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Immunomodulatory and Antioxidant Activity of a Polyherbal Formulation

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Abstract: Immunomodulatory and antioxidant activity of Guard Sansar, a Polyherbal Formulation (PHF) was assessed by carbon clearance assay and adhesion of neutrophils to nylon fibers, in Swiss albino mice and by estimation of Lipid Peroxidation (LPO), Superoxide Dismutase (SOD), catalase (CAT) and reduced Glutathione (GSH) from blood of pyrogallol-treated Wistar rats, respectively, using Levamisole as reference standard. The PHF showed significant immunomodulatory activity by increasing the rate of carbon clearance and the percent neutrophil adhesion to nylon fibers. The oxidative stress, evidenced as elevation of LPO and reduction of SOD, CAT and GSH, was reversed by pre treatment with Polyherbal Formulation. Guard Sansar possesses immunostimulatory and antioxidant activity.

Key words: Levamisole, carbon clearance, neutrophils adhesion, oxidative stress, SOD, CAT, GSH

INTRODUCTION

Stress has an impact on immune response of our body. The oxidative stress due to free radicals (H_2O_2 , oxy radicals O_2^- etc.) lead to many diseases such as atherosclerosis (Miller *et al.*, 2005), heart failure (Pacher *et al.*, 2005), shock (Ilangovan *et al.*, 2006), Alzheimer's disease, Parkinson's disease (Sudha *et al.*, 2003) and Diabetes mellitus (Rahimi *et al.*, 2005). Immune system is vulnerable to the free radical-induced oxidative stress. The cellular and humoral components of the immune system are particularly sensitive to increased levels of reactive oxygen species, which may cause premature immunosenescence (Joharapurkar *et al.*, 2004). It is essential to counteract this oxidative stress and there by enhance the immunity of body system. Allopathic drugs are available for counteracting the oxidative stress and hence improve immunity, but the side effects and prohibitive cost of these allopathic drugs makes it necessary to search for an alternative. The Ayurvedic system of medicines not only provides that alternative, but also scores over the side effects and cost factor of allopathic medicine.

Guard Sansar (GS) is a polyherbal formulation, developed by Pradhan Herbal Company, Bangalore. GS consists of extracts of seven medicinal plants, which are *Emblica officinalis*, *Tinospora cardifolia*, *Terminalia arjuna*, *Piper longum*, *Comiphora mukul*, *Nardostachys jatamansi* and *Boerhavia diffusa* as well as corals and asphaltum. These plant extracts are classified, in ayurveda, as Rasayanas which improve defense mechanism of body and possess antioxidant activity.

Earlier studies indicate that *Emblica officinalis* (Scartezzini *et al.*, 2006), *Tinospora cardifolia* (Desai *et al.*, 2002), *Terminalia arjuna* (Chander *et al.*, 2004) and *Piper longum* (Sunila and Kuttan, 2004) possess immunomodulatory and antioxidant activity. As there is a need to generate scientific evidence/preclinical data to support the clinical use of the ayurvedic medicine (s), the present study was undertaken to investigate and to validate the immunomodulatory and antioxidant activity of Guard Sansar.

MATERIALS AND METHODS

Drugs and chemicals: Polyherbal formulation (PHF) Guard Sansar was obtained as a gift from Pradhan Herbal Company, Bangalore, in September 2007. Levamisole of Khandelwal labs, Mumbai was used as reference standard drug (Meera and Mustafa, 2007). All other chemicals and reagents used were of analytical grade.

Animals: Albino rats, Wistar strain, weighing 200 ± 25 g of either sex and male Swiss albino mice weighing 22 ± 3 g, procured from National Institute of Mental Health and Neurosciences, Bangalore were used in this study. The animals were acclimatized in institutional animal house (registration No. 152/1999/CPCSEA) for 2 weeks prior to the study, in standard laboratory conditions of temperature $27 \pm 2^\circ C$ and relative humidity $60 \pm 2\%$. The animals were provided standard pellet diet (Amrut feeds, Bangalore) and water *ad libitum*. The Institutional Animal Ethics Committee's permission was obtained before starting the experiments on animals.

