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Preclinical screening of NR-A2 for antistress, anti allergic properties

Meera Sumanth*, Mrudula Yavagal

Visweswarpura Institute of Pharmaceutical Sciences, 22nd Main, 24th cross, Banashankari II Stage, Bangalore - 560 070. INDIA

Abstract

NR-A2 is a polyherbal formulation of Natural Remedies Pvt. Ltd., Bangalore, being developed for the treatment of allergic rhinitis. NR-A2 was evaluated for the antistress activity by swim endurance test in mice, anxiolytic activity using elevated plus maze, anti allergic activity using guinea pig ileum and compound 48/80 induced anaphylaxis and anti ulcer activity in rats. In an attempt to establish a relation between allergy and stress, a new experimental animal model was devised to study anti-stress activity of NR-A2 in allergy induced animals, followed by corticosterone estimation.

The formulation showed significant anxiolytic and anti-stress activity. The stress induced changes in ulcer index and in brain neurotransmitter levels were significantly decreased by NR-A2. The formulation caused excellent inhibition of histamine induced contractions in GPI and gave complete protection against degranulation in the animals treated with a prophylactic dose of 250 mg/kg p.o., one hour before Compound 48/80 injection. The anti-stress property of NR-A2 in allergy induced rat model, showed a possible link between immune system and neuroendocrine system. NR-A2 showed significant anti-stress activity in allergy induced animals and also decrease in corticosterone concentrations.

Key Words : NR-A2, antistress, anxiolytic, anti allergic, corticosterone

1. Introduction

Stress and allergy in its various manifestations have become common problems in our society now-a-day. The mortality rate of stress related allergic diseases (such as asthma, urticaria, allergic rhinitis, atopic dermatitis), cardiovascular diseases (such as angina pectoris, myocardial infarction, hypertension), CNS disorders (such as anxiety, depression, headaches), gastrointestinal disorders (such as peptic ulcer, ulcerative colitis) are increasing in the current situation [1]. STRESS basically is a reaction of mind and body against change in the homeostasis [2]. Stress alters the equilibrium of various hormones, which have a significant impact on the immune response. During stressful event, neuroendocrine hormones like

* Corresponding author
Email: meerasumanth@gmail.com

adrenocorticotropic hormone, cortisol, norepinephrine and epinephrine are released and they can alter immune functions and subsequently alter the course of immune based disease [3].

NR-A2, a polyherbal formulation of Natural Remedies Pvt. Ltd., Bangalore, being developed for the treatment of allergic rhinitis, needs to be validated for its proposed antiallergic property. The two major ingredients of NR-A2 are *Albizzia lebbeck* and *Embelica officinalis*. *Albizzia lebbeck* possess immunomodulatory [4] activity and *Embelica officinalis* is hypolipidemic, antioxidant, antiulcer, antimutagenic, and antiallergic [5]. The present study was taken up to evaluate the anti allergy and antistress activity in NR-A2. Effort to establish a relation between immune and *neruroedocrine* system by studying antistress activity of the formulation in an allergic model is also done.

2. Materials and methods

1. Drug and Chemicals: NR-A2, a polyherbal formulation, *Ocimum sanctum* used as reference antistress agent, and compound 48/80 (Sigma) were gift from Natural Remedies Pvt. Ltd., Bangalore. Horse serum (Hyclone), Triple antigen (Serum institute of India), Ketotifen (Ketasma, Sun Pharma India ltd) were used as active anaphylactic agents. Some of the other drugs and chemicals used in the study were Dichloromethane, Heparin, HPLC grade methylene chloride, HPLC grade methanol, corticosterone, (Sigma Chemicals, USA).

2. Animal: Albino rats, Wistar strain, 150-200 g and albino mice weighing 30-35 g of either sex, maintained on natural day-night cycle, at a temperature of 25°C ± 2°C, commercial pellet diet (Amrut feeds, Bangalore) and water *ad libitum* were used. The rats were divided into 4 groups of 6 animals each. Gr I rats given orally distilled water, is control, Gr II and Gr III animals were administered NR-A2, 250 and 500 mg/kg p.o. for 30 days. Similarly, albino mice were divided into 4 groups of 6 animals each. Gr I mice given orally distilled water, is control, Gr II and Gr III animals were administered NR-A2, 150 and 300 mg/kg p.o. for 30 days. The doses of formulation were calculated from the human doses of constituent plant drugs and the composition of formulation. Guinea pig, 400-450 gm was used for studying antihistaminergic activity. Institutional Animal Ethics Committee's permission was obtained before starting the experimentation.

