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RESEARCH ARTICLE

In Vivo Study of Immunomodulatory Effect of *Kalanchoe pinnata* (Lam.) Pers.

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ABSTRACT

To study immunomodulatory activity of hydroalcoholic plant extract of *Kalanchoe Pinnata* (Lam.) Pers. The test animals chosen for the present experiment were Wistar albino rats. The tests carried out were haemagglutination inhibition, delayed type hypersensitivity test, complete blood counts and histopathology (Liver and Spleen). The plant extract of *Kalanchoe Pinnata* (Lam.) Pers. was evaluated for immunosuppressive activity using Cyclophosphamide as an immunosuppressive drug and for immunostimulant activity using Septilin as an immunostimulant drug. Humoral immune response was evaluated by withdrawing blood from immunized wistar albino rats for haemagglutination inhibition test. Cell mediated immune response was studied using paw edema test conducted on immunized wistar albino rats. The blood cell counts were evaluated for generalized study of effect of the plant drugs of *Kalanchoe Pinnata* (Lam.) Pers. Histopathological examination of liver and spleen were evaluated for the plant *Kalanchoe pinnata* (Lam.) Pers. The results obtained from the study indicate that the plant *Kalanchoe Pinnata* (Lam.) Pers. possesses immunosuppressant activity *in vivo*.

KEYWORDS

Immunomodulatory activity, *Kalanchoe Pinnata* (Lam.) Pers., Wistar albino rats, Humoral immune, Cell mediated immune, Blood cell counts, Histopathology

INTRODUCTION

The immune system is involved in the etiology as well as pathophysiologic mechanisms of many diseases. Modulation of the immune responses to alleviate the diseases has been of interest for many years and the concept of 'rasayana' in Ayurveda is based on related principles[1]. Indian medicinal plants are a rich source of substances which are claimed to induce paraimmunity, the non-specific immunomodulation of essentially granulocytes, macrophages, natural killer cells and complement functions[2]. Ayurveda, the Indian traditional system of medicine, lays emphasis on promotion of health concept of strengthening host defences against different diseases[3].

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These plants, labelled as 'rasayana', have been endowed with multiple properties like delaying the onset of senescence and improving mental functions by strengthening the psycho-neuro-immune axis[4].

Kalanchoe Pinnata (Lam.) Pers. (Family: Crassulaceae) is listed in Ayurveda as rasayana plant.[5]

Hence, in the present research work, *in vivo* immunomodulatory activity of hydroalcoholic extracts of whole plant powder of *Kalanchoe Pinnata* (Lam.) Pers. was evaluated.

MATERIAL AND METHODS

Plant Material

Plant of *Kalanchoe Pinnata* (Lam.) Pers. was collected from Keshavshrishti, Maharastra. Herbarium of *Kalanchoe Pinnata* (Lam.) Pers.

was authenticated from Botanical Survey of India, Pune, India (Certificate No. BSI/WRC/Cert./2014) and collection no. HSQ 01. The plant material was washed with water to remove soil particles, dried in shade, finely powdered and then sieved through BSS mesh size 85 and stored in an airtight container at room temperature (25 ± 2° C).

Preparation of Sample Solution

About 2.0g of dried plant powder of *Kalanchoe Pinnata* (Lam.) Pers. was accurately weighed and transferred to a 100 mL stoppered conical flask. 50.0 mL of ethanol: water (1:1 v/v) was added to it and the flask was sonicated in an ultrasonic bath for 15 minutes. The flask was then shaken at 50 rpm, on a conical flask shaker overnight at room temperature (25 ± 2°C). Sample was filtered through Whatman filter paper no.1 of pore size 11 µm. The filtrate was then finally filtered using 0.45 µm nylon filters (Millipore), collected in a beaker and then evaporated to dryness on hot water bath. The final volume was then made up to 10mL with distilled water in a volumetric flask.

