

## Immunomodulatory Activity of Triphala on Neutrophil Functions

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Immune activation is an effective as well as protective approach against emerging infectious diseases. The immunomodulatory activities of Triphala (*Terminalia chebula*, *Terminalia bellerica* and *Embllica officinalis*) were assessed by testing the various neutrophil functions like adherence, phagocytosis (phagocytic index (P.I) and avidity index (A.I)) and nitro blue tetrazolium (NBT) reduction in albino rats. In recent years much attention is being focused on the immunological changes occur during stress. Noise (100 dB) stress for 4 h/d for 15 d, was employed to alter the neutrophil functions. The neutrophil function tests and corticosterone levels were carried out in eight different groups of animals, namely control, Triphala, noise-stress, Triphala noise-stress, and corresponding immunized groups were used. Sheep red blood cells (SRBC  $5 \times 10^9$  cells per ml) were used for immunizing the animals that belongs to immunized groups. In Triphala administration (1 g/kg/d for 48 d), A.I was found to be significantly enhanced in the Triphala group, while the remaining neutrophil functions and steroid levels were not altered significantly. However the neutrophil functions were significantly enhanced in the Triphala immunized group with a significant decrease in corticosterone level was observed. Upon exposure to the noise-stress, the neutrophil functions were significantly suppressed and followed by a significant increase in the corticosterone levels were observed in both the noise-stress and the noise-stress immunized groups. These noise-stress-induced changes were significantly prevented by Triphala administration in both the Triphala noise-stress and the Triphala noise-stress immunized groups. Hence our study has divulged that oral administration of Triphala appears to stimulate the neutrophil functions in the immunized rats and stress induced suppression in the neutrophil functions were significantly prevented by Triphala.

**Key words** Triphala; neutrophil; NBT reduction; phagocytosis; adherence

Everyone experiences stress, which results in the over secretion of glucocorticoids.<sup>1)</sup> Increase in glucocorticoid level leads to the suppression of immune function in both humans and animals.<sup>2)</sup> Environmental stress plays an important role in the elevation of blood glucocorticoid level, which suppresses both innate as well as acquired immune functions<sup>3,4)</sup> and results in susceptibility to infections.<sup>5)</sup>

Herbs are selected and combined for their ability to inhibit microbial overgrowth in various parts of the body and support those organ systems responsible for immune functions. In recent years, there is an upsurge in the clinical usage of indigenous drugs, because of their efficacy and being free from serious toxic effects. Moreover constant increase in the antibiotics resistant strains and various side effects caused by the synthetic drugs have prompted scientists to look for herbal immunomodulators to treat various infections.<sup>6)</sup> Herbal drugs are believed to enhance the natural resistance of the body against infection<sup>7)</sup> and their immunomodulatory activities have been reported in numerous plants.<sup>8)</sup>

Triphala is a traditional Ayurvedic herbal formulation, consisting equal parts of three medicinal plants namely *Terminalia chebula*, *Terminalia bellerica* (Family: Combretaceae) and *Embllica officinalis* (Family: Euphorbiaceae). Ayurvedic "rasayana" are widely used to enhance the natural resistance to various diseases.<sup>9)</sup> Triphala is regarded as an important rasayana in Ayurvedic medicines, as rasayana group are believed to promote health, immunity and longevity. Triphala is believed to have balancing and rejuvenating effects on the three constitutional elements in Ayurveda namely vata, pitta and kapha.<sup>10)</sup> It has been used extensively as a drug against number of diseases<sup>11,12)</sup> and also forms part of many other Ayurvedic formulations. Fruits of Triphala, are claimed to have various biological activities

such as anti viral,<sup>13)</sup> anti-bacterial, anti-allergic<sup>14)</sup> and anti-mutagenic.<sup>15)</sup> It is prescribed for various symptoms of infections, obesity, anaemia, fatigue, poor digestion, assimilation and infectious diseases like tuberculosis, pneumonia and AIDS.<sup>16)</sup>

The effect of Triphala on the neutrophil functions has never been studied. Neutrophils are professional phagocytes, constituting the prime part of our innate defense against an extensive number of potentially harmful microorganisms in our environment.<sup>17)</sup> In the present study, immunomodulatory property of Triphala in relation to the neutrophil functions was examined. The noise-stress induced changes in the neutrophil functions were investigated and the drug effects on the noise-stress induced changes in neutrophil functions were examined.