3. Experimental

3.1 Anxiolytic activity [6]: Group II and III rats were pretreated with NR-A2 for 30 days. On 30th day, each of the pretreated and control rat was placed in the center of the elevated plus maze facing one of the enclosed arms. During a 5 minute test period, the number of entries into and time spent in the open arms, the total number of arm entries and time spent at the center was measured.

3.2 Antistress activity by swim endurance test [7]: Mice of group II and III were pretreated with NR-A2 for 30 days. On the 30th day, control and pretreated animals were subjected to forced swimming in a polypropylene tank (30x45x40 cubic cm) containing water up to 15 cm height at room temperature (25 ± 2°C). The mice were removed from the water when head started dipping below the water level. The 'Swimming time' for each mouse was noted.

3.3 Anti ulcer activity [8]: Rats of group II and III were pretreated with NR-A2, 250 and 500 mg/kg p.o., respectively, for 30 days. Group IV animals received *Ocimum sanctum*, 100 mg/kg i.p. on last day. On 30th day, each rat was individually placed in plastic restrainer and restrained for two hours at 2-4°C, sacrificed by cervical dislocation and dissected. Stress

induced ulcers were evaluated using stomach and ulcer index was calculated for each animal. Whole brain was isolated to evaluate stress induced changes in brain catecholamines using HPLC method [9] with electrochemical detector-Waters 460. (flow rate 1 ml/min, detector potential 0.6 V versus Ag/KCl reference electrode, sensitivity 0.1 nA.) The amount of amines was expressed in ng/gm of brain.

3.4 Anti allergic activity

3.4.1 Anti-histaminic activity of NR-A2 using guinea pig ileum (gpi): Over night fasted guinea pig was sacrificed and about 3-4 cm long ileum was mounted, stabilized for 30 minutes by applying 500 mg tension, in Tyrode solution, with bath temperature of 32 ± 1°C, proper aeration and response magnified 10 times. A dose response curve for histamine (1 to 32 µg) was recorded first. One of the sub maximal doses of histamine was chosen. Various doses of NR-A2 administered to the tissue bath and after 5 min, the chosen sub maximal dose of histamine was given to see complete inhibition of response. Cycle was repeated with 1ng to 32 ng of pheniramine as standard antihistaminic drug. Percentage (%) response [10] was calculated as:

$$\% \text{ Reduction} = 100 \times \frac{\text{Size of response before exposure to antagonist}}{\text{Size of response after exposure to antagonist}} \times 100$$

Then the dose of antagonist producing 50 % inhibition of histamine induced GPI contraction was calculated.

3.4.2 Compound 48/80 induced anaphylaxis [11]: Rats were divided into 5 groups of 6 animals each. Control group I animals received distilled water p.o. for 30 days, Group II, III, & IV animals received NR-A2, 250 mg/kg p.o. for 30 days whereas Group V animals received Ketamine 1 mg/kg i.p. on the last day. On the last day, rats were injected 8 µg/gm body weight of Compound 48/80 I.P. Groups II, III, & IV rats received NR-A2 in the same dose at 1 hr before, 5 minutes after and 10 minutes after, compound 48/80 injection respectively. The mortality was determined during 1 hr after induction of anaphylactic shock. Mortality (%) within 60 min following compound 48/80 injection was represented as:

$$\frac{\text{Number of dead rats}}{\text{Total number of experimental rats}} \times 100$$

3.4.3 Antistress activity in allergic rat model: The rats were divided into 4 groups of 6 animals each. Group I (Control I) and Group II (Control II) animals received distilled water p.o. for 30 days. Group III animals received NR-A2 250 mg/kg p.o. for 30 days and Group IV animals received *Ocimum sanctum*, 100 mg/kg i.p. on last day. The rats of group II, III and IV were sensitized on 17th day of 30 days treatment with subcutaneous injection of 0.5 ml horse serum and 0.5 ml of triple antigen containing 20,000 million *B. purtussis* organism s.c. Animals of all the groups were subjected to stress by Swim endurance on the last day after 1hour of last dose of drugs and rechallenged with 0.5 ml horse serum and 0.5 ml triple antigen i.p. The immobility time was measured. At the end of swim endurance test, blood was withdrawn from carotid artery and corticosterone [12] levels were estimated.

