Protective Effect of *Acorus calamus* Linn on Free Radical Scavengers and Lipid Peroxidation in Discrete Regions of Brain against Noise Stress Exposed Rat

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Exposure to continuous loud noise is a serious health problem due to excess production of oxygen free radicals. In medical research, more attention is paid to the antioxidant properties of medicinal plants to minimize the harmful effects of radicals. The aim of this study was to evaluate the protective effect of both ethyl acetate and methanolic extract of *Acorus calamus* Linn against noise stress (30 d, 100 dBA/4h/d) induced changes in the rat brain. We measured the activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and the levels of reduced glutathione (GSH), vitamin C, vitamin E, protein thiols and lipid peroxidation (LPO) for the evaluation of oxidative stress status in discrete regions of the rat brain like cerebral cortex, cerebellum, pons-medulla, midbrain, hippocampus and hypothalamus. The results indicated that during exposure of noisy environment ROS generation led to increase in corticosterone, LPO and SOD, but decrease in CAT, GPx, GSH, protein thiols, vitamins C and E levels. Both the ethyl acetate and methanolic extract of *Acorus calamus* protected most of the changes in the rat brain induced by noise-stress.

Key words   *Acorus calamus*; noise-stress; rat brain; oxidative free radical; antioxidant

Free radicals arising from either the normal metabolism or induced by environmental sources interact continuously in the biological systems. Oxidants/antioxidants must be kept in balance to minimize molecular, cellular and tissue damage.[1] Reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radicals are formed in the course of cellular metabolism. A series of antioxidant compounds are present in the cells, they react with oxidizing agents and disarm them.[2] Several studies have indicated the effects of various types of stress on the antioxidant system and induction of lipid peroxidation in the brain of various stress exposed models.[3,4] Aravind kumar *et al.* reported that acute and chronic loud noise exposure generates excessive free radicals and causes disorders involving extra-auditory organs such as nervous, endocrine, and cardiovascular systems.[5,6] Among them the nervous system is relatively more susceptible to free radical damage.[7]

Pharmacological effects of the medicinal plants are related to its free-radical scavenging properties which include inhibition of lipid peroxidation, maintaining integrity and permeability of cell walls[8—10] as well as protection of neurons against oxidative stress.[11,12] Roots and rhizomes of *Acorus calamus* Linn (AC), commonly known as sweet flag, sweet grass and sweet cane (Family: Araceae), have been used in the Indian and Chinese systems of the medicine for hundreds of years for its beneficial role in improved learning performance, and its anti-aging effect.[13—15] Shukla *et al.* reported that ethanolic extract of AC prevented acrylamide-induced hind limb paralysis, decreased GSH and GST, increased dopamine receptors in the corpus striatum.[16] The ethanolic extract of AC has been reported to posses the antioxidant activity in an *in-vitro* study.[17]

But lack of studies on its effectiveness in averting noise-stress induced free radical changes in the brain in animal models forms the lacunae for this present study. Hence, we investigated the effect of ethyl acetate (EAAC) and methanolic (MAC) extract of AC on stress-induced changes in the brain antioxidant system of rats, which includes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) activity, levels of reduced glutathione (GSH), vitamins C and E, protein thiols and lipid peroxidation (LPO) in discrete brain regions (cerebral cortex, cerebellum, pons-medulla, midbrain, hippocampus and hypothalamus) and plasma corticosterone after 30 d white noise (100 dBA/4h/d) exposure.

MATERIALS AND METHODS

Animals and Chemicals   Healthy adult male Wistar strain albino rats (*n*-36), weighing between 200—220 g, were selected and maintained under standard laboratory conditions. They had free access to chow (M/s. Hindustan Lever Ltd., India) and water. Animal experiments were carried out after getting clearance from the Institutional animal ethical committee (IAEC No. 08/012/03).

The animals were divided into 6 groups with equal number of animals in each (Table 1) group.

Solvent Extraction   The dried rhizome (100 g) of *Acorus calamus* Linn was extracted with different solvents (1 : 2 w/v) at room temperature, namely ethyl acetate and methanol. Each solvent extraction was carried out for overnight and repeated three times with the same solvent. Each of these extracts were concentrated in a rotatory evaporator under reduced pressure, giving 2—3 g yield of each individual extracts, which was stored in refrigerator (4 °C) until use.[18] *Acorus calamus* was purchased from Tampcol Ltd., Chennai, India. It was identified and authenticated by The Director of Centre for Advanced Studies on Botany, University of Madras, India.

