EFFECT OF *ACORUS CALAMUS* LINN. AND IT'S ACTIVE PRINCIPLE ON NOISE STRESS-INDUCED CHANGES ON IMMUNE SYSTEM

Dharini S*[1], Gajalakshmi G[2], Sheela Devi R[1]

*[1]Department of Physiology, Dr. ALM PG IBMS, Univeristy of Madras, Taramani campus, Chennai 600113.
[2]Department of Physiology, Meenakshi Medical College and Research Institute, Enathur, Kancheepuram, Tamil nadu.

E-mail of Corresponding Author: dharinisubramani@yahoo.co.in

Abstract
Noise is an inevitable everyday stressor in our lives and is detrimental to our immune system. The aim of the present study was to investigate the potential of ethyl acetate extract of *Acorus Calamus* (AC) and its active component, α- asarone on the noise stress induced changes on immune system of Wistar albino rats. Noise stress of 100dB for 4h/day for 30 days was employed in this study to observe the effect of stress on immune system and the dosage of AC and α-asarone used were 50 mg / Kg b.wt and 9mg/Kg b.wt respectively for 45 days. Sheep red blood cells (5×10^9 cells/ml) were used to immunize the animals. Six different groups were used: Control Immunized, AC treated immunized, α-asarone treated immunized, Noise stress immunized, AC treated stress immunized, α-asarone treated stress immunized groups. The immune status was assessed by the serum cytokine levels of IL-2, IL-4, IFN-γ and the CD4, CD8 T cell lymphocyte phenotype in spleen while biochemical markers of oxidative stress- LPO, GSH in spleen and corticosterone in plasma were estimated in the immune challenged animals. Results showed that noise stress significantly increased the lipid peroxidation and corticosterone level with concomitant depletion of GSH in the immunized rats. Noise stress significantly suppressed the CD4 and CD8 T cells in spleen and the cytokines IL2, IFN-γ and enhanced IL4 levels in the serum. The supplementation with AC as well as α-asarone prevents the noise stress induced changes in the antioxidant as well as immune status in rats. This study concludes that AC restores the noise stress induced changes which may be due to its active principle α-asarone.
Keywords: Chronic noise stress, CD4, IL2, LPO, Corticosterone, *Acorus calamus*

1. Introduction

Stress brings about certain physiological changes in our body comprising a cascade of neuroendocrine events such as the HPA axis activation. Interplay between the immune, endocrine and nervous system is most commonly associated with pronounced effect of stress on immunity. Chronic stress, either physical or psychological, can have a dramatic impact on the immune system in both humans and animals.[1-5] Sensitivity of the immune system to stress is an indirect consequence of the regulatory reciprocal influences that exist between the immune system and the central nervous system. The immune system receives signals from the brain and the neuroendocrine system via the autonomic nervous system and hormones and sends information to the brain via cytokines. These connections appear to be part of a long-loop regulatory feedback system that plays an important role in the coordination of behavioral and physiological responses to infection and inflammation.

According to Babisch[6], noise activates the pituitary-adrenal-cortical axis and the sympathetic adrenal medullary axis leading to the increased adrenal gland secretions. The immune system is particularly sensitive to stress and specific effects of stress have been demonstrated by a number of studies.[7,8] Chang and Rasmussen[9] found that high intensity noise reduced interferon production and Jensen[10] and Geber *et al*.,[11] reported that various levels of noise reduced blood leukocytes, such as eosinophils, in rodents. Upon exposure to noise stress, the neutrophil functions were reported to be suppressed in immunized rats.[12]

Immunomodulation using medicinal plants is an expanding area of research. The rhizome of *Acorus calamus* Linn. has been studied to possess cardioprotective [13], anti ulcer effects[14], antistress and antioxidant property [15], antidiarrhoeal[16], dyslipidemic[17],

neuroprotective[18], wound healing[19] and hypotensive and vascular modulating effects[20]. It has been reported to prevent tonic clonic and partial seizures in mice[21], to exert beneficial effect in tibial and sural nerve transection induced neuropathic pain in rats[22] and vascular relaxant effect [23].