Chemicals

The drug, Cyclophosphamide monohydrate (purity 99.5%) was procured from Sigma-Aldrich Chemie GmbH (Aldrich Division, Steinbeim, Germany). and Septilin (Himalaya Drug Company, Bangalore) was procured from a local chemist shop. Sheep RBCs were procured from Sheep Farm, Bombay Veterinary College, Goregaon, Mumbai and were used as the antigen for the heamagglutination test. Sheep RBCs were collected in Alsever's solution, washed in pyrogen free, sterile, 0.9% normal saline, and were used (0.5×10^9 cells per ml per 100gm body weight of rat) intraperitoneally for immunization.

Experimental Protocols

The study was approved by the Institutional Animal Ethics committee, Mumbai Veterinary College, Parel, Mumbai-12. Experimental animals were handled according to the University and Legalization, regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India; vide approval number MVC/IAEC/08/2014.

Experimental Animals

Wistar strain of Male and female Albino Rats aged about 3-4 weeks, approximately weighing between 150-200gms were used in the present study.

Wistar albino rats were divided into five groups, each group consisting of four males and four females.

Group I received normal feed for all 14 days. Rest all groups were immunized with antigen, Sheep RBC on 0^{th} and 14^{th} day.

Group II received only sheep RBC

Group III received cyclophosphamide (50mg/ kg bdwt) on 1^{st} and 14^{th} day intravenously.

Group IV received Septilin (500 mg/kg bdwt) dose on all 14 days orally.

Group V received *Kalanchoe Pinnata* (Lam.) Pers. (400mg/kg bwt) extract on all 14 days orally.

Immunomodulatory Study Tests

Determination of Humoral Immune Response by Haemagglutination Inhibition (HI)

The animals were immunized by injecting 100µL of 1×10^8 SRBCs/mL intraperitoneally (i.p.) on day 0 and day 7. Blood samples were collected in eppendorf tubes from individual animals of all the groups by retrorbital vein puncture on 15^{th} day. The blood samples were centrifuged, the serum was separated. Initially, 50 µL of chilled normal saline solution was transferred to all the 96 wells of U-bottom microtitre plates, to obtain serum of each animal. Then, 50 µL of serum was placed in the first well of the same 96 well U-bottom microtitre plate and mixed. 50 µL from first well was withdrawn and added to second well. Again 50 µL of mixture was withdrawn from the second well and transferred to third well. Similar procedure was done till tenth well. Finally 50 µL from the tenth well was withdrawn and discarded. Haemaglutination titres were then performed. The reciprocal of the highest dilution

of the serum that completely inhibited agglutination (button formation) of the SRBC was taken as HI titre. (Figure 1)

Determination of Cell Mediated Immune Response (Delayed Type Hyper Sensitivity Test)

The animals of all groups were immunized by 0.1 mL of SRBC suspension containing 1x 10^8 cells, intraperitoneally, on day 0.On 14^{th} day the thickness of right hind foot pad of each rat was measured using vernier caliper (Mitutoyo, Japan).Right foot pad of each rat was injected with 1 x 10^8SRBCs.The foot pad thickness was measured again after 48hr. after the challenge. (Figure 2)

Determination of Complete Blood Count

Yaccua Tubes with rubber cap and outer caps containing sodium citrate (3.2%) as anticoagulant were used for collection of blood withdrawn by retro-orbital plexus. The cell count observed in the blood was counted using Neubauer chamber (Brand GMBH, Wertheim, Germany), followed by microscopic examination of Wright-stained smears with 100 X objective. Complete blood count was evaluated using Red blood counts, White blood counts, Neutrophil %, Eosinophil %, Lymphocyte %, Monocyte % and Platelet counts. (Table 1)

Histopathological Evaluation

The organs, liver and spleen of the animals of respective groups were collected in 10% formalin solution on final day after sacrifice by cervical dislocation.

The microtomes of the organs were prepared in wax. Staining was carried out with haematoxylin-eosin and the slides of liver and spleen were observed under light microscope (magnification at 100X). The results of these analyses were compared with that of control. (Table 2 and Figure 3).

