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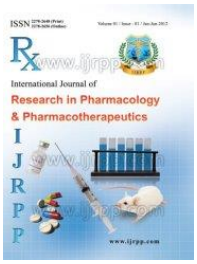

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Research Study *Open Access*

Immunomodulatory effect of leaf aqueous extract of *Desmodium triflorum*

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ABSTRACT

Desmodium triflorum, which is commonly known as "three-flowered beggar weed" is a dicotyledon, family Fabaceae. The present study was carried out to evaluate the immunomodulatory activity of aqueous extract of D. triflorum on Swiss albino rats. Delayed type hypersensitivity response and Humoral antibody titer by using injecting 0.1 ml of Sheep red blood cells (SRBCs) suspension was used to assess the activity of drug. Levamisole was given in the dose of 50 mg/kg body weight through oral route. 200 and 400 mg/kg body weight of Desmodium triflorum herbal drug was given through oral route. The extent of protection against SRBCs suspension was evaluated with drug administration by estimating total leucocyte and differential leucocyte count. aqeuous extract of D. triflorum showed pronounced immunostimulant activity by increasing the levels of total WBC count. The extract was found to be an effective immunostimulant agent.

Keywords: Desmodium triflorum, SRBCs suspension, levamizole, immunostimulant

INTRODUCTION

Traditionally medicines have been utilized since antiquity in the health care. However, with the advent of the pharmaceutical industry early in this century, the popularity of traditional medicine declined, in spite of the fact that twenty five percent of all prescription drugs still contain ingredients isolated from plants. Traditional medicine also known as indigenous or folk medicine comprises knowledge systems that developed over generations within various societies before the era of modern medicine. [1]

The traditional Chinese medicine diagnostics are based on "zheng" or "symptom", a system emphasizing the overall function of the human body. Several plants have been listed in the traditional systems of medicine and some of these are providing comprehensive relief to people suffering from various disorders. Immunology as a science probably began with the observations by Metchnikoff in 1882

that starfish when pierced by a foreign object (A rose thorn responded by coating it with cells (Latter identified as Phagocytes). Immunology – the study of the way in which the body defends itself against invading organisms or internal invaders (Tumors) has developed rapidly over the last 40 years, and particularly during the last 10 years with the advent of molecular techniques. It is now a rapidly moving field that contributing critical tools for research and diagnosing, and therapeutics for treatment of a wide range of human disease. Thus, it is an integral part of college like science course and medical studies [2,3]. The introduction of foreign substance (antigen) into the body provokes an immune reaction & for this it is essential that the body recognize it as "non self" Most antigens are first ingested & concentrated by the macrophages, & later passed to the nearly lymphocytes. The immune response is initiated by the interaction of the antigen with the receptors on the surface of the lymphocytes, and the response may be of true types.

In mammals there are five main isotypes of immunoglobulin: IgM (mostly stimulated by the primary response), IgG (for the memory response), IgA (in secretory fluids), IgE (cell- associated, often implicated in allergic reactions), and IgD (on membranes of B-lymphocytes, possibly important in recognition)**.** It is medicated by thymus-derived cells known as T cells, which interact with the antigen to reduce lymphocytes. The immune response is an essential defense mechanism against the invasion of the body by bacteria. D. triflorum has been in folkloric use for many years. Roots, leaves and the whole plant are used in Ayurvedic medicine for various treatment purposes. Roots are reported as carminative, tonic and diuretic. Leaves are antiseptic, antidiarrhoeal and galactogogue. The whole plant of D. triflorum is known to have expectorant, cooling and galactogouge properties. Faeces of wild rabbits those who have eaten the plant have been used for treatment purposes in folkloric medicine.

MATERIALS AND METHODS

Preparation of extracts of leaves of *desmodium triflorum*

Extract preparation

The collected leaves were shade dried completely and ground into powder with mechanical grinder. The powder was passed through sieve no. 60 to get uniform powdered

Solvent for Extraction

Ethanol & Aqueous

Extraction procedure

The 1 Kg of dried powder of *Desmodium triflorum* leaves was defatted with n-hexane. The defatted powder material (marc) thus obtained was extracted with sufficient quantity of Ethanol and distilled water.

Maceration process

Maceration process involves the separation of medicinally active portions of the crude drugs. The drug material is taken in a stopper container and immersed in the bulk of the solvents in the ratio of 1:2 (Drug &Solvent) and allowed to stand for 7 days in a room temperature with frequent shaking of every 30 min up to 6 hours on each day. The solvent was removed by filtration by using thin masculine cloth, distillation under reduced pressure and evaporation. The resulting semisolid mass was vacuum dried and percentage yield was calculated.