### MATERIALS AND METHODS

**Animals** The study was approved by the Institute's Animal Ethical Committee of the University of Madras (IAEC) and confirmed to national guidelines on the care and use of laboratory animals. Male albino rats (Wistar Strain), weighing 170—190 g were used for the study. Each group consisted of 6 animals. That was maintained at  $25 \pm 2^\circ\text{C}$  in the institute's animal house with food and water *ad libitum*.

**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  $5 \times 10^9$  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.

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**Experimental Groups** The following 8 groups were used for the study and each group had 6 animals. Group 1 (Control) and 2 (Control Immunized) were administered saline orally for 48 d to study the base line values of all the experiments. Group 3 (Noise-stress) and 4 (Noise-stress immunized) animals were subjected to noise-stress for 4 h/d for 15 d. The noise-stress induced changes in neutrophil functions were observed in these groups. Group 5 (Triphala) and 6 (Triphala immunized) were treated with triphala for 48 d and experiments were carried out on 49th day. The immunomodulatory activity of Triphala was assessed in these groups. Group 7 (Triphala noise-stress) and 8 (Triphala noise-stress immunized) were treated with Triphala for 48 d and were further subjected to noise-stress from 33rd day onwards with drug treatment and all the experiments were done on the 49th day. These groups were used to study the drug effect on the noise-stress induced changes in the neutrophil functions. The corticosterone level was estimated in all the experiment groups.

**Drug** Fruits of Triphala were collected and authenticated by The Chief Botanist, Tamil Nadu Medicinal plant farms and Herbal Medicine Corporation (TAMPCOL) Ltd., Chennai, India. The seedless fruits were dried under shade and powdered in the laboratory. Equal proportion of crude powder of the fruit of each plant was mixed and taken for the present study.

**Dosage** Based on our preliminary studies with different dosages (250 mg, 500 mg, 1 g) of this drug, it was found that 1 g/kg b.w dosage induced significant immunomodulatory changes. Hence 1 g/kg b.w dosage was considered for this study. Triphala powder was mixed with saline and administered orally for 48 d.

**Noise Stress Procedure** Broad band of white noise at 100 dB intensity was used in this study. The noise was 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 was used to measure the intensity of the noise generated.

In all the experiments, heparinized syringe was used to collect the blood samples from the jugular vein and stored in a silicanized tubes. Ether was used to anaesthetize the animals to collect the blood samples by using the technique of Feldman and Conforti<sup>18)</sup> to avoid stress. This procedure was conducted during the morning hours between 08.00–10.00 a.m. to avoid circadian influences. Neutrophil functions were assessed by testing adherence to nylon column, *Candida* phagocytosis and Nitro blue tetrazolium (NBT) reduction tests.

Neutrophils were identified based on the presence of the numerous fine violet colored granules with multi-lobed nucleus by staining with Leishman's stain.

#### ASSESSMENT OF IMMUNOMODULATORY ACTIVITY

**Total Leukocyte Count (TLC)** Blood sample was drawn and diluted with Turk's fluid in WBC pipette, in which red cells were lysed without affecting the leukocyte population. Leukocyte count was carried out by using an improved Neubauer's counting chamber.

**Differential Leukocyte Count (DLC)** Blood smear was made on a clean glass slide and stained with Leishman's

stain. Among the hundred leukocytes counted, different population of leukocytes were differentiated and identified based on the cell size, presence of granules, size and colour of granules and shape of the nucleus under an oil immersion preparation.

**Neutrophil Adherence<sup>19)</sup>** Neutrophil adherence was analyzed by the initial count of TLC and DLC from the blood sample. After initial count, blood sample was incubated in sterile nylon fiber column (80 mg/ml) packed in a silicanized pasteur pipette (column length 15 mm). After 15 min of incubation, blood sample was again analyzed for TLC and DLC. The product of TLC and percentage of neutrophil gives the neutrophil index (NI) of blood sample.

Percentage of neutrophil adherence was calculated by

$$\frac{(\text{neutrophil index of untreated blood samples}) - (\text{neutrophil index of treated blood sample})}{(\text{neutrophil index of untreated blood samples})} \times 100$$

gives the percentage of neutrophil adherence.

**Candida Phagocytosis<sup>20)</sup>** The phagocytic ability of neutrophil was assessed by separating the buffy coat from the blood sample. To this, the incubating medium (0.1 ml of minimum essential medium (MEM), 0.1 ml of inactivated fetal calf serum and 0.1 ml of heat killed *Candida albicans*  $2 \times 10^8$  cell/ml) was added and incubated at 37 °C for 15 min, followed by centrifugation. From the sediment, thin smears were made and stained with Leishman's stain. The number of neutrophils positive for candida in 100 cells gives phagocytic index (PI). The total number of *Candida albicans* counted within 100 neutrophils divided by 100 gives the mean particle number or the avidity index (AI).

