

## Research Paper



# Zanthoxylum Alatum Attenuates Chronic Restraint Stress Adverse Behavioral Effects Via the Mitigation of Oxidative Stress and Modulating the Expression of Genes Involved in Endoplasmic Reticulum Stress in Mice

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**ABSTRACT**

**Introduction:** The functions of the endoplasmic reticulum (ER) are important, particularly in the proteins' synthesis, folding, modification, and transport. Based on traditional medicine and our previous studies on *Zanthoxylum alatum* in lipopolysaccharide-induced depressive behavior and scopolamine-induced impaired memory, the present study explored the role of hydroalcoholic extract of *Z. alatum* (ZAHA) seeds in reducing the ER stress in mice.

**Methods:** The mice were restrained for 28 days in polystyrene tubes. ZAHA (100 and 200 mg/kg, PO) and imipramine (10 mg/kg, IP) were administered daily, 45 min before restraint from day 22 to 28. The mice were assessed by the forced swim test. Also, the antioxidant enzyme levels of Superoxide Dismutase (SOD), reduced glutathione (GSH), and lipid peroxidation (LPO) were measured in the hippocampus of mice. The expression of 78 kDa glucose-regulated protein (GRP78), 94 kDa Glucose-Regulated Protein (GRP94), and C/EBPhomologous protein (CHOP) genes was assessed by real-time PCR to explore the molecular mechanism.

**Results:** ZAHA (100 and 200 mg/kg, PO, and imipramine, IP) counteracted the stress by significantly reducing the immobility time in the force swimming test, receding oxidative stress and lipid peroxidation. The antioxidant enzyme (SOD and GSH) levels were elevated in the restraint stress group. Down-regulation of genes (GRP78, GRP94, and CHOP) compared to the chronic restraint stress group indicated stress modulating properties of the seeds in ER stress. Hesperidin, magnoflorine, melicopine, and sesamin, isolated from the active extract, were hypothesized to exert the activity.

**Conclusion:** It can be concluded that *Z. alatum* reverted chronic restraint stress through its antioxidant properties and down-regulation of genes involved in ER stress.

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## Highlights

- Chronic restraint stress causes depressive-like behavior.
- Administrating *Zanthoxylum alatum* decreases the immobility time in stressed mice in the behavioral study (forced swim test).
- *Zanthoxylum alatum* possesses antioxidant properties, thus destroying the free radical and increasing antioxidant enzyme levels.
- *Zanthoxylum alatum* reverses chronic restraint stress by down-regulating endoplasmic reticulum stress genes like 78 kDa Glucose-Regulated Protein (GRP78), 90 kDa Glucose-Regulated Protein (GRP90), C/EBPhomologous protein (CHOP), and Caspase-12.

## Plain Language Summary

One of the main causes of stress-induced cognitive dysfunction and depression is Endoplasmic Reticulum (ER) stress. Chronic Restraint Stress (CRS) induces anxiety and depressive-like behavior in rodents. Exposure to chronic restraint stress causes ER stress in the hippocampus. In this study, we assessed the effect of hydroalcoholic extract of *Zanthoxylum Alatum* (ZAHA) seeds in chronic restraint stress in mice through behavioral and molecular study. ZAHA reduced the immobility time in the behavioral study, thereby reverting chronic stress-induced depressive behavior. ZAHA increases Superoxide Dismutase (SOD) and reduces Glutathione (GSH) levels. Significant reduction of oxidative stress and malondialdehyde formation was also evident. The markers of chronic restraint stress, i.e., GRP78, GRP90, CHOP, and Caspase-12, were upregulated during stress and down-regulated after pretreatment with ZAHA. These results indicate that *Z. alatum* counteracts stress-induced depressive-like behavior. In conclusion, *Z. alatum* is a strong agent for reverting ER stress by its antioxidant property and down-regulating GRP78, GRP90, CHOP, and Caspase-12 genes involved in the stress.

### 1. Introduction

The Endoplasmic Reticulum (ER) is a key subcellular organelle involved in the synthesis, folding, modification, and transport of proteins. Chronic ER stress impairs cognitive functions and causes weaknesses in learning and memory (Sindi, Kareholt et al., 2017), depression (Mahar et al., 2014), and anxiety (Herbison et al., 2017). Overstimulation of the hypothalamic-pituitary-adrenal axis harms the central nervous system (Marin et al., 2011). Zhang et al. (2014) proposed that ER stress due to restraining causes hippocampal apoptosis and cognitive impairments.

ER is the main site for steroids, cholesterol, and other lipids. Chronic restraint stress can cause depressive-like behavior by causing neuroinflammation and oxidative stress. Many factors are involved in this process, like pro-inflammatory cytokines and reactive oxygen species generation. The release of  $Ca^{2+}$  from ER enters the mitochondria, where they release reactive oxygen or nitrogen species (ROS/RNS). This reaction

further enhances ER stress leading to apoptosis, neuroinflammation, and neurotoxicity. ER stress-induced apoptosis is involved in the stimulation of ER-resident caspase-12, which accordingly initiates caspase-3 (Nakagawa & Yuan, 2000; Nakagawa et al., 2000). Scheper, & Hoozemans, (2015) reported that unfolded protein response up-regulates genes encoding ER chaperones, decreases translation, or increases ER-associated degradation of aggregated proteins. Bettigole, & Glimcher (2015) reported that protein kinase R-like ER kinase, inositol-requiring enzyme 1 $\alpha$ , and Activating Transcription Factor 6 (ATF6) are crucial transmembrane proteins that start unfolded protein response.

