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Evaluation of Antistress Potential of Negundin A from *Vitex negundo* in Acute Stress Induced Mice

Neha Tiwari[1*], Ashutosh Mishra[1], Ganesh Bhatt[2] and Anil Chaudhary[3]

[1]A.N.D. College of Pharmacy, Babhnan, Gonda, Uttar Pradesh, India.
[2]S.G.R.R. Dehradun, Uttarakhand, India.
[3]Cadila Pharmaceuticals LTD, Ahmedabad, Gujarat, India.

Authors' contributions

This work was carried out in collaboration between all authors. Author NT designed the study, performed the statistical analysis, wrote the protocol. Authors AC and GB wrote the first draft of the manuscript. Author AM managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Study Background: The current study has been designed to evaluate the antistress activity of bioactive compound, Negundin A from hydroalcoholic extract of the leaves of *Vitex negundo*. An attempt has been made to isolate the bioactive principle by resorting to bioactivity directed fractionation and column chromatographic technique. Isolated compound, Negundin A from leaves of *Vitex negundo* was investigated on acute physical stress stress (swimming endurance test), chemical stress (writhing test) and Acute restraint stress induced biochemical alterations in swiss mice.
Methodology: The plant leaves were extracted with hexane followed by ethanol, the extract so obtained was fractionated using chloroform to obtain chloroform soluble fraction. Negundin A was isolated from chloroform soluble fraction using column chromatography and characterized by techniques like Infrared spectroscopy (FTIR) and Nuclear magnetic resonance ^{1}HNMR. Isolated compound was screened for antistress activity. The effect was assessed by Swimming endurance

**Corresponding author: E-mail: neha_pharmaco@rediffmail.com;*

test, writhing test and estimation of various biochemical parameters in Acute restraint stress like glucose, cholesterol, triglycerides and BUN levels at different doses of 25, 50 and 100 mg/kg body weight per oral. Diazepam (2 mg/kg, ip) was used as standard drug.
Results: Negundin A treated animals showed increase in swimming endurance time and reduced number of writhes in physical and chemical-induced stress models respectively. Similarly, concomitant treatment with Negundin A at different doses showed marked decrease in blood glucose, cholesterol, triglycerides and BUN level as compared to stress control in Acute restraint stress models.
Conclusion: This is the first report of antistress activity of Negundin A and isolation of Negundin A by column chromatography from hydroalcoholic exract. The results in present research indicate that the isolated compound Negundin A of *Vitex negundo* extract has significant antistress activity against a variety of biochemical and physiological perturbations in stress models.

Keywords: Medicinal plants; spectroscopic authentication; swimming endurance test; acute restrain stress; anti- stress therapeutics.

1. INTRODUCTION

According to the World Health report, approximately 450 million people suffer from a mental or behavioral disorder, yet only a small minority of them receives even the most basic treatment. This amounts to 12.3% of the global burden of disease, and will rise to 15% by 2020. In the search for new therapeutic products for the treatment of neurological disorders, medicinal plant research, worldwide, has progressed constantly, demonstrating the pharmacological effectiveness of different plant species in a variety of animal models [1]. Stress is a biological response to aversive conditions that tend to threaten or perturb the homeostasis of the organisms. Stress has been shown to induce a marked rise in the brain levels of biogenic amines such as adrenaline and nor-adrenaline. These chemical substances are released in response to stress signals and are meant to assist the organisms to cope with stress. However, increased utilization of the amines resulting in their depletion in prolonged severe stress is responsible for fatigue, reduced stamina, lowered mood [2]. Acute stress can be exciting; it keeps us active & alert. But chronic stress can have detrimental effects on health. A wide range of events or conditions is considered physiologically stressful because the adrenals are stimulated to release stress hormones. These occurrences include calorie restriction, surgery, sleep deprivation, excessive exercise & various mental states- all of which can result in elevated cortisol & catecholamine stress hormones [3]. If the stress is extreme, the homeostatic mechanisms of the organism become deficit and the survival of the organism is threatened. Under these conditions, stress triggers a wide range of body changes called General Adaptation Syndrome (GAS). The stimuli, which produce GAS, are called the Stressors and range from physical to psychological factors including cold, heat, infection, toxins, major personal disappointment etc [4]. Drugs having antistress properties induce a state of non-specific resistance against stressful conditions. Drugs like benzodiazepines, certain CNS stimulants such as amphetamines and caffeine as well as some anabolic steroids are routinely used by people to combat stress. The incidence of toxicity and dependence has limited the therapeutic usefulness of these drugs [5]. Herbal formulations have been in use for many years not only in Asian countries but also globally for human well-being. The herbal formulations claimed to enhance physical endurance; mental functions and non-specific resistance of the body have been termed as adaptogens. The potential utility of safer and cheaper herbal medicines as antistress agents have been reported as they can withstand stress without altering the physiological functions of the body [6]. Various herbs like *Withania somnifera, Emblica officinalis, Asparagus racemosus, Ocimum sanctum, Tribulus terrestris* and *Piper longum* are claimed to have immunomodulatory, adaptogenic, anabolic effects and the ability to improve vital energy [7].