**Nitroblue Tetrazolium (NBT) Reduction Test<sup>21)</sup>** The killing ability of the neutrophils was assessed by nitroblue tetrazolium reduction test (NBT). Briefly, the blood sample was incubated at 37 °C for 30 min in a clean glass slide. After incubation, the slide was gently washed with cold saline to remove other cell populations. To this NBT medium (0.2 ml of 0.34% sucrose solution), (0.2 ml of 0.28% NBT) and 0.2 ml of inactivated fetal calf serum) was added and incubated at 37 °C for 30 min. After incubation, slide was washed with cold saline and stained with safranin. When NBT was phagocytosed by the cells, intracellular dye converts it into an insoluble blue crystalline form (Formazon crystals). One hundred cells were observed and the positive cells with the formazon granules were counted.

**Corticosterone Estimation** The plasma corticosterone level was estimated by the method of Mattingly.<sup>22)</sup> 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<sub>2</sub>SO<sub>4</sub> 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.

**Food, Water Intake and Animal Body Weight** Pre-measured quantities of water, food pellets (Hindustan) were provided to the rats *ad libitum* and the quantity consumed per day was measured everyday between 09.00 and 10.00 h. Mean of the weekly intake of water and food pellets for 48 d were calculated and represented in Figs. 1 and 2. Animal

Table 1. Effect of Triphala on Neutrophil Functions and Corticosterone Level (Mean±S.D.)

Parameters	Control	Noise stress	Triphala	Triphala noise stress
Total count (Cu.mm/ml)	18408±584.31	15317±807.26 <sup>a)</sup>	19358±953.63 <sup>b)</sup>	17178±411.60 <sup>b)</sup>
Neutrophil adherence (%)	24.00±0.89	19.17±0.98 <sup>a)</sup>	26±1.414 <sup>b)</sup>	22.83±1.33 <sup>b)</sup>
Phagocytic index (P.I) (%)	79.33±1.03	72.83±1.47 <sup>a)</sup>	81.83±2.63 <sup>b)</sup>	76.50±1.05 <sup>b)</sup>
Avidity index (A.I) (%)	2.84±0.15	2.12±0.05 <sup>a)</sup>	3.21±0.17 <sup>a,b)</sup>	2.52±0.14 <sup>b)</sup>
NBT reduction (%)	11.16±0.98	13.33±1.63	12.67±1.37	26.17±1.94 <sup>a,b)</sup>
Corticosterone (µg/dl)	50.17±1.72	63.42±3.71 <sup>a)</sup>	47.17±0.79 <sup>b)</sup>	50.21±1.74 <sup>b)</sup>

a)  $p < 0.05$  compared with control. b)  $p < 0.05$  compared with noise stress.

Table 2. Effect of Triphala on Neutrophil Functions and Corticosterone Level in Immunized Groups (Mean±S.D.)

Parameters	Control immunized	Noise stress immunized	Triphala immunized	Triphala noise stress immunized
Total count (Cu.mm/ml)	12192±677.06	10317±419.13 <sup>a)</sup>	13975±863.57 <sup>a,b)</sup>	11347±248.17 <sup>a,b)</sup>
Neutrophil adherence (%)	28.83±0.98	24.17±1.17 <sup>a)</sup>	31.33±1.97 <sup>a,b)</sup>	27.33±1.51 <sup>b)</sup>
Phagocytic index (P.I) (%)	88.00±1.27	82.00±2.09 <sup>a)</sup>	92.17±2.32 <sup>a,b)</sup>	86.17±1.17 <sup>b)</sup>
Avidity index (A.I) (%)	3.63±0.34	3.13±0.08 <sup>a)</sup>	4.36±0.26 <sup>a,b)</sup>	3.45±0.19 <sup>b)</sup>
NBT reduction (%)	32.67±1.25	25.00±1.79 <sup>a)</sup>	36.67±1.30 <sup>a,b)</sup>	31.17±1.47 <sup>b)</sup>
Corticosterone (µg/dl)	46.26±1.25	58.86±1.94 <sup>a)</sup>	41.6±1.30 <sup>a,b)</sup>	47.66±2.18 <sup>b)</sup>

a)  $p < 0.05$  compared with control immunized. b)  $p < 0.05$  compared with noise stress immunized.

body weight was also measured periodically.