4. Statistical analysis: All the data was statistical analyzed using one way ANOVA. $p < 0.01$ was considered statistically significant. The post-hock analysis was done by Dunnet's test for the significance of difference between the groups.

4. Results

1. Anxiolytic activity: As shown in Table 1, NR-A2 exhibits significant anxiolytic activity [F = 67.63 (open arm time), $p < 0.001$; Dunnet's test], by increasing the time spent in open area and the number of entries into open arm and by reducing the number of entries into closed arm and time spent in closed arm.

2. Antistress activity by swim endurance test: As shown in Table 2, NR-A2 in a dose of 150 mg/kg p.o. showed statistically highly significant increase in swimming time ($F = 55.38$; $P < 0.001$; Dunnet's test) indicating excellent antistress property.

3. Anti ulcer activity: As shown in Fig.1, NR-A2 significantly reduces ulcer index, indicating anti ulcer activity ($F = 54.71$, $P < 0.001$; Dunnet's test). Fig. 2 shows that NR-A2 in the dose of 250 mg/kg produces significant attenuation in levels of norepinephrine and dopamine in brain.

4. Anti allergic activity

4.1 Anti-histaminic activity of NR-A2 using guinea pig ileum (gpi): Fig 3 shows antihistaminic effect of formulation NR-A2. The IC_{50} values (50% inhibition concentration) of NR-A2 is found to be 3.00 ± 0.44 mcg.

4.2 Compound 48/80 induced anaphylaxis: As shown in Fig. 4, the mortality of rats injected intraperitoneally with NR-A2 one hour before compound 48/80 injection was zero.

4.3 Antistress activity in allergic rat model: As shown in Table 3, active anaphylaxis induced animals shown significantly increased levels of stress. Sensitized rats when subjected to swim endurance, swam for lesser time whereas NR-A2 treated rats showed a significant ($F = 43.062$; $P < 0.001$; Dunnet's test) increase in swimming time as compared to sensitized control rats. There is a significant increase in Plasma corticosterone in rats sensitized and subjected to swim stress as compared to the normal unsensitized rats NR-A2 pretreated, sensitized rats have shown significant ($F = 0.38$; $p < 0.01$; Dunnet's test) reduction of the increased levels of corticosterone. (Fig. 5)

5. Discussion

Allergic rhinitis (Hay fever), one of the most common diseases, is an immediate type of hypersensitivity reaction to allergens such as pollens, mould, cold temperature, stress etc. NR-A2 is a polyherbal formulation developed for the treatment of allergic rhinitis, which was evaluated for the anti allergy and antistress activity.

Anxiety is one of the causative factors of stress. The elevated plus maze test is widely used for selective identification of anxiolytic and anxiogenic drugs. Anxiolytic compounds by decreasing anxiety increases the open arm exploration time [13]. The results of our study show that the animals pretreated with formulation NR-A2 spend a longer time in open area of elevated plus maze and also, the number of entries into open arm is increased, indicating the anxiolytic property of formulation NR-A2.

Antistress activity of drugs is studied using swim endurance test. The combination of cold and restrain (immobization) has been used by some researchers [7,14] to induce stress in rats. Studies show that immobilization stress brings about alterations in plasma ACTH, Epinephrine and norepinephrine levels [15]. These neurohormones are used as excellent stress markers to evaluate antistress activity. Histamine, released during stress, [16] plays a pivotal role in modulating gastric acid back-diffusion and vascular permeability that are associated with hemorrhagic ulcer [17].

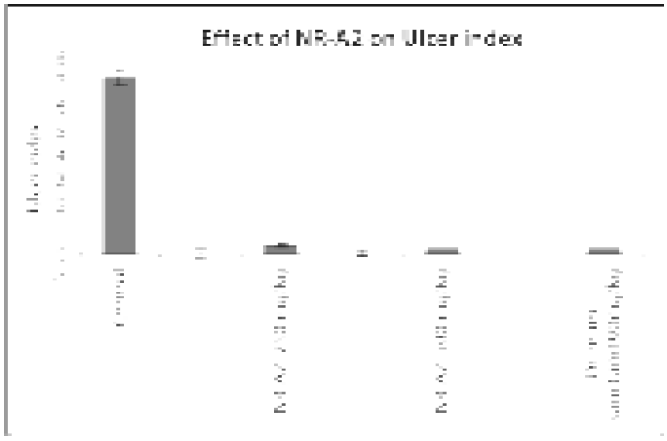

Fig. 1: Effect of NR-A2 on ulcer index
Values are Mean ± SEM, n=6, *** $p < 0.001$ as compared to control