RESULTS

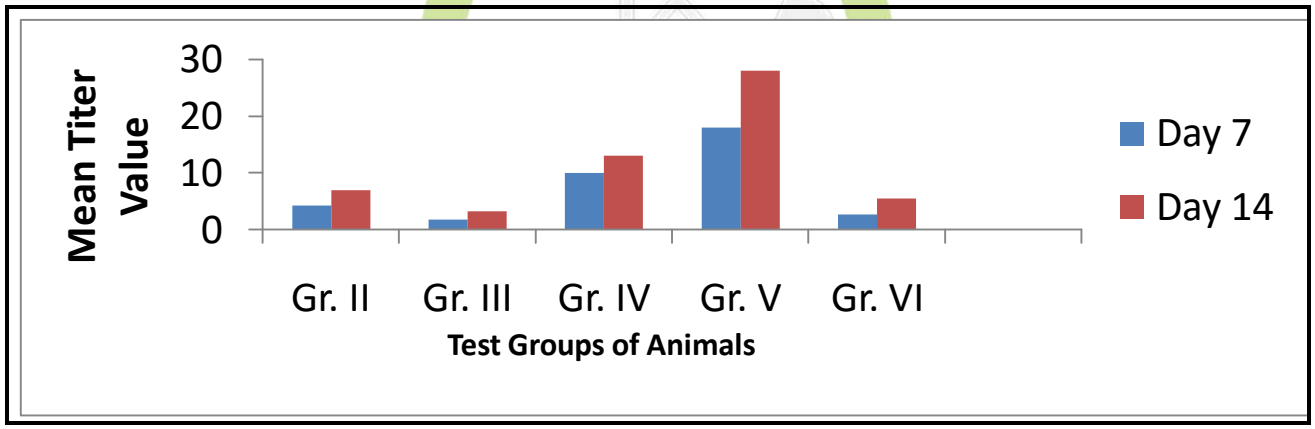


Figure 1: Graph of Mean haemagglutination inhibition titre values against different drug administration's to groups

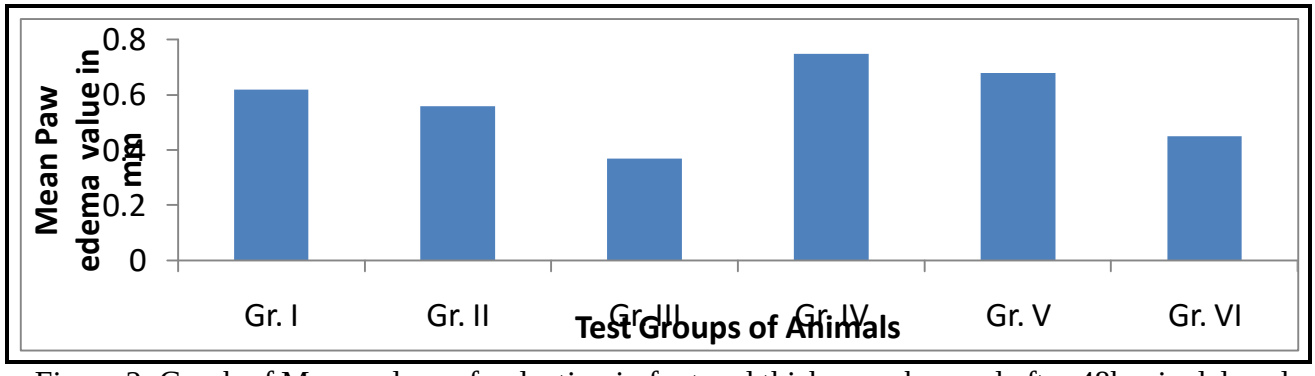


Figure 2: Graph of Mean values of reduction in foot pad thickness observed after 48hrs in delayed type hypersensitivity test against different drug administration's to groups

