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Original Article

EVALUATION OF STRESS ALLEVIATING POTENTIAL OF PERGULARIA DAEMIA ON CERTAIN IMMUNOLOGICAL PARAMETERS

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ABSTRACT

Objective: *Pergularia daemia* [PD] Forsk, commonly called "Veliparuthi" in Tamil belongs to the family of Asclepiadaceae. It has been used by villagers as traditional folk medicine for various ailments such as antipyretic, anti-venom, emmenagogue, anti-helminthic, and to treat malarial fever, infantile diarrhea. Scientific reports revealed several pharmacological properties of PD extract. Preliminary *in vitro* studies on the plant revealed the antioxidant, anti-inflammatory and antimicrobial activities of the plant. So the present study planned to investigate the *in vivo* stress alleviating the potential of PD extract on noise stress induced model of wistar albino rats.

Methods: Adult Wistar albino rats were exposed to pure tone noise stress for 45 min for one day and the effect of ethanolic PD extract on plasma corticosterone, organ weight body weight ratio of lymphoid organs and adrenal gland, immunological parameters like total leucocyte count, differential leukocyte, neutrophil adherence test and candida phagocytosis was studied.

Results: Results revealed that acute stress significantly increased the plasma corticosterone level [p<0.001] and decreased the organ weight body weight ratio of the spleen [p<0.001] and thymus [p<0.01]. It also significantly decreased the total leucocyte count [p<0.001], percentage of neutrophils [p<0.001], eosinophils [p<0.004] and lymphocytes [p<0.001], neutrophil adherence [p<0.001] and enhanced the phagocytic index and avidity index [p<0.001] in acute noise stress exposed groups. Administration of PD extract significantly prevented those acute noise stress-induced changes [p<0.001].

Conclusion: We can be PD extract protected against the noise stress induced changes in albino rats and this stress alleviating and immunomodulatory potential of the plant may be attributed to the phytochemicals and bioactive compounds present in it.

Keywords: Stress indices, Immunomodulatory, bioactive compounds, *Pergularia daemia*

INTRODUCTION

Stress is a state of psychological and physiological imbalance that occurs due to the disparity between situational demand and person's capability to react to the situation [1]. In the modern era, amidst the stressful situations that occur in the environment, noise happens to be the commonly encountered stressor. Noise is referred to a loud, unpleasant, annoying and unwanted sound that a person perceives from the environment. It is postulated that noise exposure of any kind that exceeds 90 Db is considered to be a source of stress [2]. World Health Organization gave estimation that approximately 20% of the population is constantly exposed to urban traffic noise which is greater than 65 Db and 40% of them are exposed to noise levels between 55-65 dB and these exposures resulted in various disorders [3].

A stressful episode causes activation of the hypothalamo–pituitary adrenal axis which leads to the release of cortisol, the stress hormone. Cortisol affects all systems of the body including the immune system. A long-term increase in glucocorticoid hormone causes immuno-suppression [4]. Acute as well as chronic exposure to noise stress affects the immune system [5].

In the present world, due to the increased prevalence of environmental pollutants like dust, noise and industrial waste, we are prone to many diseases that affect our immune system. A very effective immune status is essential to maintain good health. Improving our immunity can be a good step to prevent against the immune-based disorders. Our traditional medicine in India uses a variety of herbs that can be used as immune boosters and can counteract against various diseases. Many Ayurvedic herbs like Holy basil, Ginger, Ashwagandha, Triphala, cardamon, Licorice have been credited with diverse beneficial effects on immune health [6]. As the search for novel herbal immune boosters continues, the present study planned to evaluate the stress alleviating potential and immunostimulant properties of pergularia daemia on noise stress-induced immunosuppression in albino rats.

