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Original Article

Immunomodulatory potential of ethanol extract of *Spilanthus acmella* leaves.

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ABSTRACT

Aims: Ethanol extract of leaves of Spilanthes acmella (SAEE) was evaluated for its immunomodulatory potential using various models like neutrophil adhesion (NA) test, haemagglutinating antibody (HA) titre and delayed type hypersensitivity (DTH) response in rats. Methods: The coarse powder (40-mesh) of shade dried leaves (500g) of Spilanthes acmella was subjected to maceration using 95% ethanol. Preliminary phytochemical tests were conducted for SAEE to identify the various phytoconstituents. Two doses of SAEE were selected and SAEE was administered orally at doses of 250 mg/kg body weight and 500 mg/kg body weight to healthy rats. The assessment of immunomodulatory potential was carried out by testing the humoral (antibody titre) and cellular (foot pad swelling) immune responses to the antigenic challenges with sheep RBC (SRBC) and by neutrophil adhesion test. Clean tap water was served as a control in all the tests. Results: Orally administered SAEE showed a significant increase in neutrophil adhesion, haemagglutinating antibody titre (HAT) and delayed type hypersensitivity (DTH) response. In rats immunized with sheep RBC, SAEE enhanced the humoral antibody response to the antigen and significantly potentiated the cellular immunity by facilitating the footpad thickness response to sheep RBC in sensitized rats. With a dose of 500 mg/kg body weight the values of NA, HAT and DTH responses were statistically significant as compared to control. Conclusion: The study demonstrates the immunomodulatory potential of Spilanthes acmella in rats. The responses were statistically significant when they were compared to control.

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1. Introduction

Immunity, both cell-mediated (Cellular) and antibody-mediated (Humoral) are triggered by antigens. In Cellular responses, CD8+ T cells proliferate into "killer" T cells and directly attack the invading antigen while in humoral responses, B cells transform into plasma cells which synthesize and secrete specific proteins called antibodies or immunoglobulins. Antibodies bind to and inactivate a particular antigen. Most CD4+ T cells become helper T cells that aid both CMI and AMI responses. The AIDS virus uses the CD4 molecule to enter and then destroy helper T cells. A cell-mediated immune response begins with activation of a small number of T cells (Lymphocytes) by a particular antigen. Once a T cell has been activated, it can undergo proliferation and differentiation into a clone of effect or cells, a population of identical cells that can recognize the same antigen and carry out some aspect of the immune attack. The body contains not only millions of different T cells but also millions of different B cells, each capable of responding to a specific antigen. Whereas cytotoxic T cells leave lymphatic tissue to seek out and destroy a foreign antigen. In the presence of a foreign antigen, specific B cells in lymph nodes, the spleen or lymphatic tissue in the gastrointestinal tract become activated. They differentiate into plasma cells that secrete specific antibodies, which then circulate in the lymph and blood to reach the site of invasion [1,2]. Immunomodulators are biologic response modifying compounds that affect the immune response in either a positive or negative fashion. If it results in an enhancement of immune reactions is named as immunostimulation which implies stimulation of non-specific system i.e. stimulation of the function and efficiency of granulocytes, macrophages and certain T-lymphocytes [3]. Immunosupression implies mainly to reduce resistance against

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infections, stress and may be because of environmental or chemotherapeutic factors. Immunostimulation and immunosuppression both need to be tackled in order to regulate the normal immunological functioning. Hence, immunostimulating agents and immunosuppressing agents have their own standing. A number of disorders can be treated by biologic response modifiers; these include immunodeficiency diseases and autoimmune disorders. These drugs may work on cellular or humoral immune systems or both [4,5]. Spilanthus acmella commonly known as 'akarkara' is an annual hairy herb, up to 32-60 cm. tall with numerous stems of marigold yellow flowers. Stems are glandular and hairy with pungent taste. The whole plant is acrid in taste [6]. The leaves are used as immunomodulatory, adaptogenic, diuretic, tooth paste, lithotriptic, antiscorbitic, sailagogine, antibacterial, tonic and digestive [7-10]. The leaves contain alkaloids, carbohydrates, pungent amide tannins, steroids, carotenoids, provitamin A, α-carotene and β-carotene, essential oils, sesquiterpenes, and amino acid [11-17]. Preliminanary studies have reported as diuretic [18], antiinflammatory and analgesic [19], vasorelaxant and antioxidant [20]. So far no systematic study has been reported to evaluate the immunomodulatory potency of Spilanthes acmella leaves extract.Therefore, present investigation is aimed at studying the immunomodulatory potential of the ethanol extract of leaves of Spilanthes acmella in order to justify the claims that the carotenoids, provitamin A, α-carotene and β-carotene present in the leaves may be responsible for its immunomodulatory potential.