Table 1: Complete blood count results

Parameters	Time	Groups					
		I	II	III	IV	V	VI
Hamagglutination titre values	Day 7	-	4.25±0.59	1.75±0.25	10.00±1.31	18.00±2.00	2.75±0.37
	Day 14	-	7.00±0.65	3.25±0.37	13.00±1.46	28.00±2.62	5.50±0.73
Delayed Type Hypersensitivity (Foot pad thickness)	After 48 hrs	0.62±0.07	0.56±0.05	0.37±0.03	0.75±0.04	0.68±0.04	0.45±0.04
Percent Delayed Type Hypersensitivity	After 48 hrs	13.07±1.43	10.97±1.12	6.20±0.51	14.50±1.46	10.72±0.47	6.45±0.58
Red Blood Counts	Day 0	6.83±0.12	6.68±0.13	6.54±0.14	8.22±0.08	8.00±0.13	7.04±0.16
	Day 7	6.40±0.04	6.90±0.20	5.37±0.04	7.48±0.09	7.55±0.14	7.60±0.32
	Day 15	7.84±0.07	7.79±0.08	6.05±0.13	7.88±0.05	7.77±0.07	5.77±0.06
White Blood Counts	Day 0	11.18±0.74	10.36±0.99	9.69±0.49	12.24±0.32	10.36±0.33	10.54±0.59
	Day 7	11.96±0.90	10.43±0.98	1.71±0.11	11.34±0.27	11.13±0.39	2.33±0.50
	Day 15	16.8±0.75	13.2±0.31	2.23±0.15	13.0±0.40	12.2±0.34	1.56±0.68
Neutrophil Count %	Day 0	22.5±1.84	19.3±0.80	21.9±1.23	14.3±0.70	17.25±1.41	21.3±0.59
	Day 7	20.5±2.08	24.5±0.87	32.1±2.38	17.5±0.87	24.8±2.19	25.3±0.96
	Day 15	45.1±4.59	44.0±3.08	22.5±1.16	39.4±0.66	12.2±0.60	62.6±4.28
Eosinophil Count %	Day 0	0.36±0.32	0.50±0.33	0.25±0.16	0.50±0.19	0.25±0.16	0.38±0.18
	Day 7	1.13±0.35	0.75±0.37	0.13±0.13	0.63±0.18	0.38±0.18	0.38±0.18
	Day 15	0.25±0.16	0.25±0.16	0.38±0.18	0.38±0.18	0.50±0.19	0.25±0.16
Lymphocyte Count %	Day 0	69.6±1.90	63.1±1.56	57.4±2.2	68.3±1.64	64.0±2.00	59.6±2.22
	Day 7	77.3±2.14	73.0±1.04	69.6±1.88	84.6±0.94	73.9±2.14	67.1±1.33
	Day 15	61.3±4.25	40.1±3.94	33.5±1.57	58.6±3.10	39.3±0.41	40.4±0.60
Monocyte Count %	Day 0	0.38±0.18	0.38±0.18	0.25±0.16	0.50±0.33	0.50±0.27	0.38±0.18
	Day 7	0.63±0.18	0.50±0.19	0.25±0.16	0.75±0.31	0.75±0.25	0.38±0.18
	Day 15	0.63±0.18	0.25±0.16	0.13±0.13	0.75±0.16	0.63±0.18	0.13±0.13
Platelet Counts	Day 0	7.34±0.23	7.18±0.33	7.43±0.25	7.09±0.23	7.05±0.12	8.10±0.17
	Day 7	5.00±0.16	5.73±0.15	3.08±0.10	5.74±0.22	5.83±0.06	5.09±0.38
	Day 15	7.54±0.24	7.15±0.68	6.8±0.20	8.3±0.42	8.7±0.13	7.25±0.12

*Note: Values are expressed as the mean ± SEM; (n = 8) for each group. SEM - Standard Error Mean