Imipramine, a tricyclic antidepressant, reduces sadness and lethargy and improves mood and overall body tone. The drug has successively been used in various neurodegenerative disorders like anxiety or depression.

*Zanthoxylum alatum* (ZA) Roxb. (Rutaceae) is an important medicinal xerophyte, tree, or shrub, that grows up to 6 m with dense foliage and armed branched flattened prickles. It comprises about 150 genera (Nasir, 1979). The dried fruit of ZA contains an aroma, which

is present in the pericarp shell of the brown fruit wall (Latika et al., 2013). In India, the plant *Z. alatum* (Rutaceae) is found in the fierier valleys of the Himalayas from Jammu and Kashmir to Assam and Khasi hills, in the Eastern Ghats in Orissa and Andhra Pradesh, and the lesser Himalayan areas in the North-Eastern states of India, including Naga Hills, Meghalaya, Mizoram, and Manipur (Kala et al., 2005). *Z. alatum* is known for its curative properties as a traditional remedy for various ailments. It is a carminative, stomachic, and anthelmintic drug (Singh, & Singh, 2011). Its fruit and seeds are used for curing fever and dyspepsia as an aromatic tonic. Fruits extract is beneficial in roundworm infestation. It is also used in treating cold and cough, tonsillitis, headache, fever, vertigo, diarrhea, and dysentery (Geweli et al., 2008). Ethanolic extract of ZA possesses antioxidant (Batool et al., 2000) and anti-inflammatory activity (Sati et al., 2011). The essential oil of ZA has antispasmodic, antimicrobial, cytotoxic, and phytotoxic properties (Barkatullah et al., 2013). Essential oils of ZA seeds have various chemicals, including alkaloids, flavonoids, flavonol glycosides, lignins, phenolics, sterols, terpenoids, fatty acids, alkanolic acids, and amino acids (Kalia et al., 1999). In our previous studies, we reported its antidepressant (Barua et al., 2018), anticholinergic, antihistaminic, antiserotonergic activity (Saikia et al., 2017), and memory-enhancing property (Saikia et al., 2018). However, the seeds have a strong aroma and contain essential oil. Thus, we aimed to explore the modulation of genes involved in ER stress at a molecular level in the chronic restraint stress model in mice. The 78-kDa Glucose-Regulated Protein (GRP78) and 94-kDa glucose-regulated protein (GRP94) genes are crucial to keep the ER functions. Sharma et al. (2018) reported that inhibition of Protein Kinase RNA (PKR)-like ER kinase expression in the hippocampus could improve hippocampal-dependent memory and undo memory decline in mice. The GRP78, GRP94, ATF6, XBP1, ATF4, and CHOP gene expression increased in the hippocampus of rats with learned helplessness (Timberlake, & Dwivedi, 2016). To correlate its behavioral parameters, a few important stress markers, namely CHOP, GRP78, GRP94, and Caspase-12, were also investigated. We theorized that *Z. alatum* treatment could ameliorate the chronic restraint stress-induced depressive-like behavior and cognitive impairment.

## 2. Materials and Methods

### Chemicals

Imipramine hydrochloride was procured from Sigma-Aldrich Corporation (St. Louis, USA). Ethanol, ether, and acetonitrile were procured from Merck (M) and 2-thiobarbituric acid from “HiMedia.”

### Laboratory animals

We obtained 30 healthy male Swiss albino mice (30±5 g) from the animal house of the Department of Pharmacology and Toxicology, College of Veterinary Science, Khanapara, Assam. The Institutional Animal Ethics Committee (IAEC) of the College of Veterinary Sciences, Assam Agricultural University, Khanapara, permitted the study protocol (No.770/ac/CPCSEA/FVSc, AAU/IAEC/15-16/367). The animals were familiarized with the Lab condition for two weeks before conducting the experiment. The mice were kept in polypropylene cages, and food and clean drinking water were provided ad libitum. They were maintained in a standard laboratory condition (12:12 h light/dark cycle at an ambient temperature of 22°C-25°C and 30% relative humidity) according to the National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals.

### Plant material and preparation of extract

Seeds of *Z. alatum* were obtained from Arunachal Pradesh from July to August. The seeds were identified by Dr. I.C. Barua, Principal Scientist, Department of Agronomy, Assam Agricultural University. The voucher specimen was kept in the herbaria (5109, dated-25.09.2014) for future reference. The seeds were cleaned and dried in the shade for a week and then grinded in an electric grinder and powdered. Next, 250 g of powder was soaked in 1000 mL of hydroalcoholic solution (ethanol and water in the ratio of 70:30) for 72 h in a beaker; the mixture was stirred with a sterile glass rod till it became colorless. The filtrate was evaporated using a rotary evaporator (Buchi R-210, BÜCHI Labortechnik AG, Switzerland) to remove the solvent. The recovery percentage was 19.71% w/v. Phytochemical screening of *Z. alatum* disclosed the presence of steroids, glycosides, alkaloids, diterpenes, and triterpenes (Kalia et al., 1999).

## Identification of active compound

We used an ultrahigh-performance liquid chromatography UHPLC system with an ESI OrbitrapMS/MS to spot the phytoconstituents in the hydroethanolic extract of *Z. alatum* (Kumar et al., 2016). The mobile phase of solvent A: water with formic acid (0.01%) and solvent B: 100% acetonitrile were used with a steady flow rate of 0.3 mL/min by subsequent gradient method. It began with 95% A for 2 min, then gradually reduced to 5% A in 6 mins and hold at 5% A for 1 min, then to the starting conditions, 95% A for 1 min. Samples (5  $\mu$ L) were injected onto a Hypersil GOLD C18 column (150 x 3.00 mm, Thermo, USA). For the identification, by simultaneous screening at 275 nm, 366 nm, and 200-400 nm, we used a photodiode array detector. Also, we used it for the analysis of the full mass peak and fragmentation pattern of the phytoconstituents mass spectrometer. The observed mass-to-charge ratio of the sample was compared with the literature and mass databases that were the primary tool for the characterization of the phytoconstituents (Figures 1, 2, and Table 2).

