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Research Article

IMMUNOMODULATORY ACTIVITY OF VYAGHRIHARITAKI AVALEHA ON HUMORAL ANTI-BODY FORMATION

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Abstract

The aim of the study was to find out the immunomodulatory effect on humoral anti-body formation of Vyaghriharitaki avaleha (VHA). Standard procedure of Doherty (1981) was followed. Wister strain albino rats of either sex weighing between 160-240 g were selected and divided into 3 groups. First group received tap water serving as the normal control. Second group was the SRBC control group. Calculated doses of the sample Vyaghriharitaki avaleha was administered to third group. Results showed Vyaghriharitaki avaleha is having good immunostimulant activity.

Key words: Vyaghriharitaki Avaleha, Immunostimulant.

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INTRODUCTION

Vyaghrihareethaki avaleha is a purely poly herbal compound formulation used for the management of various diseases of respiratory system like asthma, bronchitis and it is said have rasayana activity. The WHO and International Asthma Council (IAC) consultation report published in 1998 on implementation of Asthma Guidelines, highlights that wherever there is the use of Traditional Medicines in Asthma care, the conventional therapy should not be stopped because of lack of evidence of safety and efficacy of these therapies. Pharmacological researches are essential to give a strong scientific base regarding the safety and efficacy of the Ayurvedic treatment. Hence in the present study Vyaghriharitaki Avaleha (VHA) was taken to prove its immunomodulatory activity with scientific basis in experimental models. Standard procedure of Doherty (1981) was followed to find the effect on humoral anti-body formation to prove its immunomodulatory activity.[1] Parameters studied were Body weight initial and before sacrifice, Ponderal changes i.e. Weight of spleen and thymus, Antibody titer,[2] Haematological parameters like Hemoglobin, WBC, Neutrophils, Lymphocyte, Eosinophil, Monocyte, RBC, MCV, MCH, MCHC, Serum biochemical parameters like Total protein, Albumin, Globulin, AG ratio, Serum IgG and histopathology of thymus, spleen, lymph node.

MATERIAL AND METHODS

Test Drug

Vyaghriharitaki avaleha (VHA)

Dose fixation:

The human dose of Vyaghriharitaki Avaleha is 20 g per day. The dose for rat was calculated by extrapolating the human dose to animals based on the body surface area ratio by referring to the standard table of Paget and Barnes (1964).[3] On this basis the rat dose was fixed to be 1.8 g/kg rat. Prior to administration, the test drug was suspended in distilled water with appropriate concentration depending upon the body weight of animals. This was administered to animals orally with the help of gastric catheter sleeved to syringe.

Dose for Rats

Human dose × 0.018 for rat weighing 200 g. i.e. 20× 0.018 = 0.36 g / 200 g / day. Conversion to dose/kg body wt. = 1.8 g / kg body wt

Animals

Wister strain albino rats of either sex weighing between 160-240 g were taken from the animal house attached to I.P.G.T. & R.A., Gujarat Ayurved University, Jamnagar. The selected animals were kept under acclimatization for 7 days before dosing. Six animals were housed in cage of poly-propylene with stainless steel top grill. The dry wheat (post hulled) waste was used as bedding material and was changed every morning. The animals were exposed to 12hour light and 12 hour dark cycle. The relative humidity was 60 to 70 % and the ambient temperature was 22 ± 02 ºC during the time of experimentation. Amrut brand rat pellet feed supplied by Pranav Agro Ltd. was provided throughout the study period except on previous night of dosing i.e. overnight fasted before dosing. The drinking water was given ad libitum in polypropylene bottles with stainless steel sipper tube.

Permission for experiment

Institutional Animal Ethics Committee has approved the experimental protocol (Approval number: IAEC 06/09-11/PhD/01).

Animal grouping

The selected animals were grouped into three groups randomly irrespective of sex and each group comprised of six animals.

1) Group 1 : Water control
2) Group 2 : SRBC control
3) Group 3 : Vyaghriharitaki avaleha (VHA) : 1.8 g / kg for rats

Route of drug administration: Oral

The stock solution of suitable concentration was prepared with distilled water, freshly just prior to administration. The test drug and vehicle to control were administered according to the body weight of the animals by oral route with the help of gastric catheter of suitable size sleeved to a syringe nozzle.