Table 1: Data showing the extractive values of dried leaf powder of *Desmodium triflorum*

Plant name	Part used	Method of extraction	Solvent	Colour of extract	Nature of extract	% yield of extract
Desmodium	Leaf	Maceration	Ethanol	Dark brown	Semisolid	4.56
			Aqueous	Dark	Semisolid	21.86

triflorum	brown

Solubility parameters extracts of desmodium triflorum

Very soluble : One part of soluble in less than one part of the solvent
Free soluble : 1:10
Soluble : 1:30
Sparingly soluble : 1:100
Slightly soluble : 1:1000
Very slightly soluble : 1:10,000
Practically insoluble : More than 10,000

Table 2: Solubility of Desmodium *triflorum* extracts in various solvents

Plant Extracts	Water	Hot water	1% v/v Tween 80	0.9 % CMC	1% DMSO
Aqueous	Free soluble	Free soluble	Sparingly soluble	Soluble	Sparingly soluble
Ethanol	soluble	Free Soluble	Sparingly soluble	Sparingly soluble	Sparingly soluble

Based on solubility test with pharmacological inert solvent, water was taken as vehicle used for pharmacological and other studies.

PRELIMINARY PHYTOCHEMICAL SCREENING OF *DESMODIUM TRIFLORUM*

The therapeutic potentials of plant and animal origin are being used from the ancient times by the simple process without the isolation of pure compounds i.e. in the form of crude drugs or the galenicals prepared from them. The pharmacological action of crude drug is determined by the nature of its constituents. Thus the plant species may be considered as a biosynthetic laboratory not only for the chemical compounds e.g. carbohydrates, proteins and fats that are utilized as a food by humans and animals, but also for a multitude of compounds including alkaloids, flavonoids, glycosides etc. which exert definite pharmacological effects. These chemical compounds are responsible for the desired therapeutic properties. To obtain these pharmacological effects, the plant materials are used as such in their crude form or may be extracted with suitable solvents to take out the desired components and the resulting principle being employed as therapeutic agents. The phytochemistry of herbal drugs embraces a thorough consideration of these chemical entities that are termed as constituents. As the herbal drugs contain so many chemical compounds, it is essential to single out those responsible for the therapeutic effect to be called as active constituents.

By considering the above facts, it is necessary to evaluate the nature of extract before evaluating the biological activity of the same. We have been selected such extract for pharmacological activity which contain large number of chemical constituents. Hence for this purpose, we have to go for following tests to evaluate the chemical nature of extracts qualitatively.

EXTRACT SELECTION FOR IMMUNOMODULATORY ACTIVITY IN RATS

Based on the literature, the plant / extracts possess flavonoid, tannin and phenolic compounds might have Immunomodulatory, wound healing property and avoiding excess animal usage, so we have selected the extract having the high content of flavonoid, tannin and Phenolic compound such as Aqueous extract of *Desmodium triflorum*. The aqueous extract selected also based on the yield.[4]

ACUTE TOXICITY

The acute toxicity study was carried out with aqueous extract of *Desmodium triflorum* as per OECD 423 Guidelines. Wister albino mice with weight ranging (25-30g) were taken for the experiment. The

animals were made into a group of 3 each, dose of *Desmodium triflorum* aqueous extract were given according to the body weight (mg/kg), starting dose of 5 mg /kg was given to the first individual animal, no death was occurred and higher doses were given to next group of animals. The animals were observed for a further 14 days for any signs for delayed toxicity.

Table 3: Acute toxicity study of aqueous extract of leaf *of Desmodium triflorum* based on OECD 423 guidelines

S. No	Number of animals	Dose in mg/kg	Report
1	3	5mg/kg	No death
2	3	50mg/kg	No death
3	3	500mg/kg	No death
4	3	2000mg/kg	No death

Table 4: Study period and observation parameters

Initial once observation	First 30 min and periodically 24 hr
Special attention	First 1-4 hr after drug administration
Long term observation	Up to 14 days
Direct observation parameters	Tremors, convulsions, salivation, diarrhea, lethargy, Sleep and coma.
Additional observation parameters	Skin and fur, eyes and mucous membrane, respiratory, circulatory, autonomic and central nervous systems, somato motor activity and behavior pattern etc.