## Drug treatments and experimental design

### Acute toxicity study

The acute toxicity study was conducted following the protocol of Organization for Economic Cooperation and Development guidelines for testing chemicals (OECD 423). The extract was fed orally at 2000 mg/kg to 3 mice, and the percentage of mortality, if any, was noted. They were observed for the next 14 d for mortality or gross abnormality with the given doses. Based on the acute toxicity study, 100 and 200 mg/kg oral doses were selected for the present study.

### Experimental Design

Five groups (n=6) of experimental animals were restrained for six hours every day for 28 days in 50 mL polystyrene tubes. They were divided into the following groups: group I, or normal control, which received vehicle (Tween-80 and saline PO); group II, negative control, or restraint group; group III, or standard group, which received imipramine 10 mg/kg IP; group IV, or ZAHA 100 mg/kg PO; and group V, or ZAHA 200 mg/kg PO.

### Stress protocol

The extract was given per os from the 22nd day of restraint till the 28th day (Chiba et al., 2012). Administration of drugs was done for 7 days 45 min prior to

stress. Imipramine (10 mg/kg, IP) was administered to group III. A forced swim test was performed for the behavioral study. After that, the mice were sacrificed by cervical decapitation immediately after the last day of restraint. Their hippocampus was dissected carefully for further biochemical and molecular analysis and kept at -80°C till further estimation.

## Behavioral studies

To evaluate the despair behavior of mice, we performed the forced swim test (Porsolt et al., 1977) 24 h after the last day of restraint. We kept each animal in an unpreventable chamber of measurement 10 cm in diameter filled up with water (25°C) up to 15 cm for a complete time span of 6 min; an initial 2 min were considered as the acclimatization period, and perception for the last 4 min was considered to conduct the stressful behavior. The immobility duration of mice was recorded using Any Maze apparatus (Stoelting Co., USA).

### Body weight

To study the effect of stress on food intake, the body weight of the animals was taken at the beginning and the end of the experiment.

## Oxidative stress analysis

### Lipid peroxidation and enzyme assays

The lipid peroxide in the brain homogenate was assessed by the thiobarbituric acid reactive substances (Ohkawa et al., 1979) method at 532 nm (Multiskan GO, ThermoFisher Scientific). The method of Bradford (1976) was employed to measure the total protein. Reduced glutathione (GSH) was measured with the slight modification of the Moron et al. (1979) method and superoxide dismutase (SOD) by Marklund and Marklund's (1974) method.

## Quantitative real-time PCR

The mRNA expression levels of GRP94, GRP78, CHOP, and Caspase-12 genes were assayed by real-time PCR (Applied Biosystems). Messenger RNA was isolated from the hippocampus using TRIzol (Ambion), followed by 1  $\mu$ g of mRNA as reverse transcribed using the RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific India Pvt). The resultant cDNA was amplified separately with specific primers for GRP94, GRP78, CHOP, and Caspase-12 using a standard protocol (Applied Biosystems 7500 Real-Time PCR system). Table 1 lists the primers (ILS primers, India).

### Statistical analysis

The results are expressed as Mean±Standard Error of the Mean. Statistical analysis was performed by 1-way analysis of variance followed by Dunnett's post hoc test in Graph Pad Prism software version 5.0 (version 5.0, Graph Pad Software Inc., San Diego, CA, USA). All results were considered statistically significant when  $P<0.05$ .

## 3. Results

### UHPLC-ESI OrbitrapMS/MS analysis to identify phenolic compounds

The gradient method was used to identify the phyto-constituents in *Z. alatum* extract. The chromatogram was recorded at 365 nm. Figure 1 shows the chromatographic representation. The peaks were identified by comparing the retention time (RT),  $\lambda$  max, and mass spectra of the *Z. alatum* extract from the literature and database. Peaks with RT (min) of 1.70, 2.08, 9.31, and 9.53 (peaks 1-4) were recognized as hesperidin, magnoflorine, melicopine, and sesamin (Figure 2).

The m/z of the hesperidin (C<sub>28</sub>H<sub>34</sub>O<sub>15</sub>) was 610.565 [M-H]<sup>+</sup> (calculated: 610.565), of the magnoflorine (C<sub>20</sub>H<sub>24</sub>NO<sub>4</sub>) was 341.09201 [M-H]<sup>+</sup> (calculated: 342.415), of the melicopine (C<sub>17</sub>H<sub>15</sub>NO<sub>5</sub>) was 314.19891 [M+H]<sup>+</sup> (calculated: 313.309), and of the sesamin (C<sub>20</sub>H<sub>18</sub>O<sub>6</sub>) was 353.09232 [M-H]<sup>+</sup> (calculated: 354.358) (Bhatt, Sharma, Kumar, Sharma, & Singh, 2017; Kumar et al., 2014).

### Effect of *zanthoxylum alatum* on the duration of immobility in the forced swim test

A significant increase in the immobility time (128±2.66 s,  $P<0.001$ ) was observed compared to the normal control group (49.50 ±1.36 s). However, ZAHA pretreatment at 100 and 200 mg/kg reduced stress, as shown by the immobility time (92.53±2.92 s and 75.99±2.56 s,  $P<0.001$ ). A similar result was observed with imipramine (Figure 3).

