analysis. Data are expressed as mean ± standard deviation.

## Collection of serum and tissues

The animals were anesthetized by chloroform and sacrificed by cervical dislocation at the end of the treatment period. Cardiac puncture method was employed for collecting the blood, and the clotted blood was centrifuged at 5000 rpm for 10 min to obtain the serum. The brain was excised from the skull, thoroughly washed in ice-cold saline, and used for biochemical and histopathological analysis.

## Biochemical analysis

The collected brain tissues were used for the estimation of protein,[15] enzymic, and nonenzymic antioxidants, namely superoxide dismutase (SOD),[16] CAT,[17] glutathione peroxidase (GPx),[18] and lipid peroxidation (LPO).[19] The collected serum was used for the estimations of triglycerides[20] and serum cholesterol.[21]

## Histopathological analysis

The histopathological analysis was performed with the excised brain tissue to evaluate the neuronal plasticity.[22]

## Statistical analysis

Data are expressed as mean ± standard error of the mean. Statistical analysis was performed using standard *t*-tests, and the $P < 0.05$ was considered statistically significant.

## RESULTS

The preliminary phytochemical analysis of the aqueous extracts of *E. littorale* revealed the presence of alkaloids, flavonoids, saponins, carbohydrates, proteins, phenols, glycosides, and tannins. High-performance thin-layer chromatography (HPTLC) profiling of glycosides [Figure 2] after derivatization showed a brownish zone at visible light mode which confirmed the presence of glycoside, swertiamarin in the sample, and the standard used.

FST shows the status of depressive condition in the experimental animal groups [Figure 3]. In the normal control, the immobility time is very less compared to other groups as they are normally active. During the course of study in a week time, the rate of immobility can be found to be lesser than that of the 1st day of the experiment due to their acclimatization. Depression was induced in Groups II to IV with MIBK (indicated as C) and Groups V to VII by sodium orthovanadate, a PTP inhibitor (indicated as I), respectively, followed by treatment with the standard antidepressant, fluoxetine, and the plant extract. The graph indicates the impact of PTP inhibitor in inducing depression with the increased rate of immobility compared to the normal group. As illustrated in the graph [Figure 3], it can be observed that the status of depression reduced on treatment and a marked reduction can be observed with the treatment of plant extract in comparison with fluoxetine-administered group.

In the biochemical analysis, the level of total protein content was found to be low in depression induced by MIBK (50 ± 3.86 μg) and PTP inhibitor (54 ± 4.18 μg) as compared with the normal control group (100 ± 3.85 μg). On comparison with the depression control, the groups administered with fluoxetine (Group III - 74 ± 4.15 μg; Group V - 60 ± 4.04 μg) and plant extract showed increased total protein levels (Group IV - 100 ± 4.41 μg; Group VII - 70 ± 4.41 μg) [Table 1]. The levels of SOD were found to have least activity against superoxide radicals in depression-induced groups (Group II - 0.06 ± 0.01 [U/g]; Group V - 0.12 ± 0.39 [U/g]) and was found to increase on treatment with fluoxetine (Group III - 0.68 ± 0.14 [U/g]; Group VI - 0.18 ± 0.04 [U/g]) and plant extract (Group IV - 0.62 ± 0.14 [U/g]; Group VII - 0.68 ± 0.12 [U/g]) [Table 2]. The activity of catalase (CAT) was found to be higher in depression groups induced by MIBK (4.0 ± 3.50 IU) and by the PTP inhibitor (7.8 ± 4.20 IU). The CAT activities were high on administration with fluoxetine (Group III - 5.4 ± 3.54 IU; Group VI - 9.6 ± 4.33 IU) and plant extract (Group IV - 8.2 ± 4.0 IU; Group VII - 7.8 ± 4.14 IU) [Table 2]. Thus, the study shows higher levels of GPx in both depression-induced groups (Group II - 400 ± 4.17 IU; Group V - 400 ± 3.54 IU) and depression-treated groups (Group III – 400 ± 3.96 IU; Group IV - 200 ± 3.87 IU; Group VI - 400 ± 4.26 IU; and Group VII - 200 ± 4.06 IU) compared to the normal control (200 ± 4.0 IU) [Table 2].

