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ORIGINAL ARTICLE

Zanthoxylum alatum Attenuates Lipopolysaccharide-Induced Depressive-like Behavior in Mice Hippocampus

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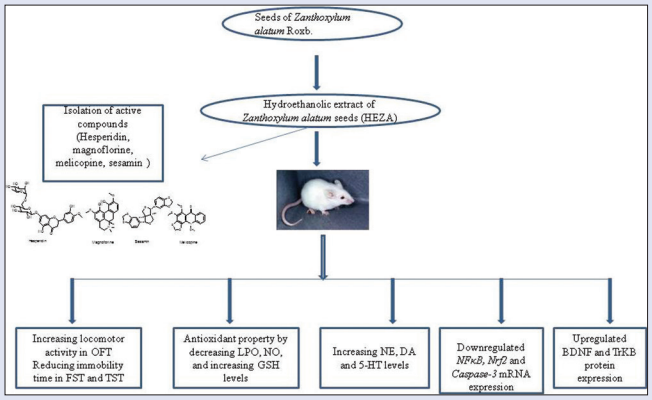
ABSTRACT

Background: *Zanthoxylum alatum* (ZA) is used ethnomedicinally for the treatment of various diseases. It is used as a nerve tonic in weak patients and in vertigo. **Objective:** The beneficial effect of hydroethanolic extract of ZA seeds (HEZA) on lipopolysaccharide (LPS)-induced depression-like behavior in mice was studied with its possible underlying mechanisms. **Materials and Methods:** HEZA (100 and 200 mg/kg, p.o.) and imipramine (10 mg/kg, i.p) were administered for 14 consecutive days, followed by LPS (0.83 mg/kg i.p) injection, 30 min posttreatment. Behavioral studies including the open field test, forced swimming test, tail suspension test and sucrose preference test were performed after 24 h of LPS administration. Levels of pro-oxidant markers (Lipid peroxidation (LPO), Nitric oxide (NO) and antioxidant enzyme viz. Glutathione (GSH), Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx) were estimated biochemically; Norepinephrine (NE), Dopamine (DA) and 5-hydroxytryptamine (5-HT) levels by high-performance liquid chromatography; nuclear factor- κB (*NFκB*), nuclear factor (erythroid-derived 2)-like 2 (*Nrf2*) and *caspase-3* mRNA expression using reverse transcription-polymerase chain reaction; the proteins of brain-derived neurotrophic factor (BDNF) and tropomyosin receptor kinase B (TrkB), in hippocampal tissue were evaluated by Western blot. Hesperidin, magnoflorine, melicopine, and sesamin were few phytoconstituents identified by liquid chromatography–electrospray ionization–tandem mass spectrometry studies. **Results:** Administration of LPS produced significant depression-like behavior by reducing the locomotor activity in open field test, increasing the immobility time in forced swim test and tail suspension test, increased LPO, NO, decreased GSH, SOD, CAT, GPx levels; lowered NE, DA and 5-HT level, upregulated *NFκB*, *Nrf2* and *Caspase-3* mRNA expression and downregulated BDNF, TrKB protein expression. Pretreatment with imipramine and HEZA reversed LPS-induced behavioral patterns, inhibited oxido-nitrosative stress, elevated antioxidant enzyme levels, and normalized the NE, DA, and 5-HT levels. The treatment also suppressed the mRNA expression of *NFκB, Nrf2,* and *Caspase-3* gene with upregulation of BDNF protein expression, the molecular marker for depression. **Conclusion:** Antidepressant-like activity of HEZA is assumed to be mediated by antioxidant property, impending neuroinflammation, alteration of monoaminergic responses, and preventing BDNF depletion.

Key words: Antioxidant, behavioral studies, imipramine, lipopolysaccharide, *Zanthoxylum alatum*

SUMMARY

- Hydroethanolic extract of *Zanthoxylum alatum* diminishes lipopolysaccharide-induced depressive-like behavior *in vivo*
- The extract exerts the antidepressant-like activity by improving antioxidant property, impending neuroinflammation, alteration of monoaminergic responses, and preventing brain-derived neurotrophic factor depletion
- Automated liquid chromatography–electrospray ionization–tandem mass spectrometry data revealed the presence of hesperidin, magnoflorine, melicopine, and sesamin in hydroethanolic extract of *Zanthoxylum alatum* seeds.



Abbreviations used: HEZA: Hydroethanolic extract of *Zanthoxylum alatum* seeds; LPS: Lipopolysaccharide; OFT: Open field test; FST: Forced swim test; TST: Tail suspension test; SPT: Sucrose preference test; LPO: Lipid peroxidation; NO: Nitric oxide; GSH: Glutathione; SOD: Superoxide dismutase; CAT: Catalase; GPx: Glutathione peroxidase; HPLC: High-performance liquid chromatography; *NFκB*: Nuclear factor-κB; *Nrf2*: Nuclear factor (erythroid-derived 2)-like 2; RT-PCR: Reverse transcription-polymerase chain reaction; BDNF: Brain-derived neurotrophic factor; TrkB: Tropomyosin receptor kinase B; 5-HT: 5-hydroxytryptamine; NE: Norepinephrine; DA: Dopamine; PCA: Perchloric acid; LC-ESI-MS/MS: Liquid chromatography–electrospray ionization–tandem mass spectrometry; ZA: *Zanthoxylum alatum.*

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INTRODUCTION

Traditional medicine has been used for thousands of years by most of the world population.[1] According to the World Health Organization, almost 80% of the population in developing countries depends on traditional medicinal plants[2] for primary health care, since they play vital roles in the prevention of diseases. *Zanthoxylum alatum* Roxb. (*Rutaceae*) (ZA) is an important small medicinal xerophyte, tree, or shrub, occupying an important place in the history of Indian system of medicines. It is distributed in tropical and temperate region of the world;[3,4] in India, from the warmer valleys of the Himalaya, Jammu and Kashmir to Assam and Khasi in the Eastern Ghats in Orissa and Andhra Pradesh and the lesser Himalayan regions in the northeast part of India, i.e., Naga Hills, Meghalaya, Mizoram, and Manipur,[5] it is widely available, and it has immense popularity for diverse medicinal properties by local people. Seeds are shining black,[6] with bitter taste,[7] commonly known as Indian Prickly Ash, Nepal pepper, or toothache tree, locally Tejphal (Hindi), Tejowati (Sanskrit), Mukthrubi (Manipur), Timur (Nepal).[8] In indigenous system of medicine, the bark, fruits, and seeds of ZA are used as carminative, stomachic, and anthelmintic.[8] The fruits and seeds are used as an aromatic tonic in fever and dyspepsia. Fruit extract is effective in expelling the roundworms. In Nepal folk medicine, ZA is widely used to treat cold and cough, tonsillitis, headache, fever, vertigo, diarrhea, and dysentery.[9] Powdered fruit mixed with *Mentha* species and table salt, eaten with boiled egg, is a good remedy for curing chest infection and digestive problems.[10] ZA as nervous system stimulant used in weak, debilitated patients, also in disorders like rheumatoid arthritis and paralysis.[11]