Benzodiazepines are still the most frequently used drugs for the treatment of generalized anxiety disorder despite their undesirable side effects such as muscle relaxation, sedation, physical dependence, memory disturbance, and interaction with other drugs [8]. Herbal medicine is the oldest form of healthcare known to mankind and it will not be an exaggeration to say that use of herbal drugs for human healthcare is probably as ancient as mankind [9].

The demand for more and more drugs from plant sources is continuously increasing. It is therefore essential for systematic evaluation of plants used in traditional medicine for various ailments. Hence, there is need to screen medicinal plants for promising biological activity. In our study, we choose the leaf portion of *Vitex negundo* to evaluate its biological activity [10]. *Vitex negundo (verbenaceae)* is commonly known as Nirkundi or Nallanocci. It is an aromatic large shrub or small tree about 3 m in height with quadrangular branches and almost found throughout india, ascending to 1500m in the outer Himalaya, fairly common in waste lands, on road side, the banks or streams or in moist places near deciduous forests [11]. *Vitex negundo* L. (Verbenaceae), is an important source of natural drugs from ancient time (Ayurvedic and Unani Systems of Medicine) and a number of pharmacological and medicinal activities and properties have been attributed toit by several studies. [12] Almost all the parts are employed, but the leaves and the roots are important as drugs [8].

Water extract of mature fresh leaves exhibited anti inflammatory, analgesic and antihistamine properties [13]. Leaves of this plant have shown mosquito repellent effects [14] as well as antiulcerogenic [15], antiparasitic [16], antimicrobial [17] and hepatoprotective potentials [18,19]. The leaf extract is used aganist fungal infection and in inflammatory conditions [20]. The seed extracts of *V. negundo* interfere with male reproductive function without producing adverse toxicity in other vital organs [11]. Some studies have also been done on antimicrobial activity of *V. negundo* along with some other Indian medicinal plants [10]. This study has been undertaken to evaluate their effect on experimentally induced stress and to find its probable mechanism of action including its antistress potential.

2. MATERIALS AND METHODS

2.1 Plant Material

The leaves of *Vitex negundo* were collected from the High Altitude Plant Physiology Research Centre (HAPPRC), Srinagar, Uttarakhand, India in the month of march 2011 and deposited in National Botanical Research Institute, Lucknow, India for taxonomical authentication. The leaves were air dried for 20 days and crushed into a coarse powder with a grinder and passed through 40-mesh sieve. They were stored in a well closed container separately.

2.2 Preparation of the Extract

The air dried and powder rhizome (2 kg) *Vitex negundo* was extracted with ethanol: water (60:40, v/v) under reflux at room temperature. After exhaustive extraction the combined extracts were concentrated under reduce pressure. A known quantity of the crude hydroalcoholic extract (940 g) was suspended in water and extracted successively with n hexane, chloroform, ethyl acetate and n-butanol. The chloroform soluble fraction (5.7 g) of hydroalcoholic exract was loaded on to column packed with silica gel (60-120), and eluted using n-hexane-ethyl acetate and then ethyl acetate-methanol in increasing order of polarity.