The suspension of ethyl acetate extract of AC (EAAC) and methanolic extract of AC (MAC) for injection was prepared by dissolving it in 3% Tween80 and all animals received

Table 1. Different Groups of Animals and the Experiment Schedule

Groups	Drug injected (i.p.)	Volume/dose	No. of days	Noise stress
1. Vehicle control	Tween80	400 μl/d	30	No
2. EAAC alone	EAAC	50 mg/kg/400 μl/d	30	No
3. MAC alone	MAC	50 mg/kg/400 μl/d	30	No
4. Vehicle stress	Tween80	400 μl/d	30	Yes (4 h/d)
5. EAAC+stress	EAAC	50 mg/kg/400 μl/d	30	Yes (4 h/d)
6. MAC+stress	MAC	50 mg/kg/400 μl/d	30	Yes (4 h/d)

Table 2. Effect of Ethylacetate and Methanolic Extracts of *Acorus calamus* (50 mg/kg Body Weight) on LPO and Protein Thiol Levels in Various Rat Brain Regions after Exposure of Chronic Noise Stress

	Vehicle control	EAAC alone	MAC alone	Vehicle stress	EAAC+ stress	MAC+ stress
Lipid peroxidation (μmol of MDA formed/mg protein)						
Cerebral cortex	1.71±0.02	1.73±0.07	1.71±0.05	2.69±0.04*	1.98±0.08*,#	2.02±0.08*,#
Cerebellum	1.73±0.04	1.74±0.05	1.73±0.09	2.68±0.07*	2.05±0.06*,#	2.00±0.07*,#
Midbrain	1.35±0.07	1.35±0.07	1.36±0.12	2.36±0.09*	2.00±0.07*,#	1.67±0.05*,#
Pons medulla	1.67±0.04	1.66±0.09	1.69±0.15	2.65±0.10*	2.01±0.10*,#	1.75±0.07#
Hippocampus	1.28±0.05	1.27±0.09	1.28±0.11	2.29±0.05*	1.76±0.09*,#	1.74±0.08*,#
Hypothalamus	1.23±0.03	1.25±0.03	1.24±0.05	2.11±0.09*	1.66±0.08*,#	1.54±0.04*,#
Protein thiols (μg/g tissue)						
Cerebral cortex	5.58±0.07	5.60±0.03	5.61±0.04	3.47±0.04*	5.41±0.06*,#	4.87±0.03*,#
Cerebellum	5.60±0.07	5.63±0.06	5.59±0.06	3.97±0.05*	5.54±0.05#	5.13±0.06*,#
Midbrain	5.77±0.07	5.79±0.04	5.78±0.05	3.89±0.04*	5.72±0.03#	5.26±0.03*,#
Pons medulla	5.23±0.03	5.25±0.03	5.26±0.03	3.79±0.05*	4.98±0.06*,#	4.91±0.06*,#
Hippocampus	5.14±0.02	5.17±0.03	5.15±0.02	2.98±0.06*	5.10±0.07#	5.12±0.06#
Hypothalamus	4.52±0.03	4.54±0.04	4.51±0.04	2.56±0.03*	4.48±0.03#	4.15±0.04*,#

Data are expressed as mean±S.D. for six rats in each group. *Significant ($p<0.05$) *vs.* vehicle control. #Significant ($p<0.05$) *vs.* vehicle stress.

400 μl of the suspension intraperitoneally (i.p.). The animals received drug/vehicle half an hour prior to the noise stress procedure for 30 d.

Chemicals used in this experiment were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and all chemicals used were of analytical grade.

Noise Stress Procedure Broad band (White) noise was produced by a white noise generator and amplified by an amplifier (40 W) which was connected to a loudspeaker located at 30 cm above the animal cage. The intensity of the sound was measured by a sound level meter (Cygnet systems-D 2023 Serial No. F02199, India) and maintained at 100 dBA intensity with background noise level at 30±2 dBA due to ventilation system.[19] Animals were exposed to noise (100 dBA 4 h/d for 30 d) at 09:00 to 13:00 h. The level of 100 dBA was chosen as it was comparable with the noise level detected in discos and some industrial workplaces.[20]

Specimen Preparation Animals were killed by cervical dislocation on the 31st day (in order to avoid the acute noise stress effect of 30th day in noise stress exposed animals), brain was removed and placed in ice-cold saline. Various parts of the brain regions were dissected out in ice cold (4 °C) conditions according to the method of Glowinski and Iversen.[21] Ten percent homogenate was prepared using ice cold Tris–HCl buffer (0.1 M, pH 7.4) in a motor driven Teflon-glass tissue homogenizer. The homogenate was centrifuged at 2000 rpm, 4 °C for 15 min and the supernatant was used for the biochemical analysis.