In this study, AC and its active component α-asarone is studied in search for an antidote against noise stress which is inevitable in our lives and for a new immunomodulator by assessing the IL-2, IL-4, IFN-γ cytokine levels, CD4, CD8 cell lymphocyte phenotype in spleen and LPO levels during noise stress. To determine the best potential impact of AC and α-asarone on an immune response, the highly immunogenic, non-infectious SRBC was used as a stimulating agent in this study.[24]

2. Materials and methods

2.1. Animals: The study was conducted on male Wistar albino rats (180-200 g). All the protocols were approved by the Institute's animal ethical committee of the University of Madras (IAEC No. 08/033/07). The animals were maintained under standard laboratory conditions: temperature 25±2°C and photoperiod of 12hr. Commercial pellet diet and water were given *ad libitum*.

2.2. Drug and Dosage: *Acorus Calamus* Linn. was purchased from Tampcol Ltd, Chennai, India and was identified and authenticated by The Director, Centre for Advanced Studies on Botany, University of Madras, Chennai, India. The dried rhizome of the *Acorus calamus* Linn. (100 g) was blended in a blender, extracted at room temperature in a soxhlet apparatus with ethyl acetate. The extract was concentrated in a rotatory evaporator under reduced pressure, giving a 5 % yield, which was stored in refrigerator (4 °C) until use.[25] Drug α-asarone was purchased from Fluka chemical company. The suspension of both AC and α-asarone for injection was prepared by dissolving it in 3% Tween 80 to the required volume.[26] Both AC and α-asarone was administered intraperitoneally (i.p.) at a dosage of 50mg/Kg b.wt at a dosage of 9 mg/Kg b.wt respectively.

2.3. Experimental groups: The albino rats were divided into 6 group, each group consisting of 6 animals. The groups are given in table 1.

Table 1. Experimental groups

Group I	Vehicle Control animals- received 3%Tween80(400µl) for 30 days, immunized on 26th day
Group II	Ethyl Acetate extract of AC (AC)- 50mg/Kg b.wt. for 30 days, immunized on 26th day
Group III	α-asarone- 9mg/Kg b.wt. for 30 days, immunized on 26th day
Group IV	30 days noise stress exposed animals, immunized on 26th day
Group V	15 days AC pre-treatment and 30 days noise stress + simultaneous AC treatment, immunized on 26th day
Group VI	15 days α-asarone pre-treatment and 30 days noise stress + simultaneous α-asarone treatment, immunized on 26th day

2.4. Noise Stress Procedure: Broad band of white noise at 100 dB intensity was used in this study. Rats were exposed to noise produced using a white noise generator and amplified by an amplifier (40 W) that was connected to a full range loud speaker fixed 30 cm above the animal cages. Sound level meter (Cygnet systems-D 2023 Serial NO. F02199, Chandigarh city, India) was used to measure the intensity of the noise generated and was maintained at 100 dB intensity.[27]

2.5. Immunization: The sheep red blood cells (SRBC) were used to immunize the animals, which were collected in a sterile Alsever's solution and washed thrice with pyrogen free normal saline and adjusted to 5×10^9 cells per ml. The animals were immunized by injecting 20% (1 ml) SRBC intraperitoneally (i.p). The day of immunization was considered as day 0. On the 5th day, the blood samples were collected to carry out the immunological parameters. Blood samples were drawn from jugular vein by the stress free method of Feldman and Conforti.[28]

2.6. Corticosterone Estimation: The plasma corticosterone level was estimated by the method of Mattingly.[29] To 1 ml of plasma, purified dichloromethane (7.5 ml) was added and gently shaken for 5 min. To the sediment (supernatant discarded), fluorescence reagent (2.5 ml) (ethanol and concentrated H_2SO_4 in the ratio 3: 7) was added and shaken vigorously for 20 s. The resulting fluorescence of the acid layer was read at excitation 470 nm and emission 530 nm in fluorescence spectrophotometer.