In the estimation of LPO in brain tissues, the formation of malondialdehyde was lower in groups with

**Table 1: Estimation of total proteins**

| Experimental groups | Concentration (μg) |
|---|---|
| Normal control (Group I) | 100±3.85 |
| Depression (MIBK) (Group II) | 50±3.86[a,*] |
| Depression (C) + fluoxetine (Group III) | 74±4.15 |
| Depression (C) + plant (Group IV) | 100±4.41[b,*] |
| Depression (inhibitor) (Group V) | 54±4.18[c,*] |
| Depression (I) + fluoxetine (Group VI) | 60±4.04 |
| Depression (I) + plant (Group VII) | 70±4.41[d,*] |

Values are expressed by mean±SD of six samples (*statistically significant $P<0.05$). Group comparison: [a]Group I versus Group II, [b]Group II versus Group IV, [c]Group I versus Group V, [d]Group V versus Group VII. C=Chemically induced depression using MIBK, I=PTP inhibitor (sodium orthovanadate), MIBK=Methyl isobutyl ketone, PTP=Protein tyrosine phosphatase, SD=Standard deviation

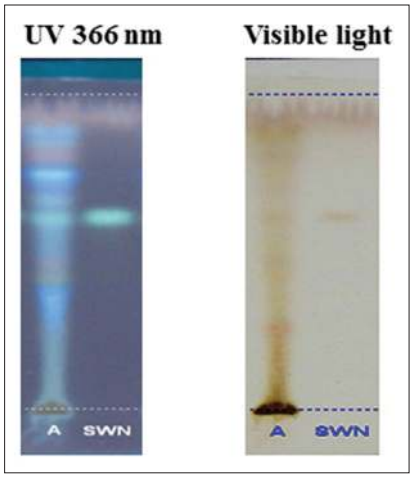


**Figure 2: High-performance thin-layer chromatography of swertiamarin after derivatization. In the figure, SWN indicates swertiamarin and A indicates plant extract**

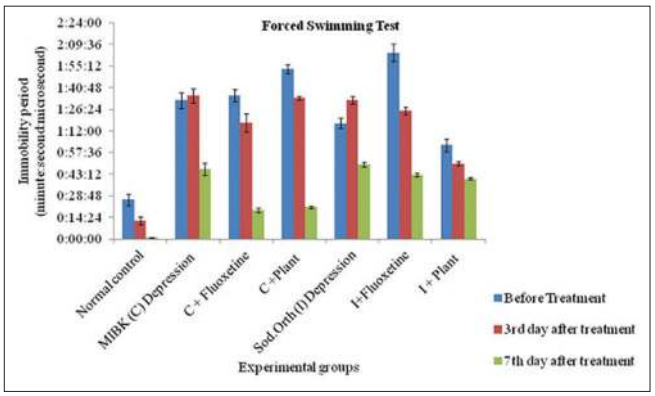


**Figure 3: Immobility periods of experimental animals by forced swimming test to determine the status of depression**

depression induced by both MIBK (0.12 ± 0.03 IU) and inhibitor (0.25 ± 0.04 IU). The values turned higher for the drug (Group III - 0.38 ± 0.04 IU; Group VI - 0.32 ± 0.04 IU) and plant extract-administered groups (Group IV - 0.38 ± 0.03 IU; Group VII - 0.57 ± 0.04 IU) when compared with the depression controls. Serum lipid profile analysis showed higher triglyceride levels (Group II - 128 ± 4.07 mg/dl; Group V -122.6 ± 4.19 mg/dl) and low levels of serum total cholesterol (Group II - 125 ± 4.28μg; Group V - 110 ± 4.28μg) in depression groups whose levels become lower on treatment with fluoxetine (Group III – 122 ± 4.40 mg/dl; Group VI - 82.6 ± 4.47 mg/dl triglycerides and in cases of cholesterol Group III - 20 ± 4.28 μg; Group VI - 85 ± 4.35 μg) and plant extract (Group IV - 125.3 ± 3.79 mg/dl; Group VII - 122 ± 3.72 mg/dl triglycerides and in cases of cholesterol Group IV - 185 ± 4.48μg; Group VII - 65 ± 4.28 μg) [Table 3].

The histopathological examination of the hippocampus in depression groups showed less prominent, disorganized neuronal, and glial cells compared to the normal rats, whereas the treatment with fluoxetine and plant extract resulted in the rejuvenation of normal cellular organization (Data not shown).

## DISCUSSION

HPTLC has shown the presence for swertiamarin which is hypothesized to possess antidepressant and anticholinergic activity due to the presence of a secoiridoid glycoside called swertiamarin which is unique to the *Gentianaceae* family.[23] Swertiamarin can be further subjected to structure elucidation and docking studies to reveal the interactions of the glycoside with its receptors to understand its role as an antidepressant which could further enter into clinical trials.[24] In the present study, the activity of SOD and CAT was less during depression and raises to normal level on treatment with *E. littorale*. GPx on contrary to previous reports is high during depression and reduces to normal during treatment with *E. littorale* which can be surmised that in depressed state, genetic polymorphism of GPx with increased proline alleles (Pro197Leu GPx) may be responsible for the abnormal high GPx activity. Enzyme expression studies are required to show whether abnormal antioxidant enzyme expression pattern prevails in depressive conditions.[25] Moreover, the increased activity can be probably due to increased production of ROS which usually occurs due to the activities of SOD and CAT.[26]