### Effect of *zanthoxylum alatum* on the body weight

The mice's mean body weight decreased (-3.94±0.11 g,  $P<0.001$ ) in the restraint group compared to the normal control group (2.61±0.62 g). Imipramine (10 mg/kg, IP) caused significant gain (0.49±0.11 g,  $P<0.001$ ) in the body weight. Also, ZAHA pretreatment at both doses significantly increased (-2.46±0.21 g,  $P<0.05$ ) the body weight

compared to the chronic restraint group (-1.16±0.07 g,  $P<0.001$ ) (Figure 4).

### Effect of *Zanthoxylum alatum* on lipid peroxidation

We found a significant increase (7.78±0.64 nM/mg protein,  $P<0.01$ ) in the malondialdehyde (MDA) level in the chronic restraint group compared to the normal control group (3.99±0.52 nM/mg protein). Imipramine showed significant decline (4.17±0.38 nM/mg protein,  $P<0.01$ ) in MDA level. ZAHA at both doses significantly reduced the MDA level (5.13±0.88 nM/mg protein,  $P<0.05$ ; 4.51±0.73 nM/mg protein,  $P<0.01$ ) compared to the chronic restraint group (Figure 5a).

### Effect of *Zanthoxylum alatum* on oxidative enzymes

Here we discuss the effect of pretreatment on reduced glutathione (GSH) and superoxide dismutase (SOD).

#### Reduced glutathione

There was a significant reduction in the GSH level (0.92±0.29 µg/mg protein,  $P<0.01$ ) in the stress group when compared to a normal control group (3.20±0.22 µg/mg protein). Imipramine significantly increased GSH level (2.90±0.16 µg/mg protein,  $P<0.01$ ). Pretreatment with ZAHA at both doses, significantly increased GSH level (2.38±0.36 µg/mg protein,  $P<0.05$ ) compared to the chronic restraint stress group (2.56±0.66 µg/mg protein,  $P<0.01$ ) (Figure 5b).

#### Superoxide dismutase

A significant decrease in the SOD level (1.01±0.32 U/mg protein;  $P<0.001$ ) was recorded in the stress group compared to the normal control group. Imipramine as a standard drug increased SOD level (2.95±0.36 U/mg protein,  $P<0.01$ ). Pretreatment with ZAHA (100 and 200 mg/kg) significantly increased SOD (2.38±0.28 U/mg protein,  $P<0.05$ ) compared to the stress group (2.71±0.20 U/mg protein,  $P<0.01$ ) (Figure 5c).

### Effect of *Zanthoxylum alatum* on mrna expression by real-time polymerase chain reaction

In the restraint group, significant upregulation of the hippocampal genes, i.e., GRP94 ( $P<0.001$ ), GRP78 ( $P<0.001$ ), CHOP ( $P<0.001$ ), and Caspase-12 mRNA ( $P<0.001$ ) compared to the vehicle control group were observed. The ZAHA treatment significantly down-regulated the expression of the GRP94, GRP78, CHOP, and Caspase-12 mRNA compared to the control group ( $P<0.001$ ,  $P<0.05$ ,  $P<0.001$ , and  $P<0.001$  for 100 and

**Table 1.** Oligonucleotide primer sequences for target genes used in real-time polymerase chain reaction

Gene of Interest	Primer Sequences
GRP94	Forward 5'- TGGGTCAAGCAGAAAGGAG-3' Reverse 5'- TCTCTGTTGCTTCCCGACTT-3'
GRP78	Forward 5'- GTTTGCTGAGGAAGACAAAAAGCTC-3' Reverse 5'- CACTTCCATAGAGTTTGCTGATAATTG-3'
CHOP	Forward 5'- GGAGCTGGAAGCCTGGTATGAGG-3' Reverse 5'- TCCCTGGTCAGGCGCTCGATTCC-3'
Caspase-12	Forward 5'- GAAGGAATCTGTGGGGTGAA-3' Reverse 5'- TCAGCAGTGGCTATCCCTTT-3'
β-actin	Forward 5'- AGCCATGTACGTAGCCATC-3' Reverse 5'- CTCTCAGCTGTGGTGGTGA-3'

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200 mg/kg of ZAHA), but ( $P < 0.01$ ) for 100 mg/kg of ZAHA in the CHOP gene expression (Figure 6a-d).

#### GRP94 gene expression

GRP94 gene expression in the restraint group was up-regulated significantly ( $P < 0.001$ ) compared to the normal control group. On the contrary, a significant down-regulation ( $P < 0.001$ ) in the expression of the gene was observed in the imipramine, ZAHA (200 mg/kg and 100 mg/kg) treated groups (Figure 6a).

#### GRP78 gene expression

The level of expression GRP78 was upregulated significantly ( $P < 0.001$ ) in the restraint group compared to the normal control group. On the other hand, a significant down-regulation of the gene was observed in the standard drug (imipramine) treated group ( $P < 0.001$ ), ZAHA (100 mg/kg) ( $P < 0.05$ ) and ZAHA (200 mg/kg) ( $P < 0.05$ ) compared to chronic restraint stress-induced group (Figure 6b).

#### CHOP gene Expression

CHOP gene expression in the restraint group also up-regulated significantly ( $P < 0.001$ ) compared to the normal control group. Conversely, a significant down-regulation

( $P < 0.001$ ) in the expression of the gene could be observed in the imipramine, ZAHA (200 mg/kg and 100 mg/kg) ( $P < 0.01$ ) treated groups compared to the restraint group (Figure 6c).