2. Materials and Method

2.1.Plant material

Leaves of Spilanthes acmella (Family-Compositae) collected from local areas of Hubli, Karnataka (India) and authenticated by Dr. Ganesh Hegde, Professor and Head, Dept. of Botany, Karnataka University, Dharwad, Karnataka, dried in shade, crushed to coarse powder were used for studies.

2.2.Processing of Plant Material [21]

Dried coarse powder (40-mesh) leaves (500g) of Spilanthes acmella was placed in a glass stoppered conical flask and macerated with 100 ml ethanol shaking frequently, and then allowing it to stand for 18 hours. Filter it rapidly through whatman No. 1 filter paper, concentrated by rotary evaporator, dried over a desicator to obtain greenish brown colored residue (3.6% w/w) was subjected to preliminary phytochemical analysis.

2.3.Phytochemical investigation [22]

Qualitative Phytochemical tests were done by Harbone method which revealed the presence of alkaloids, carbohydrates, tannins, steroids, carotenoids, sesquiterpenes, amino acids etc.

2.4.Drugs and Chemicals

All the drugs, chemicals, and reagents were procured from S.D. Fine Chemicals, (Mumbai, India). All the chemicals were of analytical grade.

Test sample was prepared by suspending 20 mg dried ethanol extract in distilled water(100ml) using 2% Tween 80 as suspending agents.

Phosphate Buffer Salt Solution (PBS): 50ml of 0.2M of KH2PO4 (27.218g KH2PO4 in 1000ml water) was add in 34.7ml of 0.2 Na2HPO4 and volume was made up to 200 ml.

Sheep Red Blood Cells (SRBC): SRBC collected in PBS (PH-7.2), washed three times in 30 ml of pyrogen free 0.9% normal saline were used in a dose of 0.25 ml/100 gm body weight i.p. for immunization and for challenge as an antigen.

2.5.Acute Toxicity Study

Healthy albino mice of either sex weighing 25-30g, maintained under controlled conditions of temperature (20 –250C) and humidity (55%) were used for toxicity study as per Up & Down or Staircase method [23]. The maximum no-lethal and the minimum lethal dose are thus determined using only about 10 mice, once the approximate LD50 or the range between the maximum non-lethal and minimum lethal dose is found, a final and more reliable LD50 assay is planed using at least 3 or 4 dose levels within this range with longer number of animals in each group. LD50 is expressed in term of mg/kg. The maximum no-lethal dose was found to be 5000mg/kg body weight; hence 1/10th of the dose was taken as effective dose (500mg/kg body weight) for SAEE.

2.6.Treatment

Albino Wistar male rats (200-250g) procured from CPCSEA approved breeder (Reg. no. 126/1999/CPCSEA dated 29.6.1999), were used for the study. Animals were housed properly under standard conditions of temperature (23± 2°C), 12 h light/dark cycles and fed with standard pellet diet (Amrut rat and mice pellet Sagli) and water ad libitum. Fresh SRBC in PBS (PH-7.2) were obtained from authentic source. The animals were divided into three groups consisting of four animals each. A group of four untreated rats was taken as control (Group I). The extract was fed orally for 14 days at a dose of 250 mg/kg body weight (Group II) and 500 mg/kg body weight (Group III) for assessment of immunomodulatory effect. On 14th day, all groups of rats were challenged with SRBC (0.25 ml/100 gm) i.p.

2.7.Experimental Models

2.7.1.Neutrophil adhesion (NA) test [24]

On 14th day of extract treatment, blood samples were collected (before challenge) by puncturing the tail vein into heparanized vials and analyzed for total leucocyte counts (TLC) and differential leucocyte counts (DLC), After initial counts, blood samples were incubated with 80mg/ml of nylon fibres for 15 min. at 370C. The incubated blood samples were again analyzed for TLC and DLC. The product of TLC and The percentage of neutrophils in the treated and untreated blood was determined and the difference was taken as index of neutrophil adhesion. Percent neutrophil adhesion was calculated as below.

$$\text{Neutrophil Adhesion (\%)} = \frac{Niu - NIt}{Niu} \times 100$$

Where NIu = Neutrophil index of untreated blood sample.
NIt = Neutrophil index of treated blood sample.