A total of 76 fractions, 250 ml each,were collected. These were pooled, based on similar thin layer chromatograms to get 5 fractions (A1-A6). The A5 (2.5 g) was subjected on to column packed with silica gel (60-120), and eluted using n-hexane-ethyl acetate (3:1) as the mobile phases. A total of 20 fractions, 100 ml each,were collected. These were pooled, based on similar thin layer chromatograms to get 5 fractions (A1.1-A6.5). The active fraction A5.5 which shows three compounds in TLC (Rf: 0.56, 0.63, 0.73) was successively rechromatographed on a silica gel column with using solvents (Toluene: ethyl acetate: chloroform / 6:2:2, v/v/v). Finally obtained fraction was (18-20) having single compound confirmed by TLC (Chloroform: Ethyl acetate/ 8:2, v/v).

2.3 Preliminary Phytochemical Investigations

The major secondary metabolites like, alkaloids, flavonoids, saponins, phenols, terpenoids, anthraquinones, proteins and aminoacids, carbohydrates and glycosides were assessed according to the standard procedure described by Harborne [11].

2.4 Spectroscopic Authentication

Spectral analysis FTIR, ^{1}H NMR and Mass of Isolated compound Quercetin was performed at Sophisticated Analytical Instrument Facility, Central Drug Research Institute, Lucknow, India to authenticate the functional group, molecular weight and molecular formula. FTIR spectra were recorded on Perkin Elmer Spectrum RX1 using alcohol. ^{1}H NMR (400 MH_z) spectra were recorded on Bruker Advance 400 in $CDCl_3$ with

tetramethylsilane as internal standard. The FAB mass spectra were recorded on a Jeol SX102/Da-600 mass spectrophotometer/Data System using Argon/Xenon 6 Kiev, 10 mA0 as the FAB gas. The accelerating voltage was 10 KV.

2.5 Experimental Animals

Albino mice (20-25 g) were bought from the Animal House of Siddhartha Institute of pharmacy, Dehradun, Uttarakhand, India. The animal room was maintained on a 12-h light and dark cycle with a constant temperature and humidity. Standard pellet food and tap water is available ad libitum. All animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the Department of Pharmacology of the Siddhartha Institute of Pharmacy, Dehradun, Uttarakhand, India. The experiments were conducted in a sound proof laboratory. All the experimental procedures and protocols (03/IAEC/SIP/11) used in the study were reviewed by the Institutional Animal Ethics Committee.

2.6 Experimental Design

The experimental animals were divided into five groups with six animals in each group as follows:

- Group I (normalcontrol) - recieved normal saline in a dose of 10 ml/kg, p.o.
- Group II (positive control) - recieved Diazepam, 2 mg/kg, i.p.
- Group III (test)-recieved isolated compound Negundin A, 25 mg/kg, p.o.
- Group IV (test)-recieved isolated compound Negundin A, 50 mg/kg, p.o.
- Group V (test)-recieved isolated compound Negundin A, 100 mg/kg, p.o.

2.7 Assessment of Antistress Activity

2.7.1 Forced swimming test

Swiss albino mice were randomly divided into 5 groups of 6 animals each. Treatment groups were pretreated with test drug extract in three different doses (25, 50 and 100 mg/kg, p.o.) for 10 days by oral route. Control group was treated with 0.2% sodium carboxy methyl cellulose in saline; while positive control group received diazepam (2 mg/kg, ip). On the 10^{th} day, the animals were allowed to swim till exhausted in a propylene tank of dimension 24 cm* 17 cm* 14 cm, filled with water to a height of 10 cm. The end point was taken when the animals drowned and 'swimming time' for each animal was noted. The mean swimming time for each group was recorded for 30 min [21].

2.7.2 Writhing test (chemical-induced stress)

Treatment groups and treatment schedule was same as that of swimming endurance test mentioned above. At the end of 10^{th} day; all the animals from treatment and control groups were administered 0.1 ml of 6% glacial acetic acid by intraperitonial route. Number of writhes was observed in all groups [22].

