Role of *Elsholtzia communis* in counteracting stress by modulating expression of *hspa14*, C/EBP homologous protein, nuclear factor (erythroid-derived 2)-like-2 factor, Caspase-3, and brain-derived neurotrophic factor in rat hippocampus

Chandana Choudhury Barua, Pompy Patowary, Arundhati Purkayastha, Prakash Haloi, Manab Jyoti Bordoloi

**Abstract:**

**OBJECTIVE:** *Elsholtzia communis* (Collett and Hemsl.) Diels has been widely distributed and is reported for many therapeutic effects. The present study aims to investigate the antistress activity of the leaf extract and its possible molecular mechanism.

**MATERIALS AND METHODS:** Hydroethanolic extract of leaves of *E. communis* (100 and 200 mg/kg, p.o.) were administered for 7 days to stress-induced male Wistar rats. The experimental animals were divided into five groups (*n* = 6). The mRNA/protein profile of few stress responsive chaperones (*hspa14*), endoplasmic reticulum stress markers (C/EBP homologous protein [*CHOP*]), antioxidant regulating genes (nuclear factor (erythroid-derived 2)-like-2 factor [*Nrf2*]), apoptotic factors (Caspase-3) in rat hippocampus were studied by polymerase chain reaction and immunoblotting.

**RESULTS:** The stress-related genes such as *hspa14*, *CHOP*, antioxidant gene *Nrf2*, apoptotic gene Caspase-3 which were overexpressed in the stress control group were significantly suppressed following administration of the extract at both the doses and the standard drug Ginseng. Likewise, brain-derived neurotrophic factor which is closely related with stress, was downregulated in the stress control group, was found to be upregulated following treatment with the extract and the standard drug Ginseng.

**CONCLUSION:** Our findings clearly indicate that *E. communis* was able to counteract stress. Hence, it has the potential to develop as adaptogen and also as a replacement/substitute of the popularly used drug, Ginseng or Ashwagandha, which is on the verge of extinction or becoming endemic due to overuse.

**Keywords:** Antioxidant, apoptosis, endoplasmic reticulum stress, heat shock protein

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**Introduction**

Stress generally involves disruption of homeostasis, and both the peripheral sympathoadrenomedullary and central monoaminergic systems are triggered by various psychosocial and physical stressors.[1] It triggers a number of molecular pathways which involves a number of...
key proteins whose upregulation and downregulation regulate the entire mechanism. Heat shock proteins (hsp) are a family of proteins that maintain cellular homeostasis during environmental stress (oxidative, chemical, and pathogenic stresses). Its expression is not often detected in cells under normal growth conditions but is induced by physiological and environmental stress and is involved in cell protection and tissue repair.

Endoplasmic reticulum (ER) under stress expresses a few ER markers triggering apoptosis to protect the organism by removal of damaged cells. One of the prime markers of the ER stress-mediated apoptosis pathway is C/EBP homologous protein (CHOP), a major protein which participates in ER stress-mediated apoptosis. CHOP also triggers apoptosis by the activation of the Bcl-2 family of proteins, ultimately activating the apoptotic protein Caspase-3, catalyzing the specific cleavage of many key cellular proteins. It is essential for normal brain development through death stimulus. Caspase-3 is also required for some typical hallmarks of apoptosis.

Oxidative stress occurs when there is an imbalance between reactive oxygen species (ROS) production and antioxidant defense system. Thus, to normalize the condition, nuclear factor (erythroid-derived 2)-like-2 factor (Nrf2), a transcription factor which functions as the key controller of the redox homeostatic gene regulatory network, comes into play. Under oxidative and electrophilic stresses, the Nrf2 signaling pathway is activated to augment the expression of a multitude of antioxidant and phase II enzymes that restore redox homeostasis.

Brain-derived neurotrophic factor (BDNF), a member of neurotrophin family, is expressed at the highest levels in the hippocampus and cerebral cortex. BDNF is one of the key contributors in the development, survival, maintenance, and plasticity of central nervous system neurons. Therefore, to study the changes in BDNF, following different stresses and subsequently the effect of the plant extract on the expression of BDNF protein, we studied this protein to derive the neuroprotective effect of our plant extract against stress.

**Materials and Methods**

**Collection of plant material**
The fresh leaves of the plants were collected, and herbaria (Barcode no. CAL000027003) of the plant was prepared by Dr. I. C. Barua, Taxonomist and Principal Scientist, Department of Agronomy, Assam Agricultural University, Jorhat, Assam, and authenticated from Central National Herbarium, BSI, Howrah, West Bengal.