Depression is a complex psychiatric most prevalent mental disorder characterized by affective, cognitive, physiological impairments that lead to abnormal behavior. Structural abnormality in the brains of patients with mood disorders has been related to abnormal brain-derived neurotrophic factor (BDNF) function.[12] In experimental animals, administration of bacterial lipopolysaccharide (LPS) has been extensively used to reproduce a series of endocrinological and metabolic alterations and behavioral depression such as reduced food intake, social exploration, and sexual behavior.[13-16] Seeds of ZA has diverse medicinal properties among various locals of each state, besides culinary use, also used as nerve tonic and toothache and in patients with vertigo, which formed the basis of this study. The present study also focuses on identifying compounds from hydroethanolic extract investigated for its putative antidepressant property and to understand the underlying mechanism of action.

MATERIALS AND METHODS

Drugs and chemicals

LPS from *Escherichia coli,* strain 055: B5, serotonin hydrochloride, dopamine (DA) hydrochloride, (±)-norepinephrine (NE) (+)-bitartrate salt, and imipramine hydrochloride was procured from Sigma-Aldrich Corp., St Louis, USA. The drugs were prepared fresh on the day of the experiment. All other chemicals used were of analytical grade.

Plant collection and identification

The dried seeds of ZA were collected from local market of Arunachal Pradesh in August 2014. Seeds were identified by taxonomist Dr. Iswar Chandra Barua, Principal Scientist, Department of Agronomy, Assam Agricultural University, Jorhat, Assam, and a voucher specimen (5109 dated September 25, 2014) was deposited in the herbarium.

Preparation of hydroethanolic extract

Cleaned seeds of ZA were shade dried, powdered mechanically, weighed, and stored in airtight container. Then, 250 g of powdered material was soaked in 700-ml ethanol and 300-ml distilled water (70:30) for 72 h in a beaker; the mixture was stirred every 18 h using a sterile glass rod. Filtrate thus obtained three times with the help of a 70-mm Whatman filter paper No. 1 was evaporated in rotary evaporator (BUCHI, ROTAVAPOR, R-210; Switzerland) under reduced pressure left a dark-brown residue. It was stored in airtight container at 4°C until use (percentage with respect to dry powder was 19.43% w/w).

Ultra-high-performance liquid chromatography–electrospray ionization Orbitrap tandem mass spectrometry analysis

The ultra-high-performance liquid chromatography system coupled with an electrospray ionization Orbitrap tandem mass spectrometry was utilized for the screening of the phytoconstituents present in the hydroethanolic extract of ZA seeds (HEZA).[17] The mobile phase of solvent A: water with formic acid (0.01%) and solvent B: 100% acetonitrile was used with a constant flow rate of 0.3 ml/min by following the gradient method. The gradient program began with 95% A for 2 min, then slowly decreased to 5% A within 6 min, and held at 5% A for 1 min, again to the starting conditions, 95% A for 1 min. Samples (5 µl) were injected on to a Hypersil Gold C18 column (150 mm × 3.00 mm, Thermo, USA). The photodiode array detector was used for the identification by simultaneous screening at 275, 366, and 200–400 nm, and mass spectrometer was used for the analysis of the full mass peak and fragmentation pattern of the phytoconstituents in HEZA. The observed mass-to-charge ratio of the samples was compared with the literature and mass databases, which is the primary tool for characterization of the phytoconstituents.

Animals

Male Swiss albino mice (18–22 g) were obtained from the animal house of the Department of Pharmacology and Toxicology, College of Veterinary Science, Khanapara, Assam. They were housed in polypropylene cages and acclimatized for a week under standard conditions of temperature (22°C ± 3°C) and humidity (50% ±10%), with free access to standard pellet diet and water *ad libitum*. The protocols were approved by the Institutional Animal Ethical Committee (IAEC) of College of Veterinary Science, Assam Agricultural University (770/ac/CPCSEA/FVSc, AAU/IAEC/15-16/367). Laboratory animal handling and experimental procedures were performed in accordance with the guidelines recommended by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forest and Climate Change, Government of India.

Acute toxicity studies

Acute oral toxicity study was performed according to the Organization for Economic Co-operation and Development (423). The overnight-fasted mice (n = 3) were orally administered HEZA at the limit dose of 2000 mg/kg and observed continuously for behavioral, neurological, and autonomic profiles for 2 h and then 24 and 72 h and thereafter up to 14 days for any lethality, moribund state, or death. The limit test was repeated in another group of mice (n = 3) for confirmation and toxic class of LD_{50} determination. Acute oral toxicity study showed no toxicity or moribund stage due to the extract. This suggested that nonobservable adverse-effect dose level is more than 2000 mg/kg and approximate LD_{50} is >2500 mg/kg.

Experimental design

The mice were divided into five groups. Each group consists of 6–10 animals:

- Group 1: Vehicle control, receiving Tween 80 and saline (p.o)
- Group 2: Negative control, receiving saline and LPS (0.83 mg/kg; i.p)
- Group 3: Standard or positive control, receiving imipramine (10 mg/kg; i.p) and LPS (0.83 mg/kg; i.p)
- Group 4: Receiving HEZA (100 mg/kg; p.o) and LPS (0.83 mg/kg; i.p)
- Group 5: Receiving HEZA (200 mg/kg; p.o) and LPS (0.83 mg/kg; i.p).

The control, LPS-treated, imipramine-treated, and HEZA-treated groups were administered respective treatments once daily for 14 consecutive days. Then, LPS (0.83 mg/kg, i.p.) was injected to mice 30 min after drug administration except control group. After 24 h of LPS challenge, open field test (OFT), forced swimming test (FST), tail suspension test (TST), and sucrose preference test (SPT) were performed for the behavioral parameters. Mice were later sacrificed, and brains were quickly removed and hippocampus was isolated and stored at −80°C until analysis.[18]

Open field test

OFT was done in mice with minor modifications[19] for studying locomotor activity. They were individually kept in a clean and fresh cage like the home cage but devoid of bedding or litter that was divided into 12 virtual quadrants, and locomotor activity was measured by counting the number of line crossings and rearing during 5-min period.

Forced swim test

FST was performed to assess the despair behavior of the mice[20] using Porsolt's FST apparatus (Stoelting Co., IL 60191, USA). Water temperature was maintained at 28–30°C ± 1°C. Mice were placed in an inescapable cylinder for 6 min during the test session and video recorded using ANY-maze software (Stoelting Co., IL 60191, USA). Immobility time was counted for the last 4 min. Mice were considered immobile when ceased struggling, remained floating motionless, and only made those movements necessary to keep their head above the water.

