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EFFECT OF *PHYLLANTHUS VASUKII* PARTHIPAN *ET AL.,* SP. NOV. (PHYLLANTHACEAE) AS AN IMMUNO STIMULANT AGENT AGAINST THE DELAYED TYPE HYPERSENSITIVE RESPONSE AND HUMORAL ANTIBODY RESPONSE TO SHEEP RED BLOOD CELLS IN SWISS ALBINO RATS.

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ABSTRACT

In the present study, immunomodulatory activities of Phyllanthus vasukii, a medicinal plant from Eastern Ghats were determined by Haemaglutination antibody (HA) titre, Delayed-type hypersensitivity (DTH) tests, Hematological parameters such as Hb, RBC and WBC and liver marker enzymes. T. bilirubin, SGOT, SGPT and ALP were carried out to determine specific and non-specific immune responses. The ethanol extracts of root and aerial parts of P. vasukii increased the body weights and organ weights in a dose dependent manner. In this study, the humoral antibody responses to SRBC was increased in both root and aerial parts of the plant. These extracts decreased DTH immune response significantly at the higher concentration of the extracts. However, no significant changes were found in hematological and liver marker enzymes after treatment of rats with the extracts. Here humoral antibody response to SRBC challenge was found to be significantly improved/increased by both root and aerial extracts of the plant. The plant root and aerial parts clearly indicated a dose-dependent decrease in antibody response. These results were compared with the standard and control values, and revealed the significant immune suppressive effect of the plant extracts at the humoral immunity level.

KEY WORDS

Phyllanthus vasukii, Hematological parameters.

Introduction

The efficiency of human immune system against microbial agents could be influenced by pathogenic exogenous and endogenous factors. Innate immune system is the first defence against infecting pathogen in to the host. The phagocytic cells and polymorphonuclear neutrophil(PMN) cells are responded first cells to move towards the infection site where the host get infected and destroys microbes. There are some natural herbal drugs and synthetic agents functioning as immunomodulators that act on the immune system [1]. In the immunomodulation, the immune system of the host is either suppressed or stimulated and normalized the immune system. The immunomodulators are considered as biological response modifiers, improves the host defence mechanism against pathogenic microbes of regulating a balance between regulatory and effector cells. In India, herbal drugs are very easily available, affordable and less potent than synthetic prescription immunomodulators and also cause minimum side effects. The Indian Ayurveda formulations have been developed some drugs for these diseases where they either activate the host defence mechanism or can selectively suppress the immune system like autoimmune disorder and hypersensitivity. These immunomodulatory properties of medicinal plants could provide an alternative to conventional synthetic drug therapy and increased resistance of microorganisms to antibiotics [2]. Several medicinal plant extracts such as *Panax ginseng, Alpinia galangal* (Zingiberaceae*) Ocimum sanctum* (Lamiaceae) and also specific active principles isolated from plants like

Cordiofolicoside A, curcumin, flavopiridol, combrestatin, lycopene and polysaccharides exhibited potent immunomodulatory properties [3-5]. The present study was aimed to evaluate the immunomodulatory activity of P. *vasukii* ethanol extract.

Materials and Methods

The roots and healthy aerial parts (without seed) of *P. vasukii* species were collected from Namakkal, Southern Eastern Ghats of Tamil Nadu state, Republic of India. They were thoroughly washed there itself with running water, brought to lab and shade dried. The plant was identified by Prof. Rajendran at the department of Botany, Bharathiar University, Coimbatore, India and authenticated. The voucher specimens were deposited at the Herbarium of Botany Department, Bharathiyar University, Coimbatore.

Preparation of extracts

The shade dried materials were pulverized separately to fine powders. These powders were defatted with petroleum ether and then extracted with ethanol in a soxhlet apparatus. After cooling, the extracts were evaporated to dryness and kept under refrigeration for further study.

