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Research Article

EVALUATION OF IMMUNOMODULATORY ACTIVITY OF AQUEOUS EXTRACT OF A POLYHERBAL FORMULATION BY *IN-VIVO* METHOD

MANJULADEVI.K[2*], PRAMOD REDDY.G[1], A.R.KOTHAI[2], THENMOZHI.M[2], DHANALAKSHMI.M[3], SARUMATHY.S[3]

[1]Department of Pharmacology, Siddha Central Research Institute (SCRI), Chennai.[2]Department of Pharmacology, Swamy Vivekanandha College of Pharmacy, Tiruchengode.,[3]Swamy Vivekanandha College of Pharmacy, Tiruchengode E-mail: munchpharma@gmail.com.

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ABSTRACT

The objective of the present study was to evaluate the immunomodulatory activity of aqueous extract of polyherbal formulation (RA-1). The assessment of immunomodulatory activity was carried by testing the humoral (Haemagglutination antibody titer (HA) model & zinc sulphate turbidity test) and cell mediated immunity (Delayed type hypersensitivity reaction model) using aqueous extract of RA-1, at 200 & 400mg/kg/day, p.o.b.w. for 21 days in healthy albino rats.. RA-1 showed a significant ($P<0.05$) increase in both the primary and secondary HA titer values and increase in serum immunoglobulin levels when compared with control which indicates it's stimulatory effect on humoral immunity. On other hand, in delayed hypersensitivity test, RA-1 significantly ($P<0.01$) potentiated the DTH response when comparing with control, indicates its triggering effect on cell mediated immunity. From the above results, it was concluded that the aqueous extract of RA-1 was safe at dose of 2000mg/kg. The active constituents like flavonoids, terpenoids & glycosides were found to be present in aqueous extract of RA-1 and it also has good stimulatory effect on both humoral and cell mediated immunity. This study comprises of safety, preliminary phytochemical screening and immunomodulatory activity of aqueous extract of RA-1.

Keywords: Immunomodulatory activity, HA titer, DTH, Zinc sulphate turbidity test.

INTRODUCTION

Immune system has evolved to protect the host from invading pathogens and to eliminate diseases[1]. Immune system not only protects against infection but also limits excessive response that might lead to autoimmune diseases[2]. Autoimmune diseases can be treated using immunomodulators. Immunomodulators used in the treatment of these disease conditions are associated with adverse effects & drug interactions[3].

Herbal preparations have been used as an immunomodulator in traditional medicine[4]. Several herbal preparations that can enhance the body immune status are extensively used in the indigenous system of medicine. There is an upsurge in the clinical usage of indigenous drugs because they are associated with fewer side effects[5].

A polyherbal formulation (RA-1) being prescribed for rheumatoid arthritis patients at Govt. Siddha Hospital, Arumbakkam, Chennai is suggested to possess immunomodulatory activity. There is no pharmacological & scientific data available to substantiate their effects. Therefore, this study was undertaken to evaluate the acute toxicity and immunomodulatory activity of the above polyherbal formulation (RA-1) in experimental animals.

MATERIALS AND METHODS

Chemicals

Dextrose, Sodium citrate, Sodium chloride, Citric acid and ethanol were used in this study was gifted samples from Siddha Central Research Institute (SCRI), *Ocimum sanctum*, linga chenduram and ingredients of RA-1 were procured from local market. A diagnostic kit used in biochemical evaluation was purchased from Siemens's Health Care Diagnostics Ltd.

Sample preparation

Collection of plant material

The polyherbal formulation RA-1 was prepared according to the method described in "Formulary of Siddha Medicines".

List of plants present in the RA-1 formulation[6]: -

Cardiospermum halicacabum L.
Tinospora cordifolia (Willd.) HK.f. & Th
Alpinia officinarum Hance
Withania somnifera (L.) Dunal
Glycyrrhiza glabra L.
Linga chenduram.

The ingredients of RA-1 were procured from local market, Chennai and authenticated by the Department of Pharmacognosy, SCRI. All the ingredients were thoroughly washed with tap water. They were shade-dried for ten days and powdered mechanically and mixed in proportion as per procedure in Formulary of Siddha Medicines.

Preparation of extract

The extraction process was carried out by cold maceration. The extract was filtered through Whatman filter paper no.1 to remove all unextractable matter, including cellular materials and other constituents that are soluble in extraction solvent. To obtain a concentrated extract, the crude extract was evaporated by keeping it in desiccator. The solidified extract was stored in air tight container in the refrigerator at 4-6°C for further use.

