ANTI-APOPTOTIC ROLE OF *SCOPARIA DULCIS* ON NOISE – INDUCED EXPLORATORY AND LOCOMOTOR BEHAVIOR IN WISTAR ALBINO RATS

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Abstract:
Exposure to noise stress is inevitable and stressful experience can alter the neuroendocrine axis which is detrimental to the neurobehavior of the animal. To date, there is no medication to combat this environmental stressor. Targeting herbal remedies with potent antioxidative and anti-apoptotic effects could help narrow down the therapeutic approach against this illness. To induce phschology stress, animals were exposed to noise stress (100 dB/4 h/day) for 30 days. This study showed that elevated corticosterone level at 100dB indicates that noise is a psychological stressor. Corticosterone can have rapid or long-term influences on neural circuits and can significantly influence behavior. In the open-field test, locomotor function of the animals was observed via increased rearing and decreased peripheral and central ambulation. Increased immobilization is a sign of despair and depressive behavior. The influence of increased dopamine, norepinephrine, and serotonin level when exposure to noise could have altered the emotional behavior of the animal. On the other hand, increased expression of Bcl-2 by 53.39% when compared to Bax in the noise-exposed group indicates the capability of the animal to stress response. Therapeutic strategies of *S. dulcis* via their antioxidative and anti-apoptotic properties can act as an antidote for this pervasive environmental stressor.

Keywords: Corticosterone; Locomotor; Bcl-2; Apoptosis; Noise; Scoparia dulcis.

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Introduction

With an ever-expanding population and urbanization, noise exposure is inevitable and insidious. Noise can cause nervous, endocrine, and cardiovascular disorders, increased blood pressure, hypertension, and sleep disturbances [1-4]. Stressor like noise affects the HPA axis through the limbic system via the auditory pathways [5]. On exposure to stress, Robert et al.[6] reported that the body release glucocorticoids. However, overstimulation of these glucocorticoids can trigger free radical generation causing oxidative damage to the cell they are meant to protect. Noise at 100 decibels/4 hours/day can generate unwarranted free radicals in the brain[7]. High PUFA content also makes the neurons susceptible to noise-induced lipid peroxidation, reduced dendritic count, decreased memory and cognition[8]. In addition, exhaustion, defeat, motor coordination, mood change, depression, and anxiety are common factors associated to noise-induced illness [9-12].

To date, there is no medication to combat this environmental stressor. Targeting herbal remedies with potent antioxidative and neuroprotective effects could help narrow down the therapeutic approach to dealing with this illness. *S. dulcis* (Scrophulariacae) is known to have neuro-protective, cytoprotective,[13] acetylcholines-terase,[14] nephroprotective,[15] antimicrobial, anti-fungal, [16, 17] analgesic, antipyretic [18] antidiabetic, antihyperlipidemic[19] sedative, hypnotic[20] antisick-ling, [21] antioxidative and anti-inflam-matory properties [7, 9, 22]. The therapeutic properties is related to the presence of active compounds live Flavonoids, Diterpenoids, polyphenols etc. Favones, favonols, favan-3-ols, favanones and Diterpenoids like scopadulcic acids A and B, scopadulciol, scopadulin, scopadiol and scoparic acids A-E [23-25] are the falvanoids present in the plant. There are a number of terpenoids, such as alpha-amyrin, betulinic acid, dulcioic acid, friedelin, glutinol, and ifflaionic acid [26-28]. The plant also contained compounds like tannins, coumarins, phenols, saponins, steroids, and sugars. *S. dulcis* major constituents, like flavonoids and terpenoids, possess neuro-protective activity. The aims of this study is to determine the neuroprotective role of *S. dulcis* in mediating behaviour and understand its influence on the mitochondrial apoptotic proteins when exposed to noise stress.

Materials and method

Chemicals

The analytical-grade chemicals utilised in this study were purchased from Sigma-Aldrich and Sisco Research Laboratory in India. The secondary antibodies were acquired from (Merck, India), and the primary antibodies were purchased from Sigma Aldrich, USA. Purchased a DAB system from Pierce, USA. Dihydroxybenzy-lamine, norepinephrine, dopamine, and serotonin standards were purchased from Sigma Aldrich, USA.