Instruments used

Instruments used were weighing scale, monopan balance, sterilizer, surgical instruments, cotton, syringe, needle, catheters, beaker and filter paper.

METHODOLOGY

Wister strain albino rats of either sex weighing between 160-240 g were selected and divided into 3 groups. First group received tap water serving as the normal control. Second group was the SRBC control group. Calculated doses of the sample Vyaghriharitaki avaleha was administered to third group. The drugs were administered for 10 consecutive days. On third day, sheep's blood was collected from the city slaughter house in a sterilized bottle containing Alsever's solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% sodium chloride) aseptically so that agglutination of blood does not take place. The collected sheep blood was thoroughly washed with sterile normal saline through repeated centrifugation until the supernatant fluid became colorless and made to 20% SRBC solution. This sensitizing agent was injected subcutaneously in the dose of 0.5ml/100 g of body weight to the rats.

On the 11th day the animals were sacrificed by ether over dose and the blood was collected in sterile test tubes. Serum was separated from it and the components in it were inactivated by incubating it for 30 minutes at 56°C temperatures in a serological water bath.

The micro-titer plate was filled with 0.1 ml sterile normal saline and serial two fold dilutions of 0.1 ml of the serum in sterile saline solution were made in the micro-titer plate up to 16 times. 0.1 ml of thrice saline washed 3% SRBC was added to each well of the tray. Blood from the same animal (Sheep) was used for both sensitization and to determine antibody titer. The trays were covered and placed in refrigerator overnight. Antibody titer (heamagglutination titer) was noted on the next day. The titer was converted to log 2 values for easy comparison.

Spleen, thymus and lymph nodes were dissected out from the animals and their weight was recorded. Tissues were transferred to 10% formaldehyde solution for fixation and later on processed for histological studies.

Parameters studied

Ponderal changes

Body weight at the start and end of the study, weight of thymus and spleen were recorded.

Haematological and serum biochemical parameters:

To estimate haematological parameters 0.08ml blood was mixed with 0.02ml of EDTA (33.33 mg/ml) and fed to the auto analyzer (ERBA CHEM-5, Trans Asia). The parameters measured were; Hemoglobin, WBC,

Neutrophils, Lymphocyte, Eosinophil, Monocyte, PCV, RBC, MCV, MCH, MCHC.

For estimation of biochemical parameters, serum was separated from collected blood and requisite quantity of serum was fed to the auto analyzer (Fully automated Biochemical Random Access Analyzer, BS-200; Lilac Medicare Pvt. Ltd., Mumbai) which was automatically drawn into the instrument for estimating different parameters. Total protein (Biurate method),[4] Albumin, Globulin (BCG Dye method),[5] AG ratio were estimated.

Histopathological Studies

Fixation

It is the process of preserving, hardening and preventing post-mortem changes of the tissues. The tissues were excised out immediately after sacrificing, cleaned off extraneous tissues, cut into pieces of such appropriate thickness that the fixative readily penetrated throughout the tissue to be fixed. Tissue transferred to 10% formaldehyde solution (37-40% formaldehyde) and allowed to remain in it till they were taken up for processing.

Tissue Processing

Tissue processing involves dehydration, clearing, infiltration of the tissue with paraffin. The usual dehydrating agent is ethyl alcohol; acetone and isopropyl alcohol can also be used. Following dehydration tissue was transferred to a paraffin solvent, which is miscible with the dehydrating agent as well. These are known as clearing agents such as choloroform and xylene. Tissue were thoroughly washed by placing them under running tap water and then conveyed through a series of following solvents as per schedule for dehydration, clearing and paraffin infiltration.

Alcohol 60%	- 20 min.
Alcohol 70%	- 20 min.
Alcohol 80%	- 20 min.
Alcohol 90%	- 20 min.
Alcohol 95%	- 20 min.
Isopropyl Alcohol	- 20 min.
Acetone (2 changes)	- 20 min. each
Choloroform (3 changes)	- 20 min. each
Melted paraffin wax (60º C)	- 40 min.