From the observation aqueous extract of leaves of *Desmodium triflorum* were screened for acute toxicity study by OECD guidelines 423 for determining the LD50. The results showed that LD50 was found to be 2000mg/kg. Therefore its ED50 was found to be 200mg/kg and study was carried out with 200 & 400 mg/kg [5].

IMMUNOMODULATORY EXPERIMENTAL PROTOCOL

Twenty four rats were divided into four groups of six animals each.

Group-I : Control Receive distilled water as vehicle

Group-II: *Desmodium triflorum* aqueous extract was administered at a dose 200mg/kg/day by oral route for 14 days

Group-III: *Desmodium triflorum* aqueous extract was administered at a dose of 400mg/kg/day by oral route for 14 days

Group-IV : Standard – Levamisole was administered at a dose of 50mg/kg/day by oral route for 14 days.

Experimental procedure

STUDY OF DELAYED TYPE HYPERSENSITIVITY RESPONSE [DTH] [6-9].

The fresh sheep blood was collected from Veterinary College, Vijayawada. It was washed three times with normal saline via centrifugation. The suspension was adjusted to 1 X 108. The animals were immunized by injecting 0.1 ml of SRBCs suspension, containing 1X 108 cells (1.0 X 108 SRBC/ml) intra peritonially on day 0. On Day 8, after immunization the thickness of the right hind footpad was measured using a Vernier caliper. The rats were then challenged by injection of 1 X 108 sub SRBCs in the left hind footpad. The footpad thickness was measured again after 24 hours of challenge. The difference between the pre- and post challenge footpad thickness, expressed in mm was taken as a measure of the DTH response.

The following formula was used to measure the DTH response.

Left foot pad challenged with antigen-Right foot pad control X 100 Left foot pad challenged with antigen.

HUMORAL ANTIBODY TITRE

The animals were immunized by injecting 0.1 ml of SRBCs suspension, containing 1X 108 cells, intraperitoneally, on day 0. Blood samples were collected in micro centrifuge tubes from individual animals of all the groups by retro orbital vein puncture on day 14. The blood samples were centrifuged and the serum separated. Antibody levels were determined by the hemagglutination technique.

Method for Serial dilution

This was performed by using 96 wells (12x8) U bottomed titreplate. The wells were marked from I to XII. In the first (I) and last well (XII) 25 microliter of serum collected from treated animals was added and inactivated at 56 degree Celsius for 30 minutes. Afterwards to all the wells except well number XII, 25 microliter of PBS was added.25 microliter was taken from first well and added to 2nd well again 25 microliter from second well was taken and added to third well and continued the same procedure up to well number XI. After this 25 microliter of sample from well number XI was discarded. Finally 25 microliter of 1% SRBC was added to all the wells and was kept at room temperature for two hours.

TOTAL LEUKOCYTE COUNT

It has got three graduations. Two graduations 0.5 and 1 are present on the stem of the pipette and the third mark 11 is placed just above the bulb. Blood is drawn up to mark 0.5 and the rest of the bulb is filled by sucking up diluting solution up to the mark 11, the bulb of the pipette is so constructed that it holds exactly 20 times the volume of fluid contained in the stem of the pipette up to mark 1. Although fluid is drawn up to 11, the dilution of the blood will be 20 because the last part of the fluid remains locked up in the stem and is not available for dilution.

The counting chamber

The ruling area consists of 9 square millimeters. The central of the smallest squares are separated by triple lines in which RBC will be counted. The side of each square for counting WBC is 1⁄4 mm.

Diluting fluid for WBC (Turks fluid)

Commonly the fluid is made up as follows:

Glacial acetic acid	-1.5ml
1%solution of gentian violet in water	-1ml
Distilled water	-98ml

The glacial acetic acid haemolysis the red cells, while the gentian violet stains the nucleus of leukocytes.

Method of counting W.B.C

The white cells are counted in four corners of 1 square millimeter ruled area on both sides. The white cells are recognized by the retractile appearance and by the slight color given to them by the stain contained in the diluting fluid. The cells touching the left side and upper side of boundary line are not counted.

Calculations

The area of the smallest square mm3square	=1/16
Volume of smallest square	=1/160 mm3
Total number of square counted	=16×4=64
Total number of cells counted	= X 64/160
mm3 of diluted blood contains	=X cells
So, 1 mm3 of diluted blood contains cells	=160/64×X
1 mm3 of undiluted blood contains X cells	=160/64×20×

DIFFERENTIAL LEUKOCYTE COUNTS

A thin blood film was made on a clean, dry, glass slide. It was dried fixed and stained to differentiate the different types of leukocytes. Hundred leukocytes were counted and percentage of different leukocytes was calculated

Composition of leishman's stain

It contains a mixture of methylene blue and eosin dissolved in acetone free methanol.