Plant Identification and extraction

The plant *S. dulcis* was authenticated (NIS/ MB/ 62/2012) and deposited in the National Institute of Siddha herbarium. At 60° C 500 g of Scopria dulcis leaves were extracted with 1.5 L of methanol using the Soxhlet apparatus. The extract was lyophilized, dried, and filtered using Whatman No. 1 filter paper.

Animals

Wistar strain, healthy adult male albino rats weighing 180–220 g were kept in conventional lab cages with two rats per cage. The Institutional Animal Ethical Committee gave its approval with IAEC no. 01/20/2013, dated February 20, 2013. Animal experiment was carried out in accordance with institutional and national laws protecting the welfare of animals.

Noise stress induction procedure

Two loudspeakers (15W each) mounted 30 cm above the cage and powered by a white-noise generator (0-26 kHz) created the noise. A sound level metre D2023 (S.NO-F02199; Cygnet Systems, Gurgaon, Haryana, India) was used to measure the noise level. The animal was exposed for 4 h/day for 30 days to create a stress model.

Experimental groups

Animal experiment was carried out between 8 and 10 am in order to prevent changes caused by circadian rhythm and metabolism. Four groups of six animals each were formed by randomly dividing the animals.

Group I the control animals received saline (0.9%) orally and were kept in the noise cage without noise stimulation.

Group II exposed to 100 dB broadband white noise for 4 h daily for 30 days.

Group III conisist of animal administrated with methanol extract of *S. dulcis* (200 mg/kg.b.w) as per Mishra et al. [29].

Group IV conisist of animal exposed 100 dB/hr for 30 days and administrated with methanol extract of *S. dulcis* (200 mg/kg.b.w) for 48 days. After the experiment, animals were administered with ketamine and xylazine combination (90/50mg/kg.b.w.) to anaesthesia for blood and tissue sample collection.

Neurobehavioral studies

The open field test (OFT) was used to examine the emotional and locomotor state of animals [30]. According to Brown et al. [31], the open field measured 72 cm in length and 36 cm in height. The floor is divide into 16 square and each square measuring 18 x18 cm. Randomly, six rats were chosen from each group. Each rat was placed into one of the four corners of the field, independently, and given five minutes to expore the OFT. After every test, the floor was cleaned with 70% ethyl alcohol.

Estimation of corticosterone

The method involves step including ferrous iron (II), potassium hexacyanoferrate, and ferrous iron (III). First, 0.5 ml of the sample mixed with working corticosterone solutions. 0.5 ml of potassium hexacyanoferrate (III) solution was mixed with 2 ml of sulfuric acid and 2 ml of ferric chloride. The mixture was heated for 30 minutes with periodic shaking in a water bath maintained at 70± 2° C. At 780 nm, the absorbance was measured in comparison to a reagent blank [32].

Determination of neurotransmitter concentrations

Neurotransmitter concentration in the brain was estimated as per method of Wagner et al. [33]. Immediately after cervical dislocation, the brain was dissected on a ice-cold plate [34]. A high-performance liquid chromatography (HPLC) combined with electrochemical detection (ECD) was used to assess the concentrations of norepinephrine (NE), dopamine (DA), and 5-hydroxytryptamine (5-HT). Perchloric acid was used to homogenise the hippocampus. Citric acid, disodium hydrogen orthophosphate, EDTA, octane-1-sulphonic acid, sodium salt, and 14% methanol were all present in the mobile phase, adjusted to pH of 4.0 using di sodium hydrogen orthophosphate. Hippocampus homogenates were centrifugie for 2 minutes at 12,000 rpm /4 °C. The standard dihydroxy benzylamine (DHBA) was then added to the brain homogenate's supernatant, and centrifugation was again performed for 20 minutes at 12,000 rpm. A 0.22-m membrane filter was used to filtere the supernatant. To separate the monoamine, 20µl of the sample were injected into the reverse phase column LiChro CART RP-18 and isocratic pump (Model 501; Waters Association, Millipore, MA, USA) of the HPLC system, which is linked to the Rheodyne injector (Cotati, CA, USA). To detect the reaction products, electrochemical detector (Model 460, Water), connected to the HPLC system and set at a potential of +0.60 V to identify monoamine neurotransmitters. The flow rate was monitored at 0.8 ml/min and NE, DA, and 5-HT were quantified using a C-R8A data processor (Shimadzu, Kyoto) and expressed as nanograms of neurotransmitter per gram of wet weight of brain tissue.

