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Evaluation of Immunomodulatory Activity on Plant Extract

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Abstract
To study the immunomodulatory activity of methanolic extracts of ripens and dried fruits of phyllanthus acidus and pedalium murex on albino wistar rats and mice. The methanolic extracts of phyllanthus acidus and pedalium murex was administered orally at the dosage levels of 50mg/kg/day,100mg/kg/day and 200mg/kg/day body wt.in rats and mice. The assessment of immunomodulatory activity on specific & nonspecific immunity were studied by heamagglutination antibody (HA) titer, delayed type hypersensitivity (DTH) and carbon clearance test. The Levamisole is used as standard immunomodulating agents. Oral administration of phyllanthus acidus and pedalium murex showed a significant increase in the production of circulating antibody titer in response to sheep red blood cells (SRBCs).A significant ($P < 0.01$) increase in both HA titer was observed when compared to control groups. They showed significantly ($P < 0.01$) potentiated the DTH reaction by facilitating the foot pad thickness response to SRBCs in sensitized rats. Also, they evoked a significant ($P < 0.01$) increase on the phagocytic activity.

Keywords
Phyllanthus Acidus, Pedalium Murex, Phagocytic Activity

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1.INTRODUCTION
It is stated that a person who undergoes rejuvenation therapy attains longevity, memory, intellect, freedom from diseases, youth, luster, brilliance, and vak-siddhi. The rejuvenation therapy is also called Rasayana. The rejuvenation therapy suggests that the body fluids are replenished by proper medications and it is possible to achieve not only vitality and vigor but also greater resistance to diseases, memory, body strength, personal beauty and sense perceptions[1,2].

This concept of Rasayana is well known to Ayurvedic Physicians. The procedure of revitalization and rejuvenation were adopted to increase the power of resistance to disease (increased immunity)[3].

Immunology attained an important place in modern biology, particularly in medical science. It also plays an important role in the process of diagnosing the diseases. Immune mechanism is also involved in a variety of diseases such as cancer, diabetic mellitus, myocardial diseases, cirrhosis and atherosclerosis[4,5]. The use of medicinal plant products as possible prophylactic or therapeutic measure for the modulation of the immune response has become a subject of scientific investigation. It is being recognized that immunomodulatory therapy could provide an attractive alternative to conventional chemotherapy in various diseased conditions, especially situations like hyper inflammatory response, autoimmune disorders and organ or bone marrow transplantations[6].

Various research workers in the search of novel immunomodulatory compounds have screened many Indian medicinal plants.Joharpurkar[7],Arshad and Ansari[8], Wagnar[9], Wagner and Proksch[10]and Rngari *et al.*[11] have reviewed the natural products which have a reputation in ethnomedical practice

and which gives an advance picture in the field of immunomodulation.

2. MATERIALS AND METHODS:

Materials:

1. Gum acacia
2. Methanol
3. Levamisole
4. Citric acid
5. Sodium carbonate anhydrous
6. Glucose
7. Sodium citrate
8. Sodium chloride

Methods:

1. Carbon clearance test
2. Humoral antibody response to SRBC10
3. Cellular immune response

2.1 Methods of Preparation of Extracts / Formulations:

The ripen fruits and dried fruits of Phyllanthus acidus and Pedalium murex of 1kg was macerated with methanol in round bottomed flask for 7 days. The flask was shaken intermittently to ensure the efficiency of extraction. After a week, the extract was filtered and concentrated under reduced pressure. The methanolic extract obtained from the plant was kept in a dissector to remove moisture and properly stored until used.

Qualitative Phytochemical Analysis:

The crude methanolic extract of *Pedalium murex* was subject to the following chemical tests for the identification of various active constituents.

- Detection of Carbohydrates
- Detection of Glycosides
- Detection of Alkaloids
- Detection of Proteins and amino acids
- Detection of Phytosterols
- Detection of Phenolic compounds and tannins
- Detection of Fats and fixed oils.

2.2 Acute Toxicity Study (Preparation of Doses and Determination LD_{50}):

Acute toxicity studies were carried out according to the method described in the literature[14-17].Separately weighed in doses of 50 and 200 mg/kg b. w. of methanolic extract of each drug was triturated with 2% w/v slurry of gum acacia with distilled water and administered orally to albino mice of either sex. The animals were observed continuously for any change in behavioral, neurological, autonomic profile and mortality for first few hours and later 24h,48h.Based on this results obtained from this study, the doses were prepared(100,200 and 400mg/kg b. w. per day) for the present pharmacological studies.