Procedure

A blood collected from the all group of animal and thin blood film was made on a clean dried glass slide. It was dried and stained with leishman's stain solution. The drop of leishman's stain was counted & 2 minutes was allowed to fix the blood film. Fixation means nucleus and various cellular organs will be fixed without any damage to the cells or cellular organs. After 2 minutes double the quantity of distilled water was added over the slide and waited for 7 minutes. In the mean time the stain will initiate the chemical reaction. The acidic dye eosin will initiate various acidophilus structures and some neutrophyllic granules and basic dye will stain structure like nucleus, basophilic granules, and cytoplasm of the lymphocyte and monocytes. After 7 minutes the slide was washed in a slow stream of water later it was dried in air. One drop of cidarwood oil was placed over the film. The cells were identified and entered into 100 squares. This gives the % of different types of leukocytes present in rat blood.

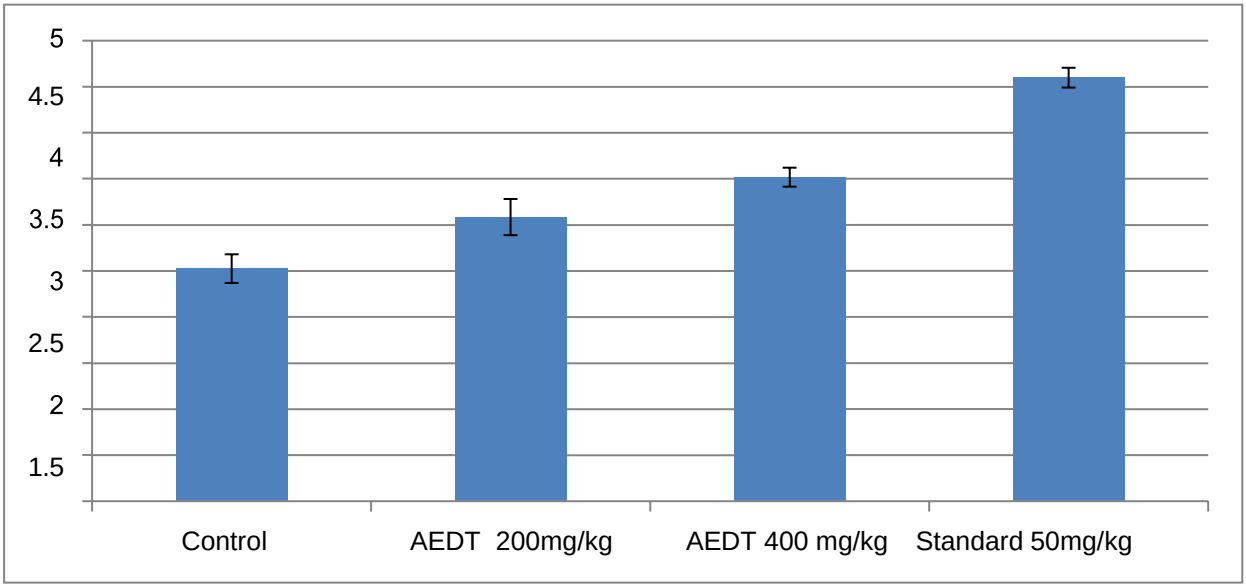
STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) followed by Dunnett's method of multiple comparisons was employed using Graph pad Instat 5.0 software. $p<0.05$, $p<0.01$ & $p<0.001$ was considered to be statistically significant.

RESULTS

Effects of *Desmodium triflorum* aqueous extract on DTH response in rats using sheep's RBCs as antigen

The effect of test extract and standard drugs on the DTH response in Wistar rats using SRBCs as antigen, administration of aqueous extract of Desmodium triflorum at the dose of 200mg/Kg, 400mg/Kg and Levamisole 50mg/Kg treatments which were given orally for 14 days showed significant increase in paw edema compared to control group. The standard drug Levamisole showed the maximum increase in paw edema volume compared to all groups and effect of 200mg/kg less significant ($p<0.05$)when compared to 400mg/kg extract($p<0.001$). The results are shown in below table:



Values are expressed as Mean±SEM, n=6. Significant (*p<0.05 & ***p<0.001) compared with treated groups Vs control.