#### Caspase-12 gene expression

The level of expression of Caspase-12 was upregulated significantly ( $P < 0.001$ ) in the restraint group, compared to the normal control group, whereas a significant down-regulation of the gene could be observed in the imipramine treated group ( $P < 0.001$ ), ZAHA (100 mg/kg) ( $P < 0.001$ ) and ZAHA (200 mg/kg) ( $P < 0.001$ ) compared to the restraint group (Figure 6d).

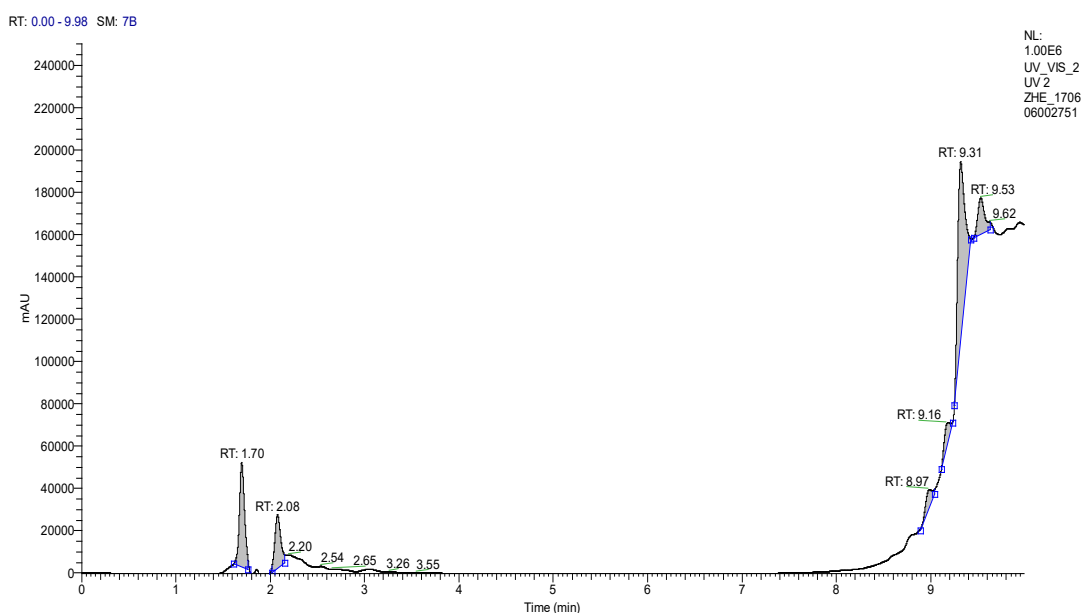
## 4. Discussion

In this study, we evaluated the effect of *Zanthoxylum alatum* on depression-like behavior induced by chronic restraint stress. We took imipramine as a reference drug to compare the effects of *Zanthoxylum alatum* because it has a strong antidepressant potential shown by previous studies (Han et al., 2011). The forced swim test is a physiological model for interpreting chronic to restraint stress-induced depressive-like behavior. In the present study, the forced swim test results showed increased immobility time with chronic restraint stress, which reflects

**Table 2.** Peak identification of *Z. alatum* hydroalcoholic extract by using liquid chromatography with tandem mass spectrometry

Row	Compounds	Retention Time (RT)	Empirical Formula	Calculated m/z	Observed m/z [M+H] <sup>+</sup>	Observed m/z [M-H] <sup>+</sup>
1	Hesperidin	1.70	C28H34O15	610.565	-----	609.15472
2	Magnoflorine	2.08	C20H24NO4	342.415	-----	341.09201
3	Melicopine	9.31	C17H15NO5	313.309	314.19891	-----
4	Sesamin	9.53	C20H18O6	354.358	-----	353.09232

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**Figure 1.** Ultra-high performance liquid-chromatography chromatogram at 365 nm of *Z. alatum* extract

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behavioral despair and depressive-like behavior in mice. Since the reduction in immobility time is considered beneficial in assessing antidepressant agents, imipramine, a tricyclic antidepressant and conventional drug for depression, was found to be effective in reducing the immobility time. ZAHA also showed a similar effect but with less potency than imipramine.

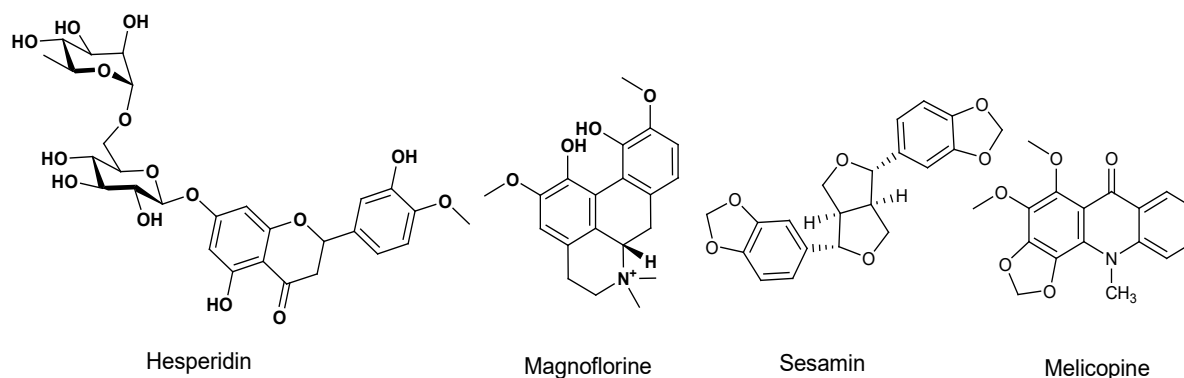
Loss of body weight in the chronic restraint group was also observed compared to the normal control group, which might be due to reluctance in eating due to restraint stress for 28 days continuously. Imipramine and ZAHA (slightly) ameliorated the loss in body weight.