Tail suspension test

It was conducted using TST apparatus (Stoelting Co., IL 60191, USA).[21] Mice were individually suspended in the hook of the TST box, 60 cm above the surface of a table with an adhesive tape placed 1 cm away from the tip of the tail in a dark room. The immobility time of each mouse was video recorded using ANY-maze software (Stoelting Co., IL 60191, USA) for 6 min, and immobility time was recorded for the last 4 min of the total observation period.

Sucrose preference test

The SPT was used to evaluate anhedonia.[22] All mice were acclimatized to drinking water and 2% sucrose solution for 5 days before LPS administration to establish a baseline sucrose preference for each mouse. Sucrose solution was filled in drinking bottle having stopper valve and placed in the home cage of animals. The relative position of bottles was changed daily to avoid development of a place preference. On the day of testing, mice were deprived of fluid and food for 2 h before testing and 48 h post-LPS, fluid content was measured, and sucrose preference was calculated using the following equation: Sucrose preference (%) = sucrose intake/(sucrose intake + water intake) × 100.

Food consumption and body weight

Food intake and body weight were recorded once daily at the onset of the dark period. Food containers were filled with 50 g of the pelleted mice chow, and intake was quantified 2 and 24 h after LPS/saline injection. Consumption of food in grams was recorded by subtracting the food remaining in the food container and on the cage floor from the amount of food measured at the preceding time point. Food spillage in the cage was ignored because it has been previously reported to be similar among rats/mice and generally weigh <1% of the food consumed.[23] Body weight was also taken at 2 and 24 h after LPS/saline injection. Both food intake and body weight were expressed in grams (g).

Assessment of oxidative stress and antioxidant status

The lipid peroxidation (LPO) end product malondialdehyde (MDA) was estimated in the hippocampus[24] using the thiobarbituric acid, and the optical density was measured spectrophotometrically at 532 nm (Multiskan Go, Thermo Fisher Scientific Oy., FI-01621, Finland). The values are expressed as ηM of MDA/mg of protein. Nitrite, an indicator of the production of nitric oxide (NO), was determined by a colorimetric assay using Griess reagent (Sigma-Aldrich Corp., St Louis, USA). The concentration of nitrite was determined from a sodium nitrite standard curve and expressed as μM of nitrite/mg of protein. Reduced glutathione (GSH) was estimated by the method of Ellman.[25] The concentration of GSH was expressed as μM of GSH/mg protein. Superoxide dismutase (SOD) activity was estimated using SOD assay kit (Sigma-Aldrich Corp., St Louis, USA) according to the manufacturer specifications. The SOD activity (units/mg of protein) was calculated using the standard plot. The catalase (CAT) activity was determined,[26] and activity was expressed as μM/min/mg protein. Glutathione peroxidase (GPx) was estimated,[27] and concentration was expressed as μM/mg of protein. The total protein was estimated by the method of Bradford.[28]

Estimation of catecholamines

Levels of NE, dopamine (DA), and 5-hydroxytryptamine (5-HT) were estimated using high-performance liquid chromatography (Thermo Scientific, Dionex Ultimate 3000 model and Chromeleon 7 software, Massachusetts, USA) coupled with an electrochemical detector (ESA Coulochem® III Detector, ESA Biosciences, Inc. Chelmsford, USA) as described by Kim *et al.*[29] A 0.3-N perchloric acid (PCA) solution is added to the tissue sample for preservation and extraction of catecholamines and acid metabolites. The tissue sample was weighed and placed into a microcentrifuge tube. For every 100 μg of wet weight, a volume of 1 ml of 0.3-N PCA is added. The samples were pulse sonicated on ice in this solution to degrade any native enzyme activity and help precipitate the proteins from the sample. The samples were then centrifuged for 10–15 min to form a pellet and clear supernatant that is free of particulates. An aliquot of the supernatant was finally diluted 1:2 with readymade MD-TM (ESA, Inc., USA) mobile phase. High-performance liquid chromatographic conditions using a normal bore column approach were as follows: flow: isocratic at 0.60 ml/min, temperature: 32°C, column: reverse-phase C18 column (MD-150, ESA, Inc. USA), guard column and holder, injection volume: 10–20 μl, partial loop mobile phase: 75-mM NaH_2PO_4, 1.7-mM 1-octanesulfonic acid, 100-μl/L triethylamine, 25-mM EDTA, and 10% acetonitrile adjusted to pH 3 with phosphoric acid, and Coulochem detector: 5011A cell: E1 at −150 mV: E2 at +220 mV and 5020 cell: EGC at +250 mV.

Reverse transcription-polymerase chain reaction analysis

Expression of nuclear factor-κB (*NFκB*), nuclear factor (erythroid-derived 2)-like 2 (*Nrf2*), and *Caspase-3* in the hippocampus of mice was studied by reverse transcription-polymerase chain reaction (RT-PCR). Tissue was homogenized using micropestle for isolation of

total RNA using TRIzol™ (Ambion). The mRNA was reverse transcribed by RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Massachusetts, USA) with random hexamer primers as per protocol of the kit. Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control for the experiment with a concentration of 10 pmol. Amplification was done in a Veriti™ thermal cycler (Applied Biosystems, California, USA). PCR condition included predenaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, variable annealing for different genes for 45 s [Table 1] and extension at 72°C for 45 min, followed by final extension at 72°C for 10 min. The PCR product was visualized in 1.5% agarose gel in 1X Tris-acetate-EDTA. The gel picture was then analyzed by Image Lab software version 5.1 (Bio-Rad, Hercules, CA, USA).

Western blotting analysis

Hippocampal tissue samples (30 mg approximately) were homogenized in 5 ml of chilled lysis buffer (RIPA buffer, Amresco LLC, Solon, OH, USA) and centrifuged at 23,000 ×*g* for 20 min at 4°C. The protein estimation of the supernatants was done by Bradford reagent (Himedia Laboratories, Mumbai, Maharashtra, India) with bovine serum albumin as the standard. Fifty micrograms of total protein were loaded on each lane of 10% gel, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using the Hoefer Mini Gel apparatus (Harvard Apparatus, Holliston, MA). Gels were electrophoresed at 150 V, and the fractionated proteins were transferred to polyvinylidene difluoride membranes (Pierce Biotechnology, Rockford, IL, USA) using semi-dry blotting apparatus (Hoefer). The membranes were blocked using 10 ml of cold blocking buffer containing 3% BSA in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at room temperature and incubated overnight (4°C) with 5 ml of 1% BSA in TBST containing antiserum rabbit polyclonal IgG (Santa Cruz Biotechnology, Inc., Texas, USA) against BDNF and tropomyosin receptor kinase B (TrkB) in 1:500 dilutions. Then, blots were washed 3 times with TBST for 15 min and incubated with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc., Texas, USA) for 1 h at room temperature. After washing with cold TBST, the color reaction on the membrane was obtained by treating the membranes with commercially available Ultra-TMB-blotting buffer. The membranes were scanned, and band intensities were quantified using ImageJ software (NIH, Bethesda, MD, USA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 5.0, GraphPad Software Inc., San Diego, CA, USA). All data were expressed as mean ± standard error of the mean. The data were statistically analyzed using one-way analysis of variance, followed by Dunnett's *post hoc* test. Results were considered statistically significant when $P > 0.05$.