Administration of Doses:

Doses of various extracts were administered orally to each animal using oral cannula fitted with a syringe of 5ml.

2.3 Methodology of immunomodulatory Studies:

Pharmacological studies to evaluate immunomodulatory activity of crude extracts(methanolic) were undertaken on swiss albino mice and Wister rats of mixed population. Experiments were carried out to determine phagocytic activity, humoral antibody response and cell mediated immune response using carbon clearance test, sheep erythrocyte agglutination method, delayed type of hypersensitivity reaction.

Humoral antibody response to SRBC10.

Mice of either sex were divided into four groups of six each. Methanolic extract
(100 mg/kg, p.o.) was administered on day 0 and continued till the day of the experiment.

All the animals were immunized by injecting 50µl of SRBCs suspension containing $5.2x10^6$ cells/ml intra peritoneal on day 0. Blood samples were collected from the orbital plexuses of individual animals on day 7 and the antibody titres were determined. Briefly, an aliquot (25 ml) of two-fold diluted sera in saline was challenged with 25 ml of 0.1% v/v SRBC suspension in microtitreplates. The plates were incubated at 37^0 C for 1 h and then observed for haemagglutination. The highest dilution giving haemagglutination was taken as the antibody titre. The mean ranks of different groups were statistically compared[18-20].

Cellular immune response:

To study the cellular immune response the edema was induced in the right paw of rats[21-26] by injecting SRBC(0.025x109 cells) in the subplanner region on 7th day, the increase in paw volume in 24 h i. e. on 8nd day was assessed by plethysmometer. The mean percentage increase in foot pad volume was considered as delayed type hypersensitivity and as an index of cell mediated immunity. The volume of the left hind paw injected similarly with phosphate-buffered saline served as a control.

Determination of phagocytic function: carbon clearance Test[27-34](Biozzi et al., 1953).

To evaluate the phagocytic activity of the reciculo-endothelial system in-vivo, a carbon clearance test was performed after completion of the drug pretreatment. On day 7, the treated rats received an intravenous injection of carbon suspension (1:50 dilution of Indian ink, Camel) in a dose of 0.5 mL/100 g body weight. Blood was withdrawn from the retro-orbital venous plexus before injection, immediately after injection and at 5 min intervals upto 20 min after injection of the carbon suspension. 0.05 mL of

blood was lysed with 4 mL of 0.1% Na_2CO_3 and the optical density was measured spectrophotometrically at 650 nm wavelength. The results were expressed as the granulopectic index, calculated by the formulae

[Log (OD_0) – log (ODt)]/t

Where OD_0 is the OD at 0 min and OD_t is the OD at t min.

2.4 Studies on *Pedalium murex*[35]:

i. Scientific Position:

The taxonomical classification are as follows:
Kingdom: *Plantae*, Plant
Phylum/Division: *Magnoliophyta,*
Class: *Magnoliopsida (Dicotyledonae),*
Subclass: *Lamiidae,*
Order: *Caryophyllales,*
Family: *Pedaliaceae,*
Genus: *Pedalium*,
Species: *P. murex L.*

ii. Distribution:

Pedalium murex (P. murex) Linn (Family: *Pedaliaceae*) is annual herb, which grows abundantly on the seacoasts, in South India, Srilanka, Ceylon, Mexico and tropical Africa. In and around Visakhapatnam the plant is very prolific after summer rains. *P.murex* has many other names, such as Telugu-*Yenugu palleru* ,Sanskrit-Brihat gokshur, Hindi-Bada goshur, English-Large caltrops.

iii. Botanical plant description:

It is a creeper that is about 2-3 feet long having branches spread all over, leaves are in pairs of 5-8 and is of irregular shape. Flowers are small and yellow colored. Fruits are round and possess 5-12 compartments and each compartment contains a seed.

The seeds contain aromatic oil. Roots are 4 to 5 inch long, brown in color and bear a fruiting. *P. murex L*. plant is a succulent herb found near seacoast of South India and some tropical areas of India. It appears during the month of July-September. It grows luxuriously in fertile soils and crop land as a weed at temperatures of 25-30 degrees.

iv. Phytochemical studies:

Preliminary chemical examination of *P.murex* revealed presence of naturally occurring different chemical constituents. Whole plant is reported to contain medicinally important. Mainly fruit contains alkaloids(3.5%-5%),stable oil, aromatic oil, resins, carbohydrates, saponins, glycosides and triterpenoids and also two important flavonoids like 2',4',5'-trihydroxy-5,7-dimethoxy flavones and triacontanyl dotriacontanoate.

v. Pharmacological studies:

Murugananthan have reported the anti-bacterial activity of *P.murex* fruits in methanolic extract against the different bacterial pathogens. International Journal of Universal Pharmacy and Life Sciences 2011;1(2):37-44.