2.7. Quantification of cytokine (IL-2, IL-4, IFN- γ): Cytokines IL-2 and IFN- γ was quantified by Enzyme Linked Immuno Sorbent Assay (ELISA) procedure using kits from Bender MedSystem (Vienna City, Austria), according to manufacturer's protocols. Briefly, 50 µl of ELISA diluents is pipetted into antibody coated wells (anti-IL-2, anti-IFN), followed by100 µl of each standard and 50 µl of test samples (serum), shaken for 5 seconds to mix the contents in the wells, covered with plate sealer and incubated for 2 hrs at room temperature. After incubation, contents of the wells were aspirated and washed three times with wash buffer. After complete removal of the wash buffer in the final wash, 100 µl of detection solution was added, covered and incubated for 1 hr. The wells were washed three times with wash solution and 100 µl of substrate reagent was added and incubated for 30 minutes in dark. The color development was arrested by adding 100 µl of stop solution and the absorbance was read at 450 nm with a reference wavelength of 570 nm.

2.8. Lymphocyte Phenotype in Spleen (CD4, CD8 cells): The spleen samples were teased on a wire mesh for single suspension in PBS (including 0.05% EDTA and excluding Mg^{2+} and Ca^{2+}). Splenic erythrocytes were lysed by two consecutive incubations (5 and 3 mnts at 37C) of the spleenocytes from spleen suspension in ammonium chloride (0.83% NH_4Cl in 0.01 M Tris-HCl, pH-7.2). Cells were washed resuspended in cold PBS. The CD4 or CD8 expressed lymphocytes from spleen suspension ($1x10^7$) were isolated by incubating with anti-CD4 or anti-CD8 conjugated magnetic microbeads (Miltenyi Biotech, Bergisch-Gladbach, Germany). After a wash with PBS, cells were passed through a separation column placed in a magnetic field. The retained cells were collected by flushing out using a Plunger and then CD4 or the CD8 cells were enumerated

2.9. Lipid peroxidation (LPO): LPO was determined by the procedure of Ohkawa *et al*., [30] LPO level was indirectly estimated by determining the accumulation of thiobarbituric acid reactive substances (TBARS), thiobarbituric acid to generate a colored product, which absorbs at 532 nm. Protein estimations were carried out as suggested by Lowry *et al*.

2.10. Reduce Glutathione (GSH): The GSH was assayed by the method of Moron et al.[31] The development of relatively stable yellow color, when 0.2M 5, 5'-dithiobis-(2-nitro benzoic acid) (DTNB) solution is added was read at 420 nm. The values are expressed in µ mol/mg protein.

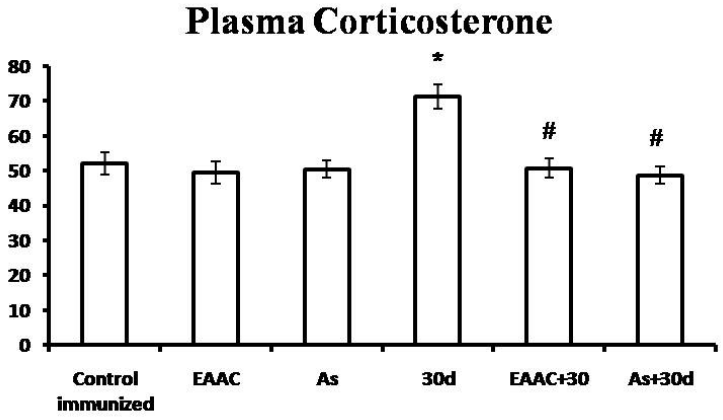
3. Statistical Analysis:

All the data were statistically analyzed using one way ANOVA followed by Tukey's multiple comparison tests when there was a significant difference by fixing the level of significance at $p < 0.05$.