2.7.3 Acute restraint stress

Swiss mice were divided randomly into 5 groups, each group containing 6 mice. Group I mice received 0.2% sodium carboxy methyl cellulose in saline; (vehicle control). Group II mice were treated with diazepam (2 mg/kg, ip) and stress; (positive control). Group III mice were treated with Negundin A 25 mg/kg, p.o. and stress. Group IV mice were treated with Nigundin A 50 mg/kg, p.o. and stress. Group V mice were treated with Nigundin A 100 mg/kg, p.o. and stress.

After pretreatment with Negundin A for 7 days, the fore limbs and hind limbs of mice were tied separately and then together securing them with adhesive tape thereby immobilizing them for 2 h on last day. After the induction of stress for 2 h, blood was collected and plasma glucose, triglyceride, cholesterol and cortisol and BUN levels were estimated [22].

2.8 Statistical Analysis

All data are expressed as the mean ± S.E.M and were obtained from four distinct experiments. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Dunnett's test. The significant difference was set at $p < 0.05$.

3. RESULTS

3.1 Extraction and Isolation

The compound was extracted with ethanol: water (60:40) at room temperature. The quantity of extract obtained after concentration under reduce pressure was approximately 940 g. The physical appearance of concentrated extract was dark green syrupy. The chloroform fraction of dark green syrup was subjected to column

chromatography. Single compound was isolated from fraction (A5.5) confirmed by TLC. The TLC examination confirmed that it was a single compound known as Negundin A. The Rf (Retention factor) value of isolated compound was found to be 0.60.

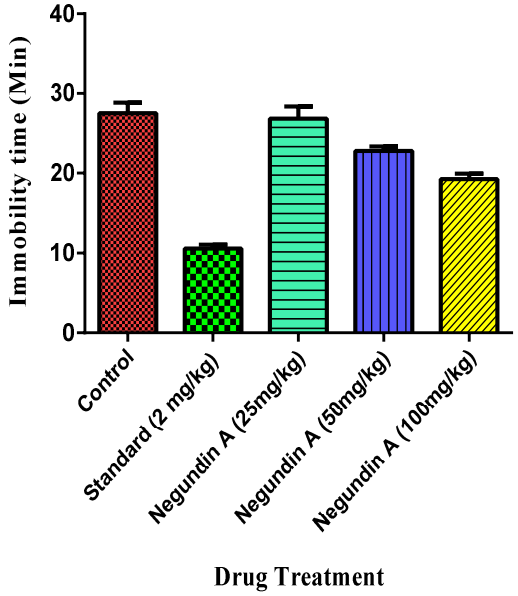

Fig. 1. Immobility time (Sec) in forced swimming test (Bar 1: Control; Bar 2: standard 2 mg/kg; Bar 3 negundin A 25 mg/kg; Bar 4: Negundin A 50 mg/kg; Bar 5: Negundin A 100 mg/kg). Value represents mean ± S.E.M, $p < 0.05$, n=6

3.2 Structure Elucidation of Isolated Compound from Leaves of *Vitex negundo*

The FTIR spectra showed sharp peaks at 2852 cm^{-1} indicating C=H stretching of alkenes. Medium and weak peaks were observed at 1451 cm^{-1} and 1386 cm^{-1} indicating C=C stretching of aromatic ring. Strong peak of ether C-O stretching was observed at 1135 cm^{-1}. Sharp peak of tetra substituted benzene ring was observed at 842 cm^{-1}.

1H NMR $CDCl_3$ at 400 MHZ, triplet at δ 6.20 ppm was assigned to 8 protons of fused aromatic compound supported by mass and IR spectra. 1H NMR displayed 6 protons of terminal methyl at δ 3.66 ppm. Chemical shift of the 2 protons adjacent phenol group appeared at δ 5.17 ppm, supported by mass spectroscopy.

Mass spectroscopy showed the molecular ion at m/z 353 (M+1) corresponding to molecular formula ($C_{20}H_{16}O_6$) 352, as supported by ^1H NMR, on the basis of their analysis.