Immunoblotting

Radioimmunoassay buffer (RIPA) (Sigma) and protease inhibitor were used to prepare the tissue lysate. On 10% SDS-PAGE, equal amounts of protein (60 g) were electrophoretically separated and then transferred to the PVDF membrane (Millipore, USA). The membranes were treated in blocking buffer containing 5% skimmed milk for 2 hours to prevent nonspecific binding. Primary Bcl-2 and BAX protein (Biovision) antibodies were used to probe membranes. The membrane was later incubated with Horseradish peroxidase-conjugated secondary antibodies (1:10,000) from Merck. Using the an ECL kit (Millipore, USA) bands were develop in a Chemi Doc image scanner from Bio-Rad an Quantity One software (Bio-Rad, USA) was used to measure the band intensity. The membranes were stripped and reprobed (1:5000) with β-actin that serve as an internal control.

Histology

Histology was done in accordance with Bancroft and Cook [35]. Animals were given 90/50 mg/kg bw of ketamine and xylazine to anaesthetized the animal. Phosphate-buffered saline and 10% buffered formalin were transcardially infused into the rats and the brain was removed and preserved in formalin until processed for histology. The formalin pigments from the fixed tissue were then washed away with running water before dehydrating with ascending grade of alcohols. The tissed was then impregnated with paraffin wax. The block was sliced with a thickness of 7 to 10 m were cut using a Spencer Lens rotatory microtome (model no. 820, New York, USA).

Statistical analysis

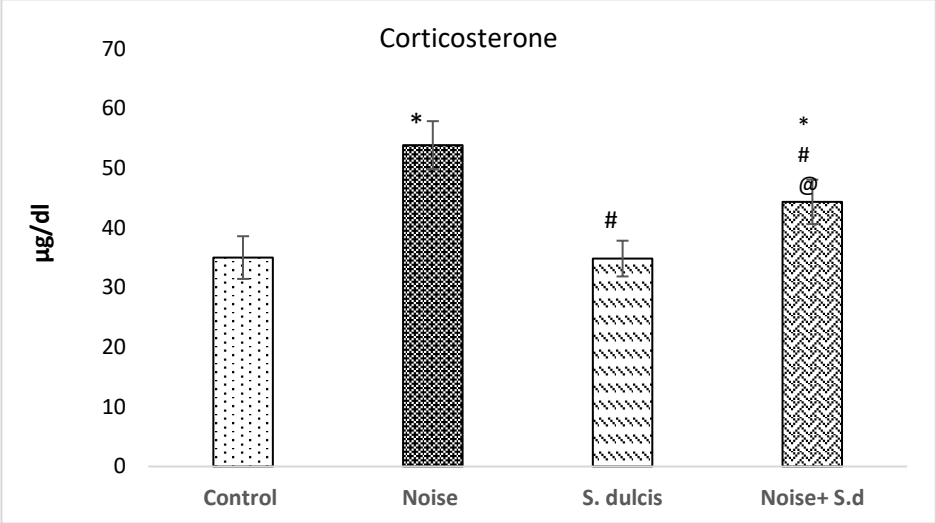
Each group included 6 animal and the data were reported as mean standard deviation (SD). The

SPSS for Windows statistical package (version 20.0, SPSS Institute Inc., Cary, North Carolina) was used to analyse all of the data. Statistical significance differences was determine using One-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests with the significance fixed at p 0.05 between the various groups.

Result and Discussion

Elevated corticosterone level in this study **(Fig:1)** indicates that noise at 100dB is a psychological stressor.