4. Results

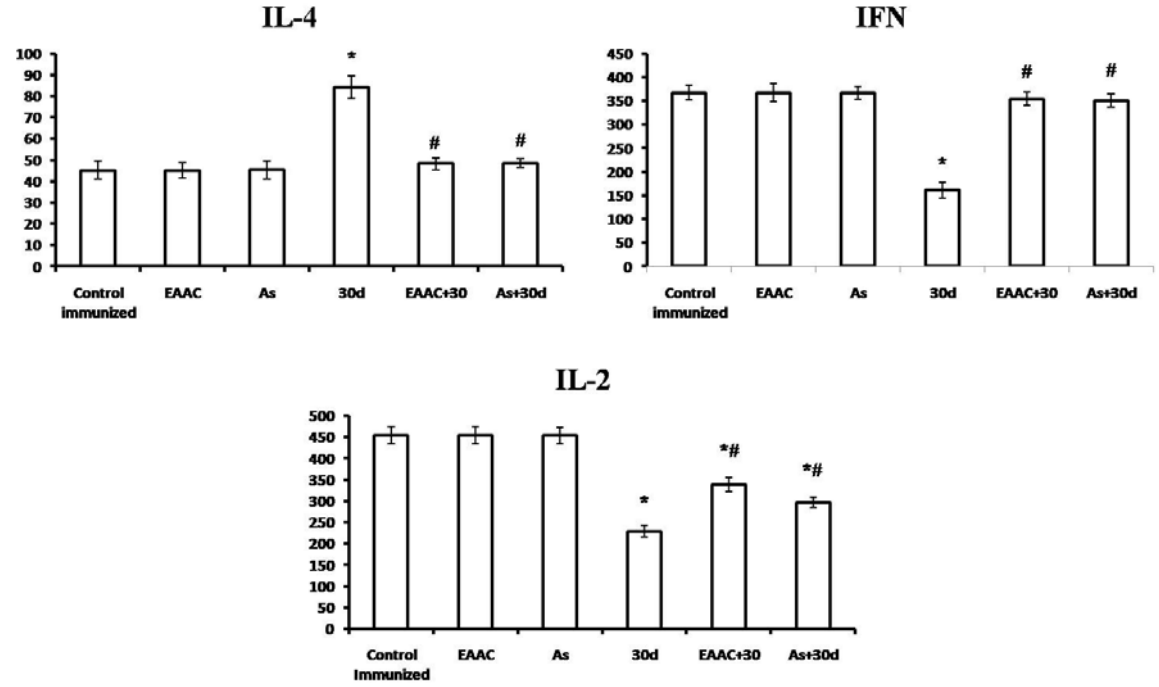
The AC and α-asarone treated group of animals did not show any variation from the control animals and therefore treated as controls.

4.1. Plasma Corticosterone: Graph1 depicts that chronic stress significantly increases the corticosterone levels in the plasma in comparison to normal groups. The corticosterone levels of AC and α-asarone treated stress exposed groups were maintained at normal levels.



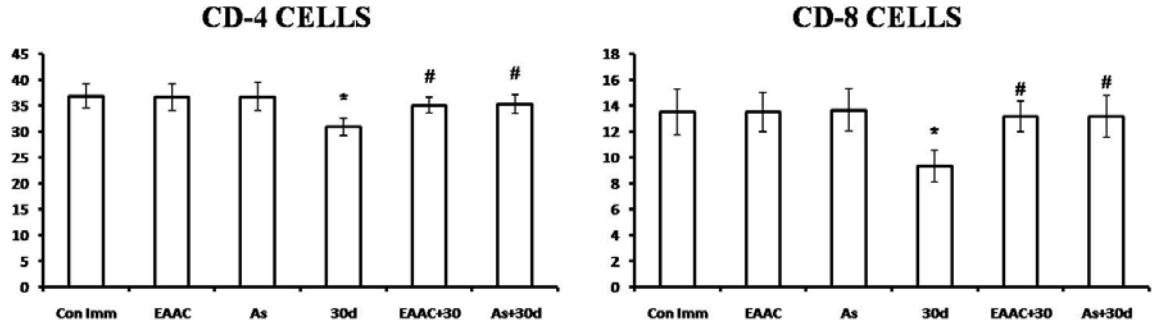
Graph 1. * indicates significance compared with control, # indicates significance compared with stress exposed group.

