

Estimation of Lipid Peroxidation levels in Rat Brain During Stress Due to Withdrawal from Chronic Ethanol Consumption under the Influence of Ginger (*Zingiber officinale Roscoe*)

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Abstract: Withdrawal from long term ethanol intake resulted in elevated levels of malondialdehyde (MDA), an index of free radical generation and lipid peroxidation in brain. Peroxidation indices serve as indicative markers for the oxidative stress within the cell. The process of lipid peroxidation has been proposed to be especially damaging because it is self propagating and because brain is relatively enriched in polyunsaturated fatty acids, the substrates for lipid peroxidation. This study is undertaken to evaluate the protection of ginger against stress induced due to withdrawal from ethanol resulting in oxidant production, especially in the brain which is never been documented. From the studies on the changes in lipid levels and peroxidation indices it has been observed and concluded that ginger has a significant outcome in reducing oxidative stress related to withdrawal. This has wide implications as withdrawal stress damages neural biomolecules-DNA (nucleolar and mitochondrial), RNA and protein. This is important because of the considerable evidence that formation of oxidants, damaging cellular molecules such as DNA, is a major contributor to aging and other degenerative diseases of aging such as brain dysfunction and neurodegeneration.

Key words: Oxidative Stress • Lipid Peroxidation • Brain • Ethanol Withdrawal • Ginger Extract

INTRODUCTION

The fact that withdrawal from ethanol administration resulted in oxidative stress much worse than ethanol intake has been well documented. Also, oxidative stress is more intense during EW than during antecedent ethanol exposure [1-3]. Oxygen radicals can attack proteins, lipid membranes and nucleic acids, thereby disrupting cellular functions and integrity. Brain is the target for different stressors because of its high sensitivity to stress induced degenerative conditions. Oxidative damage to proteins and lipids can ultimately lead to outcomes such as disorganization, dysfunction and destruction of membranes, enzymes and proteins [4-6] of the neural tissue. EW consistently produced higher $O_2^{\bullet}$ and protein carbonyl contents than ethanol exposure. Rats experiencing EW suffer acute distress due to an abrupt transition from ethanol-induced suppression of glutamate-mediated excitation to withdrawal-induced neuronal hyperexcitability [7, 8]. Several studies reported

that EW caused oxidative stress in brain areas vulnerable to ethanol, such as cerebellum and cortex [9, 10]. The pro-oxidant nature of ethanol intoxication-withdrawal also has been demonstrated in a clinical study where cerebrospinal fluid of withdrawn alcoholics contained higher concentrations of $O_2^{\bullet}$ and excitatory amino acids than control subjects. Excess ROS cause neurological damage [11].

Brain has a high amount of polyunsaturated fatty acids (PUFAs) and high content of free ions. Specifically, peroxidation of membrane lipids may cause impairment of membrane function, decreased fluidity, inactivation of membrane-bound receptors and enzymes, increased permeability to ions and possibly eventual membrane rupture [12, 13]. Protein is one of the important biochemical components of the brain in vertebrates. The specific neuronal functions such as transmission are extensively mediated by proteins [14]. If the oxidative stress is particularly severe, it can produce cell death [6, 15]. Death can occur by necrosis, but in a number of

cell types, such as neuronal cells, a mild oxidative stress can trigger the process of apoptosis, activating the intrinsic suicide pathway present within cells [16, 17].

In this study, taken the well established antioxidant potential of ginger, a culinary herb with proven medicinal properties against a wide range of ailments, a comprehensive evaluation of the role of Ginger (*Zingiber officinale*, ZO) in curtail of alcohol withdrawal stress mediated oxidative product formation has been elucidated through detailed studies with respect to its *in vitro* antioxidant property.

MATERIAL AND METHODS

Procurement of Chemicals and Animals

Drugs and Chemicals: Standards like BHT, Ascorbic acid, Citric acid and BSA were purchased from Sigma Chemicals (St. Louis, MO, USA). All other chemicals used are of analytical grade unless otherwise mentioned.

Collection of Plant Material: Aqueous ginger extract is prepared from locally available ginger roots. Ginger rhizomes were purchased fresh from the local markets and authenticated by the staff, Department of Botany, Sri Venkateswara University, Tirupati, India.