**Preparation of extract**
Powdered leaves of *E. communis* (250 g) were soaked in 1000 ml ethanol and water in the ratio 70:30 for 72 h in a beaker, and mixture was stirred every 18 h using a sterile glass rod. Filtrate obtained was subjected to rotary evaporator (Buchi R-210, BUCHI Labortechnik AG, Switzerland) to remove the solvent. The recovery percentage was found to be 19.71% w/v.

**Isolation of Compounds from Elsholtzia communis**
Dried leaves of *E. communis* were powdered and were macerated with aqueous ethanol (1:1). After 24 h, the filtrate of the extracts was evaporated to dryness under reduced pressure at 50°C (Rotary evaporator). The process was repeated for 4 times until the filtrate becomes colorless, which was designated as hydro-ethanol extract of *E. communis*. The extract was fractionated with hexane followed by chloroform for 3 consecutive times. Hexane, chloroform, and remaining fractions were evaporated to dryness.

Chloroform fraction of this hydro-ethanol extract (30 g) was chromatographed over a column of 600 g of silica gel (60–120 mesh) starting with the solvent system hexane, and 200 ml fractions were collected in the following order: Fr. 1–3 (hexane), Fr. 4–14 (1:10, ethyl acetate: hexane), Fr. 15–25 (1:7, ethyl acetate: hexane), Fr. 26–35 (1:5, ethyl acetate: hexane), Fr. 36–45 (1:3, ethyl acetate: hexane), Fr. 46–53 (1:1, ethyl acetate: hexane), Fr. 54–61 (3:1, ethyl acetate: hexane), Fr. 62–73 (5:1, ethyl acetate: hexane), Fr. 74–84 (7:1, ethyl acetate: hexane), Fr. 85–92 (10:1, ethyl acetate: hexane), Fr. 93–98 (ethyl acetate), and Fr. 99–110 (ethanol). The collected fractions were analyzed by thin-layer chromatography (TLC) in different solvent systems. Similar fractions were combined and evaporated under reduced pressure at 45°C using rotavapor.
Fractions 12–40 were combined and further fractionated using column chromatography over a column of 300 g of silica gel (60–120 mesh) starting with the solvent system hexane, and 200 ml of fractions were collected in the following order: Fr. 1–5 (1:2, ethyl acetate: hexane), Fr. 6–9 (1:1, ethyl acetate: hexane), Fr. 10–13 (2:1, ethyl acetate: hexane), Fr. 14–18 (3:1, ethyl acetate: hexane), Fr. 20–23 (4:1, ethyl acetate: hexane), Fr. 24–29 (5:1, ethyl acetate: hexane), Fr. 30–32 (6:1, ethyl acetate: hexane), and Fr. 37–46 (7:1, ethyl acetate: hexane). The collected fractions were analyzed by TLC in different solvent systems. Similar fractions were combined and evaporated under reduced pressure at 45°C using rotavapor.

Drug and treatment schedule
Ginseng (100 mg/kg, orally), the standard drug was obtained from Sigma-Aldrich (St. Louis, MO, USA). All compounds were dissolved in distilled water and Tween 80 (0.1%) solution. Drugs were prepared fresh daily before administration.

Animals
Adult male Wistar rats (170–200 g) were allowed for acclimatization to the condition overnight. Rats were housed 3–4 per cage at a constant temperature (22°C ± 2°C) and 12:12 h light/dark cycle, fed standard laboratory food, water was given ad libitum. All experiments were performed according to current guidelines for the care of laboratory animals by IAEC (Approval No. 770/ac/CPCSEA/FVSc, AAU/IAEC/10-11/72); effort was made to minimize suffering of the experimental animals throughout the study.

Experimental methods
The rats were divided into five groups (n = 6), namely, nonstress group, stress group, standard (Ginseng) drug-treated groups for 7 days. Based on the acute toxicity studies as per OECD Guidelines 423, two doses of E. communis were selected, namely, 100 and 200 mg/kg (EC 100 and EC 200). The drugs were administrated daily 45 min before stress regimen, for 7 consecutive days. Water immersion stress, the standard model for inducing stress was used. On the 8th day, the animals were anesthetized and humanely sacrificed; hippocampus was skillfully isolated from the brain for polymerase chain reaction (PCR) and immunoblotting study.