**Statistical Analysis** All the data were statistically analyzed using one way ANOVA followed by Tukey's multiple comparison tests. The level of significance was fixed at  $p < 0.05$ .

Food, water intake, animal body weight and differential count were statistically analysed by Student's "t" test  $p < 0.05$ .

## RESULTS

**Total Leukocyte Count (TLC)** Administration of Triphala did not show any significant change in the TLC in Triphala group when compared with control animals (Table 1). However, when rats were immunized with SRBC, TLC was significantly increased in Triphala immunized group with respect to control immunized animals (Table 2). Rats exposed to noise-stress, significant decrease in TLC was observed in both noise-stress and the noise-stress immunized groups when compared to their respective controls. These noise-stress induced reductions in TLC were significantly prevented in Triphala noise-stress group. Even though there was a significant increase of TLC in Triphala stress immunized group when compared to stress immunized, but it was significantly decreased from the control immunized group.

**Differential Leukocyte Count (DLC)** Oral administration of Triphala for 48 d did not show any significant changes in the different leukocyte population of neutrophil, lymphocyte and monocytes when compared with the control animals (Table 3)

**Neutrophil Adherence (NA)** Percentage of NA was not significantly changed in Triphala group when compared with control animals. NA to nylon column was significantly increased in Triphala immunized group with respect to the control immunized group (Table 2). When rats were exposed to noise-stress for 15 d, significant decrease in NA was observed in both noise-stress and noise-stress immunized groups when compared to their respective controls. These

Table 3. Effect of Triphala on Differential Leukocyte Count in Control Group (Mean±S.D.)

Parameters	Control	Triphala
Neutrophil (%)	23.67±1.44	25.00±1.21
Lymphocyte (%)	71.50±1.17	70.33±1.44
Monocyte (%)	2.33±0.49	2.67±0.49

Student's "t" test comparison  $p < 0.05$ .

noise-stress induced decreases in NA were significantly prevented in the drug administrated group of both Triphala noise-stress and Triphala noise-stress immunized groups when compared to their respective controls (Tables 1, 2).

**Phagocytic Index (P.I)** The number of neutrophil positive for candida phagocytosis was not significantly altered in Triphala group (Table 1). When rats were challenged with SRBC, significant enhancement in P.I was observed in Triphala immunized group when compared with control immunized animals (Table 2). Noise-stress significantly decreases the P.I in both noise-stress and noise-stress immunized groups. Triphala administrated rats exposed to noise-stress, the noise-stress induced decrease in P.I was significantly prevented in both Triphala noise-stress and Triphala noise-stress immunized groups with respect to their controls.

**Avidity Index (A.I)** Administration of Triphala for 48 d significantly increased the average number of candida engulfed by the neutrophils in both Triphala and Triphala immunized groups with respect to their controls (Tables 1, 2). On exposure to noise-stress, significant reduction in A.I was observed in both noise-stress and noise-stress immunized groups. When Triphala treated groups exposed to noise-stress, insignificant change in the A.I was observed in both Triphala noise-stress and the Triphala noise-stress immunized groups with respect to their controls.

**Nitroblue Tetrazolium (NBT) Reduction Test** The NBT reduction was not altered significantly in Triphala group when compared with control animals (Table 1). When

rats were immunized with SRBC's the NBT reduction was significantly increased in Triphala immunized group with respect to control immunized animals (Table 2). Rats were exposed to noise-stress, NBT reduction was not altered significantly in noise-stress group, but significant decrease in NBT reduction was observed in noise-stress immunized group with respect to their control groups. The enhancement of NBT reduction was observed in both Triphala noise-stress and Triphala noise-stress immunized group when compared with their respective controls.

**Corticosterone** Oral administration of Triphala not showed any marked changes in the corticosterone level in Triphala group. Among the rats that were immunized with SRBC, corticosterone level was found to be decreased in Triphala immunized group with respect to control immunized animals. Animals exposed to noise-stress, significant increase of corticosterone level was observed in both the noise-stress and the noise-stress immunized groups when compared to their respective controls. These noise-stress induced increases in corticosterone level were significantly prevented in both Triphala noise-stress and Triphala noise-stress immunized groups when compared to their controls (Tables 1, 2).