Phytochemical analysis

The aqueous extract of polyherbal formulation (RA-1) was subjected to preliminary phytochemical screening for the detection of various plant constituents like alkaloids, glycosides, saponin, steroids, terpenoids, tannins, carbohydrates, flavonoids, lignin etc..,

Experimental animals

Young mature Albino *Wistar* rats of either sex weighing about 180 to 220gm and Swiss albino mice of both sex weighing about 25 to 30gm were used which were obtained from the inbred colony in animal house of SCRI, Chennai. All the animals were kept in 12 hours light and 12 hours dark with constant temperature 23±2°C and relative humidity of 55-60%. The animals were provided with food and water *ad libitum* except during fasting. The present study was conducted after obtaining approval from the Institutional Animal Ethical Committee(IAEC) and this protocol met the requirement of nation guidelines of CPCSEA[7] (PROPOSAL NO: 88/PHARMA/SCRI/2010, Dated on 18th Nov, 2010).

Experimental procedure

The aqueous extract of RA-1 was subjected to acute oral toxicity test and pharmacological evaluation.

ACUTE TOXICITY STUDIES

Acute toxicity studies were carried out according to the OECD-423 guidelines[8]. Three female albino rats were randomly selected and weighed. Albino rats weighing 180-220gms were used for the study. The animals were subjected to overnight fasting with free access to water. The extract was administered in single oral dose of 2000mg/kg. Then the animals were deprived of food for 4 hours after administration of test preparation and observed for mortality and physical/behavioral changes for 14 days.

EVALUATION OF IMMUNOMODULATORY ACTIVITY

Experimental design

Twenty four albino rats of either sex with a weight between 180-220gm were chosen. The animals were divided into four groups, with six animals in each group. Group I: Control group received 0.2ml of distilled water - per orally (p.o), Group II: Standard drug group received ethanolic extract of *Ocimum sanctum* (100mg/kg, p.o), Group III: Test drug group received aqueous extract of RA-1(200mg/kg, p.o.) and Group IV: Test drug group received aqueous extract of RA-1(400mg/kg, p.o).

Preparation of Alsevere's solution

Composition:Dextrose (2.05gm), Sodium citrate (0.8gm), Sodium chloride(0.4 gm) and Citric acid(0.05 gm). All the ingredients were weighed and dissolved in 100ml distilled water. Alsevere's solution was used in the proportion of 1:2 (sheep blood:Alsevere's solution) [9].

Antigenic material: Preparation of Sheep RBCs (SRBCs)

Sheep blood was collected in the sterile Alsevere's solution. Blood was kept in the refrigerator and processed, for the preparation of SRBCs batch, by centrifuging at 2000rpm for 10 minutes and washing with physiological saline for 4-5 times and then suspending into buffered saline for further use.

Haemagglutination Antibody (HA) titer

Group I animals received vehicle only for 21days. Group II animals received standard drug 100mg/kg, Group III & IV animals were given 200mg/kg and 400mg/kg of the aqueous extract of RA-1 daily for 21 days respectively. On 7th and 14th day of the study, animals from all the groups (I to IV) were immunized and challenged respectively, with SRBCs in normal saline (0.1ml of 20%SRBCs) intraperitoneally. Blood was withdrawn on 14th and 21st day from retro-orbital plexus under mild ether anaesthesia from all antigenically sensitized and challenged animals respectively. Blood was centrifuged to obtain serum. SRBCs count was diluted with buffered saline to make it 0.1% of SRBCs.

Each well of a microtitre plate was filled initially with 20µl of saline and 20µl of serum was mixed in the first well of the microtitre plate. Subsequently the 20µl diluted serum was removed from the first well and added to the next well to get two fold dilutions of the antibodies present in the serum. Further two fold dilution of this diluted serum were similarly carried out till the last well of second row (24 well), so that the antibody concentration of any of the dilution is half of the previous dilution. 20µl SRBCs (0.1% of SRBCs) were added to each of these dilution and the plates were incubated at 37°C for one hour and examined visually for agglutination.

The highest dilution giving the agglutination was taken as the antibody titer. The antibody titers were expressed in graded manner, the minimum dilution (1/2) being ranked as one, and the mean ranks of different groups were compared for statistical significance. Antibody titer obtained on 14th day after immunization (on 7th day) and on 21st day after challenge (on 14th day) with SRBCs was considered as primary and secondary humoral immune response respectively.