Detection of stress-induced genes by reverse transcriptase polymerase chain reaction
Expression of hspa14, CHOP, and Nrf2 genes was performed using PCR technique. The PCR reaction mixture contained a total reaction volume of 25 μl composed of 12.5 μl 2X PCR Master Mix (Fermentas), 0.5 μl of each primer, 1 μl template, and 10 μl nuclease-free water. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control for the experiment with a concentration of 10 pmol. Amplification was carried out in a thermal cycler (Applied Biosystems Veriti Thermal cycler). PCR condition included pre-denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 sec, variable annealing for different genes, namely, 54°C, 56°C, 60°C, and 66°C for hspa14, GAPDH, Nrf2, and CHOP, respectively 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 TAE. The gel picture was then analyzed by Image Lab software to access the relative quantity of the bands.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis - and immunoblot analysis
Hippocampal tissue samples (30 mg approximately) were homogenized in 5 ml of chilled lysis buffer (RIPA Buffer, Amresco, USA) and were centrifuged at 23000 ×g for 20 min at 4°C. The protein concentration of the supernatants was quantified by Bradford reagent (Himedia) with Bovine serum albumin (BSA) as the standard. Fifty micrograms of total protein was loaded and separated in 10% polyacrylamide gels containing sodium dodecyl sulfate using the Hoefer Midi Gel apparatus (Harvard Apparatus, Holliston, MA, USA).[9] Gels were electrophoresed at 150 V, and the fractionated proteins were visualized by Coomassie Blue staining or transferred to nitrocellulose membrane using semi-dry blotting apparatus (Hoefer).[10] The membranes were then blocked using 10 ml of cold blocking buffer containing 3% BSA in tris buffer saline with Tween 20 (TBST) for 1 h and were incubated overnight (4°C) with 5 ml of 1% BSA in TBST containing antiserum mouse rabbit polyclonal IgG (Santa Cruz Biotechnology, Inc.) against BDNF and Caspase-3 in 1:500 dilution. After overnight incubation, blots were washed 4 times (5 min each) with 10 ml of TBST. The blots were then reacted with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc.) for 1 h. After rinsing with cold TBST, the color reaction on the nitrocellulose membrane was obtained using commercially available Ultra 3,3′,5,5′-tetramethylbenzidine-Blotting Buffer (Sigma).

Statistical analysis
Results are presented as mean ± standard error of the mean. Statistical analysis was performed by one-way
Results

Isolation of compounds from Elsholtzia communis
Combined fractions of 1-9 of E. communis/chloroform fraction/fractions 12-40 were subjected to preparative TLC using the solvent system ethyl acetate: hexane (1:2). A pure compound was isolated, and 1H NMR, 13C NMR, IR and MS spectra were recorded for this pure compound. The study of these spectroscopic data allowed to identify the compound as structure [Figure 1].

Stress regulating genes
The underlying molecular mechanisms were attempted to study, since stress is a manifestation of multiple external factors and in addition to biochemical alterations, various genes are either upregulated or downregulated. Biochemical alterations such as glucose, triglycerides, and cholesterol were estimated simultaneously.[11] We also explored few of the gene expression study which is stress responsive. Consecutive administration of E. communis altered the profile of stress responsive chaperones (hspa14), ER stress markers (CHOP), oxidative stress responsive gene regulating antioxidant (Nrf2), neuronal factors (BDNF) and apoptotic factors (Caspase-3). GAPDH and β-actin were taken as internal controls. Real time-PCR and western blotting were selectively performed to access the expression pattern of the candidate genes involved in various pathways of stress responsive mechanism.

Stress responsive chaperones (hspa14)
Hspa14 were upregulated in stress group as compared to nonstress group, whereas, in Ginseng, EC 200 and EC 100 treated group significant (P < 0.001) downregulation was observed as compared to the stress group [Figure 2].

Endoplasmic reticulum stress markers (C/EBP homologous protein)
A significant suppression in the expression of CHOP mRNA in Ginseng-treated group (P < 0.001) followed by EC 200 (P < 0.001), and EC 100 (P < 0.01) treated groups was observed when compared to stress group in rat hippocampus. Even though the expression of CHOP did not differ significantly (P < 0.001) in the stress group as compared to nonstress group, an increasing trend could be observed [Figure 2].

Oxidative stress responsive gene regulating antioxidant (nuclear factor (erythroid-derived 2)-like-2 factor)
Nrf2 activity showed significant (P < 0.001) upregulation of its mRNA in the hippocampus of rats in the treated groups (Ginseng, EC 100, EC 200). The stress group showed significant (P < 0.001) downregulation of Nrf2 gene on comparison to nonstress group [Figure 2].