**Food, Water Intake and Animal Body Weight** Oral administration of Triphala for 48d influences the food and water intake significantly in Triphala group when compared with control animals (Figs. 1, 2). Though the animal body weight was significantly reduced in the initial stage of the drug administration, but it was gradually increased and insignificant with that of controls was observed from fourth week onwards (Fig. 3).

## DISCUSSION

Immune activation is an effective as well as protective approach against emerging infectious diseases.<sup>23)</sup> Basic research on natural substances with immunomodulating properties are performed by stimulating the non specific innate immunity.<sup>7)</sup> In our study, neutrophil functions were examined. Immunized animals were used, because dynamic and complex nature of the immune system in which a drug effect can be detected more reliably after immune challenge. Noise has an ever-increasing impact on human daily life and stress-related illnesses are more frequently observed.<sup>24)</sup> Noise-stress has high environmental and clinical relevance,<sup>25)</sup> moreover environmental stresses are more reliable to study the natural defenses.<sup>26)</sup> In this study noise-stress was used to alter the neutrophil functions, as noise exceeding 90 dB is considered as a stress.<sup>27)</sup> Stress result in over secretion of glucocorticoids,<sup>28)</sup> hence plasma corticosterone level was estimated.

Circulation of Immune cells is essential for maintaining an effective immune defense network.<sup>29)</sup> TLC was found to be increased in the Triphala immunized group, this may be due to the fall in the corticosterone levels. When rats exposed to noise-stress, significant reduction in the TLC population was observed. This reduction in TLC may be due to the increased glucocorticoids levels, which affects the circulation pattern of immune cells.<sup>30)</sup> Jensen and Rasmussen (1963) reported the reduction of TLC in mice was observed after stress exposure<sup>31)</sup> and in rats it was reported by Dhabhar *et al.* (1995).<sup>32,33)</sup> When Triphala treated rats exposed to noise-

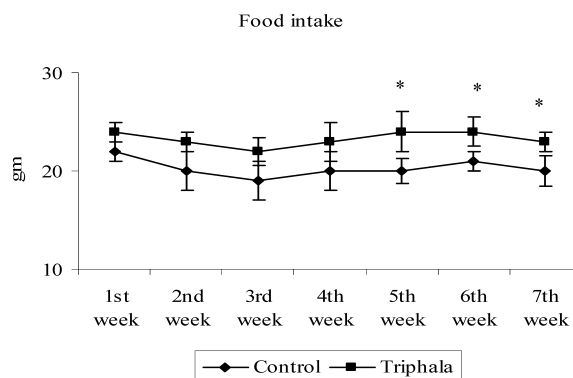


Fig. 1. Food Intake

\* $p < 0.05$ , versus control (mean  $\pm$  S.D.).

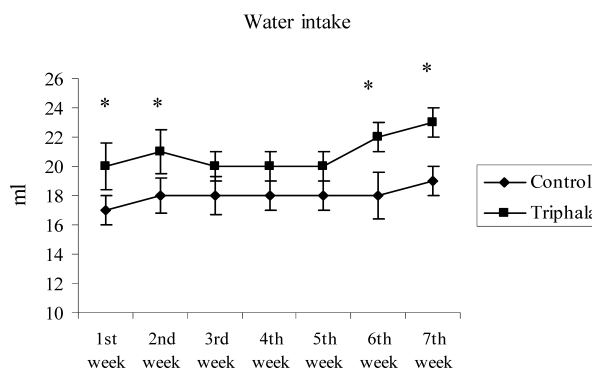


Fig. 2. Water Intake

\* $p < 0.05$ , compared with control (mean  $\pm$  S.D.).

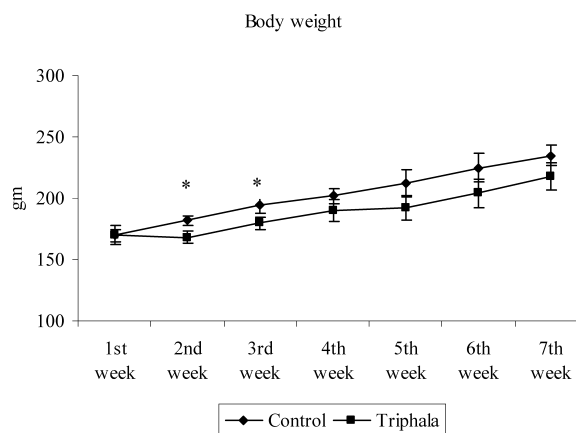


Fig. 3. Body Weight

\* $p < 0.05$ , compared with control (mean  $\pm$  S.D.).

stress, maintains the normal circulation of TLC, this may be due to the insignificant elevation in the corticosterone level.