4.2. Serum Cytokines: As shown in the graph 2, noise stress used in this study significantly increased the IL-4 levels in the serum while a significant decrease was observed in the serum IL-2 and IFN-γ levels. The mean values of IL-4 and IFN-γ in the stress exposed group of animals, pretreated with AC as well as α-asarone were similar to the controls. The stress-induced effect on IL-2 was significantly suppressed, but not completely reversed by both AC as well as α-asarone treatment to stress exposed groups.



Graph 2. * indicates significant compared with control, # indicates significant compared with stress exposed group.

4.3. CD4 and CD8 T Lymphocyte Phenotype in Spleen cells: The number of CD4 and CD8 cells in the spleen of stress exposed rats were significantly lower than the unstressed control groups. This stress-induced decrement in the both CD4 and CD8 cells was prevented by the administration of both AC and α-asarone to the stress exposed groups are summarized in graph 3.

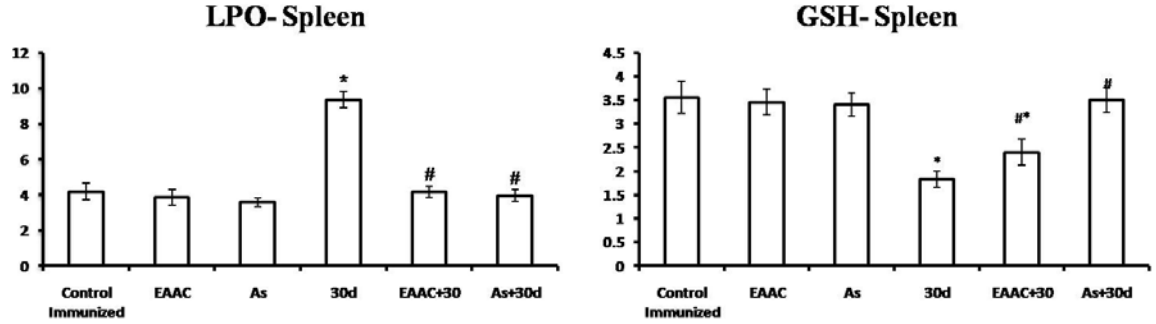


Graph 3. * indicates significance compared with control, # indicates significance compared with stress exposed group.

4.4. Antioxidant Activity: Concentrations of lipid peroxides and glutathione (GSH) quantified in the spleen homogenates of different groups are summarized in **graph 4**. LPO level of the stressed group was significantly higher than that of control groups. This stress induced elevation

of lipid peroxides was significantly lower in AC and α-asarone treated groups.

Stress induced elevation of LPO level in the stress exposed group was accompanied by lower levels of GSH. However, while α-asarone treatment to stress exposed groups were observed to maintain the splenic GSH level at normal levels, AC could not increase the GSH to normal levels though it increased it significatly from the stress exposed group.



Graph 4. * indicates significance compared with control, # indicates significance compared with stress exposed group.

5. Discussion

The increased corticosteroid levels are often used as an index of stress.[32] Elevated levels of glucocorticoids do have negative effects on the cognitive abilities of animals, causing neuronal cell death and reducing neurogenesis.[33] It is a known fact that corticosteroids act as an immune suppressor in both humans and animals.[34] A significant increase in the plasma corticosterone levels after chronic stress has been reported.[35-37] The present study also observed an increase in the plasma corticosteroid levels after 30 days of noise exposure which is well in agreement with the earlier reports. Hence the elevation of corticosteroid observed in this study suggests that it may not be beneficial to immune system. This also indicates that the animals do not adapt to stress even after repeated exposure to noise and points out the continuous activation of HPA axis. However, the observed increase in the plasma corticosterone level in the stressed animals was well prevented on administration of both AC and α-asarone.