Shelke *et al.,* reported the anti-microbial activity of aqueous and ethanolic extract of *P.murex* on bacteria Bacillus subtilus and fungi, aspergillus Niger were determined using cup and plate method. International Journal of research in Ayurveda and Pharmacy 2011;2(4):1255-1257.

Madhu babu *et al.,* reported antioxidant activity of methanol extract of fruits of *P.murex* (MEC)by using carbon tetrachloride and intoxicated rat liver as the experimental model. International Journal of Pharma and Biosciences 2011;2(1):622-628.

Hemalatha *et al*., reported the aphrodisiac activity of ethanolic extract of *P.murex* fruit during an oral glucose tolerance test was performed. Asian Pacific Journal of Tropical Biomedicine 2012:1-4.

Balamurugan *et al*., reported the anti-hyperlipidemic potential of the ethanolic extract of fruits of *P.murex* at doses of 200 and 400mg/kg/p.o.in high fat diet fed rats. International Journal of Pharmacology 2008;4(4):310-313.

Sreedevi *et al*., reported the nephroprotective activity of the ethanolic and aqueous extracts of fruits of P.murex against Gentamicin-induced renal toxicity in rats. International Journal of Drug. Dev & Res 2011;2(2):40-46.

vi. Medicinal uses:

The plant is sweet, cooling, mucilaginous, diuretic and inflammatory and used to treat digestive, carminative, tonic, spasmodic affections, amenorrhoea, and vitiated conditions of pita, inflammation and general debility and may be useful in developing new formulations with more therapeutic and economical value. Hence, the present study is undertaken based on traditional claim and the presence of medicinally active phytochemical constituents which promote the plant to have immunomodulatory activity.

vii. Plant Collection:

Pedalium murex was collected from Narayanagiri District of Warangal, South India in the month of February 2020.

viii. Preparation of Extract:

The powder drug 1kg was macerated with methanol in round bottomed flask for 7 days. The flask was shaken intermittently to ensure the efficiency of extraction. After a week, the extract was filtered and concentrated under reduced pressure. The Methanolic extract obtained from the plant was kept in a dissector to remove moisture and safely store until used.

ix. Qualitative Phytochemical Screening:

The methanolic extract of *Pedalium murex* was screened for the following phytochemical constituents using standard procedure. (Singh, 1985 & Tyler, 1981).

- Detection of Carbohydrates
- Detection of Glycosides
- Detection of Alkaloids
- Detection of Proteins and amino acids
- Detection of Phytosterols
- Detection of Phenolic compounds and tannins
- Detection of Fats and fixed oils.

x. Phytochemical Screening of *Pedalium Murex*

The plant extract was subjected to preliminary phytochemical screening for the detection of various plant constituents present. The term qualitative analysis refers to the establishing and proving the identity of a substance. The active ingredients, after isolation, can be incorporated into the modern medicine system for the development of the newer formulations for the respective ailments.

The systematic investigations of the plant material for its phytochemical behavior involve four different stages:

i) Procurement of raw material and quality control.
ii) Extraction, Isolation, Purification, and characterization of the constituents of Interest.
iii) Investigation of biosynthetic pathways of the compound.
iv) Quantitative evaluation.

2.5 Studies on Phyllanthus Acidus:

i. The Botanical Classification of the Plant:

Kingdom: Plantae
Division : Mgnoliophyta
Class : Mgnoliopsida
Order : Mglgiphiales
Family : Phyllanthaceae
Tribe : Phyllanthaceae
Subtribe : Flueggeinae
Genus : Phyllanthus
Species : *Phyllanthus acidus.*

Other scientific names are:

Cicca disticha Linn.
Cicca acida Linn
Averrhoa acidaLinn.
Cicca acidissima Blanco
Phyllanthus distichus MuelL-Arg.
Phyllanthus acidissimus MuelL-Arg
Phyllanthus acidus Skeels

Alternate Names:

Names of this tree in Indian and other languages include:

amalika in Sanskrit
aamla in Hindi
aamla in Gujarati
aavalaa (or *awla*)in Marathi
avaalo in Konkani
sunhlu in Mizo
amala in Nepali
*amloki*in Bengali
amlakhi in Assamese
amla in Oriya
Aula in Punjabi
nellikka in Malayalam
heikru in Manipuri
sohmylleng in Khasi
usiri (or *usirikai*)in Telugu
nellikkai nellikkaai or *nellikaayi*)in Tamil and Kannada

Also found are the names *emblic*, *emblic myrobalan*, *malacca tree* and the variants in spelling *aola*, *ammalaki*, *aamvala*, *aawallaa*, *dharty*, *nillika*, and *nellikya*.

ii. Origin and Distribution

This species is believed to have originated in Madagascar and to have been carried to the East Indies. Quisumbing says that it was introduced, into the Philippines in prehistoric times and is cultivated throughout those islands but not extensively. It is more commonly grown in Indonesia, South Vietnam and Laos, and frequently in northern Malaya, and in India in home gardens. The tree is a familiar one in villages and on farms in Guam, where the fruit is favored by children, and occurs in Hawaii and some other Pacific Islands.

It was introduced into Jamaica from Timor in 1793 and has been casually spread throughout the Caribbean islands and to the Bahamas and Bermuda. It has long been naturalized in southern Mexico and the lowlands of Central America, and is occasionally grown in Colombia, Venezuela, Surinam, Peru and Brazil. Formerly an escape from cultivation in South Florida, there are now only scattered specimens remaining here as curiosities.

iii. Description

This is a curious and ornamental shrub or tree, 6 1/2 to 30 ft (2-9 m) high, with spreading, dense, bushy crown of thickish, rough, main branches, in general aspect resembling the **Bilimbi** (q.v.). At the branch tips are clusters of deciduous, greenish or pinkish branchlets 6 to 12 in (15-30 cm) long, bearing alternate, short-petioled, ovate or ovate-lanceolate, pointed leaves 3/4 to 3 in (2-7.5 cm) long, thin, green and smooth on the upper surface, blue-green with a bloom on the underside; altogether giving the impression of pinnate leaves with numerous leaflets. There are 2 tiny, pointed stipules at the base of each leaf. Small, male, female, and some hermaphrodite, 4-parted, rosy flowers, are borne together in little

clusters arranged in panicles 2 to 5 in (5-12.5 cm) long, hanging directly from leafless lengths of the main branches and the upper trunk, and the fruits develop so densely that they form spectacular masses. The fruit is oblate with 6 to 8 ribs; is 3/8 to 1 in (1-2.5 cm) wide; pale-yellow to nearly white when fully ripe; waxy, fleshy, crisp, juicy and highly acid. Tightly embedded in the center is a hard, ribbed stone containing 4 to 6 seeds.

iv. Medicinal Uses:

In India, the fruits are taken as liver tonic, to enrich the blood. The sirup is prescribed as a stomachic; and the seeds are cathartic. The leaves, with added pepper, are poultice on sciatica, lumbago or rheumatism. A decoction of the leaves is given as a sudorific. Because of the mucilaginous nature of the leaves, they are taken as a demulcent in cases of gonorrhea.

The root is drastically purgative and regarded as toxic in Malaya but is boiled and the steam inhaled to relieve coughs and headache. The root infusion is taken in very small doses to alleviate asthma. Externally, the root is used to treat psoriasis of the soles of feet. The juice of the root bark, which contains saponin, gallic acid, tannin and a crystalline substance which may be lupeol, has been employed in criminal poisoning.The acrid latex of various parts of the tree is emetic and purgative.

v. Phytochemical Studies:

The fruit is a very rich source of vitamin C according to most if not nearly all references, this is probably not the case [Ghosal, 1996]. It was proposed that superior effect of the mistaken "vitamin C" component is actually the more stable and potent antioxidant effect of the tannins that appeared to be the vitamin. A repeated laboratory test showed that every 100g of fresh fruit provides 470 - 680mg of vitamin C. The vitamin value of amla increased further when the juice was extracted from the fruit. The dehydrated berry provided 2428 - 3470mg of vitamin C per 100g. Its mineral and vitamin contents include calcium, phosphorous, iron, carotene, thiamine, riboflavin, and niacin. The seeds of the Indian gooseberry contain a fixed oil, phosphatides and an essential oil. The fruits, bark, and the leaves of this tree are rich in tannin.