From the spectral data the structure of the compound VN was elucidated as Negundin A with molecular formula $C_{20}H_{16}O_6$.

3.3 Forced Swimming Test

Mice pretreated with Negundin A at 25 mg/kg did not show significantly increased swimming endurance time,whereas at doses 50 mg/kg and 100 mg/kg of isolated compound Negundin A significantly decrease the immobility time as compared to the control group. Diazepam also significantly reduced the immobility time compared to the vehicle control (Table 1).

3.4 Writhing Test

Negundin A significantly decreased the number of writhes in mice compared to the vehicle control group and a dose dependent effect was observed (Table 1).Reduction in number of writhes was found to be 30.5±2.1, 18.7±1.5 and 10.7±2.5 with 25, 50 and 100 mg/kg of isolated compound Negundin A from *Vitex negundo*. Diazepam produced 4.6±1.09 reduction in no. of writhes.

Table 1. Effect of negundin A on immobility time and number of writhings

Treatment	Mean immobility time	Number of writhings (min)
Control	28.23±1.38	41.90±1.16
diazepam 2 mg/kg	10.59±0.46*	3.08±0.52*
negundin A 25 mg/kg	26.83±1.52	30.50±2.13
negundin A 50 mg/kg	22.78±0.55	19.45±0.90
negundin A 100 mg/kg	19.29±0.65*	10.43±0.56*

Note: n=6, the observation are mean ± S.E.M and data were analyzed using graph prism pad as statistical unit.
** $p < 0.05$ (ANOVA followed by Dunnett's test)*

Table 2. Effect of negundin a on biochemical parameter on acute restraint stress induced in albino mice

	Control	Acute restrain stress	Negundin A 25 mg/kg	Negundin A 50 mg/kg	Negundin A 100 mg/kg	Diazepm 2 mg/kg
Glucose (mg/dl)	80.07±2.08	148.65±1.89	127.34±2.65	117.65 ±1.43	102.24±0.54	85.77±1.87
Cholesterol (mg/dl)	55.08±1.72	90.43 ±1.72	84.23±2.88	75.09 ±1.60	67.53 ±1.87	58.00±1.09
Triglycerides (mg/dl)	69.65±2.98	105.12±2.33	94.22±1.05	89.14±2.28	80.44 ±0.56	70.54±1.90
BUN (mg/dl)	25.03±2.60	55.41±1.72	49.89±2.88	40.08±1.68	33.18±1.11	28.09±1.06

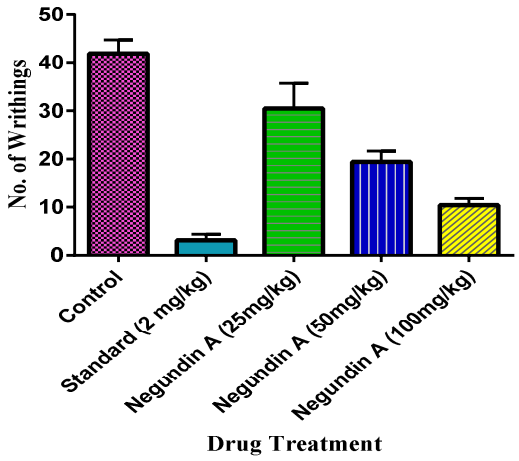

Fig. 2. No. of writhings in Writhings test (Bar 1: Control; Bar 2: Standard 2 mg/kg; Bar 3 negundin A 25 mg/kg; Bar 4: Negundin A 50 mg/kg; Bar 5: Negundin A 100 mg/kg). Value represents mean ± S.E.M, $p < 0.05$, n=6

3.5 Acute Restraint Stress

Mice treated with Negundin A (25 mg/kg) did not significantly ($p<0.05$) reduced the blood glucose level elevated by stress, however 50 and 100 mg/kg dose significantly reduced the blood glucose level as compared to stress control (Table 2). Reduction of increase cholesterol and triglycerides and BUN level following stress by Negundin A at the dose of 100 mg/kg was comparable to vehicle control.