Next the tissues were placed in paraffin wax to prepare tissue blocks, which are oriented so that sections are cut in desired plane of the tissue. Tissues are fixed to metal object holder after trimming them to suitable size.

Section Cutting

A smear of 5% Mayer's egg albumin was prepared and smeared on to the slide and dried. The tissue sections of the 6 μm thickness were cut with the help of Spencer type rotating microtome. The tissue sections were put on slide and drops of water and then sections were floated on slide between 50-60 ^{0}C. Water dried off and slide dried on hot plate at about 50 ^{0}C for 30 min. This section is ready for staining.

Staining Procedure

Reagents

Mayer's haematoxylin stain: Dissolve 50 g of ammonium or potassium in a liter of water without heating. Then dissolve haematoxylin 1.0 g in this solution, further add 0.2 g sodium iodate, 1.0 g citric acid and 50 g chloral hydrate, shake until all of them are in solution form. The final colour of stain is reddish violet.

Eosin Stain : 2% w/v in Alcohol : After fixing the section on slide, the sections were stained by serially placing them into following reagents :

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Xylol (2 changes)	-	3 min.
Acetone	-	3 min.
Alcohol 95%	-	3 min.
Running water	-	3 min.
Haematoxylin stain	-	20 min.
Running water wash	-	20 min.
Eosin working solution	-	2 min.
Alcohol 95% (3 changes)	-	3 min.
Acetone (2 changes)	-	3 min.
Xylol (2 changes)	-	3 min.

After passing through the above reagents and stains, the slides are mounted with DPX (Diphenyl Phthalein Xylene) and cover slips are placed taking care to avoid formation of air bubbles during mounting the slides. The slides were viewed under Trinocular Research Carl Zeiss Microscope (Germany) at various magnifications to note down the changes in the microscopic features of the tissues studied. [6]

Statistical analysis

The obtained data have been presented as Mean±SEM, difference between the groups was statistically determined by student's t test for paired and unpaired data for the treated group with the level of significance set at $P < 0.05$. The level of significance was noted and interpreted accordingly.

OBSERVATIONS AND RESULTS

Effect on humoral immunity (antibody formation)

Data related to the effect of test drug on body weight have been provided in Table 1. Statistically significant decrease in body weight was observed in SRBC control rats in comparison to normal control rats. The body weight decrease observed after SRBC injection was significantly reversed by treatment with the test drugs. Infact significant increase was observed in comparison to the initial body weight in the test group.

Table 2 shows the data pertaining to effect of test drug on weight of thymus and spleen. An apparent increase in relative weight of thymus was observed in SRBC group in comparison to normal control group. In VHA treated group elevation in weight of thymus was observed however the observed increase was found to be statistically non-significant in comparison to SRBC control rats. An apparent and statistically non-significant increase in spleen weight was observed in SRBC injected rats. The spleen weight in the test sample administered groups was not affected to significant extent.

Data related to effect of test drug on different haematological parameters have been provided in Table 3. SRBC sensitization significantly decreased eosinophil and monocyte count in comparison to normal control group. The test drug significantly reversed the decrease observed in eosinophil and monocyte counts. The test drug VHA showed significant decrease in WBC count in comparison to SRBC control group. The test drug showed significant decrease in platelet count in comparison to SRBC control group. Other haematological factors were not affected significantly by either SRBC sensitization or treatment with test drug.

Table 4 shows data pertaining to effect of test drug on different biochemical parameters in SRBC pre-sensitized rats. SRBC sensitization significantly increased total protein. Treatment with test drug did not affect the total protein level to significant extent in comparison to SRBC control group. In VHA treated group significant increase in serum albumin level was observed. Table 5 shows data pertaining to effect of test drug on antibody formation against SRBC - in rats. Administration of test drug apparently increased the anti-body titre value in comparison to SRBC control rats.