Pergularia daemia is now emerging as one of the herbal medicinal plants with a good antioxidant and anti inflammatory properties. It is hispid fetid smelling lactiferous twinner grown around the tropical regions of India. The plant was used by villagers for various ailments as an emmenagogue, anti-helminthic, emetic, expectorant, antiseptic and anti-venom antipyretic and a good appetizer. The stem bark is used to treat malaria, infantile diarrhea, rheumatic swellings, delayed childbirth, menstrual problems. Dried leaf is used to treat bronchitis, cough and whooping cough [7].

Many pharmacological properties of PD like hepatoprotective [8], antimicrobial [9], antifertility [10], diuretic [11], psycho-pharmacological [12], anti-inflammatory [13] activities have been screened by scientists. The present study was planned to investigate the stress alleviating potential of *Pergularia daemia* against noise stress induced changes in albino rats.

MATERIALS AND METHODS

Plant collection

The plant PD was collected from Karuppur, Salem district, Tamil Nadu, India and the voucher specimen was deposited in the Herbarium and authenticated in CAS Botany, University of Madras. [Authentication No. MUCASB-H106] dated 12.1.2012 by Dr. K.

Murugesan, Professor in Botany, University of Madras, Guindy campus.

Preparation of ethanolic extract of *Pergularia daemia*

The aerial parts consisting of leaves stem and flowers were taken and washed with water to remove the dust and the sand. It was shade-dried at room temperature. The dried portion of the plant was ground in an electric blender, and its fine powder was collected by sieving.

The ethanol extract of the powder was obtained by treating it with 95% ethanol in a soxhlet apparatus by continuous heat extraction for 72 h. At the end of extraction, the extract was concentrated to dryness under vacuum on a rotary evaporator at 40 ° C and the final product was stored.

Drug delivery

The suspension of PD extract was prepared by dissolving it in 5 % tween 80 [10 gm/100 ml]. A pilot study was conducted to find out the effective therapeutic dose of PD extract. Different doses of PD extract like 100 mg/kg, 200 mg/kg, and 300 mg/kg were administered. The minimal effective dosage of PD extract as anti stress was found to be 200 mg/kg b.w. The Lethal dose of the ethanolic extract [EE] and ethanol fraction [EFEE] PD extract in albino rats was reported to be 2000 mg/kg b.w.[8]. One tenth of the maximum dose of PD extract tested for acute toxicity ie., 200 mg/kg b.w. was also considered in our study. PD extract was administered at a dose of 200 mg/kg b.w. to the experimental animals orally using a gavage.

Animal groups

Healthy male adult wistar albino rats, weighing 150–170 gm were selected. All the animals were maintained at standard laboratory conditions under 12 h light/dark cycles at 25±2 °C and were provided with food and water *ad libitum*. All experimental procedures were approved by the Institutional Animal Ethical Committee IAEC No is [03/007/2014] under the guidelines of Committee for Purpose of Control and Supervision of Experiments on Animals [CPCSEA], Government of India.

The animals were divided into five groups, each containing 6 animals

Group I: Control group-Animals were not exposed to noise stress. They were sacrificed to obtain a baseline data of all parameters.

Group II: Acute noise stress group-Animals were exposed to noise stress for 45 min and sacrificed immediately. These animals were used to evaluate the effect of acute noise-stress-induced in all parameters

Group III: Acute noise stress with PD treated group-Animals were treated with PD extract at a dose of 200 mg/kg b.w. for 7 d, subjected to noise stress for 45 min on the 8th day and sacrificed immediately. These animals were used to determine the effect of PD extract on acute noise-stress-induced changes in all the parameters.

Group IV: PD treated alone for 7 d group-Animals were treated with PD extract only at a dose of 200 mg/kg b.w. for 7 d and sacrificed on 8thday. These animals were used to find out the effect of PD extract alone on all the parameters.

Group V: Vehicle control group-Animals were treated with 5 % tween 80 alone for 7 d and sacrificed on the 8th day. These animals were used to find out the effect of the vehicle [5 % tween 80] alone on all parameters.