Fig: 1. Effect of *S. dulcis* on corticosterone level when exposed to 30 days of noise stress.



Values are expressed as Mean ± SD, N=6, "*" compared with control; "#" compared with noise "@" Compared with *S. dulcis* control. Significance at p < 0.05. The symbols represent statistical significance: *, # & @ < P 0.05

Noise can activate the limbic system to release amygdaloid Corticotropin-releasing hormone (CRH), which influences the neuroendocrine and behavioural responses [36]. In addition to the amygdala, the hippocampus is also a component of the limbic system that involves in learning, memory, and behavioural and emotional responses. The hippocampus is sensitive to stress due to the high concentration of glucocorticoid and mineralocorticoid receptors [37]. High glucocorticoid levels can alter the plasticity and structural integrity of the hippocampus and prefrontal cortex, [38] which can ultimately influence behaviour. Altered emotional and locomotor behaviour observed using the open field test on exposure to 30 days of noise does not only involves CRH and glucocorticoid stimulation. Other factors, such as neurotransmitter regulation, can also influence the behaviour of the animals. Increased hippocampal norepinephrine levels on noise exposure

(Table:1) indicate the involvement of sympathetic stimulation in response to stress. The dopaminergic reward system is essential for monitoring and coping with stressful events [39]. It is also notable that dopamine

(Table: 1) can act as a percussor for synthesizing more epinephrine and norepinephrine as a compensatory response to adapt to stress [9].

Table: 1. Effect of *S. dulcis* on neurotransmitter levels (ng/g) of tissue in hippocampus when exposed to 30 days of noise stress.

Neurotransmitter	Control	Noise	*S. dulcis*	Noise +Sd
Norepinephrine	10227.11±845.97	17044.57±1172.20*	11972.26±1128.00$^{\#}$	14300.44±882.86$^{*\#@}$
Dopamine	828.97±63.49	1513.75±67.86*	972.79±99.65$^{\#}$	1249.88±118.30$^{*\#@}$
Serotonin	2097.25±346.91	3138.21±185.40*	2164.73±236.18$^{\#}$	2715.89±200.53$^{*\#@}$

Values are expressed as Mean ± SD, N=6, "*" compared with control; "#" compared with noise "@" Compared with *S. dulcis* control. Significance at p < 0.05. The symbols represent statistical significance: *, # & @ < P 0.05.

Noise-induced exploratory, locomotor and emotional status

In the open field test, rearing is an exploratory behaviour where the animal stands on its hind legs to analyze the environment [40]. Locomotion is motivated by exploration, and rearing is an inquisitive character that indicates the animal's innate behaviour to explore its surroundings. Decreased rearing and increased faecal pellet **(Table:2)** observed in the noise-exposed group indicate the altered exploratory and emotional behaviour of the animal. Hippocampal morphology **(Fig: 2)** and rearing have showed that hippocampal intrapyramidal and infra-pyramidal mossy fibres are directly linked [40, 41]. In addition, decreased peripheral square crossing indicates decreased locomotor activity, and decreased central square crossing indicates anxiety-like behaviours in the animal **(Table:2).**

Table:2. Effect of *S. dulcis* on exploratory, locomotor and emotional behavior of Wistar albino rats when exposed to 30 days of noise stress.