Fig1: Effects of *Desmodium triflorum* aqueous extract on DTH response in rats using sheep's RBCs as antigen

HUMORAL ANTIBODY TITRE

Administration of aqueous extract of Desmodium triflorum at the dose of 200mg/Kg and 400mg/Kg and Levamisole 50mg/Kg treatments which were given orally for 14 days showed highly significant increase in antibody titre values compared to control group. But when the effect was compared between extract group 400 mg/kg showed marked effect. The results are shown in below table.

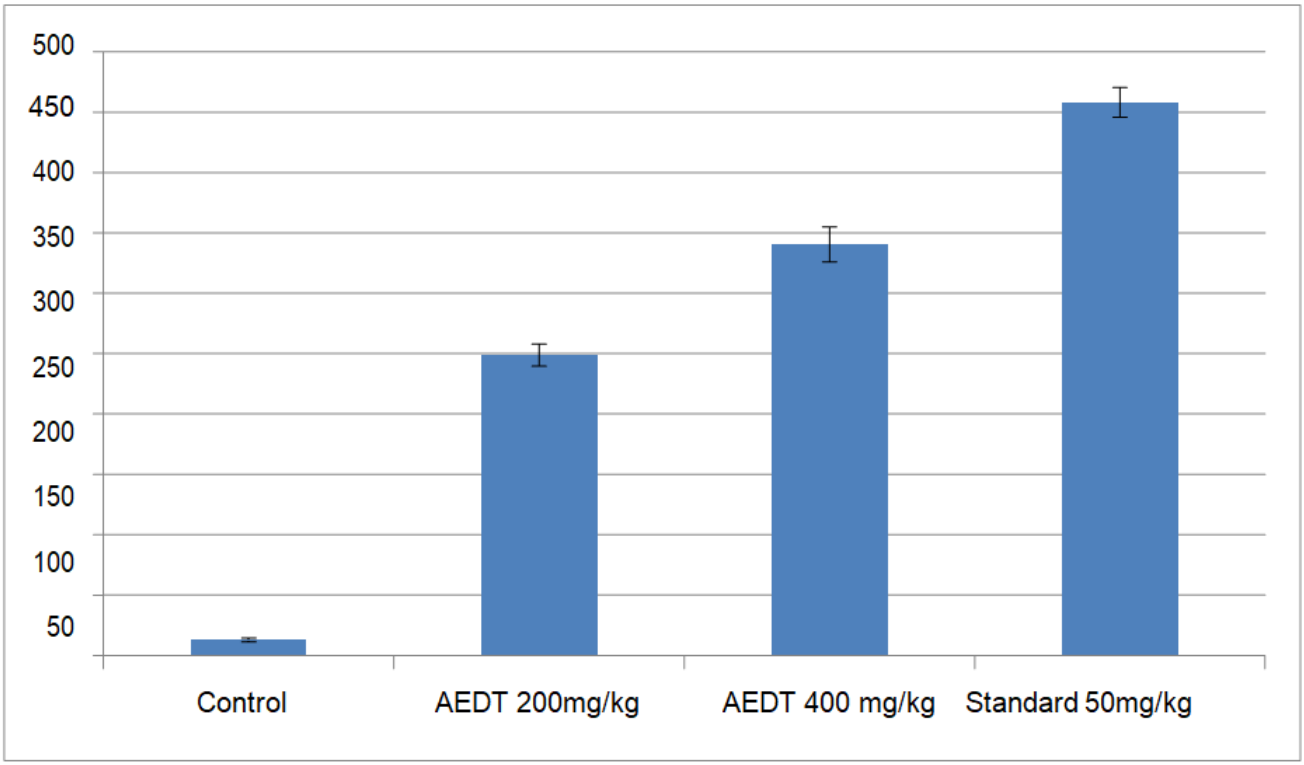
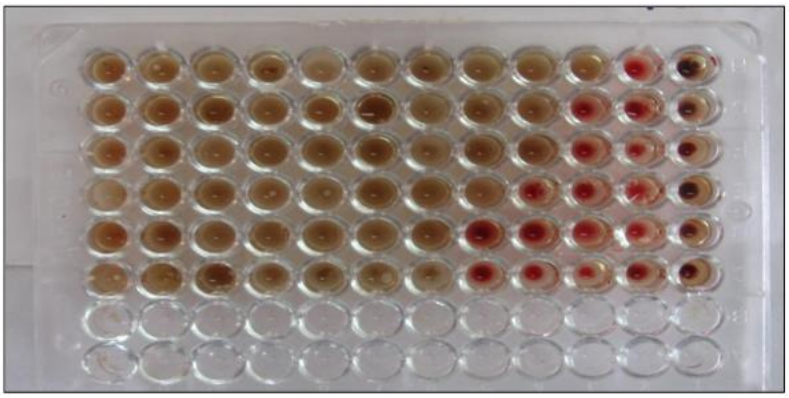
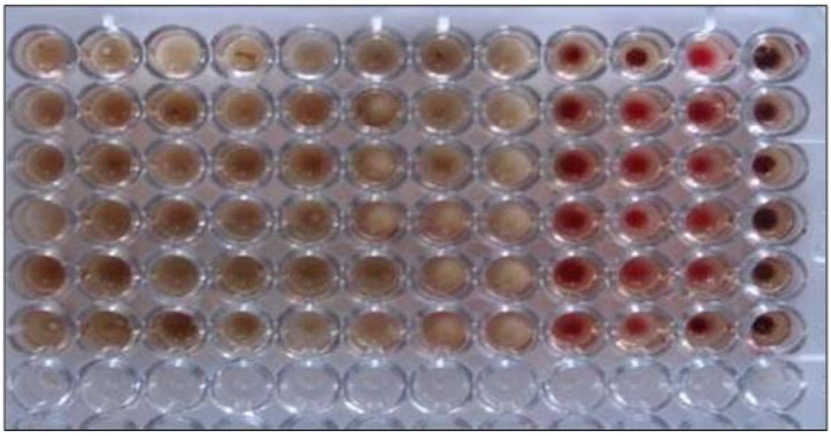