Noise stress induction

Animals were exposed to pure tone noise with the intensity of 100 dB and frequency of 10 KHz. The pure tone noise was obtained by using a function generator and amplified by an amplifier. The amplifier was connected to two loudspeakers located 30 cm above the cage. A sound level meter is used to measure the intensity of noise. The background noise level in the room was found to be 44±2 dB due to the ventilation system.

All experiments were carried out in the forenoon between 9 am to 10 am to avoid circadian rhythm variations. The blood samples were collected by using the technique of Feldman and Comforti [1980] [14]. 3 ml of blood was collected in a heparinized syringe and used for immunological protocols and used for estimation of plasma corticosterone. The spleen, thymus, lymph node and adrenal gland were removed, blotted and weighed.

Evaluation parameters

Stress indices

Determination of plasma corticosterone

Plasma corticosterone was determined by the method of Mattingly [15]. Free and protein-bound corticosterone were extracted into dichloromethane and shaken with the sulphuric acid-ethanol reagent. A resulting fluorescence reagent was prepared. A corticosterone stock solution was prepared at 100 mg/dl and was diluted to produce concentrations ranging from 10–100 µg/dl. 7.5 ml of dichloromethane was added to all the plasma samples and corticosterone standards, and the resulting dichloromethane extract phase was then transferred to a tube and allowed to react with 2.5 ml of fluorescent reagent [concentrated sulphuric acid: absolute ethanol] [70:30] and the tubes were thoroughly mixed. An aliquot of the lower phase was removed, and the fluorescence was measured at an excitation 470 nm and emission 530 nm with a spectro-photofluorometer. The results were expressed as µg/dl of plasma.

Organ weight/body weight ratio of lymphoid organs and adrenal gland

The total body weight of the animal before the animal sacrifice was taken and then later the spleen, thymus, lymph node and adrenal gland were removed, blotted and weighed. The organ weight was calculated by dividing respective organ weight with body weight [g] and multiplying with 100 [16]

Immunological parameters

Determination of total leucocyte count [TLC]

Blood was taken up to 0.5 marks in a WBC pipette and was diluted with Turks fluid up to 11 marks. The bulb of the pipette was gently rotated between palms and was kept for 10 min allowing the staining of nucleus and granules in the cells. Then the diluted blood was charged in an improved Neubauers counting chamber, and the counting of the cells was done.

Determination of differential leucocyte count [DLC]

An ideal blood smear was prepared and stained with Leishman stain for 2 min and fixed with distilled water for 7 min. Then the slide was washed and dried. The different types of leucocytes viz., neutrophils, eosinophils, basophils, monocytes and lymphocytes were counted under oil immersion objective [100 X] and the results were expressed in percentage.

Neutrophil adherence

Neutrophil adherence (NA) was determined by the method of Srikumar *et al.* 2005 [17]. After doing TLC and DLC, the remaining blood sample was incubated with 80 mg/ml of nylon fiber column which was packed in a siliconized pasteur pipette of 15 mm length under sterile conditions. After a brief period of incubation, the blood was reanalyzed for TLC and DLC. The Neutrophil index of that blood sample was given by the product of TLC and the percentage of neutrophils.

Neutrophil adherence

$$= \text{NI of untreated sample} - \frac{\text{NI of treated sample}}{\text{NI of untreated sample}} \times 100$$

[NI-Neutrophil index]

Candida phagocytosis

The phagocytic ability of neutrophils was determined by the method of Archana *et al.* 2000 [18]. 0.5 ml of heparinised blood was

centrifuged, and buffy coat was obtained. To this buffy coat, heat killed *Candida albicans* was added and incubated for 15 min at 37 °C. Using the sediment, ideal smears were prepared and stained with Leishman stain. In this duration of time, the phagocytes start engulfing the *Candida albicans*. The ingested *Candida albicans* were deeply stained and counted inside the neutrophils under oil immersion objective lens [100X]. The phagocytic index (PI) is given by the number of neutrophils positive for candida ingestion among 100 neutrophils. The avidity index (AI) is expressed as a total number of *Candida albicans* counted within 100 positive cells divided by 100.