Experimental animals

Normal healthy male Wistar albino rats (180-240 g) were used in the present study. All the rats were housed under standard environmental conditions at room temperature (25±2°C) and relative humidity of 42-55%, light and dark period of 12:12 hours. These rats were fed with feed of standard pellet diet and water ad libitum prior to the animal studies. In this study, standard pellet diet of Gulmohur brand, M/s Hindustan Lever Ltd. Mumbai, India was used. Animal ethical committee approved the experimental protocols based on the guidelines of CPCSEA (Committee for the purpose of control and supervision of Experimental animals) and the Institutional Animal Ethical Committee (01/2016/IAC/KASC).

Acute toxicity

Acute oral toxicity study was performed as per OECD – 423 guideliness (acute class method) on Swiss albino rats. No mortality was observed upto a dose of 2000 ma $kg^{-1}$of *P. vasukii* root and aerial parts ethanol extract. Therefore 200 and 400 mg kg^{-1} doses of the plant extract were chosen for the present study.

Experimental design

The rats were divided into two groups for each extract, totally four groups along with fourth group which was treated as standard dry, dexamethasone, at 10 mg kg^{-1} body weight. Each group consists of five rats. The control Group I was provided with normal saline and the treatment groups were provided with the root and aerial parts ethanol extract of *P.vasukii* at the dose of 200 and 400 mg kg^{-1} body weight (Group II and III) for five days, respectively . Group IV rats were given dexamethasone. All the rats were humanized 24 hours after the last dose. Body weight (%) and relative weight of spleen, liver and kidney (organ weight/100 g b.w. were determined for the rats.

Immunomodulatory tests

All the rats were divided into four groups of five rats each on the 10^{th} day of the initiation of the experiment. The rats were immunized with 0.2 mL of 2 % v/v ($4x10^8$ mL^{-1}). Sheep red blood cells (SRBC) were used for the assessment of delayed – type hypersensitivity (DTH) response and haemagglutinin titre (HR) assay.

Delayed type hypersensitivity response

The rats were treated with the extract at a dose of 200 and 400 mg kg^{-1} body weight /day for 14 days. On the tenth day, 0.2 ml of 2% v/v ($1x10^8$ mL^{-1}) Sheep Red blood cells were injected intraperitonally into each rat. On the fifth day of immunization, all the rats were again given another dose of $1x10^9$ cells (2% v/v) of SRBC injected subcutaneously into the left plantar tissue of each rat paw (hind foot paw). The right foot paw was also injected with the same volume of saline, which served as the trauma control for non-specific swelling. Twenty-four hours after injection, the thickness of the left paw of each treated rats were measured with a plethysmometer [6].

Haemagglutinin titre (HT) assay

A procedure described by Bin-Hafeez *et al*. (2001) was employed for determining the haemagglutinin (HT) assay. The SRBC agglutination test was conducted to study the humoral antibody response against antigen. Swiss albino rats (n= 6) were immunized by injecting 0.2 mL of $5x10^9$ SRBC/mL intraperitonially (I.P) on the day 0. Ethanol root and aerial part extracts of *P.vasukii* were administered to the rats at the doses of 200 and 400 mg kg^{-1} body weight for five days.

On the fifth day after immunization, blood was collected from the heart of the rats for serum

preparation. Serial two-fold dilution of serum was prepared in PBS at 7.2 in 96 well microtitre plates and mixed with 50 mL of 1% SRBC suspension in PBS now, all the plates were kept at room temperature for two hours. The value of antibody titre assigned to the highest serum dilution showed visible haemagglutination. In this study, antibody levels were determined by the haemagglutination technique. As the data obtained were subjected to statistical analysis. Here, the reciprocal of the highest dilution of the test serum agglutination was taken as the titre.

Hematological and liver marker enzymes

Red blood cell (RBC) count, white Blood cell (WBC) count, and hemoglobin Hb content were measured from tail vein blood. Total billirubin was determined after the procedure described by Balistri and Shaw, (1987). Serum Glutamate Pyruvate Transaminase (SGPT)and Serum Glutamate Oxalo Transaminase (SGOT) were determined by the method of Reitman and Frankel (1957) Alkaline Phosphatase (ALP)was determined by the method of King and Armstrong, (1934).