Biochemical Estimation Lipid peroxidation activity was indirectly estimated by determining the accumulation of thio-barbituric acid reactive substances, in the tissue homogenate by the method of Ohkawa *et al.*[22] The superoxide dismutase

activity was measured as the degree of inhibition of auto-oxidation of pyrogallol at the alkaline pH according to the method of Marklund and Marklund.[23] The activity of catalase was measured as the amount of hydrogen peroxide consumed per minute per mg of protein as per the method of Sinha.[24] Glutathione peroxidase was assay by measuring the amount of reduced glutathione consumed in the reaction mixture according to the method of Rotruck *et al.*[25] The Reduced glutathione was assayed by development of relatively stable yellow color, when 200 mM 5,5′-dithiobis-(2-nitro benzoic acid) solution was added according to the methad of Moran *et al.*[26] The protein thiol was estimated by the method of Sedlack and Lindsay.[27] Vitamin C and vitamin E were estimated by the methods of Omaye *et al.*[28] and Desai[29] respectively. Protein was estimated by the method of Lowry *et al.* using BSA as standard.[30] Plasma corticosterone was estimated according to the method of Mattingly.[31]

Statistical Analysis All data were expressed as mean ±S.D. The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS version 10.0 (SPSS, Cary, NC, U.S.A.). When there was significant difference, Tukey's multiple comparisons were performed by fixing the significance level at $p<0.05$.

RESULTS AND DISCUSSION

Free radical-induced lipid peroxidation has been associated with many neurodegenerative diseases. Exposure of noise-stress for 30 d significantly increased the LPO level and SOD activity and concomitantly decreased the activities of CAT, GPx, levels of GSH, vitamin C, vitamin E and protein thiols in all the regions of the brain that were tested.

Lipid peroxidation serves as a marker of oxidative stress. As shown in Table 2. there was a significant ($p<0.05$) increase in LPO after 30 d exposure to noise-stress in all the brain regions. Brain cells are the most vulnerable to free radical damage caused by lipid peroxidation compared to other tissues, owing to their highest percentage of unsaturated fats.[32] Administration of EAAC and MAC effectively decreased LPO levels in all the brain regions. This clearly showed that AC extracts effectively reduced the oxidative stress.

The Significantly increased SOD and decreased CAT and GPx (Table 3) activities observed in the rat brain after 30 d noise exposure, were similar to that of earlier reports using different stressors.[33] Administration of EAAC and MAC normalized significantly the activities of SOD, CAT and GPx in almost all brain regions of noise exposed animals.

The glutathione status of a cell could be taken as the most accurate single indicator of the health of the cell, as the GSH depletion determines the vulnerability to oxidant attack which was observed in noise-stress (Table 4) exposed group. Vitamins C and E levels (Table 4) were decreased statistically ($p<0.05$) in vehicle stress group after 30 d noise expo-

Table 3. Effect of Ethylacetate and Methanolic Extracts of *Acorus calamus* (50 mg/kg Body Weight) on Activity of SOD, CAT and GPx in Various Rat Brain Regions after Exposure of Chronic Noise Stress