RESULTS

Identification of phenolic compounds

The rapid screening of the compounds present in the HEZA was confirmed by chromatographic analysis using liquid chromatography–electrospray ionization–tandem mass spectrometry.[30] Peaks with RT (min) of 1.70, 2.08, 9.31, and 9.53 (peaks 1–4) were identified as hesperidin, magnoflorine, melicopine, and sesamin [Figure 1]. The *m/z* of the compound (1) hesperidin was found 610.565 $[M-H]^+$ (calculated: 610.565) with empirical formula $C_{28}H_{34}O_{15}$. The compound (2) magnoflorine ($C_{20}H_{24}NO_4$) was 341. 09201 $[M-H]^+$ (calculated: 342.415). The *m/z* of the compound (3) melicopine was 314.19891 $[M+H]^+$ (calculated: 313.309) with empirical formula $C_{17}H_{15}NO_5$. The compound (4) sesamin ($C_{20}H_{18}O_6$) was 353.09232 $[M-H]^+$ (calculated: 354.358).[31,32] The chromatographic representation is illustrated in Table 2.

Acute toxicity studies

Acute oral toxicity studies revealed no lethality or any toxic reactions up to the end of the study period. HEZA was safe up to a dose level of 2000 mg/kg of body weight (limit test), and the observed LD_{50} for oral administration of the extracts was more than 2500 mg/kg. Hence, the two doses, 100 and 200 mg/kg body weight used in the study, were also free from toxic effects.

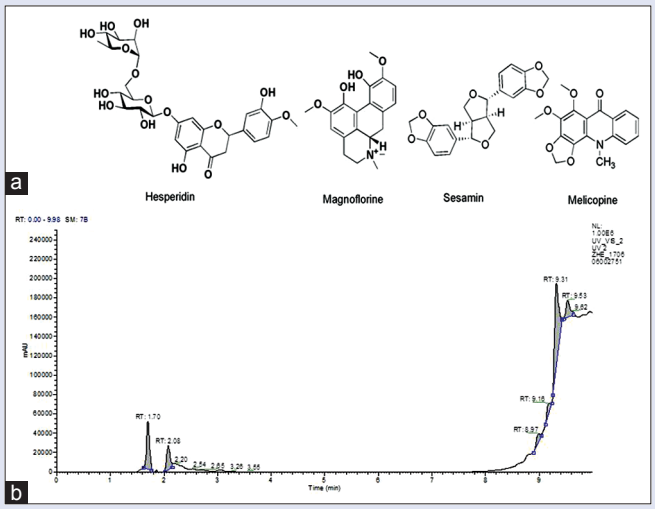

Figure 1: (a) Chemical structure of the identified compounds from hydroethanolic extract of *Zanthoxylum alatum* seeds. (b) Ultra-high-performance liquid chromatography chromatogram at 365 nm of hydroethanolic extract of *Zanthoxylum alatum* seeds. RT: Retention time

Table 1: Oligonucleotide primer sequences for target genes used in reverse transcription polymerase chain reaction

Gene of interest	Primer sequences	Annealing temperature (°C)	Product size (bp)
NFκB-p65	FP: 5'- AACAAAATGCCCCACGGTTA -3'	60	115
	RP: 5'- GGGACGATGCAATGGACTGT -3'		
Nrf2	FP: 5'- CAGCATGTTACGTGATGAGG -3'	60	105
	RP: 5'- GCTCAGAAAAGGCTCCATCC -3'		
Caspase-3	FP: 5'- TGGTGATGAAGGGGTCATTTATG -3'	60	152
	RP: 5'- TTCGGCTTTCCAGTCAGACTC -3'		
GAPDH	FP: 5'-AGGTTGTCTCCTGTGACTTC-3'	60	95
	RP: 5'-CTGTTGCTGTAGCCATATTC-3'		

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

Behavioral assessment

Effect of hydroethanolic extract of Zanthoxylum alatum seeds pretreatment on lipopolysaccharide-induced alteration on line crossings and rearing in open field test

In OFT, there was a significant reduction in both numbers of line crossings ($P < 0.001$) and rearing ($P < 0.05$) in LPS-treated mice than the vehicle-treated group, showing reduced locomotor activity. The standard drug imipramine ($P < 0.001$) and test drug HEZA (100 mg/kg; $P < 0.05$ and 200 mg/kg; $P < 0.01$) significantly improved number of line crossings dose dependently; however, it is nonsignificant in the number of rearings when compared with LPS-challenged mice [Figure 2].

Effect of hydroethanolic extract of Zanthoxylum alatum seeds pretreatment on lipopolysaccharide-induced increase in immobility time in forced swim test and tail suspension test

LPS-treated mice showed a significant ($P < 0.001$) increase in the duration of immobility both in FST and TST in comparison to the vehicle-treated group. Conversely, pretreatment with imipramine and HEZA (100 and 200 mg/kg) significantly ($P < 0.001$) reversed LPS-induced increase in immobility time [Figure 3], showing antidepressant-like effect of HEZA.

Effect of hydroethanolic extract of Zanthoxylum alatum seeds pretreatment on lipopolysaccharide-induced anhedonic behavior

LPS-treated mice showed a marked reduction in sucrose consumption as compared to vehicle-treated mice. However, both imipramine and HEZA (100 and 200 mg/kg) pretreatment showed improvement in sucrose consumption in LPS-challenged mice, but this effect was not significant [Figure 4].

Effect of hydroethanolic extract of Zanthoxylum alatum seeds pretreatment on lipopolysaccharide-induced alteration in food consumption and body weight

LPS administration also decreased food consumption ($P < 0.05$) and body weight ($P < 0.001$) in mice compared to the vehicle-treated group [Figure 5]. Imipramine ($P < 0.001$) and HEZA (100 mg/kg; $P < 0.05$ and 200 mg/kg; $P < 0.01$) although significantly improved body weight; however, neither imipramine nor HEZA showed any significant increase on food intake.