Statistical analysis

The results were expressed as mean±SEM. The data were analyzed in SPSS version 15 by One ways ANOVA. The significance among groups was determined by Tukey's multiple comparison tests and the significance level was fixed at p<0.05.

RESULTS

Stress indices

Plasma corticosterone

Animals exposed to acute noise stress for 45 min showed a significant increase in plasma corticosterone level [p<0.001] compared to control. Pretreatment with PD extract for 7 d significantly prevented the changes caused by acute noise stress [p<0.001] [table 1]

Organ weight/body weight ratio of lymphoid organs

There was a significant decrease in the organ weight/body weight ratio of the spleen [p<0.001] and thymus [p<0.01] in acute noise stress group compared to control. Pretreatment with PD extract significantly prevented the decrease in organ weight/body weight ratio of spleen and thymus. There was no significant change in the organ weight/body weight ratio of lymph node and adrenal gland in all the groups compared to control. PD extract alone and vehicle control did not show any significant change in any lymphoid organ and adrenal gland [table 1].

Immunological parameters

Total leucocyte count

TLC showed a significant decrease in acute noise stress [p<0.001] compared to control. PD treatment significantly increased the TLC [p<0.001], thus reverting it back to normal [table 2]. PD extract alone and vehicle control did not show any significant change in TLC.

Table 1: Effect of PD extract treatment on acute noise stress induced changes in stress indices

Groups	Plasma Corticosterone [µg/dl]	Organ wt/body wt ratio [Spleen]	Organ wt /body wt ratio [thymus]	Organ wt/body wt ratio [lymph node]	Organ wt/body wt ratio [adrenal gland]
Control	36.52±0.36	3.92±0.04	1.14±0.05	0.08±0.02	0.219±0.02
Acute noise stress	92.09±0.44*	3.22±0.03*	1.07±0.04*	0.09±0.01	0.220±0.02
Acute noise stress with PD treatment	65.99±0.39#	3.89±0.03#	1.15±0.05#	0.09±0.01	0.220±0.02
PD treatment alone	65.99±0.39	3.91±0.03	1.14±0.05	0.09±0.01	0.221±0.02
Vehicle control	37.09±0.30	3.92±0.04	1.14±0.04	0.09±0.01	0.222±0.02
P value	(p<0.05)	(p<0.05)	(p<0.05)	(p<0.05)	(p<0.05)

Values are expressed as mean±SEM. "*"indicates significance compared to control and #"indicates significance compared to acute noise stress group. Significance level was fixed at p<0.05

Table 2: Effect of PD extract treatment on acute noise stress induced changes in total leucocyte count

Groups	Total leucocyte count [cells/m^3]
Control	14388.33±7.57
Acute noise stress	7421.67±7.34*
Acute noise stresses with PD treatment	13611.67±6.91#
Pd treatment alone	15263.33±7.38
Vehicle control	14678.33±6.71
P value	(p<0.05)

Values are expressed as mean±SEM. "*"indicates significance compared to control and #"indicates significance compared to acute noise stress group. Significance level was fixed at p<0.05

Differential leucocyte count

DLC showed a significant decrease in the percentage of neutrophils [p<0.001], eosinophils [p<0.004] and lymphocytes [p<0.001] in acute noise stress groups compared to control. There was no significant change in basophils and monocytes. PD extract treatment significantly increased the neutrophils [p<0.001], eosinophils [p<0.02], lymphocytes [p<0.05] as it reduces the impact of acute noise stress on the animal. Vehicle control and PD alone did not show any change in all the parameters [table 3].