	Vehicle control	EAAC alone	MAC alone	Vehicle stress	EAAC+ stress	MAC+ stress
Superoxide dismutase (Units/min/mg protein)						
Cerebral cortex	0.545±0.03	0.557±0.03	0.532±0.02	0.627±0.02*	0.550±0.03#	0.545±0.04#
Cerebellum	0.931±0.02	0.921±0.06	0.927±0.05	1.049±0.11*	0.934±0.05#	0.948±0.03#
Midbrain	0.764±0.02	0.778±0.05	0.763±0.05	1.107±0.06*	0.898±0.05*#	0.918±0.03*#
Pons medulla	0.753±0.01	0.768±0.06	0.763±0.07	1.315±0.08*	1.158±0.05*#	0.991±0.06*#
Hippocampus	1.824±0.07	1.834±0.10	1.808±0.05	2.229±0.09*	2.070±0.07*#	1.959±0.08#
Hypothalamus	4.367±0.08	4.387±0.09	4.360±0.17	5.381±0.04*	4.621±0.07*#	4.818±0.15*#
Catalase (μmol of H_2O_2 consumed/min/mg protein)						
Cerebral cortex	4.88±0.05	4.83±0.07	4.88±0.11	3.47±0.06*	4.47±0.04*#	4.03±0.05*#
Cerebellum	6.07±0.11	6.06±0.13	6.03±0.09	5.12±0.09*	5.63±0.08*#	5.64±0.20*#
Midbrain	5.59±0.05	5.60±0.13	5.70±0.44	4.86±0.10*	5.33±0.04#	5.29±0.08#
Pons medulla	5.67±0.06	5.66±0.09	5.66±0.22	4.78±0.11*	5.48±0.08#	5.17±0.08*#
Hippocampus	5.88±0.12	5.80±0.09	5.77±0.10	4.54±0.08*	5.15±0.08*#	5.13±0.08*#
Hypothalamus	6.48±0.05	6.44±0.12	6.43±0.11	5.48±0.13*	5.92±0.10*#	5.75±0.09*#
Glutathione peroxidase (μmol of GSH oxidized/min/mg protein)						
Cerebral cortex	1.66±0.06	1.65±0.05	1.64±0.08	1.06±0.06*	1.44±0.06*#	1.37±0.03*#
Cerebellum	2.76±0.16	2.76±0.12	2.78±0.08	1.90±0.14*	2.41±0.08*#	2.27±0.03*#
Midbrain	2.36±0.10	2.37±0.11	2.35±0.05	1.75±0.13*	2.17±0.06*#	1.98±0.08*#
Pons medulla	2.33±0.05	2.33±0.05	2.31±0.09	1.79±0.06*	1.99±0.09*#	2.02±0.05*#
Hippocampus	1.73±0.12	1.75±0.07	1.76±0.09	1.33±0.05*	1.53±0.06*#	1.60±0.08#
Hypothalamus	1.79±0.11	1.76±0.10	1.77±0.05	1.05±0.05*	1.52±0.06*#	1.33±0.02*#

Data are expressed as mean±S.D. for six rats in each group. *Significant ($p<0.05$) *vs.* vehicle control. #Significant ($p<0.05$) *vs.* vehicle stress.

Table 4. Effect of Ethylacetate and Methanolic Extracts of *Acorus calamus* (50 mg/kg Body Weight) on GSH, Vitamin C and E Level in Various Rat Brain Regions after Exposure of Chronic Noise Stress

	Vehicle control	EAAC alone	MAC alone	Vehicle stress	EAAC+ stress	MAC+ stress
Reduced glutathione (μmol/mg protein)						
Cerebral cortex	0.492±0.004	0.498±0.007	0.500±0.025	0.347±0.012*	0.423±0.009*#	0.418±0.005*#
Cerebellum	0.415±0.012	0.410±0.011	0.412±0.022	0.315±0.017*	0.403±0.011#	0.386±0.017*#
Midbrain	0.469±0.016	0.480±0.013	0.473±0.022	0.339±0.017*	0.404±0.014*#	0.394±0.011*#
Pons medulla	0.537±0.009	0.544±0.028	0.539±0.014	0.354±0.010*	0.480±0.019*#	0.397±0.018*#
Hippocampus	0.427±0.006	0.429±0.004	0.422±0.067	0.265±0.015*	0.392±0.019#	0.379±0.014#
Hypothalamus	0.339±0.009	0.335±0.030	0.334±0.048	0.234±0.010*	0.322±0.007#	0.293±0.016*#
Vitamin C (μg/g tissue)						
Cerebral cortex	470.4±40.17	479.7±24.49	467.7±41.74	330.7±24.90*	465.2±17.75*#	411.2±38.80*#
Cerebellum	342.1±24.99	348.8±20.94	345.8±18.56	211.8±22.40*	347.4±21.18#	318.9±22.50#
Midbrain	387.4±25.94	389.2±41.00	386.1±29.52	274.3±23.89*	391.6±20.70#	354.8±21.57#
Pons medulla	284.8±25.12	290.5±29.06	298.2±27.18	225.8±16.35*	286.2±18.56#	266.3±18.56#
Hippocampus	391.6±21.78	389.9±25.59	390.6±25.03	276.9±17.15*	370.6±35.38#	353.6±34.88#
Hypothalamus	345.2±27.86	348.0±22.54	334.9±23.01	225.9±19.44*	331.9±20.21#	325.5±32.04#
Vitamin E (μg/g tissue)						
Cerebral cortex	19.76±1.50	20.14±1.40	19.51±1.81	12.15±1.40*	18.56±1.21#	16.74±0.76*#
Cerebellum	11.97±1.26	12.00±1.40	11.98±1.22	8.49±0.86*	12.17±1.01#	12.02±0.72#
Midbrain	12.11±0.72	12.40±0.50	12.14±0.43	8.41±1.02*	11.78±1.07#	11.85±0.74#
Pons medulla	14.07±1.33	13.95±0.78	13.90±1.14	8.92±0.90*	13.66±0.43#	13.15±0.84#
Hippocampus	17.64±0.79	17.65±0.86	17.68±0.91	11.24±0.87*	17.19±0.92#	15.77±0.92*#
Hypothalamus	12.07±1.48	12.14±1.12	12.15±1.11	8.06±1.06*	11.84±0.98#	11.50±0.78#