Biochemical estimation

Effect of hydroethanolic extract of Zanthoxylum alatum seeds pretreatment on lipopolysaccharide-induced alteration in oxidative stress and antioxidant parameters

LPS treated mice showed significant ($P < 0.01$) increase in LPO and NO levels in the hippocampus, as compared to vehicle-treated group. Pretreatment with imipramine (10 mg/kg; $P < 0.05$) and HEZA (200 mg/kg; $P < 0.01$) showed a significant reduction in LPO content in the hippocampus than vehicle-control group. Likewise, imipramine (10 mg/kg; $P < 0.001$) and HEZA (200 mg/kg; $P < 0.01$) pretreatment significantly reduced NO after 24 h of LPS administration than vehicle-control group. In addition, the GSH level due to LPS treatment was elevated either by imipramine ($P < 0.001$) or HEZA (200 mg/kg; $P < 0.01$) pretreatment. Decreased SOD activity in the hippocampus ($P < 0.05$), 24 h following LPS treatment than vehicle-treated group, was reversed by both imipramine and HEZA (100 and 200 mg/kg) nonsignificantly. LPS-challenged mice showed decreased CAT activity, which was increased by pretreatment with imipramine ($P < 0.05$), but HEZA pretreatment did not show any significant elevation at both the dosages. A significant reduction ($P < 0.01$) in GPx content in the hippocampus due to LPS treatment was reverted by imipramine ($P < 0.05$) and not by HEZA at both the dosages. The graphical representations are shown in Figure 6.

Table 2: Peak identification of hydroethanolic extract of *Zanthoxylum alatum* extract seeds using liquid chromatography-mass spectrometry/mass spectrometry

Compounds	RT	Empirical formula	Calcd *m/z*	Obsd *m/z* $[M+H]^+$	Obsd *m/z* $[M-H]^+$
Hesperidin	1.70	$C_{28}H_{34}O_{15}$	610.565	-	609.15472
Magnoflorine	2.08	$C_{20}H_{24}NO_4$	342.415	-	341.09201
Melicopine	9.31	$C_{17}H_{15}NO_5$	313.309	314.19891	-
Sesamin	9.53	$C_{20}H_{18}O_6$	354.358	-	353.09232

RT: Retention time

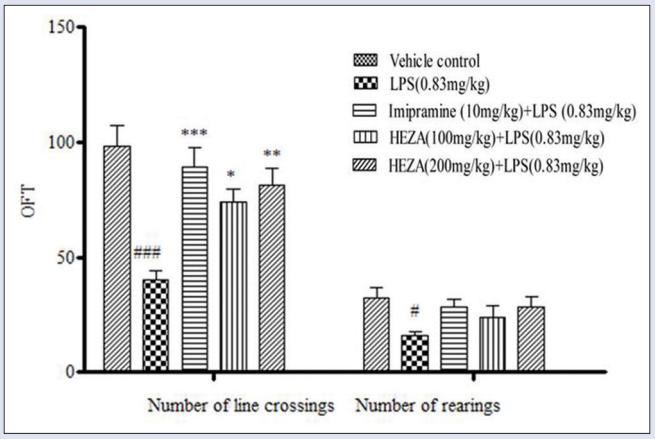

Figure 2: Effect of HEZA pretreatment on LPS-induced changes in behavioral parameter on OFT. Values represent the mean ± standard error of the mean ($n = 6$). $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$ compared with vehicle control. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with LPS-challenged group. LPS: Lipopolysaccharide; HEZA: Hydroethanolic extract of *Zanthoxylum alatum* seeds; OFT: Open field test

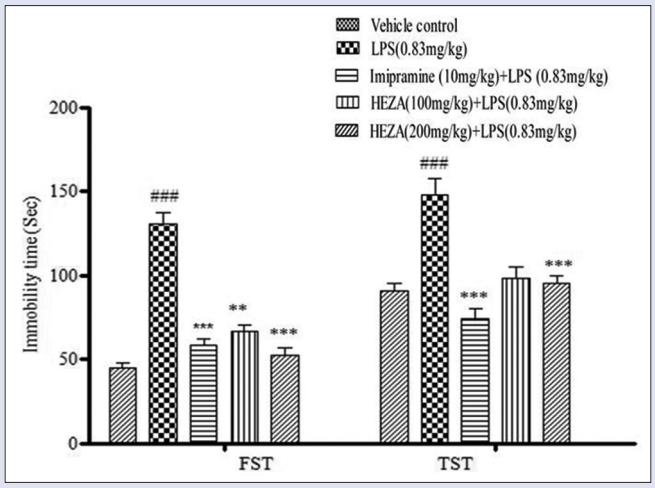

Figure 3: Effect of HEZA pretreatment on LPS-induced changes in behavioral parameter on FST and TST. Values represent the mean ± standard error of the mean ($n = 6$). $^{\#\#\#}P < 0.001$ compared with vehicle control. $^{**}P < 0.01$; $^{***}P < 0.001$ compared with LPS-challenged group. HEZA: Hydroethanolic extract of *Zanthoxylum alatum* seeds; LPS: Lipopolysaccharide; FST: Forced swim test; TST: Tail suspension test

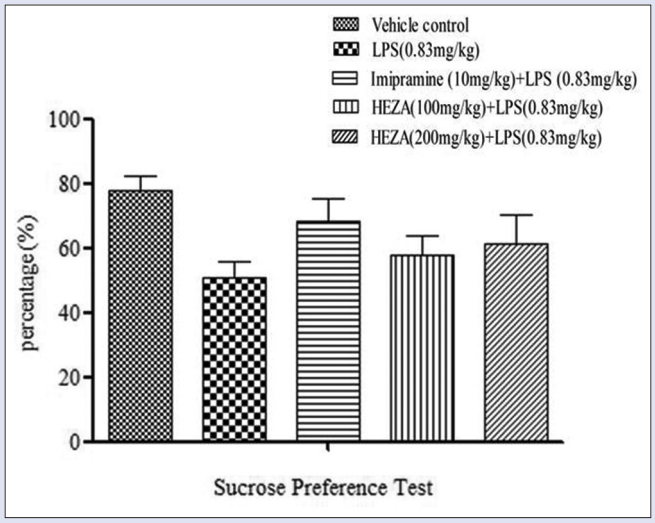

Figure 4: Effect of HEZA pretreatment on LPS-induced changes in SPT. Values represent the mean ± standard error of the mean (n = 6). HEZA: Hydroethanolic extract of *Zanthoxylum alatum* seeds; LPS: Lipopolysaccharide; SPT: Sucrose preference test