Experimental

Carbon clearance assay: Swiss albino mice were divided into 4 groups of 6 animals each. Control (group 1) animals were administered the vehicle, group 2, 3 and 4 animals were administered the PHF 390, 780 mg kg⁻¹ and reference standard levamisole, 50 mg kg⁻¹ P.O., respectively for 5 days. The dose of the polyherbal formulation was calculated from human dose (3 g day⁻¹) 48 h after the last treatment, mice were injected carbon ink suspension (0.1 mL) via the tail vein. Thereafter, blood was drawn (in 5 µL EDTA solution) from the retro-orbital plexus at 0 and 15 min, a 25 µL sample mixed with 2 mL of 0.1% sodium carbonate solution and absorbance was read at 660 nm using spectrophotometer (Schimadzu 1601). The phagocytic index was calculated using the Equation:

$$\text{Phagocytic index} = \frac{k(\text{sample})}{k(\text{control})}$$

$$K = (\text{Log}_e \text{OD}_1 - \text{Log}_e \text{OD}_2) / 15$$

Where:

- OD₁ = Optical densities at 0 min
- OD₂ = Optical densities at 15 min (Jayathirtha and Mishra, 2004; Ghule *et al.*, 2006)

Neutrophil adhesion: Swiss albino mice were divided into 4 groups of 6 animals each. Control (group 1) animals were administered the vehicle, group 2, group 3 and group 4 animals were administered the PHF 390, 780 mg kg⁻¹ and reference standard levamisole, 50 mg kg⁻¹ P.O., respectively, for 14 days. On 14th day, blood was collected from the retro-orbital plexus into heparanized vial and analyzed for total leucocyte count (TLC) and differential leucocyte count (DLC). There after, blood was incubated with 80 mg mL⁻¹ of nylon fibers for 15 min at 37°C and analyzed for TLC and DLC. The product of TLC and % Neutrophil gives Neutrophil index (NI). Percent neutrophil adhesion was calculated as shown:

$$\text{Neutrophil adhesion (\%)} = \frac{NI_u - NI_t}{NI_u} \times 100$$

Where:

- NI_u = Neutrophil index of untreated blood sample
- NI_t = Neutrophil index of treated blood sample (Fulzele *et al.*, 2002)

Pyrogallol induced immunosuppression: Albino rats, Wistar strain were divided into 5 groups of 6 animals each. Group 1 (Control) animals were administered the vehicle only, group 2 (Pyrogallol Control) animals were administered the vehicle and Pyrogallol 50 mg kg⁻¹ I.P., group 3, 4 and 5 animals were administered the PHF

270 mg kg⁻¹, 540 mg kg⁻¹ and reference standard Levamisole 50 mg kg⁻¹ P.O., respectively, for 28 days. The animals of group 3, 4 and 5 were also administered Pyrogallol 50 mg kg⁻¹ intraperitoneally, for 28 days. The dose of polyherbal formulation was calculated from human dose (3 g day⁻¹) using conversion factor (0.018 for rats and 0.0026 for mice) based on body surface area. On 14th day, the rats were sensitized with sheep red blood cells [0.5×10⁹ cells (100 g⁻¹ i.p.)]. On 21st day, blood was withdrawn from retro orbital plexus, 25 µL of blood was mixed with SRBC [0.025×10⁹ cells (100 g)⁻¹] and the analyzed for presence/absence of humoral immune response (Joharapurkar *et al.*, 2004).

On 28th day, the blood was withdrawn from retro orbital plexus and centrifuged at 8000 rpm for 20 min. The supernatant was discarded and the pellet was dissolved in adequate quantity of 0.1 M phosphate-buffered normal saline to get 5% suspension of RBC.

Lipid peroxidation (LPO) estimation: Two milliliter of 28% trichloroacetic acid was added to 2.0 mL of RBC suspension and centrifuged. One mL of 1% thiobarbituric acid was added to the supernatant, heated in boiling water bath for 60 min, cooled and absorbance was read at 532 nm. Lipid peroxidation was calculated using the molar extinction coefficient of malondialdehyde (MDA) 1.56×10⁵ and expressed in terms of nanomoles of MDA g⁻¹ Hb (Stocks and Dormandy, 1971).