Delayed Type Hypersensitivity (DTH) Response

The drug treatment was exactly the same as described above for HA titer. On the 21st day, 0.03ml of 20% SRBCs was injected in sub plantar region of right hind paw and normal saline in the left hind paw in same volume. Foot pad oedema in rats was used for the detection of cellular immune response. The foot pad reaction was assessed at 0, 1, 3, 12 and 24 hours. On the 22nd day, the thickness of the right hind paw was measured using digital plethysmograph (which indicates that the oedema formed is due to hypersensitivity reaction). The foot pad reaction was expressed as the difference in thickness in mm between the right and left hind paw [10].

Histopathology

On the 22nd day of the experiment, the animals were sacrificed and organs like spleen, liver, kidneys, and thymus were isolated, washed using 0.9% saline and preserved in 10% formalin solution for 2 days and sent for histopathological study.

Zinc Sulphate Turbidity Test

The rats were divided into different groups as described in the animal grouping, six hours after the last dose, blood was collected and the serum was used for the estimation of immunoglobulin levels using method serviced by Mullen[11].

Zinc sulphate solution preparation

The triple distilled water was boiled for 15 minutes to remove dissolved CO_2 and was used to prepare Zinc sulphate solution (208mg/ L $ZnSO_4.7H_2O$). The ZnSO4 solution was kept in aspirator bottle to protect uptake of carbon dioxide. This was achieved by the insertion of soda lime into the stopper. A tubing to deliver 6ml per vial was connected to the aspirator bottle.

Test procedure

A control vial containing 6 ml distilled water and the test vial containing 6ml zinc sulphate solution were taken and added to 0.1 ml serum sample. The solutions were gently shaken to ensure complete mixing and reading was taken spectrophotometrically at 580nm[12].

STATISTICAL ANALYSIS

The results were expressed in Mean ± S.D. The statistical significance within the groups were analyzed using Student-'t' test and between the groups were assessed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests using online software Vassar stats and the $P<0.05$ was considered as statistically significant.

RESULTS

Preliminary phytochemical screening

Aqueous extract of RA-1 found to contain terpenoids, flavonoids, glycosides, carbohydrates, alkaloids, saponins, phytosterols, fixed oils & fats, tannins & phenols, proteins, gums, mucilage, and lignin.

Acute oral toxicity study

The polyherbal extract of RA-1 did not exhibit toxic effects at 2000mg/kg body weight. In this study no sign and symptoms of toxicity & no mortality were observed. Based on this, 200mg/kg and 400mg/kg were selected for the further studies as per OECD guidelines.

Evaluation of immunomodulatory activity

Haemagglutination antibody (HA) titer

The effect of aqueous extracts of RA-1 on primary and secondary immune response, on HA titer shows significant ($p<0.05$) difference between control and test group (Table 1 and Figure 1).

Zinc sulphate turbidity test

The aqueous extracts of RA-1 have significant ($p<0.05$) increase in serum immunoglobulin levels when compared with control (Table 2 and Figure 2).

Delayed Type Hypersensitivity (DTH) Response

The aqueous extracts of RA-1 showed significant ($p<0.01$) increase in foot pad edema when compared with standard group (Table 3 and Figure 3).

Relative body weight of spleen and thymus

The aqueous extracts of RA-1 have significant ($p<0.05$) increase in relative body weight of spleen and thymus (Table 4 and Figure 4).

Table 1:

GROUPS	MEAN HAEMAGGLUTINATION ANTIBODY (MHA) TITER VALUE		STUDENT - 't' TEST
	Primary Immune Response (14th day)	Secondary Immune Response (21st day)	
GI(Control)	4.044 ± 0.68	4.7365 ± 0.68	**P = 0.1082**
II(Standard)	5.892 ± 0.72688*	7.047 ± 1.02*	**P = 0.0475**
GIII(TEST)	5.198 ±0.955*	6.238 ± 0.759*	**P = 0.063**
GIV(TEST)	5.083 ± 0.3578*	6.007 ± 0.566*	**P = 0.0073**
One way ANOVA	**P = 0.0023**	**P = 0.0005**	**-**

The values are expressed as (Mean ± S.D.) n=6,* P<0.05 comparing GI Vs GII, GIII & GIV.