Neuronal factor (Brain-derived neurotrophic factor)
There was upregulation of the expression of the neuronal factor (BDNF) in the hippocampus in Ginseng-treated group (P < 0.001) followed by EC 200 (P < 0.001) as compared to the stress group [Figure 3].
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Apoptotic factors (Caspase-3)
Caspase-3 expression in the hippocampus of rats was significantly ($P < 0.001$) declined in Ginseng-treated group followed by EC 200 and EC 100 as compared with the stress group [Figure 3].

Discussion

Triterpenes, which include phytosterols and phytoecdysteroids, are thought to encompass adaptogenic role in mammals and in humans.\[12\] The hydroethanolic extract of $E.\ communis$ showed the presence of triterpenes, glycosides, and steroids in our phytochemical studies.

Water immersion stress is extensively used for induction of stress in neurological studies.\[13\] We also delved into the underlying molecular mechanism affecting stress in animals, and few important genes were taken into consideration. Hsp70 is a well-known member that protects cells and tissues from different pathological conditions; it is reported that stress can induce hsp expression.\[14\] $E.\ communis$ showed downregulation of hspa14. Similar report by Chowdhuri et al.\[15\] also showed upregulation of hspa14 in stressed animals.

$CHOP$ is a pro-apoptotic ER stress protein, a key pathologic event in neurological disease processes and neuronal cell death.\[16\] The stressed animal showed upregulation of $CHOP$ and expression of ER stress responsive markers, ultimately triggering the apoptotic

![Figure 2: Effect of Elsholtzia communis on mRNA expression of C/EBP homologous protein, nuclear factor (erythroid-derived 2)-like-2 factor, hspa14, in hippocampus of control and experimental group of rat. (a) Lane 1: Ladder, Lane 2-6: Nonstress, Stress, Standard, EC 100, EC 200. (b) Quantitative expressing mRNA level was assessed using ImageJ software and is expressed as percentage fold change when compared with the internal control (Glyceraldehyde 3-phosphate dehydrogenase). Results are represented as mean ± standard deviation ($n = 3$). Statistical significance was determined by one-way analysis of variance followed by Tukey Post hoc Test. Where ***$P < 0.001$ compared with control group. ###$P < 0.001$ compared with stress group.]

![Figure 3: Effect of Elsholtzia communis on protein expression of brain-derived neurotrophic factor and Caspase-3 in hippocampus of control and experimental group of rat. (a) Lane 1-5: Non Stress, Stress, Ginseng, EC 100, EC 200. (b) Quantitative expressing the protein level was assessed using ImageJ software and is expressed as percentage fold change when compared with the internal control (β-actin). Results are represented as mean ± standard deviation ($n = 3$). Statistical significance was determined by one-way analysis of variance followed by Tukey post hoc Test. Where ***$P < 0.001$ compared with control group, ###$P < 0.001$ compared with chronic stress group.]

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pathway. Hydroethanolic extract of *E. communis* induced downregulation of the *CHOP* mRNA, accordingly combating the ER stress response.

Apoptosis in excess is related to cellular degeneration by oxidative stress, frequently associated with aging and pathogenesis of neurodegenerative conditions.[16] Expression of Caspase-3 is a frequently activated death protease catalyzing the specific cleavage of many key cellular proteins. Significant downregulation of Caspase-3 in *E. communis*-treated group indicates that the extract might impart positive effect in counteracting stress.

When oxidative stress occurs, there is an imbalance between ROS production and antioxidant defense system. To normalize the condition, *Nrf2*, which functions as the key controller of the redox homeostatic gene regulatory network, comes into play. The stress group showed upregulation of the *Nrf2* mRNA, triggering the expression of antioxidant genes which facilitate to combat the oxidative stress. *E. communis*-treated group showed downregulation in the expression of *Nrf2*, possibly due to its antioxidant property. The plant possesses *in vitro* antioxidant activity,[17] hence the plant's antistress property might be imparted due to the phytoconstituents as well as antioxidant activity.

Stress and glucocorticoids are reported to decrease the expression of BDNF in the hippocampus and dentate gyrus.[8,18] Decline in the expression of BDNF during stress leads to loss of normal plasticity, damage and leads to the loss of neurons. *E. communis* in both doses (100 and 200 mg/kg) showed a significant increase in the expression of BDNF thus confirming its antistress activity. The cytoprotective and antioxidant property of *E. communis* may be responsible for the neuroprotection against cell death and the deleterious effects of stress.