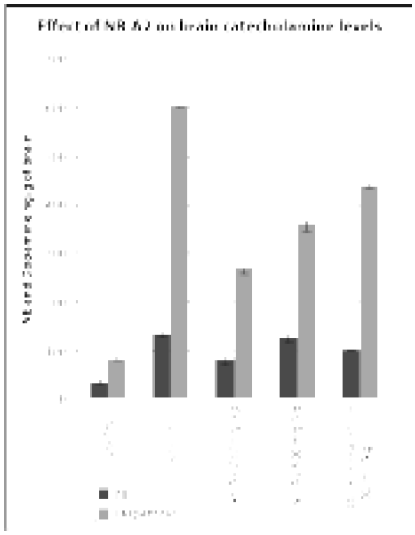

Fig. 2: Effect of NR-A2 on Norepinephrine and dopamine levels in brain
Values are Mean ± SEM, n=6, *** $p < 0.001$ as compared to control

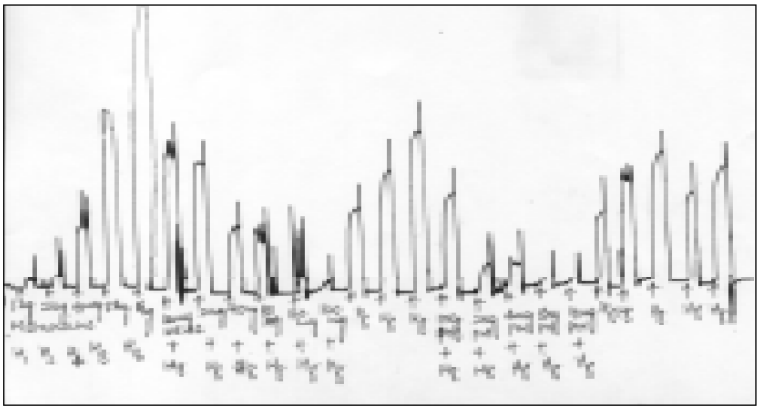

Fig. 3: Antihistaminic Activity of NR-A2 using Guinea pig ileum
H_8 = Reference Dose of histamine, PM = Pheniramine Maleate

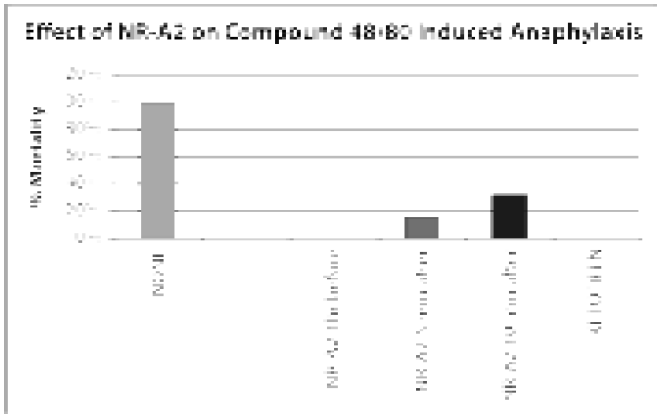

Fig. 4: Effect of NR-A2 on compound48/80 induced anaphylaxis in rats

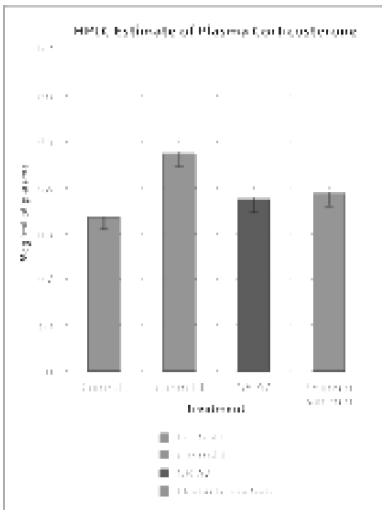

Fig 5: Effect of NR-A2 on plasma corticosterone levels in active anaphylaxis induced rats
Values are Mean ± SEM, n=6, *** p < 0.001 as compared to control
Control I-Unsensitized, stressed rats,
Control II- Rats sensitized with 0.5 ml horse serum and 0.5 ml triple antigen s.c., stressed