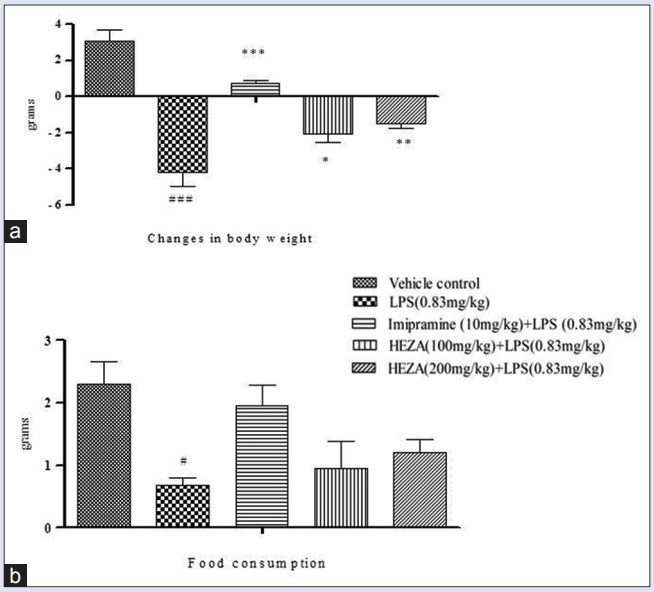

Figure 5: Effect of HEZA pretreatment on LPS-induced changes in (a) body weight (b) food consumption after 24 h of LPS administration. Values represent the mean ± standard error of the mean (n = 6). $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$ compared with vehicle control. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with LPSchallenged group. HEZA: Hydroethanolic extract of *Zanthoxylum alatum* seeds; LPS: Lipopolysaccharide

Effect of hydroethanolic extract of Zanthoxylum alatum seeds pretreatment on lipopolysaccharide-induced alteration in norepinephrine, dopamine, and 5-hydroxytryptamine concentrations

The catecholamine levels mainly NE, DA, and 5-HT concentrations in mice hippocampus of LPS-challenged group significantly ($P < 0.001$) reduced compared to vehicle-treated group. Pretreatment with HEZA reverted LPS-induced decline in NE (200 mg/kg; $P < 0.001$), DA (100 mg/kg, $P < 0.05$; 200 mg/kg, $P < 0.001$), and 5-HT (100 and 200 mg/kg; $P < 0.001$) concentrations. Similarly, imipramine ($P < 0.001$) also antagonized LPS-induced decline in catecholamine and 5-HT levels in the hippocampus [Figure 7].

Reverse transcription-polymerase chain reaction analysis

Effect of hydroethanolic extract of Zanthoxylum alatum seeds pretreatment on lipopolysaccharide-induced alteration in nuclear factor-κB, nuclear factor (erythroid-derived 2)-like 2, and Caspase-3 gene expressions

The RT-PCR analysis of *NFκB* (inflammatory marker), *Nrf2* (oxidative stress marker), and *Caspase-3* (apoptosis marker) gene expressions is shown in Figure 8. The mRNA expression levels of *NFκB* ($P < 0.001$), *Nrf2* ($P < 0.05$), and *Caspase-3* ($P < 0.05$) genes were significantly upregulated in the hippocampal tissues of LPS treated mice. HEZA significantly downregulated the mRNA expression of *NFκB* (100, 200 mg/kg; $P < 0.001$), *Nrf2* (100, 200 mg/kg; $P < 0.01$), and *Caspase-3* (200 mg/kg; $P < 0.05$) genes in the hippocampus of mice like imipramine ($P < 0.001$).

Immunoblotting assay

Effect of hydroethanolic extract of Zanthoxylum alatum seeds pretreatment on lipopolysaccharide-induced alteration in brain-derived neurotrophic factor and tropomyosin receptor kinase B protein expression

The immunoblotting assay of BDNF and its receptor TrkB, shown in Figure 9, revealed downregulation of BDNF and TrkB protein expression in LPS-treated mice. HEZA upregulated BDNF (200 mg/kg; $P < 0.05$) and TrkB (100 and 200 mg/kg; $P < 0.001$) protein expression; however, imipramine ($P < 0.001$) was better in upregulating its expression.

DISCUSSION

In our study, we have observed that hesperidin, magnoflorine, melicopine, and sesamin were identified from HEZA. There are several studies that reported hesperidin has beneficial antioxidant and neuroprotective effects.[33] Magnoflorine has cytotoxic, antiviral,[34] anxiolytic,[35] antioxidant, and anti-inflammatory[36] activities. Melicopine is acridone alkaloid with cytotoxic and antimalarial property.[37] Sesamin has anti-nociceptive, anti-inflammatory,[38] and antihypertensive activities[39] and reported to ameliorate the effects of chronic electric foot shock-induced spatial and habit learning memory deficits by regulating both NMDA receptor and dopaminergic neuronal systems.[40]

Depressive-like behavior following 24-h LPS-challenged mice was studied by OFT, FST, TST, and SPT. In OFT, LPS-treated group showed a significant reduction in the central as well as peripheral crossings, rearing movement, and increased immobility time. HEZA pretreatment antagonized the LPS-induced reduction in movement and increased immobility time. In FST and TST, the LPS-treated mice showed a significant increase in the duration of immobility in comparison to the vehicle-treated group. Our findings are consistent with the findings of Mello *et al.*,[41] where LPS-treated mice after 24 h showed increased immobility duration both in FST and TST than vehicle-treated group. Conversely, pretreatment with imipramine and HEZA significantly reversed LPS-induced increase in immobility time. Anhedonic behavior is one of the basic characteristic features of depression which was assessed by reduced consumption of sucrose solution.[42] Reduction in the preference to sucrose and marked anhedonia in LPS-treated mice than imipramine and HEZA showed antidepressant activity of our compound. In a similar study, Jangra *et al.*[43] also reported that mangiferin, a natural glucosyl xanthone, increased