Antioxidant enzymes can stabilize or deactivate free radicals and inhibit oxidative damage. This phenomenon is depicted by reduced SOD and GSH levels in the hippocampus, one of the possible mechanisms in depression

pathophysiology (Ahmad et al., 1993). These free radicals react with membrane lipids rich in polyunsaturated fatty acid and form lipid peroxidation, generating Malondialdehyde (MDA) that causes cell membrane damage.

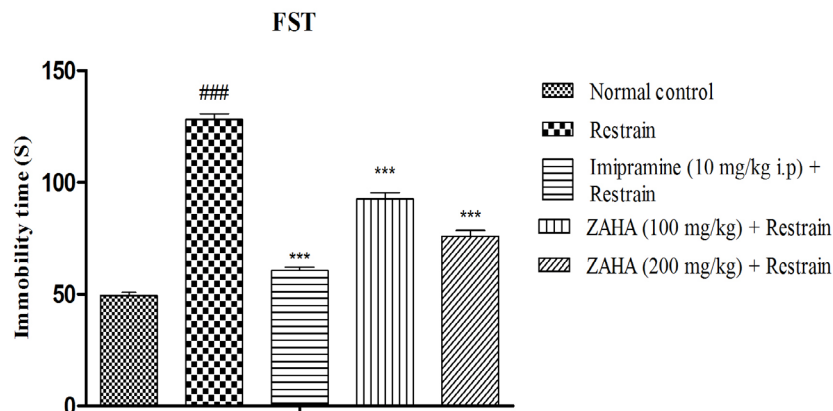
In our study, we found that imipramine and ZAHA significantly reduced oxidative stress and malondialdehyde formation. The results are very promising in this model, and the test compound showed potential in counteracting chronic stress, and the resultant depressive behavior is also taken care of. This effect is due to their diverse phytochemicals, which were identified in our study.

According to previous studies, up-regulation of ER stress-related genes in depression could compensate for the harmful effects of prolonged stress, glucocorticoid release from vulnerable brain regions such as the hippocampus, and so on (Bown et al., 2000; Ishisaka et al.,



**Figure 2.** Chemical structure of the isolated compounds from *Z. alatum* extract

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**Figure 3.** Effect of Hydroalcoholic Extract of *Z. alatum* (ZAHA 100 & 200 mg/kg) and imipramine on immobility time revealed by forced swim test in mice in ER stress

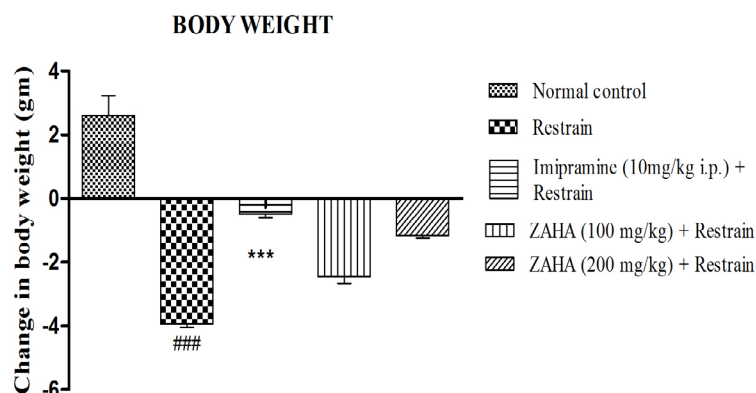
Values are presented as Mean±SEM. Six animals are in each group. ###P<0.001 compared to normal control; \*\*\*P<0.001 compared to the chronic restraint group.

2011; Barua et al., 2018). Studies also show ER stress is responsible for memory impairment under different pathophysiological situations (Zhang et al., 2014; Jangra et al., 2016; Barua et al., 2018).

The phytochemical study reveals hydroalcoholic extract contains alkaloids, glycoside, triterpenes, tannic acid, etc. Its chemical analysis was reported by various other researchers (Akhtar et al., 2009; Ranawat et al., 2010). We identified magnoflorine, melicopine, sesamin, and hesperidin in our study. Hesperidin possesses antioxidant, anti-inflammatory, neuroprotective effects, and anti-carcinogenic activities (Cho, 2006; Roohbakhsh et al., 2015). Magnoflorine has cytotoxic, antiviral (Mo-

hamed et al., 2010), antioxidant (Li, & Wang, 2014), and anti-amnesic properties (Koch et al., 2017). Melicopine is an acridone alkaloid with cytotoxic and antimalarial activity (Wang et al., 2014). Sesamin has antinociceptive, anti-inflammatory (Monteiro et al., 2014), and anti-chronic stress activity (Zhao et al., 2016).