Extract Preparation: Whole rhizome of ginger is thoroughly washed, sliced, grated and grind to fine paste. A weighed quantity (30g) of the paste is subjected to continuous hot extraction in soxhlet apparatus using double distilled water. The extract is evaporated under reduced pressure using rotary evaporator and then lyophilized to give an extract sample and stored at 4°C for further studies.

Animals: The study involved young (3-4 months old; 200-220g) male albino rats of wistar strain purchased from Sri Venkateswara Traders Pvt. Limited, Bangalore, maintained in the animal house of the department in polypropylene cages. Standard conditions of humidity (50± 9% relative humidity), room temperature (25-28°C) and 12 h light/ dark cycle (6:00 AM to 6:00 PM) were maintained. A standard rodent diet (M/s Hindustan Lever Ltd., Mumbai) and water were provided *ad libitum*. All experimental procedures were approved by the CPCSEA on Animal Care, Govt. of India, bearing the CPCSEA No. 438 / 01/a / CPCSEA / IAEC / SVU / KSR-1 (dt: 11.09.2008).

Treatment Protocol: After a two weeks adaptation period, animals were divided, six per group and grouping involved 3 batches, first batch includes control group given normal saline. Second batch involves two groups of rats given 20% Alcohol (p.o.) at a dose of 2gm/kg body weight and another group was set to receive alcohol at same dose along with ginger extract (200 mg/kg body weight) while third batch was also treated the same way as the second batch except that they are allowed for withdrawal. All the treatments were carried out for 6 weeks with the exception that withdrawal groups were allowed for 3 days of withdrawal from the drug after the last dose.

Tissue Collection: After the experimental period, the animals were sacrificed by cervical dislocation. The whole brain is removed, washed with ice-cold saline, blotted, dried in liquid nitrogen and immediately transferred to the ice chamber at -80°C. Cerebrum, Hippocampus region, Pons Medulla and Cerebellum were separated as described by Nayak and Chatterjee [18].

Biochemical Assays

Preparation of Synaptosomes: Synaptosomal fraction is prepared from the brain homogenate following the method of Hajos [19]. In brief the preparation is as follows, after sacrifice, the brain regions were dissected out on ice. The tissue is minced and homogenized gently in 10 volumes w/v of ice-cold 0.32M sucrose with a Teflon homogenizer. After centrifugation of the homogenate at 1000g for 10 min at 4°C, the supernatant is again centrifuged at 12,500g for 20 min. The pellet is finally resuspended in 10 volumes of 0.32M sucrose and used as the crude synaptosomal fraction.

In Vitro Antioxidant Studies

Reducing Power Assay: The reducing power is determined according to the method previously described by Oyaizu [20]. A higher absorbance of the reaction mixture indicated greater reducing power.

Fe²⁺ Chelation Capacity: The ferrous ion chelating activity is evaluated by a standard method of Dinis *et al.*, [21]. EDTA is used as a positive control.

FRAP Assay: Determination of ferric reducing/antioxidant power assay (FRAP) is carried out according to the method of Benzie and Strain [22].

Lipid Metabolism

Estimation of Lipid Profiles: Phospholipid levels are estimated according to Zilversmith and Davis [23]. The total cholesterol content and Triglycerides (TG) levels were estimated as described by Natelson [24].

Estimation of Lipid Peroxidation Indices: Lipid peroxidation (LPO) was determined by estimating MDA levels as described by Ohkawa *et al.*, [25]. Lipid Hydroperoxides (LOOH/ LHPs) were estimated according to the method of Jiang *et al.*, [26].

Data Analysis: Statistical analyses are carried out by one-way ANOVA with multiple comparison measurements used to assess the statistical significance of effects using the Statistical Package for Social Sciences (SPSS) software 12.0. Scheffe's *post hoc* analyses were used when appropriate.

RESULTS

In vitro antioxidant studies are given from figures a1-a3.

Reducing Power Assay: The reductive abilities of *Zingiber officinale* extract and the standard BHT are measured as the absorbance at 700nm plotted against concentration. Ginger extract has great reducing capabilities compared to BHT (Fig. a1).

The absorbance (A=700nm) was plotted against concentration of sample. Each value represents mean $\pm$ S.D. (n = 6).

Metal Chelating Activity/ Fe^{2+} Chelation Assay: Effects of ginger rhizome extract and standard Citric acid on ferrozine- Fe^{2+} complex formation. IC_{50} of extract = 49.58 ± 0.84 μ g/ml. The IC_{50} value of citric acid is 60.96 ± 0.05 μ g/ml (Fig.a2).