Margination of neutrophils from the blood stream requires a firm adhesion, which is mediated through the interactions of the  $\beta 2$  integrins present on the neutrophils.<sup>34,35)</sup> The  $\beta 2$  integrin stored in the cell granules to be up regulated for a firm adherence.<sup>36)</sup> Adherence to nylon column was increased in the Triphala immunized group. This may be due to the up-regulation of  $\beta 2$  integrins and also by decreased corticosterone level. Reports suggest that oral administration of *Haridradi ghrita* (main ingredients of Triphala) significantly increases the neutrophil adherence to nylon column.<sup>37)</sup> De-

crease in the percentage of NA was observed in noise-stress groups, either internalization or shedding of  $\beta 2$  integrins may be the reason for this decrease.<sup>38)</sup> Increased glucocorticoids level may also lead to this decrease in the neutrophil adherence.<sup>39)</sup> As corticosterone level was not altered by noise-stress in the Triphala treated group. This could be the possible reason for normal neutrophil adherence.

Phagocytosis by neutrophils constitutes an essential arm of the host to defense against foreign antigens. Neutrophils have receptors for fragment crystallizable (Fc) and complement component (C3b) which are involved in the uptake of foreign antigens. Both PI and A.I was enhanced in the immunized groups of Triphala, this enhancement may be due to the fall in corticosterone level. When rats were exposed to noise-stress, decrease in both PI and A.I was observed. Evidence emerged that prolonged stressors can substantially decrease serum complement levels in animals.<sup>40)</sup> Chohan *et al.* (2001) reported that mice exposed to chronic auditory stress results in the suppressed *in-vitro* and *in-vivo* activity of phagocytic.<sup>41)</sup> The corticosterone levels were found to be normal in the Triphala treated stress exposed groups, which could be the possible reason for no significant change in PI and A.I.

NBT reduction test relies on the generation of bactericidal enzymes (like NADPH-oxidase) in neutrophils during intracellular killing. These enzymes are necessary for normal intracellular killing against foreign antigens. During intracellular killing, the cellular oxygen consumption increases and glucose metabolism reduces the colorless NBT to blue formazan.<sup>42)</sup> Triphala administrated group immunized with SRBC, showed increase in NBT reduction. This may be due to increase in the bactericidal enzymes within the neutrophils. The generation of the bactericidal enzymes may be affected by the noise-stress, results in the decreased NBT reduction. The generation of bactericidal enzymes may not be affected by the noise-stress in Triphala treated groups, this may be the reason for insignificant changes in the NBT reduction.

During stress, limbic system of the brain triggers the hypothalamus to secrete corticotropin-releasing hormone (CRH).<sup>43)</sup> The CRH triggers the pituitary gland to secrete adrenocorticotrophic hormone, which activates the adrenal glands to release corticosterone secretion in the blood stream.<sup>44)</sup> Decrease in the corticosterone level was observed in the Triphala immunized group. This may be one of the reasons for the stimulation of neutrophil functions was observed in this group. Exposed to noise-stress, corticosterone levels were raised. Kugler *et al.* (1990) showed an increase in plasma corticosterone levels for up to four weeks from the day of stress exposure.<sup>45)</sup> Increased corticosterone levels may be the reason for significant suppression of neutrophil functions in stress exposed groups. Reports conform that increase in corticosterone level suppresses both innate as well as acquired immune functions.<sup>46,47)</sup> As there was no significant elevation in the corticosterone level was observed in Triphala treated stress exposed groups, this could be the reasons for maintaining the normal neutrophil functions.

Food and water intake were significantly increased in the Triphala administrated groups. Chawla (1982) reported that Triphala improves digestion,<sup>48)</sup> this may be one of the reason for an increased food and water intake. The animal body weight was reduced in the initial stage of the drug administration, may be due to hypocholesterolaemic action of

Triphala,<sup>49)</sup> however animal body weight was gradually increased from 4th week of drug administration.

The outcome of this result confirms that, treatment with Triphala for 48 d enhanced the A.I in Triphala group and appears to stimulate the neutrophil functions with decreased corticosterone level in the Triphala immunized groups. When rats were exposed to noise-stress for 15 d, neutrophil functions were significantly suppressed and the corticosterone levels were increased. This noise-stress induced suppression in the neutrophil functions and increased corticosterone levels were significantly prevented by Triphala.

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