Table: 1 Effect on body weight

Treatment	Initial body weight (g)	Final body weight (g)	Actual change in body weight (g)	% change in comparison to control
Control	196.500 ± 09.32	221.667 ± 11.38$^{\alpha\alpha}$	25.167 ± 05.10	--
SRBC control	206.333 ± 14.49	194.667 ± 12.16	-11.667 ± 08.51$^{\#\#}$	146.35 ↓
VHA	179.333 ± 02.57	196.667 ± 04.46$^{\alpha\alpha}$	17.333 ±02.86**	248.56 ↑

Data: Mean ± SEM, ↑- Increase, ↓- Decrease,
$^{\alpha\alpha}$ P<0.01, (comparison to initial body weight, Paired t test)
$^{\#\#}$ P<0.01(comparison to normal control group, Unpaired t test)
** P<0.01(comparison to SRBC control group, Unpaired t test)

Table: 2 Effect on relative weight of thymus and spleen

Parameters	Control	SRBC Control	VHA
Thymus(mg/100g)	160.67 ± 9.83	178.47±06.17	182.69 ±19.51
Spleen(mg/100g)	214.00 ± 13.18	216.19±12.47	214.49 ±09.75

Data: Mean ± SEM, ↑- Increase, ↓- Decrease

Table: 3 Effect on different hematological parameters:

Parameters	Normal control	SRBC control	VHA
Hb (g/dl%)	15.12±00.22	15.47±00.36	15.08±00.32
WBC (10^3/Cumm)	7680.00 ± 566.92	7540.00 ±229.35	6280.00± 193.39**
Neutrophil (%)	17.80±03.14	21.50±02.32	19.00±01.48
Lymphocyte (%)	76.40±02.94	75.00±02.30	77.00± 01.79
Eosinophil (%)	03.17±00.17	02.20±00.37$^{\#}$	03.00±00.00*
Monocyte (%)	02.67±00.21	01.50±0.22$^{\#\#}$	02.50±00.22*
PCV (%)	49.02±00.61	49.37±01.65	46.87±00.89
RBC (10^6/cumm)	08.46±00.10	08.57±00.36	08.05±00.16
Platelet (10^3/ul)	1229.50 ±51.25	1345.00 ± 107.05	966.33± 39.44**
MCV	57.97±00.39	57.72±00.61	58.23±00.60
MCH	17.88 ±00.19	18.13 ±00.43	18.75±00.14
MCHC	30.85±00.19	31.40±00.57	32.18±00.19

Data: Mean ± SEM, ↑- Increase, ↓- Decrease,
$^{\#}$ P<0.05, $^{\#\#}$ P<0.01 (comparison to normal control group, Unpaired t test)
* P<0.05, ** P<0.01 (comparison to SRBC control group, Unpaired t test)

Table: 4 Effect on different serum biochemical parameters:

Parameters	Control	SRBC Control	VHA
Total protein (g/dl)	06.35 ± 0.13	07.43 ± 0.42	07.52 ± 0.13
Albumin (g/dl)	03.17± 0.06	03.57± 0.17	04.18 ±0.095
Globulin (g/dl)	03.18 ±0.18	03.87 ±0.47	03.33 ±0.20
A:G Ratio	01.01±0.07	00.99±0.12	01.27 ±0.10

Data: Mean ± SEM, ↑- Increase, ↓- Decrease

Table: 5 Effect on Antibody formation

Treatment	Antibody titer (Log_2 values)	Percentage change
SRBC Control	3.93 ± 0.23	--
VHA	4.62 ± 0.23	17.67 ↑

Data: Mean ± SEM ↑ - Increase

Table 6: Histopathological observations

Organs	Normal control	SRBC control	VHA
Thymus	Normal	Normal	Normal
Spleen	Normal	Normal	Increased white pulp proportion
Lymph node	Normal	Normal	Normal

Effect on cytoarchitecture of different organs

Microscopic examination of thymus, spleen and lymph node sections from different groups was carried out at different magnifications. Sections from normal control group were compared to sections from SRBC control rats. The sections from test drug administered and SRBC injected rats were compared to SRBC control rats.