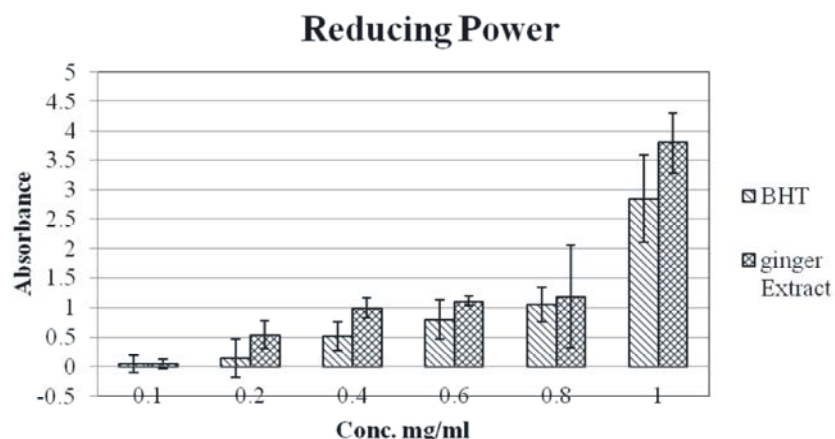


Fig a1: The Reducing power assay of *Zingiber officinale* extract and the standard BHT.

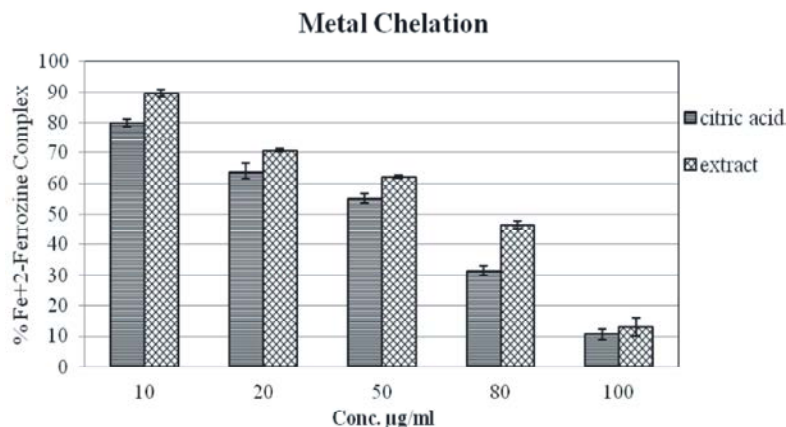


Fig a2: The Metal Chelating capabilities of *Zingiber officinale* extract and the standard Citric acid.

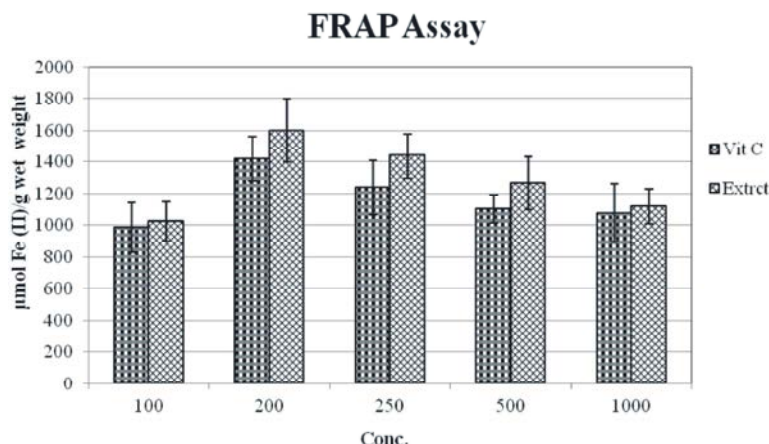


Fig a3: FRAP Assay of ginger extract in comparison with the standard Vit.C

The data is expressed as percentage inhibition of chromogen formation. The results are mean $\pm$ S.D. of six parallel measurements. IC_{50} of extract = 49.58 ± 0.84 μ g/ml. The IC_{50} value of the standard is 60.96 ± 0.05 μ g/ml.)