Table 3: Effect of PD extract treatment on acute noise stress induced changes in differential leucocyte count

Groups	Neutrophil %	Eosinophil %	Basophil %	Lymphocyte %	Monocyte%
Control	21.50±0.24	9.17±0.18	0.16±0.11	0.50±0.12	0.50±0.12
Acute noise stress	18.00±0.22*	7.67±0.15*	0.16±0.11	0.30±0.12*	0.50±0.12
Acute noise stress with PD treatment	20.00±0.24#	8.67±0.15#	0.16±0.11#	0.30±0.12#	0.50±0.12
Pd treatment alone	21.80±0.20	9.00±0.15	0.16±0.11	0.50±0.12	0.50±0.12
Vehicle control	21.50±0.23	9.00±0.15	0.16±0.11	0.50±0.12	0.50±0.12
P value	(p<0.05)	(p<0.05)	(p<0.05)	(p<0.05)	(p<0.05)

Values are expressed as mean±SEM. "*"indicates significance compared to control and #"indicates significance compared to acute noise stress group. Significance level was fixed at p<0.05

Neutrophil adherence

Neutrophil adherence to nylon column significantly decreased [p<0.001] in acute noise stress group compared to control and treatment with PD extract significantly increased the NA [p<0.001]. Vehicle control and PD alone did not show any change in NA [table 4]

Phagocytic index

The number of neutrophils positive for candida phagocytosis was significantly enhanced in acute noise stress group compared to control [p<0.001]. Treatment with PD extract significantly decreased the PI compared to acute noise stress group [p<0.001]. Vehicle control and PD alone did not show any change in PI [table 4].

Avidity index [AI]

Animals exposed to acute noise stress showed a mild increase in the average number of candida engulfed by neutrophils reflected in the AI compared to control [p<0.001]. Treatment with PD extract significantly decreased the AI compared to acute noise stress group [p<0.001]. Vehicle control and PD alone did not show any change in AI [table 4].

Table 4: Effect of PD extract treatment on acute noise stress induced changes in neutrophil adherence and candida phagocytosis

Groups	Neutrophil adherence [%]	Phagocytic index [%]	Avidity index
Control	23.23±0.16	65.42±0.18	2.49±0.05
Acute noise stress	9.38±0.15*	67.79±0.15*	3.71±0.07*
Acute noise stresses with PD treatment	18.62±0.21#	65±0.17#	3.07±0.08#
Pd treatment alone	24.67±0.17	65.71±0.18	2.44±0.04
Vehicle control	23.58±0.16	65.19±0.14	2.5±0.04
P value	(p<0.05)	(p<0.05)	(p<0.05)

Values are expressed as mean±SEM. "*" indicates significance compared to control and" ±"indicates significance compared to acute noise stress group. Significance level was fixed at p<0.05

DISCUSSION

Stress indices

Plasma corticosterone

Exposure of animals to noise stress for 45 min produced a highly significant increase in plasma corticosterone which coincides with the previous reports by Sembulingam *et al.* 1997 and Manikandan *et al.* 2005 [19,20]. This is due to the activation of the hypothalamic-pituitary-adrenal [HPA] axis, where the corticotrophin releasing hormone [CRH] from the hypothalamus initiates the release of adrenocorticotrophic hormone [ACTH]. ACTH stimulates the release of corticosterone from the adrenal cortex. Pretreatment with PD extract significantly decreased the plasma corticosterone levels, thus preventing the impact of noise stress on the animal.

Organ weight/body weight ratio of lymphoid organs and adrenal gland

Acute exposure to noise stress showed a significant decrease in weight of spleen, thymus, and no change in the weight of lymph node and adrenal gland. During stress, stimulation of sympathetic nervous system results in a splanchnic vasoconstriction that shifts blood to peripheral circulation, thereby showing a decrease in weight of spleen and thymus [21].

Immunological parameters

Total leucocyte count

In the present study, exposure to acute noise stress produced a highly significant decrease in total leucocyte count compared to control. This is in coincidence with the previous reports by Sembulingam *et al.* [21]. This decrease in TLC may be attributed to the acute rise in glucocorticoid secretion which affects the circulation of the cells. PD extract treatment significantly maintained the normal circulation of TLC which may be due to the insignificant elevation in the level of glucocorticoids.