2.7.2.Haemagglutinating Antibody (HA) titre [25]

Rats of group II and III were pretreated with SAEE for 14 days and each rat was immunized with SRBC (0.25 ml/100 gm body weight i.p.), including control rats. The animlas were treated with SAEE for 14 more days and blood samples were collected from each rat on day 15 for HA titre. The titre was determined by titrating serum dilutions with SRBC. The micro titre plates were incubated at room temperature for 2 hours and examined visually for agglutination. The highest number of dilution of serum showing haemagglutination was expressed as HA titre.

2.7.3.Delayed Type Hypersensitivity (DTH) response [26]

DTH response was determined by the significantly decrease or increase in paw volume. All the three groups of SRBC immunized rats were challenged by subcutaneous administration of SRBC 0.25ml/100g body weight in right hind foot pad on 28th day and 0.2 ml of 0.9% normal saline was similarly injected into left hind foot pad as control. The cell mediated immune response was measured at 24 h after SRBC challenged on 28th day in terms of increase in paw volume (plethysmometrically). The DTH response was expressed as the mean percent increase in paw volume between the right foot pad injected with SRBC and left foot pad injected with normal saline.

2.8.Statistical Analysis

The values were expressed as mean ± SEM. The results were analyzed by using ANOVA and Student's 't'test. Statistical significance on comparison with control group are indicated by *mark *P<0.01, was considered significant.

3.Results

Although immunomodulatory agents of plant and animal origin enhance the immune responsiveness of the body against a pathogens by activating primarily the non-specific immune system i.e. stimulation of the function and efficiency of the macrophages and other complement, they should be subjected to systematic studies to substantiate the therapeutic claims made with regard to their clinical utility. The yield of the SAEE was found to be 16.62% w/w. The preliminary phytochemical investigation revealed the presence of alkaloids, carbohydrates, tannins, steroids, carotenoids and sesquiterpenes (Table 1). Neutrophil Adhesion Test is an indicative of the marginalization of phagocytic cells in the blood vessels, i.e. an indication of immunostimulation. The % neutrophil adhesion in control group animals was 32.12, in SAEE treated group animals at doses 250 mg/kg body weight, it was 35.91 whilst for SAEE treated group animals at doses 500 mg/kg body weight, it was 52.06*.As is evident from the results There was no significant increase in neutrophil adhesion at a dose 250 mg/kg body weight however, showed significant (P<0.01) increase at a dose 500 mg/kg body weight as compared to control, suggesting possible immunostimulant action of the SAEE (Table 2). For haemmagglutinating antibody (HA) titer, the animlas were treated with SAEE for 14 more days and blood samples were analyzed from each rat on day 15 for HA titre and values were significantly (P<0.01) increased at different doses (250mg/kg and 500 mg/kg body weight) for SAEE (6.43±0.0333* and 8.59±0.0318*) as compare to control(4.57±0.0057), suggesting possible immunostimulant action of the SAEE (Table 3). DTH response Using SRBC as an Antigen, on 28th day after +24h of challenge in the control group animals was 2.02±0.0142 while in SAEE treated group animals at different doses 250 mg/kg and 500mg/kg body weight were 4.76±0.0217* and 8.45±0.0328*, therefore, the peak edema after +24 h of challenge was the evaluating parameter. Ethanol extract (500 mg/kg body weight) was statistically most significant (P<0.01) compared to control treatment in increasing the delayed-type hypersensitivity response (Table 4). Thus, the results obtained with SAEE treatment were found to be significantly comparable with that of the control, which showed a significant immunomodulatory potential.

Table 1. Preliminary phytochemical analysis of ethanol extract of Spilanthes acmella leaves.

Chemical Constituents	Ethanol extract
Alkaloids+	
Carbohydrates+	
Flavonoids-	
Tannins +	
Amino acids+	
Glycosides-	
Steroids+	
Sesquiterpenes+	
Carotenoids+	

+ = Positive, - = Negative

Table 2. Effects of ethanol extract of Spilanthes acmella leaves on neutrophil adhesion in albino rats.

Groups (A)	TLC (10^3/ mm^3)		Neutrophil % (B)		Neutrophil Index (A x B)		Neutrophil Adhesion
Control	UB	FTB	UB	FTB	UB	FTB	(%)
	3.7± 0.05	2.7± 0.05	38.3± 0.33	35.66± 0.33	141.8± 2.51	96.2± 1.35	32.12
Treated (250 mg/kg)	5.1± 0.08	4.23± 0.03	52.33± 1.45	40.66± 0.66	268.7.7± 5.35	172.2± 4.20	35.91
Treated (500 mg/kg)	4.0± 0.05	2.6± 0.09	50.33± 0.33	36.6± 0.33	201.3± 1.87	56.53± 0.33	52.06*

The values are expressed as mean ± SEM. The significance on comparison with control group is indicated by * mark. *P<0.01

Table 3. Effects of ethanol extract Spilanthes acmella leaves on H.A. titre to antigenic challenge by SRBC in albino rats.