Ferric Reducing Antioxidant Potential assay/FRAP

Assay: The antioxidant potential value of the extract through FRAP Assay is found to be 670.23 μ g/ml, much higher than that of the standard 553.40 μ g/ml (Fig. a3).

The results are mean $\pm$ S.D. of six parallel measurements. The antioxidant potential value of the extract is 670.23 μ g/ml, was higher than that of the standard 553.40 μ g/ml.

The Changes in Lipid Profiles During Withdrawal Stress Can Be Summarized as Follows (Figs.b1-b3):

Phospholipid Content: The changes in the phospholipids content in different regions of rat brain during EW and pre-treatment with extracts of ginger is represented in Fig.b1.

The percent change of depletion in the phospholipids content during ethanol-induced withdrawal can be represented as:

PL: HC (-42.62)>CB (-40) > PM (-38.65) > CC (37.48)

The phospholipid content is increased in all the brain regions due to pre-treatment with the extract of ZO and withdrawal.

Total Cholesterol: The changes in the Total cholesterol content in different regions of rat brain during EW induced stress and pre-treatment with aqueous extract of ginger is represented in Fig.b2:

The percent change of depletion in the Total cholesterol content during withdrawal-induced stress due to cessation of ethanol can be represented as:

TC: HC (-50)>CB (-47.14)>PM (-37.48)>CC (-26.63)

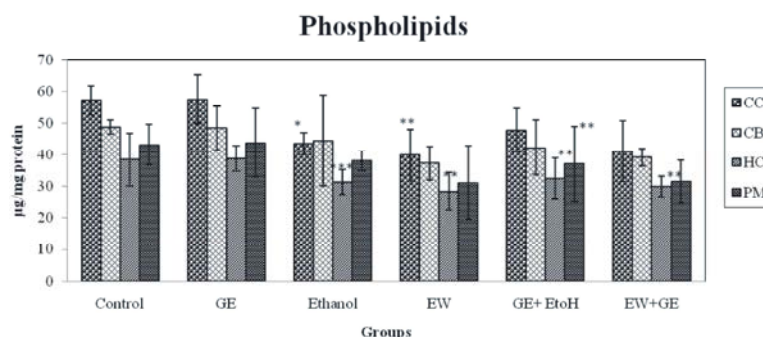


Fig b1: Phospholipids content in different regions of rat brain during EW and pre-treatment with extract of *Zingiber officinale*

All the values are mean, $\pm$ SD of six individual observations

*Significant at $P < 0.001$, ** significant at $P < 0.01$, ***significant at $P < 0.05$ in comparison with the control.

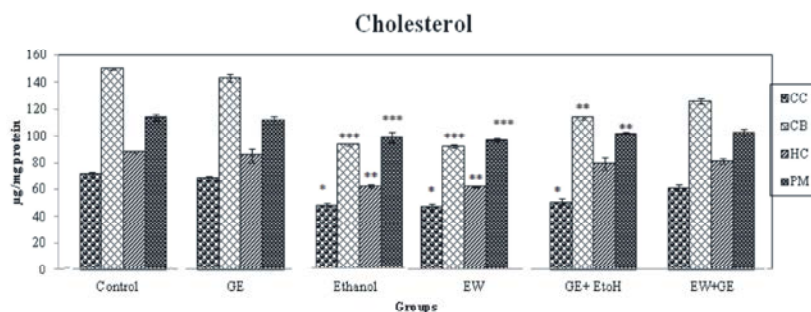


Fig b2: The changes in the Total cholesterol content in different regions of rat brain during EW induced stress and pre-treatment with aqueous extract of *Zingiber officinale*

All the values are mean, $\pm$ SD of six individual observations

*Significant at $P < 0.001$, ** significant at $P < 0.01$, ***significant at $P < 0.05$ in comparison with the control.