**Table 2: Estimation of superoxide dismutase, catalase, and glutathione peroxidase**

| Experimental groups | Concentration | | |
|---|---|---|---|
| | SOD (U/g) | CAT (IU) | GPx (IU) |
| Normal control (Group I) | 0.70±0.04 | 4.0±3.27 | 200±4.0 |
| Depression (MIBK) (Group II) | 0.06±0.01[a,*] | 4.0±3.50[a] | 400±4.17[a,*] |
| Depression (C) + fluoxetine (Group III) | 0.68±0.14 | 5.4±3.54 | 400±3.96 |
| Depression (C) + plant (Group IV) | 0.62±0.14[b] | 8.2±4.0[b] | 200±3.87[b,*] |
| Depression (inhibitor) (Group V) | 0.12±0.39[c,*] | 7.8±4.20[c] | 400±3.54[c,*] |
| Depression (I) + fluoxetine (Group VI) | 0.18±0.04 | 9.6±4.33 | 400±4.26 |
| Depression (I) + plant (Group VII) | 0.68±0.12[d,*] | 7.8±4.14[d] | 200±4.06[d,*] |

Values are expressed by mean±SD of 6 samples (*statistically significant $P<0.05$). A Unit of CAT is measured by the amount of hydrogen peroxide consumed/min/mg protein and a unit of GPx is measured by μg of glutathione reduced/min/mg protein. Group comparison: [a]Group I versus Group II, [b]Group II versus Group IV, [c]Group I versus Group V, [d]Group V versus Group VII. C=Chemically induced depression using MIBK, I=PTP inhibitor (sodium orthovanadate), SOD=Superoxide dismutase, CAT=Catalase, GPx=Glutathione peroxidase, SAD=Standard deviation, MIBK=Methyl isobutyl ketone, SD=Standard deviation

**Table 3: Estimation of lipid peroxidation, triglycerides, and serum total cholesterol**

| Experimental groups | Concentration | | |
|---|---|---|---|
| | LPO (IU) | Triglycerides (mg/dl) | Cholesterol (μg) |
| Normal control (Group I) | 0.25±0.03 | 98.6±3.68 | 310±4.04 |
| Depression (MIBK) (Group II) | 0.12±0.03[a] | 128±4.07[a,*] | 125±4.28[a,*] |
| Depression (C) + fluoxetine (Group III) | 0.38±0.04 | 122±4.40 | 20±4.28 |
| Depression (C) + plant (Group IV) | 0.38±0.03[b,*] | 125.3±3.79[b] | 185±4.48[b,*] |
| Depression (inhibitor) (Group V) | 0.25±0.04[c] | 122.6±4.19[c,*] | 110±4.28[c,*] |
| Depression (I) + fluoxetine (Group VI) | 0.32±0.04 | 82.6±4.47 | 85±4.35 |
| Depression (I) + plant (Group VII) | 0.57±0.04[d,*] | 122±3.72d | 65±4.28[d,*] |

Values are expressed by mean±SD of 6 samples (*statistically significant $P<0.05$). A unit of LPO is measured by n moles of malondialdehyde formed/mg protein. Group comparison: [a]Group I versus Group II, [b]Group II versus Group IV, [c]Group I versus Group V, [d]Group V versus Group VII. C=Chemically induced depression using MIBK, I=PTP inhibitor (sodium orthovanadate), MIBK=Methyl isobutyl ketone, LPO=Lipid peroxidation, SD=Standard deviation

Another noted outcome in this present study is the reduced level of LPO during depression. Sodium orthovanadate besides as a PTP inhibitor, when administered in lower and optimum doses, was used in protecting cells against direct action of LPO on brain acetylcholinesterase thereby preventing the major complications in diabetes, namely cholinergic neural dysfunction.[27] Studies reveal that the serum high-density lipoprotein and total cholesterol levels are lower during major depression[28,29] as indicated in the present study. Hypertriglyceridemia is another metabolic cause for depression[30] which is revealed in this study with increased serum triglyceride levels during depression. Another metabolic factor focused in depression is the altered cholesterol transport to the liver from tissues. Hence, the lipid metabolism is involved in depression which warrants further investigations. Evaluation of lipid profile is essential for establishing the relationship between depression and the cardiovascular disease for early diagnosis and prevention at its first phase.[31,32]

Depression is more than a mental disorder causing important alterations of the oxidative stress affecting the whole organism. Currently, the ongoing researches mostly target the analysis of the significant association that depression has with cardiovascular diseases, cancer and why people suffering from depression die at a younger age. As a systemic disease, depression studies look forward for successful animal models to understand the pathophysiology and to study the novel antidepressant strategies.[33,34]

## CONCLUSIONS

The present study has put forth a new insight for developing animal models of depression using PTP inhibitor (sodium orthovanadate), the importance of SHP2 inhibitors in identifying downstream targets of cell signaling and the natural or synthetic compounds that interact with them for developing novel therapeutics for depression. Ultimately, this study has also established the antidepressant potential of *E. littorale* which could be further studied to identify the pharmacologically active antidepressant compound.

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### Conflicts of interest

There are no conflicts of interest.

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