Table No. 1: Effect of NR-A2 on Anxiolytic activity (Time in seconds)

Groups	No. of entries in open arm	Time spent in open arm	No. of entries in closed arm	Time spent in closed arm	Time spent at center
Control	3.00 ± 0.25	44.16 ± 3.00	5.66 ± 0.42	220.83 ± 3.74	35.83 ± 3.51
NR-A2 (250 mg/kg)	4.33 ± 0.42	98.33 ± 4.01***	2.33 ± 0.21***	135.83 ± 8.50***	68.33 ± 6.28**
NR-A2 (500 mg/kg)	3.62 ± 0.33	81.66 ± 2.10***	3.16 ± 0.30***	113.33 ± 13.52***	75.00 ± 12.78***
Ocimum sanctum (100 mg/kg)	6.54 ± 0.42	109.17 ± 3.51***	2.80 ± 0.30***	119.17 ± 4.16***	71.66 ± 5.2**

Values are Mean ±SEM, n = 6, one way ANOVA , F = 67.63
*** p< 0.001 as compared to control, ** p< 0.01 as compared to control,

Table No. 2: Effect of NR-A2 on swim endurance test in mice

Treatment	Swimming time (minutes)
Control	14.83 ± 1.046
NR-A2 (150 mg/kg)	30.08 ±1.55***
NR-A2 (300 mg/kg)	45.33 ± 3.807***
Ocimum sanctum (100mg/kg)	28.33 ± 9.972***

Values are Mean ± SEM. n=6, one way ANOVA, F = 55.38 *** p< 0.001 as compared to control,

Table No. 3: Antistress activity of NR-A2 in active anaphylaxis induced rats

Treatment	Swimming time (minutes)
Control I(unsensitized)	142.00 ± 8.38
Control II(sensitized,stressed)	48.67 ± 3.60**
NR-A2 (250 mg/kg)	108.33 ± 7.60***
Ocimum sanctum (100 mg/kg)	74.17 ± 3.51

Values are Mean ± SEM. n=6, one way ANOVA, F = 43.062, **P < 0.001 as compared to control I, *** P < 0.001 as compared to control II

The intensity of ulcers induced in cold restrain stress is more [18]. The increased swimming time in swim endurance test and decreased ulcer index, attenuation in levels of norepinephrine and dopamine in brain of pretreated animals, suggest the antistress activity of formulation NR-A2.

Allergic rhinitis is an immediate type of hypersensitivity reaction. Assessment of IC_{50} of a histaminergic antagonist using guinea pig ileum and mortality of rats due to compound 48/80 induced anaphylaxis are some of the best models for studying the anti-allergic activities of a drug. The major clinical manifestations in allergic and anaphylactic reactions are release of mediators such as histamine, cytokines and leukocytes [19]. In the evaluation of antihistaminic activity using GPI preparation, the drug competitively binds histaminenergic receptors on the smooth muscles and blocks them. Thus the peripheral histamine action is inhibited. In our studies, NR-A2 shows significant antihistaminic activity. Compound 48/80 is a potent mast cell degranulator which induces severe anaphylaxis in rats. Our study indicates that, a prophylactic treatment with NR-A2 one hour before compound 48/80 injection, provide complete protection from degranulation. One of the constituents of the formulation NR-A2 is *Albizzia lebbeck*, acts by mast cell stabilization and shows the antianaphylacatic/antiallergic activity [20,21]. Hence it may be responsible for anti-allergic activity of formulation NR-A2.

Studies indicate that allergy through immune system plays a vital role in stress.So it was thought appropriate to undertaken a study to establish a relation between stress and allergy. In our study, the sensitized rats pretreated with NR-A2 showed a considerable increase in

swimming time and reduction in corticosterone levels as compared to unsensitized control rats. A marked elevation of plasma ACTH and corticosterone concentrations in rats subjected to swim stress, which is used as a marker to study stress (Anisman *et al.*, 1998) is reported. The reduction in corticosterone levels in NR-A2 pretreated, sensitized rats indicates the antistress activity of the formulation. Thus this novel model can be useful to study antistress activity of a drug/herbal formulation in an allergic animal model.

6. Conclusion

NR-A2, a polyherbal formulation of Natural Remedies Pvt. Ltd., Bangalore, posses antistress, antiallergic properties.

7. Acknowledgement

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