Super oxide dismutase (SOD) estimation: To 50 µL of the erythrocyte lysate prepared from the 5% RBC suspension, 75 mM of Tris-HCl buffer (pH 8.2) and 30 mM EDTA were added and absorbance was read at 420 nm, using spectrophotometer (Schimadzu 1601). 2 mM of pyrogallol was added to the reaction mixture and an increase in absorbance was recorded at 420 nm for 3 min. One unit of enzyme activity is 50% inhibition of the rate of autooxidation of pyrogallol as determined by change in absorbance min⁻¹ at 420 nm (Marklund and Marklund, 1984). The protein content of lysate was estimated by Lowry's (1951) method and the activity of SOD is expressed as units mg⁻¹ protein.

Catalase estimation: Catalase (CAT) activity was determined in erythrocyte lysate using Aebi's method with some modifications. The erythrocyte lysate (50 µL) was added to a cuvette containing 2.0 mL of phosphate buffer (pH 7.0) and 1.0 mL of 30 mM H₂O₂ and absorbance was read after 1 min at 240 nm. The molar extinction coefficient of H₂O₂, 43.6 M cm⁻¹ was used to determine the Catalase activity. One unit of activity is equal to one millimole of H₂O₂ degraded per minute and is expressed as units mg⁻¹ of protein (Aebi, 1984).

Glutathione estimation: Blood glutathione was measured by addition of 0.2 mL of whole blood to 1.8 mL distilled water followed by 3.0 mL of precipitating mixture (1.67 g metaphosphoric acid, 0.2 g EDTA and 30 g NaCl to make 100 mL of solution). It was centrifuged at 5000x g for 5 min and 1.0 mL of the supernatant was added to 1.5 mL of the phosphate solution, followed by the addition of 0.5 mL of DTNB reagent. The optical density was measured at 412 nm using a Shimadzu 1601 spectrophotometer (Ellman, 1959).

On day 29, again, sheep red blood cells were injected in the sub planter region of the hind paw and an increase in the paw volume was measured after 48 h to assess cell mediated immune response.

Statistical analysis: All values were reported as mean±SEM. The results were analyzed using One-Way ANOVA, followed by Tukey's multiple comparison tests. $p = 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

As shown in Table 1, the phagocytic index and the percent neutrophil adhesion in PHF treated animals improved as compared to control. The result is comparable with that due to reference standard, Levamisole.

In pyrogallol control animals, the humoral response was negative (no clumping due to antigen antibody reaction). The humoral immune response in rats pretreated with PHF (540 mg/kg/day) and levamisole (50 mg/kg/day) was positive.

As shown in Fig. 1, the paw volume in pyrogallol control rats was increased as compared to control group. In PHF and levamisole treated animals, the paw volume was decreased when compared to pyrogallol control.

Table 2 shows that the LPO level was increased and SOD, CAT, GSH levels were decreased in pyrogallol control rats as compared to control. In PHF treated animals the LPO level was decrease and SOD, CAT, GSH levels were increased in dose dependent manner as compared to pyrogallol control. These results are comparable with that due to reference standard, Levamisole.

The immune system is a complex system. The concept of Rasayana in Ayurveda is similar to the modulation of immune response by various agents in order to alleviate the disease. Immunomodulators are being used as an adjuvant in conditions of cancer and other stress related diseases. Immunomodulatory agents of plant origin enhance the immune responsiveness of an organism against a pathogen by nonspecifically activating the immune system. Guard Sansar, a polyherbal formulation was evaluated for the immunomodulatory and antioxidant activity in the present study. The PHF, in a dose of 780 mg kg⁻¹, p.o. produced a significant rise in carbon clearance indicating stimulation of the reticulo endothelial system. When reticulo endothelial system is stimulated, there is increase in number of phagocytic cells, which engulf the antigens, indicating increase in