CHOP, GRP78, GRP94, and Caspase12, the standard indicators for stress, were upregulated following ER stress in the experimental animals. However, following pretreatment with ZAHA, the above genes were down-regulated, indicating that they counteracted ER stress. The C/EBP homologous protein (CHOP) is a transcription factor that activates at different levels

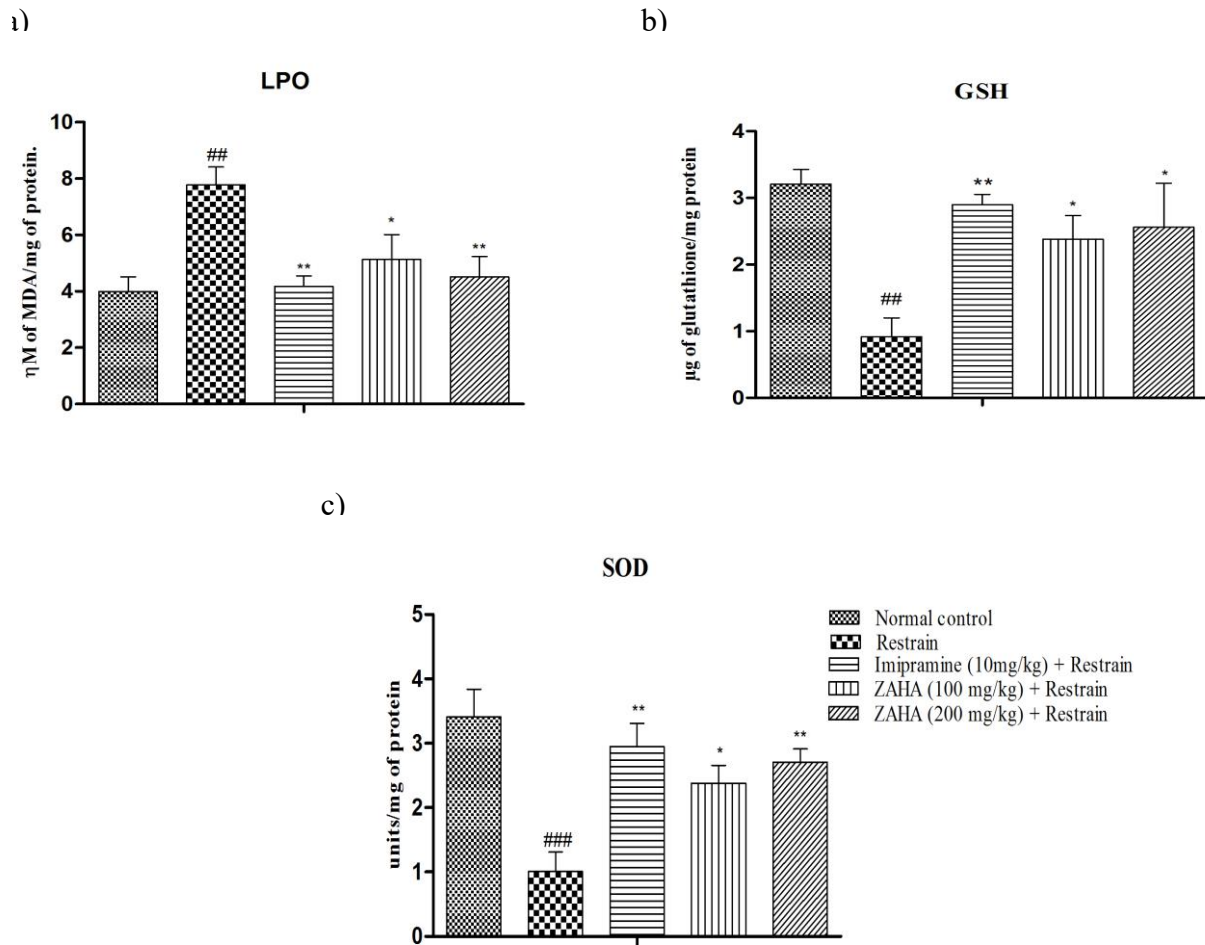


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**Figure 4.** Effect of hydroalcoholic extract of *Z. alatum* (ZAHA 100 & 200 mg/kg) and imipramine (10 mg/kg, IP) on the body weight of mice in ER stress

Values are presented as Mean±SEM. Six animals are in each group; ### P<0.001 compared to the normal control; \*\*\*P<0.001 compared to the chronic restraint group.





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**Figure 5.** Effect of ZAHA (100 & 200 mg/kg) and Imipramine (10mg/kg, IP) on LPO, GSH, and SOD levels in the chronic restraint group

Values are presented as the Mean±SEM. Six animals are in each group. ##P<0.01 and ###P<0.001 compared to the normal control. \*P<0.05 and \*\*P<0.01 compared to the chronic restraint group.