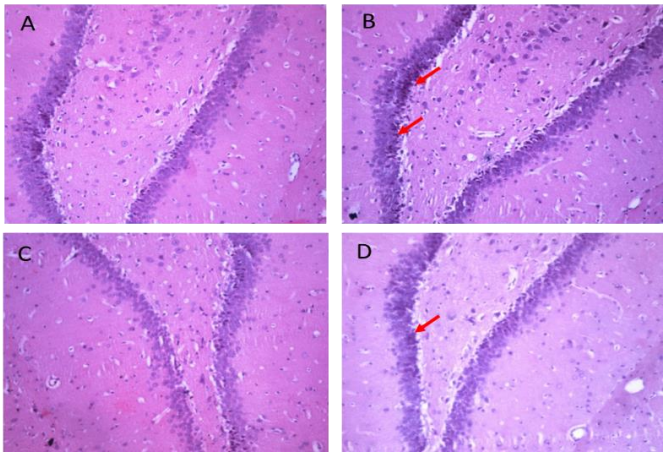
Parameters	Control	Noise	*S. dulcis*	Noise +Sd
Peripheral (Day-1)	62.66 ± 5.16	38.66 ± 3.26*	61.66 ± 5.60	46.5±3.33*#@
Peripheral (Day -30)	60.83± 3.43	41.83 ± 3.54*	59.63 ± 3.54	53.16±4.07#
Central (Day -1)	3.83± 0.75	1.16 ± 0.98*	3.66 ±1.21	3.16±0.89#
Central (Day-30)	7.66± 1.03	3.16 ± 0.75*	7.5 ±1.04	5.83±1.47#
Immobilization (Day-1)	19.83± 1.72	41.66 ± 3.26*	19.16 ±2.22	32.66±3.50*#@
Immobilization (Day-30)	20.5±2.42	31.66 ± 2.16*	21.33 ±3.82	23.33±3.38#
Grooming (Day-1)	16.0± 2.60	8.33±1.21*	14.16± 2.22	11±1.66*#@
Grooming (Day-30)	16.33±2.16	11.66±1.22*	15.0±2.19	14.5±2.07#
Rearing (Day-1)	21.66± 2.16	12.5±1.87 *	20.16±1.72	17.0±1.41*#@
Rearing (Day-30)	21.83± 2.22	13.5±1.87*	21.83±2.31	19.33±1.86#
Fecal Bolus (Day-1)	1.83±0.75	4.66 ±1.03*	1.5 ± 1.40	2.83±0.75#
Fecal Bolus (Day-30)	1.16±0.98	3.83 ±1.47*	1.33± 1.21	1.50±1.22#

Values are expressed as mean ± SD from six animals. Ambulation (peripheral and central squares) was expressed in number of square entries. Immobilization was expressed in seconds. Rearing and grooming were expressed in number of attempts. Fecal bolus was expressed in numbers. Values are expressed as Mean ± SD, N=6, "*" compared with control; "#" compared with noise "@" Compared with *S. dulcis* control. Significance at $p < 0.05$. The symbols represent statistical significance: *, # & @ < P 0.05.

This agrees with the fact that increased central area exploration reflects a low level of anxiety and sporadic peripheral square crossing indicates decreased locomotor activity [42, 43]. Motor deficits due to altered dopamine levels in the cerebellum and the striatum on exposure to noise [9] could be a factor associated with decreased locomotor function observed in this study. On the other hand, immobilization is an indicator of despair and depression [44] caused by stressful events. Increased immobility and depression are correlated, and it was reported that anti-depressant drugs reduce immobility [45]. Chronic noise stress is known to alter biogenic amine levels, and serotonin is essential for mood regulation and well-being. Increased serotonin levels observed in this study

(Table: 1) could have been related to an increase in tryptophan hydroxylase (TPH), a rate-limiting enzyme in 5-HT synthesis. Grooming, on the other hand, is a displacement response behaviour [46]. A gradual increase in grooming on the 1st and 30th day clearly shows that the animals did not adapt to the open environment even after repeated noise exposures **(Table: 2)**. It is possible that treatment with *S. dulcis* could alleviate emotional and locomotor activity due to the antioxidant properties of the plant, thereby protecting the hippocampal neurons from noise induce damages **(Fig: 2)**. *S. dulcis* displayed significant free radical scavenging and antioxidant properties [17].

Fig.2. Effect of S. *dulcis* on hippocampus histology when exposed to 30 days of noise stress.