The fruits, leaves and bark are rich in tannins. The root contains ellagic acid and lupeol and bark contains leucodelphinidin. The seeds yield a fixed oil (16%) which is brownish-yellow in colour. It has the following fatty acids: linolenic (8.8%), linoleic (44.0%), oleic (28.4%), stearic (2.15%), palmitic (3.0%) and myristic (1.0%) [Thakur et al]. The ethanol soluble fraction contains free sugars, D-glucose, D-fructose, D-myo-inositol. The acidic water-soluble fraction contains a pectin with D-galacturonic acid, D-arabinosyl, Drhamnosyl, D-xylosyl, Dglucosyl, D-mannosyl and Dgalactosyl residues [Thakur et al].

vi. Pharmacological studies of *Cicca acida(Phyllanthus acidus)*:

a. Methanolic extracts of 79 Malaysian plants were assessed for antinematodal activity against *Bursaphelenchus xylophilus. Cicca.acida* showed strong antinematodal activity. (Muhammad et al., 1997).

b. Rat fed with the extracts from *Pacidus* showed a hepatoprotective effect against acute liver damage induced by carbon tetrachloride. (Lee et al., 2006).

c. Methanolic extracts of *Eacidus* possess strong antibacterial activity *in vitro.* (Melendez et al., 2006).

d. An extract from the medicinal plant Phyllanthus acidus and its isolated compounds induce airway chloride secretion: A potential treatment for cystic fibrosis (Sousa et al., 2007).

e. Selective Antimicrobial properties of *Phyllanthus acidus leaf* extract against *Candida albicans, Escherichia coli* and *Staphylococcus aureus* using Stokes Disc diffusion, Well diffusion, Streak plate and a dilution method (Jagessar et al., 2008).

f. Antibacterial properties of tropical plants from Puerto Rico.In the study, *Phyllanthus acidus* or *Cicca acida* was one of the plants that showed the highest antibacterial activity against *E-coli* and *Staphylococcus aureus* (Melendez et al., 2006).

vii. Plant Collection:

Phyllanthus acidus was collected from Hanamkonda, District of Warangal, South India in the month of February 2020.

viii. Preparation of Extract:

The ripen fruits of 1kg was macerated with methanol in round bottomed flask for 7 days. The flask was shaken intermittently to ensure the efficiency of extraction. After a week, the extract was filtered and concentrated under reduced pressure. The methanolic extract obtained from the plant was kept in a dissector to remove moisture and properly store until used.

3. RESULTS:

3.1 PEDALIUM MUREX

The qualitative phytochemical analysis shows the presence of triterpeniods, glycosides, carbohydrates, saponins, tannins, proteins, fixed oiled and steroidal compounds in the crude methanolic extract. The results from the above chemical tests are summarized in Table 1.

Table-1: Qualitative Phytochemical Results of Pedalium Murex

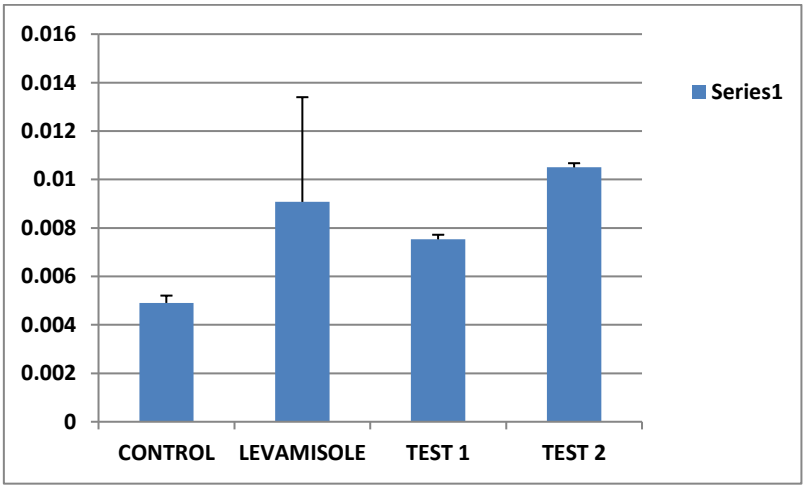
Chemical tests	Methanolic Extract of *Pedalium murex*
Alkaloids	+
Carbohydrates	-
Steroids and Sterols	-
Glycosides	-
Saponins	+
Flavonoids	+
Tannins and Phenolic	-
Triterpenoids	-
Proteins and Amino Acids	-
fixed oils	-

'+' represent presence and '-'represent absence of phytoconstituents.