Statistical analysis

All the results were expressed as mean ±standard deviation SD and the data were analysed with the help of one-way analysis of variance (ANOVA) with Punnett'spost tests. P-values ($P<0.05$) of the test data were compared with control to determine statistically significant differences. The data were analysed by the statistical analysis system SPSS (software for windows release 10.0; SPSS Inc., Chicago IL, USA).

Result

In this experiment, the ethanol extracts of root and aerial parts of *P.vasukii* effectively increased the body weight, as well as the weight of spleen, liver and kidney (Table – 1). In control rats, extracts with Dexamethasone showed similar results when compared to the plant extract.

Delayed- type hypersensitivity (DTH) response

The cell mediated immune response of *P. vasukii* extracts were assessed by DTH reaction i.e. foot paw reaction. DTH response to SRBC was calculated as the measure of paw odema thickness (mm) for 200 and 400 mg kg^{-1} b. wt. of each rat, after treatment with the extracts and compared with control. Both the root and aerial parts of extracts showed dose – dependent decrease in DTH response. The plant ethanol extracts of root and aerial parts at the dose of 400 mg kg^{-1}elicited a significant ($P<0.05$) increase in DTH response when compared with the control rats. Here, dexamethasone (Group IV) decreased DTH response, and these results were compared with the control group. The maximum effect was observed at 400 mg kg^{-1} body weight which was found to be 0.44 and 0.42 in the root and aerial extract of *P. vasukii* and 0.39 in the case of dexamethasone respectively. These results indicated that both extracts on DTH response showed reduced paw thickness as compared to the control group, confirming its stimulatory effect on T- cells (Table 2).

Haemagglutinating antibody titre activity (HA)

The haemagglutinating titre (HT) activity was used to assess humoral immune response. Table 2 depicts the haemagglutin titre (HT) values. The plant root and aerial part extracts at the doses of 200 and 400 mg kg^{-1} body weight showed 4.91, 3.75 and 7.53, 6.81 and the control value was 5.92 respectively (Table 2). Here humoral antibody response to SRBC challenge was found to be significantly improved/increased by both root and aerial extracts of the plant. Meanwhile, the mean value of HA titre of the extracts were compared to the vehicle control. The plant root and aerial parts clearly indicated a dose-dependent decrease in antibody response. The most significant results were obtained at the concentration of 400 mg kg^{-1} b.wt., at which haemagglutination titres were found to be significantly higher. These results were compared with the standard and control values, and revealed the significant immune suppressive effect of the plant extracts at the humoral immunity level.

Hematological liver marker enzymes

The plant root extract at the dose of 400 mg kg^{-1} body weight had no significant effect on Hb, RBC and WBC count as compared with the saline treated control group. The trend was also observed for the plant aerial parts (Group III) and for the dexamethasone treated rats (Group IV). Similarly, there is no significant elevation in the level of SGOT, SGPT and ALP as a result of treatment with *P. vasukii* root and aerial parts extract. Total bilirubin content was slightly increased (Table 3).

Discussion

The present study demonstrated the immunomodulatory response of root and aerial parts ethanol extract of *P.vasukii* and the results were compared with a standard drug, Dexamethasone. The study was based on assessment of humoral antibody titre (HT) foot pad odema and delayed type hypersensitivity (DTH). These extracts on body weight, and relative organs weights such as spleen liver and kidney were investigated. The results were found to be increased with increasing concentration of both extracts of the plant, but seem to be not a significant one. The main function of the immune system is to protect organisms against infectious agents and potential pathogenic pathogens which put the immune system between a healthy and diseased state in the host. There are several plants that possess immunomodulatory activity through various mechanisms including their effect concerned with different cells, mast cells, natural killer cells and co-stimulatory molecules in the body. Immunosuppressant is one of the immunomodulators that could be used for control of pathological immune response and are active in auto immune disease, immediate and delayed type of hypersensitivity immune reactions and graft rejection. In this study the immunomodulatory activities of the ethanol extract of root and aerial parts of *P. vasukii*, was evaluated. Our results have supported the traditional knowledge of the genus *Phyllanthus* and its species for its medicinal properties.