Groups	H.A. Titre
Control	4.57±0.0057
Treated (250mg/kg)	6.43±0.0333*
Treated (500mg/kg)	8.59±0.0318*

The values are expressed as Mean ± SEM. The significance on comparison with control group is indicated by * mark. *P<0.01

Table 4. Effects of ethanol extract *Spilanthes acmella* leaves on DTH response to antigenic challenge by SRBC in albino rats.

Groups	DTH response (% increase in paw volume)
Control	2.02±0.0142
Treated (250mg/kg)	4.76±0.0217*
Treated (500mg/kg)	8.45±0.0328*

The values are expressed as Mean ± SEM. The significance on comparison with control group is indicated by * mark. *P<0.01

4.Discussion

Herbal drugs are known to possess immunomodulatory properties and generally act by stimulating both specific and nonspecific immunity. Many plants used in traditional medicine have immunomodulating activities. Some of these stimulate both humoral and cell-mediated immunity, while others activate only the cellular components of the immune system. Some of these plants also suppress both humoral and cell-mediated immunity [27]. The SAEE is becoming increasingly popular for a variety of diseases and infective conditions, primarily influencing the host defence mechanism. In the present study, the SAEE when administered orally, at different doses (250 mg/kg and 500 mg/kg body weight p.o.), significantly increase the recruitment of neutrophils adhesion to nylon fibers which correlates to the process of margination of cells in blood vessels. The neutrophil adhesion was significantly increased at dose of 500 mg/kg body weight compared to untreated control. Neutrophil play an important role in host immune mechanism system. The neutrophilic phagocytic system has many advantages. They are attracted by a limited number of stimuli, which generally signal the hence of tissue injury of unknown reason. Even the foreign bodies, thermal or chemical burns, bacterial infection and other types of injuries can provoke an intense neutrophil response. The vast number of cells can be mobilized due to any set of chemotactic stimuli, moreover, neutrophils are highly effective at killing certain bacteria and their ability to digest cellular debris and exogenous particulate matter provides an important step in the healing process. This indicated that the leaves ethanol extract might have enhanced the capacity of the monocytes-macrophages system. Thus it can be suggested that the ethanol extract possess immunomodulatory activity [28]. HA titre did not show any significant change with 250 mg/kg body weight of SAEE administration. However, a significant increase (P<0.05) was observed at a dose of 500 mg/kg body weight compared to untreated control animals. The augumentation of the humoral response as evidenced by an enhancement of antibody responsiveness to SRBC in rats as consequence of both pre and post-immunization drug treatment indicates the enhanced responsiveness of macrophages and B-lymphocyte subsets involved in antibody synthesis. The DTH response, which is a direct correlate of cell mediated immunity (CMI), was found to be increased at a dose of 500 mg/kg of the SAMEE. During CMI responses, sensitized T-lymphocytes, when challenged by the antigen, are converted to lymphoblasts and secrete lymphokines, attracting more scavanger cells to the site of reaction. The infiltrating cells are thus immobilized to remote defensive (inflammatory) reaction [29]. In our studies, foot volume was enhanced after SAMEE treatment suggesting cell mediated immune enhancement. The immunostimulant activity of Spilanthes acmella, one of the ingredients in the present drug is well known [16]. SAEE was found to be highly stimulating agent for both humoral as well as cell mediated responses. This study, apart from confirming the immunostimulant activity of SAEE also, presents evidence for the presence of carotenoids, provitamin A, α-carotene and β-carotene which induce stimulation of immune response in treated animals. Therefore, the plant holds promise for being used as an immunomodulatory

5.Conclusion

In the present investigation SAEE when administered orally, significant increased in the adhesion of neutrophils to nylon fibers, increase in both, HA titre and DTH response indicate that the SAEE potentiates humoral as well as cellular immunity. So, the immunomodulatory potential of SAEE in order to justify the claims that the carotenoids, provitamin A, α-carotene and β-carotene present in the leaves which may be responsible for its immunostimulant activity. Thus it can be concluded that Spilanthes acmella possesses immunomodulatory potential. Further studies are warranted for understanding the exact mechanisms responsible for immunomodulatory potential by SAEE.

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