Data are expressed as mean±S.D. for six rats in each group. *Significant ($p<0.05$) *vs.* vehicle control. #Significant ($p<0.05$) *vs.* vehicle stress.

sure, compared with vehicle control group in all the brain regions. Vitamin C is a well known water soluble ketoacetone and plays a crucial role in the suppression of superoxide radicals.[34] In the cell dehydroascorbic acid is formed on reduction of GSH, which cycles the tocopheroxyl radicals to tocopherol. Vitamin C deficiency results in depletion of tissue tocopherol.[35] Vitamin E has been effective in blocking peroxyl-mediated chain reactions and in combination with ascorbate in scavenging superoxide radicals in lipid membranes. The administration of EAAC and MAC extracts causes significant increase in the levels of these vitamins and GSH. It could have been achieved by preventing oxidative stress in the noisy environment.

Protein thiolation is apparently induced by different mechanisms that involve thiol/disulfide exchange of one or two electron oxidations of cysteinyl residue.[36,37] The significant ($p<0.05$) decrease in protein thiols (Table 2) was observed in noise stress exposed group animals. Decreased protein thiols in brain due to oxidative damage, as reported by Nikolaos *et al.*[38] is also well in agreement with this study and further strengthens our finding. EAAC and MAC could prevent the protein oxidation most effectively in all the brain regions. This was may be due to minimizing oxidative stress produced upon noise-stress.

Plasma corticosterone (Fig. 1) level was increased in vehicle stress group, when compared to vehicle control. This was also observed in the previous study.[39] In both groups EAAC +stress and MAC+stress treatment with AC extracts along with 30 d noise stress resulted in significant decrease in the plasma corticosterone level. Body weight of the animal was measured every 5th day of this study. Significant weight loss was observed in vehicle stress group on the 20th day onwards. However, in both extract treatment groups this change was normalized (data not shown).

We conclude that *Acorus calamus* effectively prevents the noise stress-induced changes in the rat brain. This anti-stressor effect might be due to an increase in brain antioxidative capacity which in turn could be achieved by protection of decreasing GSH, vitamins C, and E levels and restoring free radical scavenger's enzymatic activity. This is the first report to provide a direct evidence for the anti-stressor effect of EAAC and MAC in animal models and thus recommend the use of the *Acorus calamus* for lifestyle-related diseases and to improve general health conditions in human subjects. Extensive safety studies should be preceded before we clinically apply *Acorus calamus* LINN as an anti-stressor.
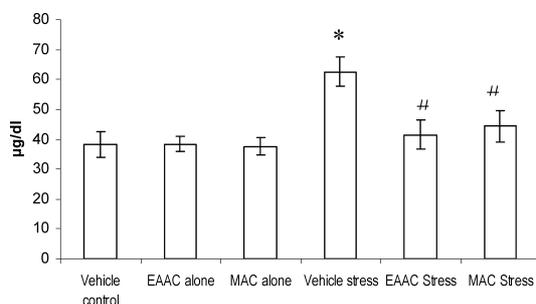

Fig. 1. Effect of Ethylacetate and Methanolic Extracts of *Acorus calamus* on Plasma Corticosterone in Rat

Each bar represents mean±S.D. of 6 rats. *Significant ($p<0.05$) *vs.* vehicle control. #Significant ($p<0.05$) *vs.* vehicle stress.

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