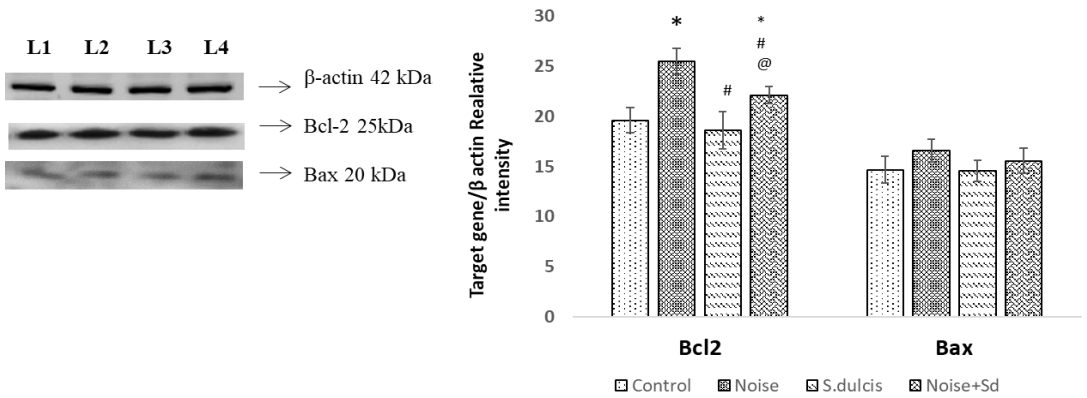


Illustrates H&E stain of hippocampus (10X) in different experimental groups. (A) Control animals (B) Noise exposure for 30 days (C) *S. dulcis* treated control (D) Noise treated with *S. dulcis* treated

Noise-induced free radical generation and mitochondrial-mediated apoptosis

Noise stress can generate unwarranted free radicals, lipid peroxidation, and altered antioxidant system in the brain [7]. The brain uses 20% of the body's total oxygen metabolism and the neurons consume 75% to 80% of the brain's total energy [47]. Oxidative phosphorylation is a source of ATP production; this also produces free radicals that disrupt mitochondrial homeostasis, a factor that can elicit apoptotic cell death. In addition, neuroinflammatory cytokines like IL-2, IF-γ, and TNF-α response to exposure to noise stress plays a vital role in maintaining neuronal integrity [22]. The antiapoptotic Bcl-2, localized on the outer membrane of the mitochondria, promotes cellular survival. On the other hand, proapoptotic Bax can program cell death by permeabilizing the mitochondrial outer membrane permeabilization (MOMP) and subsequent initiation of the caspases cascade. Increased BcL-2 protein expression **(29.88%)** on exposure to 30 days of noise stress when compared to the control indicates the intrinsic ability to promote hippocampal neuronal survival **(Fig: 3)**. The ability of the Bcl-2 expressing cells to enhance antioxidant capacity and suppresses oxidative stress [48] could be one of the factors in regulating neuronal survival. Li and Ohizumi, [49] also reported that the isolated acetylated flavone glycosides from *S. dulcis* exhibited neurotrophic or Nerve Growth Factor (NGF) potentiating action that is helpful in the treatment of neurological illnesses. Increased Bax protein expression **(13.28%)** was also observed in the noise-exposed group. However, no significant changes were observed in all the experimental groups **(Fig:3)**.

Fig: 3. Effect of S. *dulcis* on Bcl-2 and Bax protein expression in the hippocampus when exposed to 30 days of noise stress.



Values are expressed as Mean ± SD, N=6, "*" compared with control; "#" compared with noise "@" Compared with *S. dulcis* control. Significance at $p < 0.05$. The symbols represent statistical significance: *, # & @ < P 0.05.

Bcl-2 and Bax, protein expression hippocampus, were upregulated on exposure to 30 days of noise, which increased Bcl-2 by **53.39%** when compared to Bax does not favour apoptotic cell death. Chana et al.[50] reported that *S. dulcis* extract attenuated the Sf9 cells against H_2O_2-induced cell death to corroborate our findings.

Conclusion

Noise is an environmental stressor that can affect the physiological and psychological status through multiple pathways. Targeting herbal remedies with potent antioxidative and neuroprotective effects could help narrow the therapeutic approach. The ability of *S. dulcis* to normalize the HPA/SAM axis dysfunction via the reduction of corticosterone could attenuate the behavioural responses of the animals. It is also important to note that increased expression of apoptotic proteins like BcL2 and Bax indicate the neuroprotective properties of *S. dulcis.*

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Conflict of interest

The authors declare that there is no conflict of interest to reveal.

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