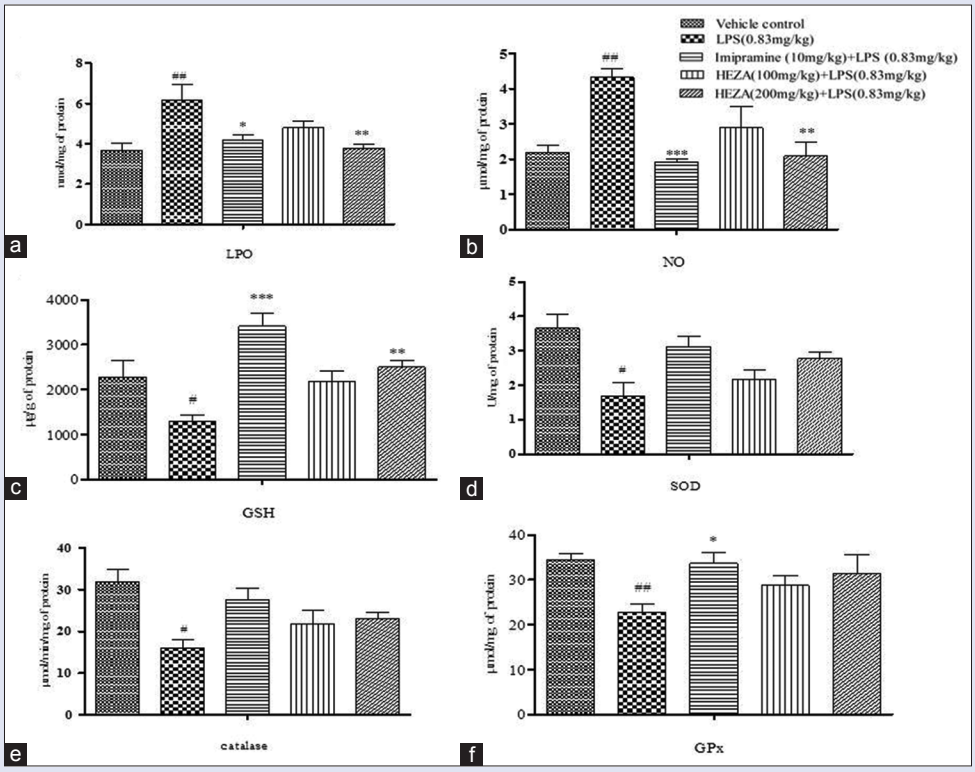

Figure 6: Effect of HEZA pretreatment on LPS-induced changes on *in vivo* antioxidant parameters (a) LPO (b) NO (c) GSH (d) SOD (e) catalase (f) GPx. Values represent the mean ± standard error of the mean; (n = 6). $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ compared with vehicle control. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with LPS-challenged group. LPO: Lipid peroxidation; NO: Nitric oxide; GSH: Glutathione; SOD: Superoxide dismutase; GPx: Glutathione peroxidase; LPS: Lipopolysaccharide; HEZA: Hydroethanolic extract of *Zanthoxylum alatum* seeds

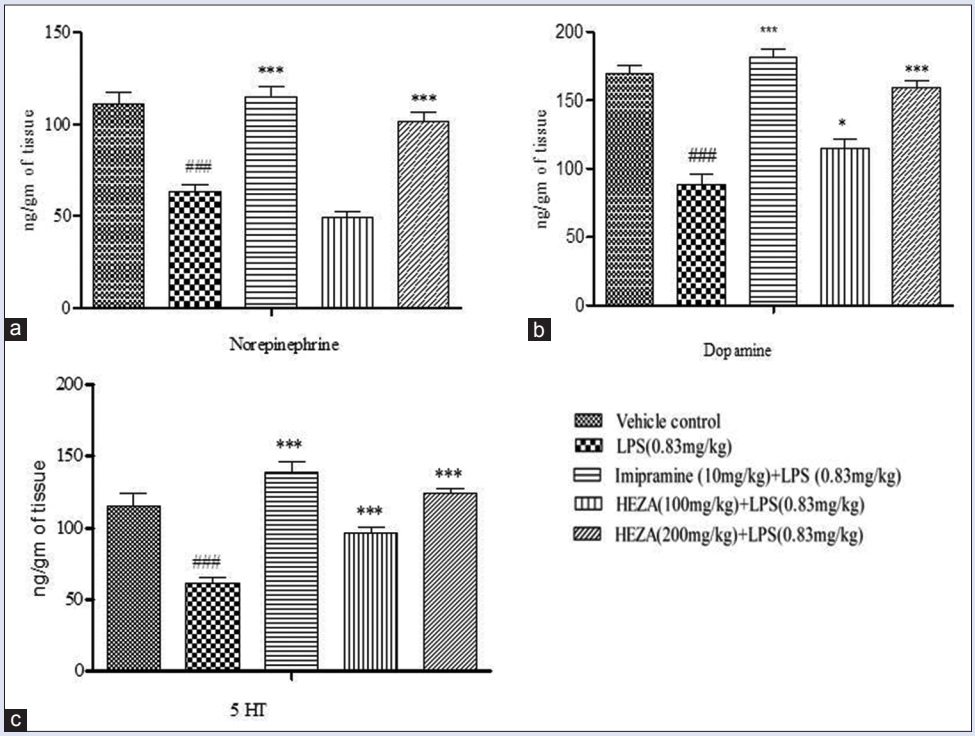

Figure 7: Effect of HEZA pretreatment on LPS-induced changes in (a) norepinephrine (b) dopamine (c) 5-HT in mice hippocampus. Values represent the mean ± standard error of the mean; (n = 6). $^{\#\#\#}P < 0.001$ compared with vehicle control. $^{*}P < 0.05$; $^{***}P < 0.001$ compared with LPS-challenged group. LPS: Lipopolysaccharide; HEZA: Hydroethanolic extract of *Zanthoxylum alatum* seeds; 5-HT: 5-hydroxytryptamine