Table 2: Histopathology Results

Testing	Histopathology of Liver	Histopathology of Spleen
Group I	Mild degree diffuse granular degeneration	No abnormalities were detected
Group II	Mild to moderate degree diffuse granular degeneration	No abnormalities were detected
Group III	Mild degree diffuse granular degeneration	No abnormalities were detected
Group IV	Mild degree diffuse granular degeneration	No abnormalities were detected
Group V	Moderate degree diffuse granular degeneration	No abnormalities were detected

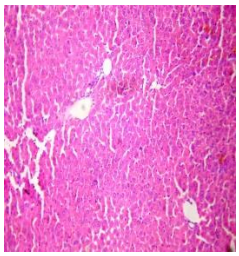
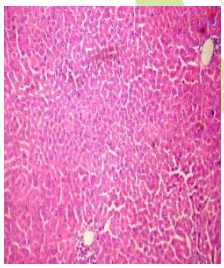
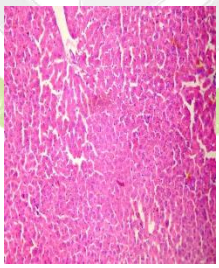
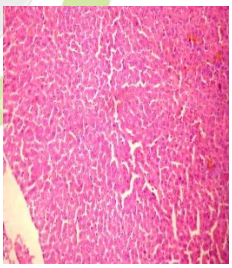
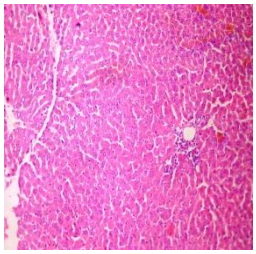
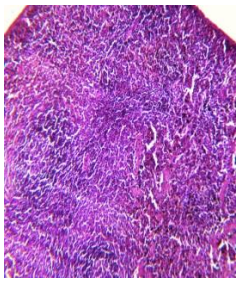
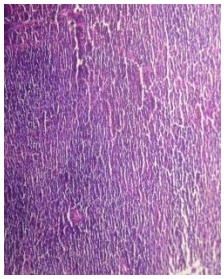
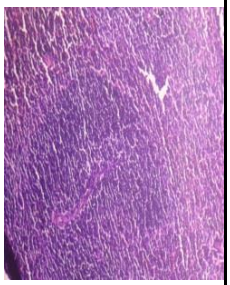
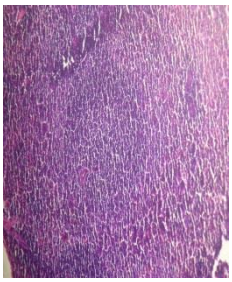
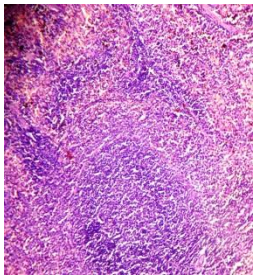
Group I	**Group II**	**Group III**	**Group IV**	**Group V**
Histology slides of Liver of Animals				
				
Histology slides of Spleen of Animals				
				

Figure3

DISCUSSION

The study was designed to evaluate the effectiveness of the hydroalcoholic extract of *Kalanchoe pinnata* (Lam.) Pers. stated to have immunomodulating property[6]. The animal model chosen for the present study were wistar albino rats. Standard cyclophosphamide was used as an immunosuppressant due to its action of DNA-alkylation leading to cell death and leucopenia.[7] Septilin was used for immunostimulation, as it has been reported to develop resistance to infections[8]. The study was performed over a period of fourteen days. Wistar albino rats were immunized on zero day to generate an immune response. The immune response was then assessed by determining the humoral immunity, cell mediated immunity and blood cell counts. Histopathological examinations of liver and spleen were done.

Immunomodulatory study was carried out with tests suggested in literature[9]. The tests conducted for determining the *in vivo* immunomodulatory activity of *Kalanchoe pinnata* (Lam.) Pers. were haemagglutination test for determining humoral immune response and delayed type hypersensitivity test for determining cell mediated immune response. The complete blood cell counts and histopathological examination of liver and spleen were also evaluated.