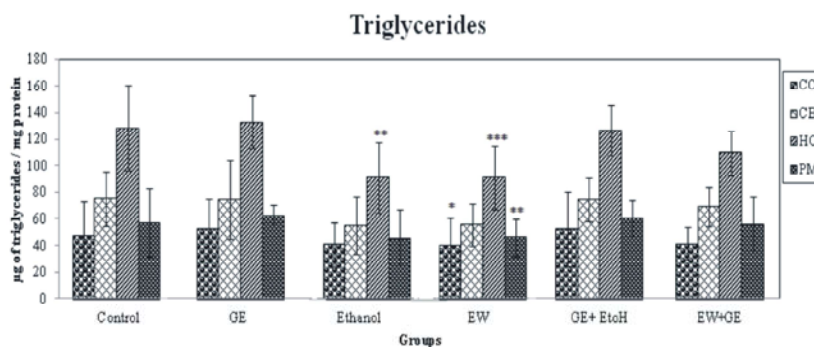


Fig b3: The changes in the triglycerides content in different regions of rat brain during ethanol induced withdrawal and pre-treatment with extract of *Zingiber officinale*

All the values are mean, $\pm$ SD of six individual observations

*Significant at $P < 0.001$, ** significant at $P < 0.01$, ***significant at $P < 0.05$ in comparison with the control.

The total cholesterol levels were significantly increased in all the brain regions during treatment with ZO extract.

Triglycerides: The changes in the triglycerides content in different regions of rat brain during ethanol-induced withdrawal and pre-treatment with extracts of ginger were represented in Fig.b3:

The percent change of depletion in the triglycerides content during ethanol-induced withdrawal can be represented as:

TG: HC (-37.51) > PM (-30.90) > CB (-29.94) > CC (-26.26)

The triglyceride content is elevated in all the brain regions during pre-treatment with aqueous extract of ginger.

The changes in lipid peroxidation indices during withdrawal stress can be summarized as follows (Figs c1 and c2):

TBARS Content: The changes in the MDA content in different regions of rat brain during EtOH-induced withdrawal and pre-treatment with aqueous extracts of ginger is represented in Fig.c1.

The percent change of elevation in the MDA content during EtOH-induced withdrawal when compared with ethanol (Positive control) can be represented as:

TBARS: CB (60.352) > HC (68.466) > CC (51.935) > PM (46.705)

Lipid Hydroperoxide Levels: The changes in the lipid hydroperoxide content in different regions of rat brain during EtOH-induced withdrawal and pre-treatment with aqueous extract of ginger is represented in fig.c2.

The percent change of elevation in the LHP content during EtOH-induced withdrawal when compared with ethanol (Positive control) can be represented as:

LHPs: CC (204.711) > CB (153.659) > HC (93.593) > PM (46.677)

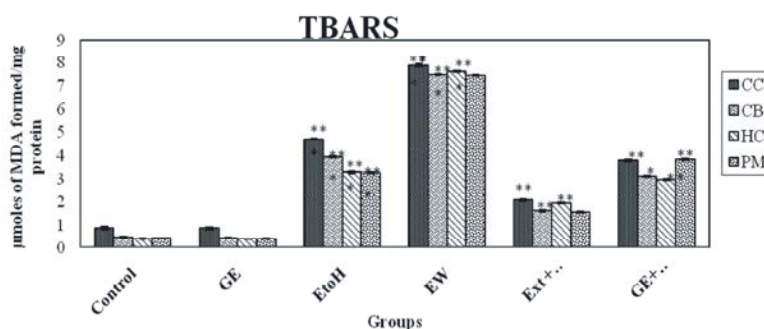


Fig. C1: The levels of MDA in different regions of rat brain during EtOH induced withdrawal and pre-treatment with aqueous extracts of *Zingiber officinale*

All the values are mean, $\pm$ SD of six individual observations

*Significant at $P < 0.001$, ** significant at $P < 0.01$, ***significant at $P < 0.05$ in comparison with the control.

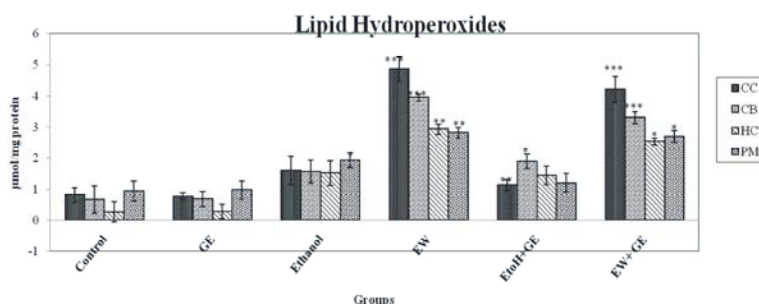


Fig. C2: The changes in the lipid hydroperoxide content in different regions of rat brain during EtOH-induced withdrawal and pre-treatment with aqueous extracts of *Zingiber officinale*

All the values are mean, $\pm$ SD of six individual observations

*Significant at $P < 0.001$, ** significant at $P < 0.01$, ***significant at $P < 0.05$ in comparison with the control.