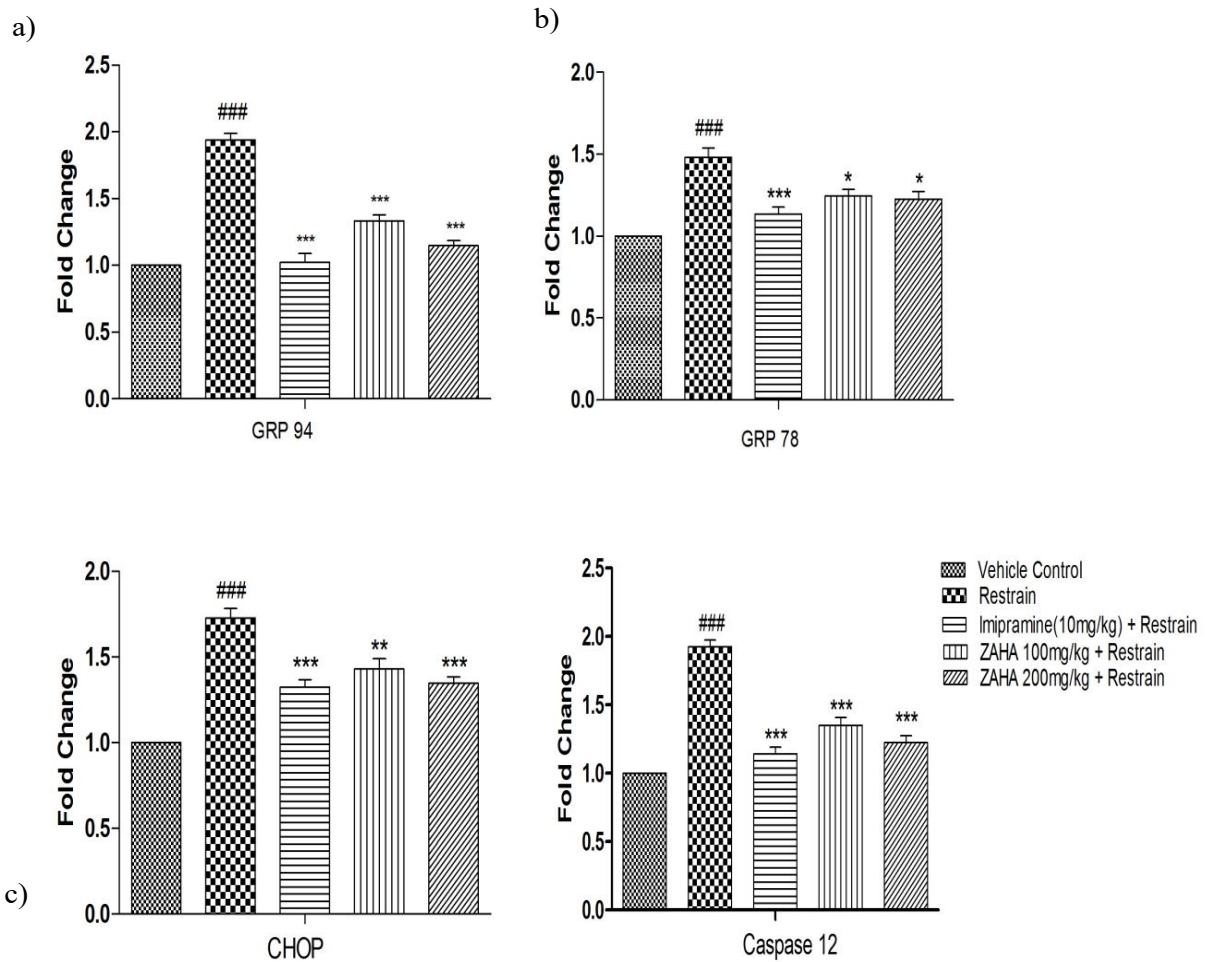
during ER. It is activated by the p38 kinase (Wang, & Ron, 1996). Deregulated CHOP movement compromises cell viability (Zhan et al., 1994), and cells without CHOP are essentially shielded from the deadly outcomes of ER stress (Oyadomari et al., 2002). This condition was observed in our study.

GRP78 is a key regulator of the ER stress response. Overexpression of GRP78 inhibits the up-regulation of CHOP, which induces apoptosis. ZAHA treatment downregulated the expression of this deleterious gene and inhibited apoptosis.

Caspase-12 shows resistance to ER stress-mediated apoptosis (Nakagawa et al., 2000; Rao et al., 2001). Activated caspase-12 can activate caspase-9, which cleaves procaspase-3. Activated procaspase-3 would lead to the

apoptosis of cells (Yingying et al., 2008). In our study, expression of Caspase-12 was also down-regulated following treatment with imipramine and ZAHA.

Glucose-regulated protein 94 is the HSP90-like protein in the lumen of the endoplasmic reticulum. This protein functions in the development and physiology of organisms as antigen-presenting cells, producing pro-inflammatory cytokines and priming the adaptive immune response (Yang et al., 2007). Thus, the amplified expression of GRP94 indicates ER-stressed cell priming for inflammatory interactions was significant in our study in the restraint group. Davide et al. (2010) reported that many recent genetic, biochemical, and cell biological studies have shed light on the functions of GRP94.



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**Figure 6.** Quantitative expression

A: GRP94, B: GRP78, C: CHOP, and D: Caspase-12 Genes by Real-Time PCR in different treatment groups viz. the normal control, restraint, imipramine, ZAHA (100 mg/kg, PO), ZAHA (200 mg/kg, PO)

Values are expressed as fold change represented as Mean $\pm$ SEM (n=3). ###P<0.001 compared to the control; \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 compared to the restrain group.

Our investigation revealed that ZAHA could restrain GRP94 and Caspase-12 expression by recovering ER stress. Treatment with methanolic extract of *Entada phaseoloides* seeds weakened ER stress in chronic stress in mice (Barua et al. 2018). Jangra et al. (2016) reported that Honokiol degenerates ER stress by down-regulating GRP78 and CHOP expressions. Barua et al. (2017) reported that *Elsholtzia communis* counteracts stress by modulating the expression of hsp14, CHOP, Nrf2, Caspase-3, and brain-derived neurotrophic factor in rat hippocampus.

## 5. Conclusion

The seed extracts of *Z. alatum* have many customary medicinal properties, including their use as nerve tonic and stimulant in debilitated patients. Its positive effect has been shown in reverting ER stress in our study. It thwarts the restraint of stress-induced depressive-like behavior in mice under its antioxidant property, down-regulating the stress markers like GRP78, GRP90, CHOP, and Caspase-12 due to an assortment of phyto-constituents present therein.

## Ethical Considerations

### Compliance with ethical guidelines

Mice were used according to the NIH Guide for the Care and Use of Laboratory Animals, and the experiments were performed following approval of the protocol by the Ethics Committee of the College of Veterinary Sciences, Assam Agricultural University (No.770/ac/CPCSEA/FVSc, AAU/IAEC/15-16/367) to minimize the suffering.

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### Authors' contributions

All authors equally contributed to preparing this article.

### Conflict of interest

The authors declared no conflict of interest.

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