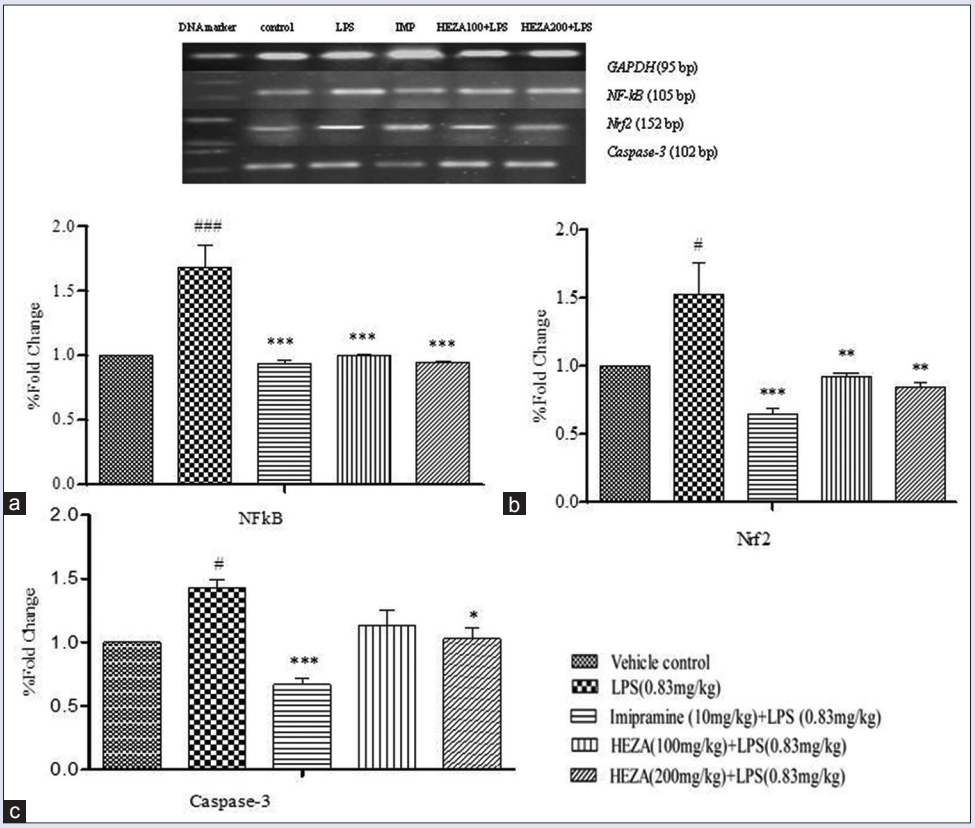

Figure 8: Effect of HEZA pretreatment on LPS-induced changes in brain (a) NFκB; B, (b) *Nrf2*, and (c) *Caspase-3* mRNA expression in the hippocampus of mice. GAPDH was used as positive control to assess the equal loading of sample. Values are expressed as mean ± standard error of the mean; (n = 6). $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$ compared with vehicle control. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with LPS-challenged group. NFκB: Nuclear factor-κB; Nrf2: Nuclear factor (erythroid-derived 2)-like 2; LPS: Lipopolysaccharide; HEZA: Hydroethanolic extract of *Zanthoxylum alatum* seeds

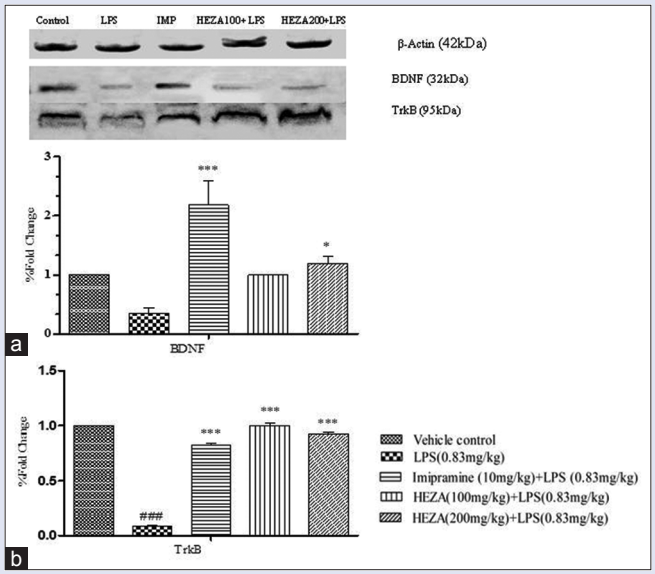

Figure 9: Effect of HEZA pretreatment on LPS-induced changes in (a) BDNF and (b) TrkB protein expression in the hippocampus of mice. β actin was used as internal control. Values are expressed as mean ± standard error of the mean; (n = 6). $^{\#\#\#}P < 0.001$ compared with vehicle control. $^{*}P < 0.05$; $^{***}P < 0.001$ compared with LPS-challenged group. BDNF: brain-derived neurotrophic factor; TrkB: Tropomyosin receptor kinase B; HEZA: Hydroethanolic extract of *Zanthoxylum alatum* seeds; LPS: Lipopolysaccharide

sucrose consumption, whereas in LPS-treated mice, sucrose preference was diminished indicating anhedonic behavior.

In the pathophysiology of depression, oxidative and nitrosative stress pathways play an important role.[44] Predisposing factors involved in the neuroprogression during depression are lipid peroxidation, high nitrite level, and lower level of antioxidant levels.[45] Antioxidant enzymes are powerful scavengers of free radicals and act as inhibitors of oxidative damage. Enzymatic antioxidants such as SOD, CAT, and GPx synergistically scavenge ROS.[46] Hippocampus of mice was affected by LPS-induced oxido-nitrosative stress, and increased LPO and NO level, in the hippocampus, indicates oxido-nitrosative stress. Decreased enzymatic antioxidants such as SOD, CAT, GPx, and GSH were evident in LPS-induced mice of all the groups without treatment. Following treatment with imipramine and HEZA, a significant reduction in LPO and NO levels was noticeable in the hippocampus. Result also showed a significant increase in GSH levels; however, SOD, CAT, and GPx levels were increased nonsignificantly, suggesting that HEZA attenuates oxido-nitrosative stress in the hippocampus. This result is in agreement with the findings of Jangra *et al.*,[43] where LPS-induced oxidative stress was reduced with mangiferin pretreatment by decreasing LPO and nitrite content. Our study shows that HEZA could protect the hippocampal cell from oxidative damage by virtue of its phytoconstituents, mainly hesperidin and magnoflorine.[33,36]

Pretreatment with imipramine and HEZA significantly elevated NE, DA, and 5-HT levels in the hippocampus of LPS-challenged mice. Imipramine is a tricyclic antidepressant involved in reuptake of NE and 5-HT. Therefore, we presume that HEZA might exert its antidepressant

effect akin to imipramine. In addition, HEZA also increased DA level in the brain; this could be due to the presence of sesamin and reported to ameliorate both NMDA receptor and dopaminergic neuronal systems.[40]

NFκB controls the expression of hundreds of genes involved in immunity, inflammation, proliferation, and defense against apoptosis.[47] Upregulation of *NFκB* observed in LPS-treated mice could be one of the reasons for pathogenesis of depression by generation of inflammatory responses. In our study, HEZA contains magnoflorine and sesamin as phytoconstituents might impart its neuroprotective and anti-inflammatory activity impending neuroinflammation.[34,36] Apoptosis in excess is related to cellular degeneration by oxidative stress, associated with aging and pathogenesis of neurodegenerative conditions.[45] Expression of *Caspase-3* is a frequently activated death protease catalyzing the specific cleavage of many key cellular proteins. Significant downregulation of *Caspase-3* mRNA expression in HEZA pretreated group indicates that the extract might reverse apoptosis too.

BDNF is a mediator involved in neuronal survival and plasticity of dopaminergic, cholinergic, and serotonergic neurons in the central nervous system.[48] Neurotrophins play an important role in regulating development and maintenance of the peripheral and central nervous system's function. Antidepressant treatment regulates levels of trophic factors in the brain. LPS induced inflammation led to significant downregulation of BDNF level in different regions of the brain.[49] Low level of hippocampal BDNF protein expression in LPS-challenged mice, which was upregulated following treatment with HEZA, provides a clue for its mechanistic pathway of the antidepressant-like effect.

CONCLUSION

It may be inferred that antidepressant-like activity of HEZA could perhaps be mediated through multiple mechanisms, i.e., inhibition of oxido-nitrosative stress, impending neuroinflammation, alteration of monoaminergic responses, and preventing BDNF depletion. There are enough reports that the phytoconstituents identified in our chemical analysis, namely hesperidin, magnoflorine, melicopine, and sesamin imparted these activities by multiple mechanisms. Since antidepressant property of the seeds is not studied earlier, hence further study on its effect other neurobiological diseases such as Alzheimer's disease and Parkinson disease will further strengthen its claim in our study.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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