Fig 2: Effect of Desmodium triflorum aqueous extract on the Humoral Antibody titer In Wistar Rats

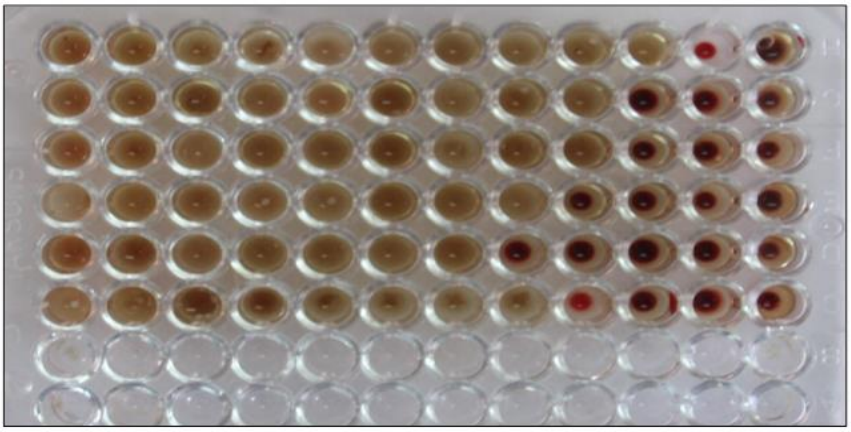
MICRO TITER PLATE



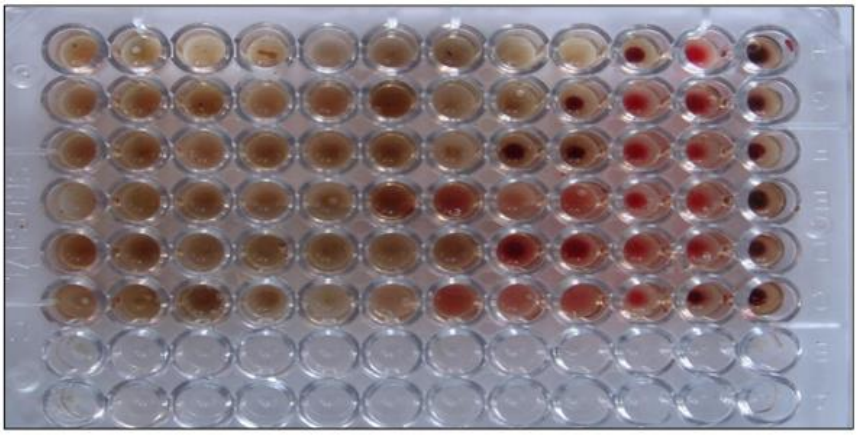
Control



Desmodium triflorum 200mg/Kg



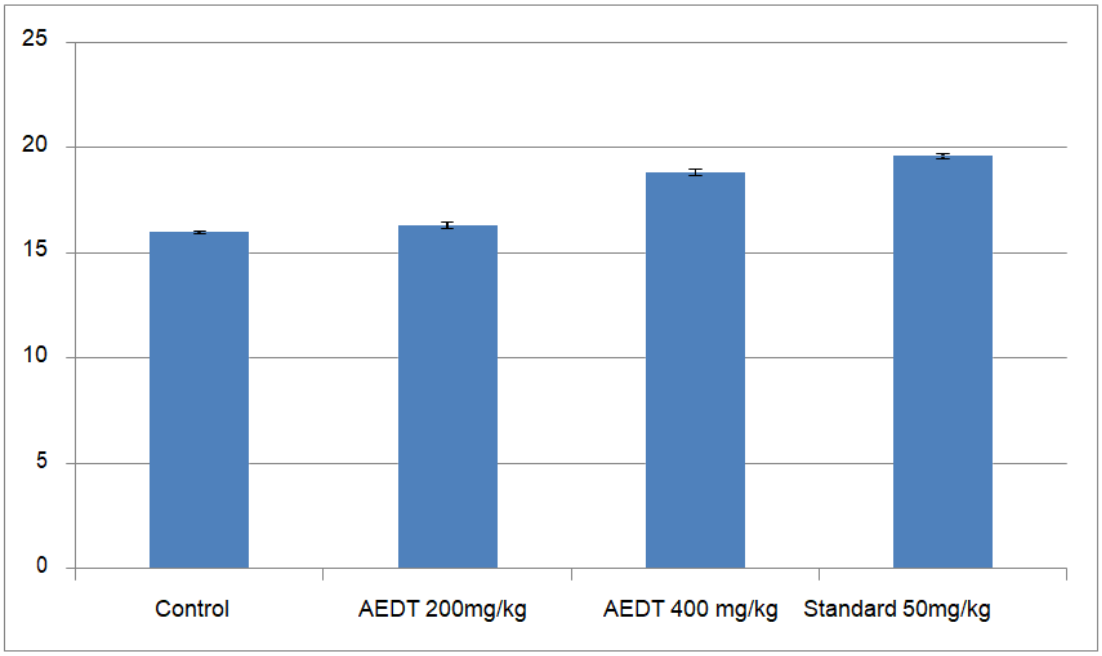
Desmodium triflorum 400mg/Kg



Standard drug – Levamisole 50mg/Kg

Total leukocyte count

The effect of administration of aqueous extract of Desmodium tiflorum at the dose of 200mg/Kg and 400mg/Kg and Levamisole 50mg/Kg treatments shows in table. The 200mg/kg showed no significant effect on TLC count compared to control group, whereas the 400mg/Kg and standard drug Levamisole 50mg/Kg showed significant increase in total leukocytes count compared to control group.