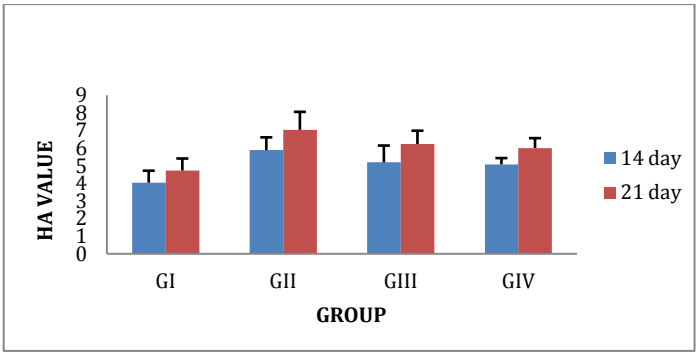
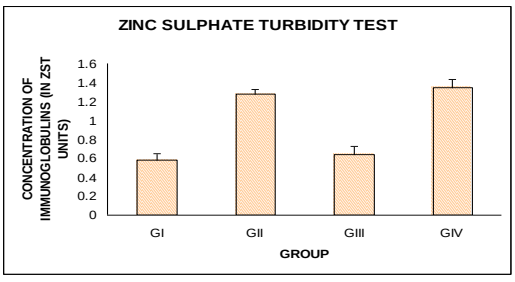


Fig 1 : GI- Control Group (vehicle)
GII - Standard group (*Ocimum Sanctum* 100mg/kg),
GIII- Test RA-1, 200mg/kg, GIV- Test RA- 1, 400mg/kg

Table 2

GROUP	SERUM IMMUNOGLOBULIN IN TURBIDITY UNITS
GI(Control)	0.582±0.0641
GII(Standard)	1.283±0.045*
GIII(TEST)	0.643±0.088*
GIV(TEST)	1.350±0.0856*
One way ANOVA	**P = 0.0000**

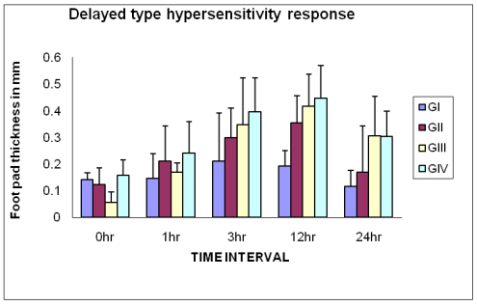
The values are expressed as (Mean ± S.D.) n= 6, * P<0.05 comparing GI Vs GII, GIII & GIV.



(Figure 2):GI- Control Group (vehicle)
GII - Standard group (*Ocimum sanctum* 100mg/kg),
GIII- Test RA-1, 200mg/kg,
GIV- Test RA- 1, 400mg/kg.

Table 3

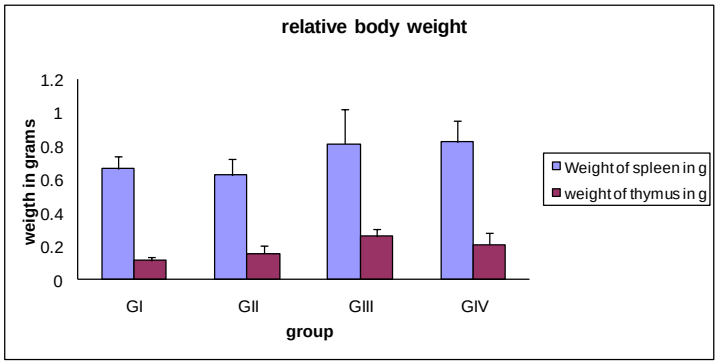
Group	Mean foot pad thickness in mm					Repeated MeasuresOne way ANOVA
	0hr	1hr	3hr	12hr	24hr	
GI(Control)	0.1417 ± .024	0.145 ± .0937	0.211 ± 0.178	0.193 ± 0.056	0.117 ± 0.059	**P= 0.447**
GII(Standard)	0.123 ± 0.061	0.212 ± 0.132	0.298 ± 0.111	0.353 ± 0.103	0.168 ± 0.173	**P=0.019**
GIII(TEST)	0.136 ± 0.04	0.168 ± 0.035	0.348 ± 0.175	0.417 ± 0.119*	0.307 ± 0.146	**P=0.001**
GIV(TEST)	0.158 ± 0.057	0.242 ± 0.116	0.395 ± 0.127	0.447 ± 0.122*	0.303 ± 0.094	**P=0.000**
One way ANOVA	**P=0.6290**	**P= 0.984**	**P= 0.2124**	**P=0.001**	**P=0.034**	



(Figure 3):GI- Control Group (vehicle)
GII - Standard group (*Ocimum sanctum* 100mg/kg),
GIII- Test RA-1, 200mg/kg, GIV- Test RA- 1, 400mg/kg

Table 4

Group	Weight of spleen in g	Weight of thymus in g
GI(Control)	0.663±0.0773	0.113±0.0225
GII(Standard)	0.626±0.0935	0.156±0.0496
GIII(TEST)	0.813±0.205	0.26±0.0438
GIV(TEST)	0.826±0.127	0.21±0.068
One way ANOVA	**P= 0.036**	**P= 0.0003**