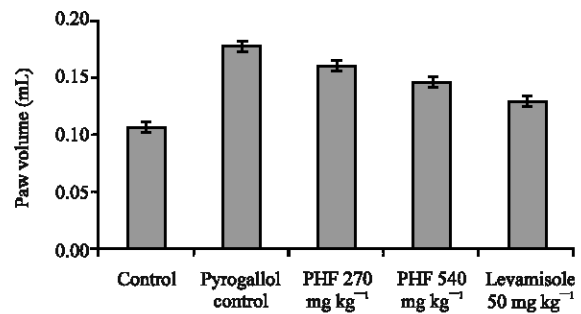


Fig. 1: Effect of PHF on paw volume in rats

Table 1: Effect of the polyherbal formulation on phagocytic index and percent neutrophil adhesion in experimental mice (Mean±SEM)

Groups	Treatments	Phagocytic index	Neutrophil adhesion (%)
1	Control	1.001±0.06600	20.658±0.754
2	Polyherbal formulation (390 mg kg ⁻¹) p.o.	1.345±0.0797*	24.773±1.230
3	Polyherbal formulation (780 mg kg ⁻¹) p.o.	1.498±0.0628**	31.876±0.764***
4	Standard reference levamisole (50 mg kg ⁻¹) p.o.	1.530±0.1021***	32.775±1.398***

n = 6; One way ANOVA followed by Tukey's multiple comparison test. *** $p < 0.001$ vs. control, ** $p < 0.01$ vs. control and * $p < 0.05$ vs. control

Table 2: Effect of PHF on oxidative stress parameters (values are mean±SEM)

Groups	Treatments	LPO (MMDA g ⁻¹ Hb)	SOD (U mg ⁻¹ protein)	CAT (U mg ⁻¹ protein)	GSH (μmol g ⁻¹ Hb)
1	Control	82.701±0.6696	25.576±0.3941	270.720±1.214	4.815±0.0472
2	Pyrogallol control	115.025±1.631***	15.111±0.3211***	172.466±1.075***	2.318±0.069***
3	PHF (270 mg kg ⁻¹) p.o.	104.370±0.892***	22.213±0.3291***	218.073±0.651***	3.826±0.078***
4	PHF (540 mg kg ⁻¹) p.o.	87.451±0.7469***	23.615±0.3896***	250.390±1.111***	4.223±0.109***
5	Levamisole (50 mg kg ⁻¹) p.o.	84.435±0.939***	24.033±0.2402***	258.946±0.846***	4.613±0.113***

n = 6; One way ANOVA followed by Tukey's multiple comparison test. *** $p < 0.001$ vs. normal control, *** $p < 0.001$ vs. pyrogallol control

immunity (Ghule *et al.*, 2006). PHF, when orally administered, significantly increased the adhesion of neutrophils to nylon fibers in a dose dependent manner. As the number of neutrophil increase, immunity of body against microbial infections is enhanced, due to chemotaxis, phagocytosis, exocytosis and both intracellular and extra cellular killing (Ghule *et al.*, 2006). The activity of PHF was compared with Levamisole, an already established immunomodulatory agent. The four plant extracts present in the PHF contribute to its immunomodulatory activity.

In this study, we have used pyrogallol to induce immunosuppression. Pyrogallol is a strong generator of free radicals (Gupta *et al.*, 2002). The free radicals on reaction with oxygen, generate reactive oxidative free radical intermediates, which repetitively attack polyunsaturated fatty acids in the biomembranes and initiate Lipid peroxidation (Boll *et al.*, 2001; Jeng-Yan *et al.*, 2003) which is a marker of oxidative stress. Thus, immunosuppression leads to oxidative stress.

In this study, in the pyrogallol control animals, there was increase in LPO and decrease in SOD, CAT GSH levels. In animals pre treated with PHF the endogenous antioxidant such as SOD, CAT GSH levels were increased and these increased endogenous antioxidants scavenge the reactive oxygen species so that LPO levels were decreased in dose dependent manner. These results are comparable with that of Levamisole.

CONCLUSION

The polyherbal formulation Guard Sansar, possess immunomodulatory and antioxidant activity. Authors declare that there is no conflict of interest.

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