The present study has revealed an overall stimulatory effect of both root and aerial parts ethanol extracts of *P. vasukii* in the immune function in rats, the humoral immunity involves interaction of B-cells with antigen and their subsequent proliferation and differentiation into antibody secreting plasma cells. Antibody functions as the effector of the humoral response by binding to antigen and neutralizing it (or) facilitating its elimination by cross- linking to form clusters that are more readily ingested by phagocytic cells [7]. This study was focused on the effect of ethanol extracts of *P. vasukii* on modulation of immune response, and the results revealed that these extracts were immunostimulating at the level of humoral and cell mediated responses. In addition, augmentation of the humoral immune response to SRBCS by plant extracts was observed as an increase in the antibody titre in rats, indicating the enhanced responsiveness of T and B lymphocyte subsets, which were involved in antibody synthesis [8,9]. The high value of HA titre obtained in the case of *P. vasukii* extracts indicated that immunomodulation was achieved through humoral immunity. Similarly, the ethanolic leaf extract of *Sonerila tinnevelliensis* and *Canscora perifoliata* also exhibited non-significant immunostimulant activities in a dose – dependent manners. Reports on the methanol extract of *Cuculigo orchioids* showed an increase in the hemagglutination titre in a dose-dependent manner and such results reveal the significance of the present study [10].

The delayed type hypersensitivity reaction was measured as an indicator of T-cell mediated immunity [11]. DTH is characterized by large influx of non – specific inflammatory cells, mainly macrophage and it is a part of the process of graft rejection, tumor immunity or immunity to several intracellular infectious microorganisms, especially those causing chronic diseases [12]. The DTH response is a direct correlation of cell mediated immunity, which are found to be higher at the concentration of 400 mg kg^{-1} body weight for both root and aerial parts of the plant. The increased activity of DTH at the time of cell mediated immunity (CMI) responses may be due to sensitized T-lymphocytes which subsequently proliferate and release cytokines. The non- significant difference in DTH response observed in the present study showed that the ethanol extracts of both root and aerial pats of *P. vasukii* has a stimulatory effect on lymphocytes and accessory cells required or the expression of the reaction and thus increases cell mediated immunity. These extracts exhibited an increase in DTH reaction and it's reflected from the increased foot pad thickness compared with control suggesting heightened infiltration of macrophages to the inflammatory site [7,13]. The ethanol extracts of root and aerial parts of *P. vasukii* enhanced hematological parameters such as Hb, RBC and WBC non- significantly. In the similar way, the biochemical parameters such as SGOT, SGPT and ALP levels except T- billirubin were increased non-significantly. This study supports the effectiveness of the extracts of *P. vasukii* as immunomodulatory agents.

Conclusion

The immunostimulatory activity of *P. vasukii* could be attributed to the presence of flavonoids, alkaloids, tannins, saponin glycosides and phenolic compounds. Therefore, the plant holds promise for being used as a strong immunestimulating agent for human health. The present investigation has also opened an avenue for further research especially with reference to the development of potent formulation for enhancement of immunity from *P. vasukii.*

Table 1. Effect of root and aerial part ethanolic extracts of *P. vasukii* on the Body and Relative Organ weight

Parameter	Body and relative organ weight (mean± SE) in gms				
Treatment	Dose mg kg^{-1}	Body weight	Spleen	Liver	Kidney
(Group-I) Control	Normal saline	21.36±0.11	0.49±0.03	4.36±0.26	1.31±0.22
(Group-II) Root extract	200	23.96±0.92	0.64±0.015ns	4.96±0.62	1.56±0.92
	400	31.16±0.36**	0.82±0.027*	6.13±0.93*	1.75±0.16*
(Group-III) Aerial extract	200	20.95±0.18	0.61±0.036ns	5.18±0.16	1.39±0.26
	400	29.16±0.56*	0.93±0.016**	5.16±0.84ns	1.62±0.29*
Dexamethasone	10 mg	22.96±0.81	0.112±0.031**	4.11±0.92	1.89±0.07**