Two subsets of T helper cells (Th cells) namely Th1 and Th2 cells regulate cellular and humoral immunity repectively.[38] Both Th1 and Th2 cells have distinct cytokine secretion pattern and immunomodulatory effects. Th1 secretes IL-2 and IFN-γ required for cell-mediated immune reponse while Th2 secretes IL-4 required for humoral immune response.[39] Th1/Th2 ratio exerts important effects on the balance of cellular and humoral immunity.[40] The present data show that noise stress is associated with a decrease in the production of Th1-type cytokines IL-2 and IFNγ and an increase in the production of the Th2-type cytokine IL-4. Such an increase in IL-4 and a concomitant decrease in IL-2 and IFN-γ on exposure to noise stress has been reported.[41] Our observations revealed that the deviated levels of the IL-4 and IFN-γ in chronically stressed rats were reversed by AC as well as α-asarone treatments while the IL-2 levels, though were markedly increased from the stressed animals, could not be completely reversed.

Almost any type of physical or mental stress can lead within minutes to greatly enhanced secretion of ACTH and consequently cortisol as well, often increasing cortisol secretion as much as 20-fold.[42] Cortisol suppresses the immune system, causing lymphocyte production to decrease markedly. The T lymphocytes are especially suppressed.[43] A variety of lymphocyte functions, including activation, proliferation, and differentiation, are sensitive to glucocorticosteroids. Although glucocorticosteroids do not affect T-cell activation, down-regulation of RNA synthesis decreases proliferation.[44] A stress as simple as overexercising for three weeks could cause lowered CD4 counts, and that they did not correct for at least three more weeks after returning to a normal exercise schedule.[45] Oluwatobi[46] observed a decrease in white blood cells and helper T-cells (CD4 cell) counts in warm water swimming stress and prolonged stress and attributed this to the buildup of the unused adrenergic stress hormones which is known to hinder the normal functioning of the immune system as well as CD4 counts. Batuman et al.,[47] observed a significant decrease in the total number of mononuclear cells, particularly

suppressor/cytotoxic (CD8) T cells, in the spleen and blood and suggested that lower levels of IL-2 production during stress could be one reason for the decreased mitogen responsiveness of T cells, often seen with stress. These results correlate with our findings that prolonged stress decreases the CD4 and CD8 cell counts significantly. Also, our study is parallel to other previous works where similar findings have been documented.[48,49] Noise stress induced decline in the CD4 and CD8 cells in spleen were significantly prevented in both AC and α-asarone treated stressed animals.

Lipids when reacted with free radicals can undergo the highly damaging chain reaction of lipid peroxidation. The damage caused by lipid peroxidation is highly detrimental to the functioning of the cell[50] and such membrane changes may be a contributing factor for the altered immune status observed in this study. The cell immediately responds to this stress by up regulating its antioxidant defense. As reported earlier[51,15], the LPO in the noise stress exposed animals in this study was also observed to increase significantly from the controls which was prevented by the administration of AC as well as α-asarone.

Glutathione plays a unique role in the cellular defense system against toxic chemicals of endogenous and exogenous origin[52]. In addition, GSH has been reported to be involved in protein and DNA synthesis, in the maintenance of cell membrane integrity, and the regulation of enzyme activity.[53] Depletion of GSH increases vulnerability to free radical induced damage. Interleukin 2-dependent functions, including T-cell proliferation, the generation of CD8+ T-cell blasts, cytotoxic T-cell activity, lymphokine-activated killer cells and natural killer cells, are particularly sensitive to glutathione depletion.[54] Thus, the decreased GSH observed in the stressed group of our study could be another reason for the decreased T lymphocyte phenotypes in the spleen of noise stress exposed animals. The increased LPO in the stressed animals could be attenuated by AC and α-asarone treatment. Though the GSH levels in the stressed animals could be alleviated on treatment with α-asarone, it could not be maintained at control levels by administration of AC.

Conclusion

Chronic stress could be detrimental to our health since it affects the immune cells and the signaling proteins, cytokines as observed in this study. Our study suggests that AC could be a potential herbal adaptogen to combat the immunosuppression mediated by noise stress. The immunomodulating effect of AC could be due to its active principle- α-asarone which was also observed to alleviate the effect of chronic stress.

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