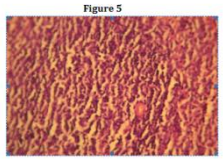


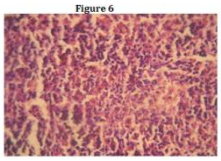
(Figure 4):GI- Control Group (vehicle)
GII - Standard group (*Ocimum sanctum* 100mg/kg),GIII- Test RA-1, 200mg/kg, GIV- Test RA- 1, 400mg/kg

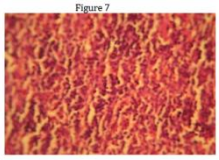
Histopathology results

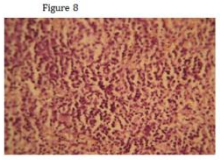
The spleen were sliced and stained with Haematoxylin-eosin stain and examined under microscope.

Figure 5 Spleen of GI shows prominent follicle and proliferating lymphocytes
Figure 6 Spleen of GII shows congestion and loosely dispersed cells
Figure 7 Spleen of GIII shows congestion with dispersed lymphocytes.
Figure 8 Spleen of GIV shows congestion and loosely dispersed cells









DISCUSSION

Immune system is a complex system, involving an interwoven network of biochemical mechanisms[13]. The modulation of immune response by various agents in order to alleviate the disease has been of interest since many years.

Immunomodulatory agents of plant origin enhance the immune responsiveness against a pathogen by activating the immune system. Such agents should be subjected to systemic studies to substantiate their pharmacological activity[14].

In this study, aqueous extract of RA-1 was screened for its safety, preliminary phytochemical screening and immunomodulatory activity.

RA-1 was safe up to the dose of 2000mg/kg as per the result of acute toxicity test. In preliminary phytochemical screening, active constituents like flavonoids, terpenoids and glycosides are found to be present in aqueous extract of RA-1. These are the principle constituents to produce immunostimulant activity. Further studies needed for the isolation of pure bioactive constituents from this extract to explore the exact mechanism of action.

The humoral immunity was tested by using indirect method (Haemagglutination antibody titer model) & direct method (Zinc sulphate turbidity test). The humoral immune system involves interaction of B cells with the antigen and their subsequent proliferation and differentiation into antibody-secreting plasma cells. Antibody function as the effectors of the humoral response by binding to the antigen and neutralizing it or facilitating it's elimination by cross-linking to form clusters that are more readily ingested by phagocytic cells. In this study, to evaluate the effect of aqueous extract of RA-1 on humoral response, its influence was

tested on SRBC specific HA antibody titer in rats and it found that the aqueous extract of RA-1(200 &400mg/kg b.w) showed an enhanced production of circulating antibody. The augmentation of the humoral immune response to SRBC antigen by increase in HA titer indicates the enhanced responsiveness of macrophages, T and B- lymphocytes subsets involved in antibody synthesis[15].

The estimation of serum immunoglobulin level is a direct measure to detect the humoral immunity. Serum immunoglobulin refers to a group of molecules produced by B-lymphocytes, they are soluble and secreted from B-cell receptors and are produced to a maximum level to encounter the invasion by an antigen, and hence they are called as antibodies[11]. In our study, aqueous extract of RA-1 (400mg/kg) shows more turbidity which in turn indicates the increased level of serum immunoglobulin when compared to the control group.

In our study, the cell mediated immunity was tested by using Delayed type hypersensitivity (DTH) response method. DTH requires the specific recognition of a given antigen by activated T-lymphocytes, which subsequently proliferate and release cytokines. These inturn, increase vascular permeability, induce vasodilatation, macrophages accumulation[16] and activation, promoting increased phagocytic activity and increased concentrations of lytic enzymes for more effective killing[17]. In this study, the SRBC (chemical sensitizer) is used to elicit contact hypersensitivity reaction in rats. Both doses of aqueous extract of RA-1(200 and 400mg/kg) found to potentiate the DTH reaction induced by SRBC. Increase in DTH reaction in rats in response to thymus dependent antigen suggests that the immunostimulatory effect of RA-1 on T-lymphocytes and accessory cell types required for the expression of the reaction[18].

The relative body weight of spleen and thymus showed an increase in RA-1 200 & 400mg/kg b.w. treated groups. This increase may be due to the increased activity of thymus and spleen. This was also confirmed by histopathological results. Thus present studies validate the traditional use of RA-1 in Siddha medicine as an immunomodulatory agent.

CONCLUSION

From the present study it was concluded that RA-1 is safe in doses up to 2000mg/kg and has good immunostimulant action when compared to the control group. Further studies are under progress to explore the mechanism of action and other pharmacological investigation on RA-1formulation.

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