DISCUSSION

Malondialdehyde, one of the lipid oxidation products can react with the free amino group of proteins, phospholipids and nucleic acids resulting in structural modification, which induce dysfunction of organ systems. A high level of lipid peroxidation products has been detected in cell degradation after cell injury or disease [27,28]. Lipid hydroperoxides (LOOHs) are major non-radical intermediates of lipid peroxidation [29]. LOOHs disrupt membrane structure/function and can be lethal to cells on this basis alone [30-34]. The elevated lipid peroxidation in the cortex, cerebellum, pons and hippocampus (Figs. c1-c2) could play an important role in accelerating onset of aging because lipid peroxidation gives rise to a series of reactive mutagens: lipid peroxides, lipid hydroperoxides, lipid alkoxyl and peroxy radicals and enals [35]. Infact, withdrawal from alcohol has been shown to induce oxidative stress in alcoholic patients and *in vivo* in rat brain after chronic intake. In the present study, lipid peroxidation levels are significantly increased in withdrawal rat cerebrum, brain stem and

cerebellum compared with controls, but both of the variables in the rat brain regions are found to be low in the ginger extract-supplemented ethanolic and withdrawal group compared with the ethanol and the withdrawal groups.

This study examined for the first time whether ginger extract could evoke protection against cellular oxidative stress and lipid peroxidation in rat brain during EW. The main findings are that daily administration of a ginger extract at 200mg/kg bw to rats consuming ethanol, 1) prevents overt increase in MDA levels during subsequent EW, 2) decreases brain contents of the oxidative stress markers and 3) preserves the resilience of cell membranes by maintaining phospholipids and triglyceride levels in the face of EW. Daily ginger administration throughout the experiment revoked protection against EW oxidative stress. Collectively, these results demonstrate that the ginger intake/ supplementation afforded significant protection against EW stress.

The present study also explores the *in vitro* properties of the extract of ginger especially the reducing capacity of a compound may serve as a significant

indicator of its potential antioxidant activity. The reducing power of the extracts were compared with standard BHT and it is found that reducing capacity of the extracts were much better than standard (Fig a1); Metal chelating capacity is significant since it reduces the concentration of the transition metal that catalyzes lipid peroxidation [36]. According to the results, the plant extract is not as good as the standard citric acid; but the decrease in concentration-dependent color formation in the presence of the extract indicates that it has iron chelating activity (Figs a2, a3).

Studies indicate that *Z. officinale* rhizome extract contains significant amounts of flavonoids and phenolic compounds. Both these classes of compounds have good antioxidant potential and their effects on human nutrition and health are quite considerable. Research has established that zingerone, another of ginger's antioxidants, neutralizes the powerful oxidant, peroxynitrite, which has also been implicated as an aggravating factor in Alzheimer's and other neurodegenerative diseases [37]. Ginger's prominent role in reducing lipid oxidation by enhancing the activities of crucial internally produced antioxidants, such as superoxide dismutase has been validated by contemporary research. In particular, melatonin found in ginger [38, 39] is not only a highly effective free-radical scavenger itself, but also stimulates production of the main antioxidant enzyme of the brain, glutathione peroxidase [40]. Moreover, El-Sharaky *et al.*, [41] reported that ginger significantly lowered lipid peroxidation and raised the levels of antioxidant enzyme activities. Furthermore, Afshari *et al.*, [42] studied the effect of dietary ginger (5% of their consumed food daily) on STZ-induced diabetic rats and reported that the MDA level in diabetic rats treated with ginger were significantly lower than in untreated group, as consumption of ginger induced attenuation in lipid peroxidation and increased catalase activity in diabetic treated rats as compared with untreated diabetic rats [43].

In conclusion, the results obtained from the present study provide a rationale for the use of the rhizome of *Zingiber officinale* for the treatment of alcohol-related disorders especially related to ethanol withdrawal. However, the ability of extract components to penetrate the brain is unclear and studies are presently undertaken in our laboratory to characterize the active ingredients of the extract and to explore the mechanism of their action in brain during ethanol withdrawal.

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Conflict of Interest: None Declared.

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