Thymus

Examination of sections of thymus from normal control rats exhibited normal cytoarchitecture. In sections from SRBC control group also normal cytoarchitecture was observed in some sections in comparison to normal control group sections. Sections from VHA treated group showed increased cellularity in comparison to SRBC control group.

Spleen

Examination of sections from normal control rats exhibited normal cytoarchitecture. The cyto-architecture of SRBC control group was found to be similar to normal control group sections. Sections from VHA treated group showed increased white pulp proportion in comparison to SRBC control group.

Lymph nodes

Examination of sections from normal control rats exhibited normal cytoarchitecture. The cytoarchitecture of SRBC control group was found to be similar to normal control group sections. Sections from VHA treated group also showed similar cytoarchitectue that of SRBC control group**.**

DISCUSSION

Respiratory disorders are considered as an allergic condition. An allergy is an immune malfunction whereby a person's body is hyperensitized to react immunologically to typically non immunogenic substances. The immunity of the patients should be improved to combat the reoccurrence nature of the disease condition. The concept of immunomodulation relates to nonspecific activation of the function and efficiency of macrophages, granulocytes, complement, natural killer cells and lymphocytes and also to the production of various effector molecules generated by activated cells. It is expected that these nonspecific effects give protection against different pathogens including bacteria, viruses, fungi etc. and constitute an alternative to conventional chemotherapy.[7][8]

Gain in body weight indicates normal progressive health status of an organism.

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Decrease in body weight is indicative of degenerative changes in the body or certain organs. In the present study pre-sensitization to animals with SRBC lead to significant decrease in body weight in 10 days. The test drug significantly attenuated the SRBC induced body weight loss. This shows that the body weight decrease observed in SRBC alone group is reversed by test drug administration. The reason for the decrease in body weight after SRBC injection needs to be discussed. The body weight decrease may be due to decreased food intake or organ degeneration. The first reason seems to be more possible than the latter. The reversal of this effect can be considered to indicate the cytoprotective effect of the test formulation. As thymus and spleen are lymphoid organs and play a vital role in immune responses, administration of antigen may bring about some changes in their anatomical and physiological features. In the present study the test drug non-significantly increased the weight of thymus. Histopathological observation of this organ shows increased cellularity in the treated group. In spleen increased white pulp proportion was observed. The observed changes in these organs may be due to immunostimualtory effect of test formulation.

Haemagglutination antibody (HA) titer is a primary parameter for studying the humoral response. In the present study the formulation enhanced antibody titre indicating the presence of moderate immunostimulant activity. Among the 12 hematological parameters studied, antigen injection leads to significant changes only in two parameters. Significant decrease in eosinophil and Monocyte counts in SRBC control group was noted.

Treatment with VHA significantly reversed the changes observed in these two factors. The eosinophil count decreases in the cases of toxicity and monocyte count decreases in the immunosuppressive reactions.[9]

As only these two parameters were changed significantly among 12 haematological parameters it may not indicate any major change for drawing any inference. Among the biochemical parameters, serum total protein level is significantly increased in SRBC control group. Test formulation administration did not affect it to significant extent.

The main ingredient of the formulation Vyaghriharitaki is Kantakari and Haritaki. Reported study on *S. xanthocarpum* (Kantakari) further confirms the traditional use of *S. xanthocarpum* as a popular complementary medicine to relieve cough and bronchial asthma.[10]

Immunostimulatory activity of aqueous extract of *S. xanthocarpum* fruits on mice gives strong evidence that the plant is an immunostimulating agent. [11] *T. chebula*s also has immunomodulatory activity.[12]

Alcoholic extract of the fruits of *P. longum* and its component piperine was studied for their immunomodulatory activity.[13]

The observed activity profile in the present study may be attributed due to presence of these ingredients in the test formulation. The present study provides evidence that the formulation Vyaghriharitaki has immunomodulatory activity which supports the rasayana effect of the test drug scientifically supporting the opinion of the Ayurvedic literature.

CONCLUSION

The test drug Vyaghriharitaki Avaleha showed immunostimulant activity. Yet various other pharmacological actions should be proved along with clinical trials in large samples to substantiate the traditional science with scientific background.

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