Differential leucocyte count

Differential leucocyte count showed a significant decrease in eosinophils, neutrophils, and lymphocytes, but did not reveal a significant change in basophils and monocytes in acute noise stressed group compared to control. This also may be caused due to the increase in plasma corticosterone level produced during acute noise stress exposure. Pretreatment with PD extract significantly prevented the acute noise stress-induced decrease in eosinophils, neutrophils and lymphocytes thus maintaining the normal circulation of leucocytes.

Neutrophil adherence test

Phagocytosis, a process that destroys the foreign organism involves three steps, margination, and attachment to the organism, engulfment and release of lysosomal enzymes from the neutrophils to kill the organism. Margination by neutrophils from the bloodstream requires a very firm adhesion. β2 integrins that are stored in the granules of neutrophils increase in number and accelerates the adhering capacity of neutrophils [17]. In the present study, acute noise stress exposure significantly decreased the neutrophil adherence to nylon and this coincides with the studies by Archana *et al.* 2013 [5]. This may be due to shedding or internalization of β2 integrins that occurs due to the increase in plasma corticosterone level.

In the present study, Pretreatment with PD extract significantly increased the adherence of neutrophils, which indicates that the process of margination of neutrophils along the blood vessels was enhanced by PD extract as it prevents the internalization of β2 integrins in the neutrophils.

Candida phagocytosis

Animals exposed to acute noise stress showed an enhanced phagocytic index and avidity index in candida phagocytosis. The response of neutrophils may be controlled by the sympathetic nervous system [22]. Neutrophils are enhanced with alpha and beta adrenergic receptors. So, increased sympathetic stimulation during exposure to noise stress initiates the c AMP mechanism, resulting in the release of lysosomal enzymes from the neutrophils. Thus, the killing ability of neutrophils is enhanced in acute noise stress exposure. Pretreatment with PD extract significantly prevented the increase in phagocytic index and avidity index. This emphasizes the stress alleviating potential of PD extract in acute noise stress treated groups. In a study reported by Hemanthkumar *et al.*, when heptane extract of PD was subjected to *in vitro* antimicrobial activity, it showed the highest zone of inhibition against the bacteria *E. coli* and *Candida albicans* [23].

Thus, the results revealed that ethanolic extract of Pergularia daemia significantly prevented the immunosuppression induced by acute noise stress. Phytochemical analysis of different extracts of PD revealed the presence of alkaloids, terpenoids, tannins, saponins, flavonoids, and glycosides [24]. Saponins are a class of glycosides that is found widely in plants having detergent properties and is stable both in alkaline and acidic media. These saponins possess antioxidant, anti-inflammatory, anti-apoptosis and immuno-stimulant properties. Tannins can inactivate and kill micro-organisms and can act as a potential anti-viral, antibacterial and

anti-parasitic agent [25]. These two phytochemicals were found to be present in the ethanolic extract of PD. The bioactive compounds in PD extract responsible for its immunostimulatory effects have been identified by Gas mass spectrometric analysis. They are 2-hydroxy-methyl ester, 2-Methoxy-4-vinylphenol, Phthalic acid di-[1-hexen-5-yl] ester, l-[+]-Ascorbic acid 2,6-dihexadecanoate and Methyl [Z]-5,11,14,17-eicosatetraenoate [26]. These bioactive compounds in PD extract may be responsible for its antistressor activity against acute noise stress.

CONCLUSION

The present study reveals that acute noise stress exposure increases the stress markers and produces alterations in various parameters of immune functions. Administration of PD extract reduced the noise stress induced changes in those animals. Thus, we conclude that PD possesses a potent anti-stressor activity with immunostimulant properties. Further immunological studies may authenticate the role of PD extract in immunomodulation and establish the effective use of the plant in stress-induced immune disorders.

CONFLICT OF INTERESTS

Declared none

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