4. DISCUSSION

Animals when subjected to a period of stress produce characteristic changes in several hormones and parameters associated with the central nervous system and the hypothalamic-pituitary-adrenal axis (HPA). HPA changes include an increase in cortisol, a reduced sensitivity of the HPA to feedback down-regulation, and a disruption in the circadian rhythm of cortisol secretion [23]. The mechanism by which stress raises serum cholesterol, triglycerides and BUN levels in stress induced animals is due to the enhanced activity of hypothalamo-hypophyseal axis resulting in increased liberation of catecholamines and corticosteroids [24]. Negundin A as well as the standard drug Diazepam significantly reduced the elevated serum cholesterol, triglyceride levels (Fig. 3) which may be due to inhibition of stimulation of sympathetic nervous system [4]. During acute restrint stress, blood glucose level increases, this is found to be significantly reduced by Negundin A which is an indication of its anti-stress activity. Negundin A also significantly reduced stress induced BUN level exhibiting anti-stress activity which is comparable to standard Diazepam. It is expected that immobility occuring in these tests will reflect a state of behavioural despair or unable to adapt the stress as seen in human [25]. In swimming endurance paradigm, animal forced to swim in water eventually assume a characteristic immobile posture which reflects a sate of tiredness, fatigue, reduced stamina or depressed mood. These signs represent he core symptoms observed in individual under intence stress. Drugs with antistress property reduced the duration of immobility of animals [21]. Hence swimming endurance time and number of writhes were used as antistress parameters for preliminary antistress activity screening of the extract. Mice pretreated with Negundin A showed significant improvement in the swimming time (Fig. 1) and reduced number of writhes (Fig. 2) in acute stress model. The isolated compound Negundin A at a dose of 100 mg /kg is found to be more effective in both chemical induced stress and swimming endurance test.

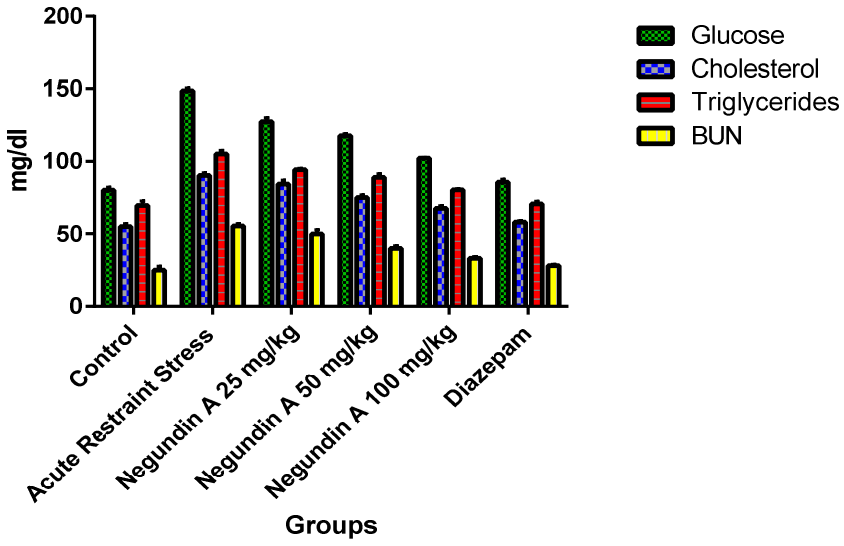

Fig. 3. Antistress activity of negundin A in cold restraint stress model. Value represents mean ± S.E.M, $p < 0.05$, n=6

5. CONCLUSION

In conclusion, this is the first report which identified the anti-stress potential of Negundin A. The present investigation indicates that Negundin A at the dose of 100 mg/kg has significant antistress activity as shown by its mitigating effects on acute stress-induced biochemical perturbations, comparable to that of diazepam, the conventional antistress agent.

These results encourage towards possible use of *Vitex negundo* as a patient friendly alternative to the present pharmacotherapy. The present finding highlights the importance of pharmacological interventions of Vitex constituent in the prevention of stress-induced neurological and related disorders.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All the authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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