This study, for the first time, confirms that leaves of *E. communis* may conceivably be considered as potential adaptogen. There are many species of *Elsholtzia*, most of them have diverse medicinal properties and few of which have already been exploited,[19] but from our present study, a new activity has been unveiled, based on the molecular study in stress-induced mechanism and thus hypothesize its antistress activity. The activity could be attributable to its antioxidant property and various phytoconstituents present therein, mainly terpenoids and flavonoids. Due to its immense popularity for other uses, this novel activity may certainly attract scientists. Isolation and identification of a pure molecule are underway, which can be used as a single molecule for drug development such as Ginseng or Ashwagandha.

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Conflicts of interest
There are no conflicts of interest.

References
1. Fuchs E, Flügge G. Chronic social stress: Effects on limbic brain structures. Physiol Behav 2003;79:417-27.
2. Lanneau D, Brunet M, Frisan E, Solary E, Fontenay M, Garrido C. Heat shock proteins: Essential proteins for apoptosis regulation. J Cell Mol Med 2008;12:743-61.
3. Oyadomari S, Mori M. Roles of CHOP/GADD153 in endoplasmic reticulum stress. Cell Death Differ 2004;11:381-9.
4. Fulda S, Gorman AM, Hori O, Samali A. Cellular stress responses: Cell survival and cell death. Int J Cell Biol 2010;2010:214074.
5. Szegedi E, Logue SE, Gorman AM, Samali A. Mediators of endoplasmic reticulum stress-induced apoptosis. EMBO Rep 2006;7:880-5.
6. Leong XF, Rais Mustafa M, Jaarin K. Nigella sativa and its protective role in oxidative stress and hypertension. Evid Based Complement Alternat Med 2013;2013:120732.
7. Birben E, Sahiner UM, Sakesken C, Erzurum S, Kalayci O. Oxidative stress and antioxidant defense. World Allergy Organ J 2012;5:9-19.
8. Licinio J, Wong M. Brain-derived neurotrophic factor (BDNF) in stress and affective disorders. Mol Psychiatry 2002;7:519.
9. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press; 1989.
10. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci U S A 1979;76:4350-4.
11. Barua CC, Patowary P, Bordoloi MJ, Purkayastha A, Barua IC, Dutta P, et al. Attenuating effect of *Elsholtzia communis* (Coll and Hrst) Diles on dysregulated HPA in stress condition. Int J Pharm 2016;8:651-8.
12. Oberdörster E, Clay MA, Cottam DM, Wilmot FA, Mclachlan JA, Milner MJ. Common phytochemicals are ecdy steroid agonists and antagonists: A possible evolutionary link between vertebrate and invertebrate steroid hormones. J Steroid Biochem Mol Biol 2001;77:229-38.
13. Salman H, Bergman M, Weizman A, Bessler H, Weiss J, Straussberg R, et al. Effect of diazepam on the immune response of rats exposed to acute and chronic swim stress. Biomed Pharmacother 2000;54:311-5.
14. Soti C, Nagy E, Giricz Z, Vígh L, Csermely P, Ferdinandy P. Heat shock proteins as emerging therapeutic targets. Br J Pharmacol 2005;146:769-80.
15. Chowdhuri DK, Parmar D, Kakkar P, Shukla R, Seth PK, Srimal RC. Antistress effects of bacosides of *Bacopa monnieri*: Modulation of Hsp70 expression, superoxide dismutase and cytochrome P450 activity in rat brain. Phytother Res 2002;16:639-45.
16. Chandra J, Samali A, Orrenius S. Triggering and modulation of
17. Barua CC, Roy JD, Pathak DC, Bordoloi M, Khta K. Pytochemical composition and in vitro anti-oxidant property of Elsholtzia communis. In: Gupta VK. editors. Bioactive Phytochemicals: Perspectives for Modern Medicine. Vol. 2. New Delhi: Daya Publishing House; 2014. p. 411-20.

18. Smith MA, Makino S, Kvetnansky R, Post RM. Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. J Neurosci 1995;15 (3 Pt 1):1768-77.

19. Harley RM, Atkins S, Budantsev AL, Cantino PD, Conn BJ, Grayer R, et al. Labiatae. In: Kubitzki K, Kadereit JW, editors. The Families and Genera of Vascular Plants. Vol 7. Berlin: Springer; 2004. p. 167-275.