Haemagglutination Inhibition test was performed using sheep RBCs as an antigen to assess effects of various treatments on humoral immune response. Haemagglutination titre values showed that hydroalcoholic extract of *Kalanchoe pinnata* (Lam.) Pers. decreased humoral immune response in group VI, administered *Kalanchoe pinnata* (Lam.) Pers.as compared to group II(only antigen). This decrease in humoral immune response is not as prominent as the decrease observed in group III, administered with cyclophosphamide. This shows that hydroalcoholic extract of *Kalanchoe pinnata* (Lam.) pers. shows immunosuppressive activity but is less immunosuppressive than a known immunosuppressant, cyclophosphamide.

Delayed type hypersensitivity response was evaluated using sheep red blood cells as an antigen to assess effects of various treatments on cell mediated immune response. The test was performed by assessing paw edema size reduction. The hydroalcoholic extract of *Kalanchoe pinnata* (Lam.) pers. administered to group VI showed reduced cell mediated response for delayed type hypersensitivity as compared with group II (only antigen). This decrease in cell mediated response is not as prominent as the decrease observed in group III, administered with cyclophosphamide. This shows that hydroalcoholic extract of *Kalanchoe pinnata* (Lam.) pers. shows immunosuppressive activity but is less immunosuppressive than a known immunosuppressant, cyclophosphamide.

Hydro alcoholic extract of *Kalanchoe pinnata* (Lam.) pers., administered to group VI showed overall decrease in counts of Red blood cells, White blood cells, Neutrophils, Eosinophils, Lymphocytes, Monocytes and Platelets as compared to group II (only antigen) but but this decrease is less as compared to the decrease in group III, administered with cyclophosphamide.

The hydro alcoholic extract of *Kalanchoe pinnata* (Lam.) pers., administered to group VI showed minimal degree diffuse granular degeneration in the liver as compared with liver of test animals of group II (only antigen).

The hydroalcoholic extract of *Kalanchoe pinnata* (Lam.) pers., administered to group VI also showed no abnormalities in spleen of test animals as compared with spleen of test animals of group II (only antigen).

Therefore, after evaluation of all the above parameters, it was observed that hydroalcoholic extracts of whole plant powder of *Kalanchoe pinnata* (Lam.) Pers. showed immunomodulating activity *in vivo*.

In a reported method[10], a similar kind of immunomodulatory study was done to assess the efficacy of hydro-alcoholic extract of flowers of *Hibiscus rosa sinensis* Linn. and ethanolic extracts of aerial parts of *Cleome gynandra* Linn. The study was done *in vivo*. The results showed immunostimulatory activity of *Hibiscus rosasinensis* Linn. and immunosuppressive

activity of *Cleome gynandra* Linn. In another reported method[11], a similar immunomodulatory study was done to assess the efficacy of saline extracts of leaves of *Aloe vera* Linn. the results showed that *Aloe vera* extract produces stimulatory effect on the humoral and cell mediated immune response in the albino mice. However, as compared to the all the above methods, in the present study, additional parameters of Complete blood count and histopathological evaluation were done to find the efficacy of plant *Kalanchoe pinnata* (Lam.) Pers. more effectively.

Thus, the literature survey revealed that these tests were not carried out for hydroalcoholic extract of *Kalanchoe pinnata* (Lam.) Pers. and in the present research work, immunomodulatory activity was proved with parameters of humoral immune response, cell mediated immune response, cell blood count and histopathological evaluation.

CONCLUSION

The dried whole plant extract of *Kalanchoe pinnata* (Lam.) Pers. showed a decrease in titre values, delayed type hypersensitivity response and complete blood count. The histopathological examinations revealed changes in histology of liver and spleen supporting the above conclusion. Hence, all of the above observations reveal modulating effect of both the extracts. Thus, the plant *Kalanchoe pinnata* (Lam.) Pers. is found to possess immunosuppressive activity.

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