Each Value is SEM ± 6 individual observations * P < 0.05, ** P < 0.01: Compared normal control vs Treated groups
NS- not significant
Group I: Control rats given normal saline Intraperitonially (IP)
Group II: Rats given root extract at the dose of 200,400 mgKg^{-1} body weight for 5 days by IP
Group III: Rats given aerial extract at the dose of 200,400 mg Kg^{-1} body weight for 5 days by IP
Group IV: Rats given Dexamethasone at the dose of 10 mg Kg^{-1} body weight for 5 days by IP

Table 2. Effect root and aerial ethanolic extracts of *P. vasukii* on DTH response compared with dexamethasone and on HT titre by using SRBC as an antigen in mice.

Treatment Groups	Parameter		
	Dose (mg kg^{-1})	Foot Pad Edema (mm)	HT titre
(Group-I) Control	Normal saline	0.30±0.018	2.96±0.016
(Group-II) Root extract	200	0.33±0.027ns	4.91±0.036*
	400	0.44±0.015*	7.53±0.051**
(Group-III) Aerial extract	200	0.31±0.054ns	3.75±0.016ns
	400	0.42±0.093**	6.81±0.056**
Dexamethasone	10 mg	0.39±0.056**	5.92±0.016**

Each Value is SEM ± 6 individual observations * P < 0.05,** P < 0.01: Compared normal control vsTreated groups
NS- not significant
Group I: Control rats given normal saline Intraperitonially (IP)
Group II: Rats given root extract at the dose of 200,400 mg Kg^{-1} body weight for 5 days by IP
Group III:Rats given aerial extract at the dose of 200,400 mg Kg^{-1} body weight for 5 days by IP
Group IV:Rats given Dexamethasone at the dose of 10 mg Kg^{-1} body weight for 5 days by IP

Table 3. Effect of root and aerial ethanolic extracts of *P. vasukii* on the Hematological and Serum Liver marker enzymes.

Parameter		Hematological (Blood)			Biochemical (Serum)			
Treatment	Dose mg kg^{-1}	Hb (g dL^{-1})	RBC (X10 6/mm^2)	WBC (X10 6/mm^2	T Bilirubin (mg dL^{-1})	SGOT (U/L)	SGPT (U/L)	ALP (U/L)
(Group-I)Control	Normal saline	13.11±0.94	4.43±0.16	5.44±0.46	0.63±0.01	31.16±0.67	28.22±0.54	131.16±4.16
(Group-II)Root extract	200	11.84±0.31*	4.01±0.67	6.22±0.92	0.72±0.02	39.22±0.91ns	37.16±0.31*	143.91±6.56
	400	13.16±0.26	4.11±0.36	8.11±0.31	0.79±0.04	44.16±0.86ns	41.16±0.37*	126.11±1.36
(Group-III)Aerial extract	200	10.84±0.011*	3.91±0.13	6.01±0.84	0.89±0.01	42.16±0.91	42.16±0.91*	153.84±5.16*
	400	12.91±0.66	4.11±0.19	6.88±0.91	0.93±0.11	43.91±0.86	49.22±0.96**	139.26±3.16
Dexamethasone	10 mg	11.84±0.011	4.26±0.81	5.92±0.36	0.71±0.62	39.16±0.31	31.13±0.62ns	132.16±4.36

Each Value is SEM ± 6 individual observations * P < 0.05,** P < 0.01: Compared normal control vsTreated groups, NS- not significant

Group I: Control rats given normal saline Intraperitonially(IP)

Group II: Rats given root ethanolic extract at the dose of 200,400 mg Kg^{-1} body weight for 5 days by IP

Group III:Rats given aerial ethanolic extract at the dose of 200,400 mg Kg^{-1} body weight for 5 days by IP

Group IV:Rats given Dexamethasone at the dose of 10 mg Kg^{-1} body weight for 5 days by IP

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