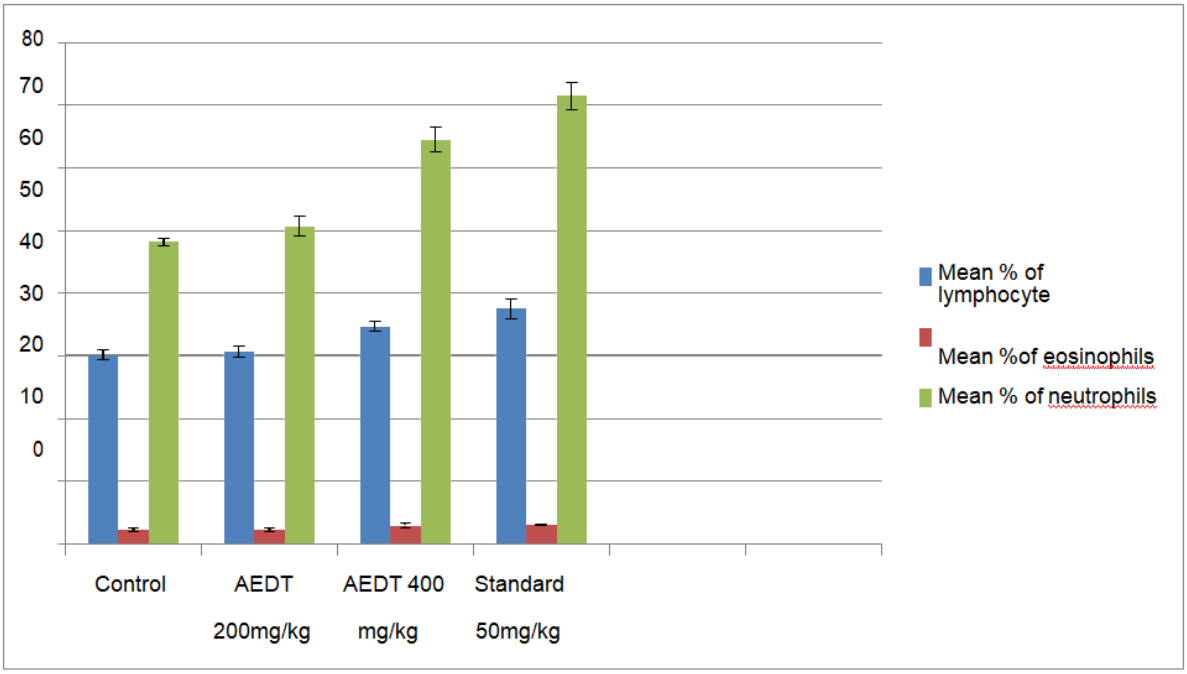


Values are expressed as Mean±SEM, n=6. Significant (***p<0.001) compared with treated groups Vs control.

Fig 3: Effect of Desmodium triflorum aqueous extract on Total Leukocytes in Wistar Rats

Differential leukocyte count

The effect of administration of aqueous extract of Desmodium triflorum the dose of 200mg /Kg, 400mg/Kg and Levamisole 50mg/Kg treatments which were given orally for 14 days. The differential leukocyte count, the lower dose 200mg/Kg of Desmodium tiflorum showed no increase in mean percentage of lymphocytes, eosinophils and neutrophils values as compared to control. The results obtained from the animals that received higher dose of aqueous extract 400mg/Kg and standard drug Levamisole 50mg/Kg showed the fact there was a highly significant increase in the mean percentage of lymphocytes and significant increase in the mean percentage of neutrophils respectively when compared to control. There is no effect in mean % increase in eosinophils count even in 400mg/Kg dose of aqueous extract of Desmodium triflorum and standard Levamisole-50mg/Kg compared to control group.



Values are expressed as Mean±SEM, n=6. Significant (***p<0.001) compared with treated groups Vs control. Mean bearing same superscripted do not differ significantly. Mean bearing different superscripted differ significantly.'a' values have significantly differ (p<0.01) from' b'. 'b' indicates significantly differ (p<0.05) from 'c'.

Fig 4: Effect of Desmodium triflorum aqueous extract on differential leukocyte count

DISCUSSION

The leaf of *Desmodium triflorum* which was studied its immunomodulatory. An immunomodulatory can be defined as a substance, biological or synthetic, which can stimulate or suppress any of the components of the immune system including both innate and adaptive arms of the immune responses. There are two main categories of immune stimulators. The specific immune stimulators are those which provide antigenic specificity in immune response, such as vaccines or any antigen; the non-specific immune stimulators are those which act irrespective of antigenic specificity to augment immune response of other antigens or stimulate components of the immune system without antigenic specificity, such immune stimulators as adjuvant and non-specific.[10]

Some plants are believed to promote positive health and maintain organic resistance against infection by establishing body equilibrium against infection by establishing body equilibrium. The concept of immune modulation 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 effectors molecules generated by activated cells. It is expected that theses nonspecific effects give protection against different pathogens including bacteria, viruses, fungi etc. and constitute an alternative to conventional chemotherapy.

The results of present study revealed that the aqueous extract of leaves of Desmodium triflorum generally revealed immune stimulatory effect on the humoral immune function and cell mediated immunity in wistar rats. No mortality and behavioral changes were observed in the treated groups up to 400 mg/kg body weight. Desmodium triflorum produced a significant, increase in DTH reaction foot pad reaction as shown above results. The increase in DTH reaction in rats might be in response to cell dependent antigen revealed the stimulatory effect on T cell.

CONCLUSION

We concluded from the result, it is possible that

the present of phytochemicals like flavonoids, tannin and saponnins reported by previous studies might be responsible for the observed immune stimulatory ability that will support for immune suppressed disease. Further, Studies are required to elucidate the extract mechanism based on molecular and genetic level responsible for immunomodulatory activity. A well-known that immune cells regulating cytokines which is necessary for proliferative phase of wound healing and immune cells like neutrophil, monocyte, macrophage and lymphocyte involved in inflammatory phase of wound healing and further pharmacological studies is going on to evaluate wound healing potential of plant extract.

The present findings are significant for the development of alternative, inexpensive and perhaps safer strategies for the treatment of disease. A detailed study is also required on structure determination of the compounds from bioactive fractions in order to find the structure activity relationship.

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