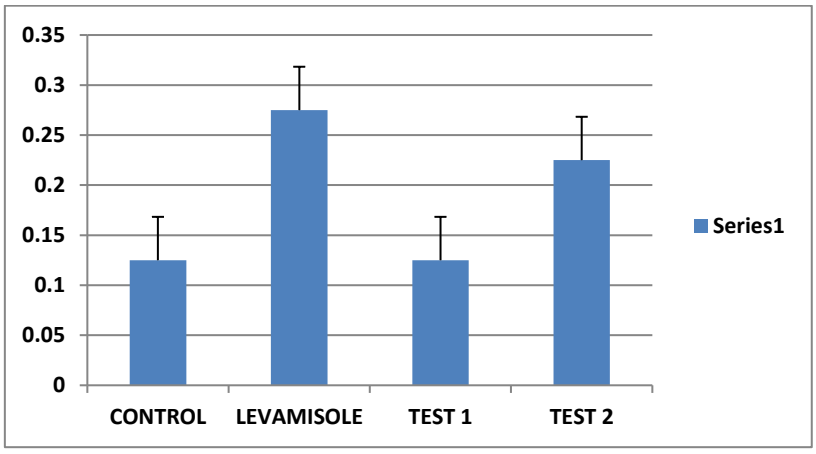
Table-2: Effect of Methanolic preparation of Pedalium Murex on CCT and DTH Response

Group	Treatment	Dose(mg/kg) for 7 Days	CCT (Mean ± SD)	DTH Response (Mean ± SD)
1	CONTROL	-----	0.004905±0.000302	0.125±0.043301
2	LEVAMISOLE	50	0.009075±0.004323	0.275±0.043301
3	TEST 1	100	0.00753±0.000189	0.125±0.043301
4	TEST 2	200	0.0105±0.000173	0.225±0.043301

n=4 for group, ***p<0.0001 very significant, **p<0.01 very significant, ***p<0.05 significant



Carbon clearance of Pedalium Murex



Delayed hypersensitivity activity of Pedalium Murex

3.2 PHYLLANTHUS ACIDUS:

The qualitative phytochemical analysis showe the presence of triterpeniods, glycosides, carbohydrates, saponins, tannins, proteins, fixed oild and steroidal compounds in the crude methanolic extract. The results from the above chemical tests are summarized in Table 3.

Table-3: Qualitative Phytochemical Results of Phyllanthus Acidus

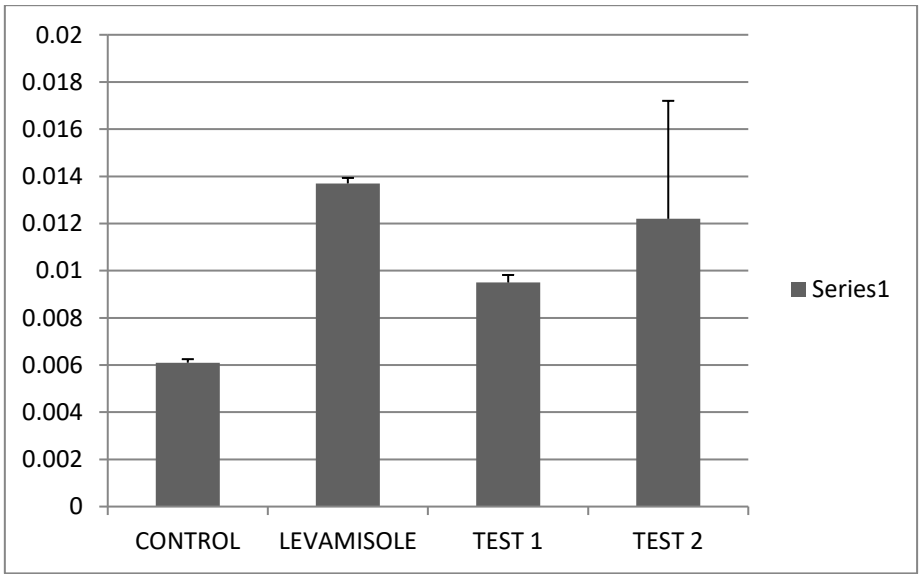
Chemical tests	Methanolic Extract of *Phyllanthus acidus*
Alkaloids	-
Carbohydrates	-
Steroids and Sterols	-
Glycosides	-
Saponins	-
Flavonoids	+
Tannins and Phenolic	+
Triterpenoids	+
Proteins and Amino Acids	-
fixed oils	-

'+' represent presence and '-'represent absence of phytoconstituents.

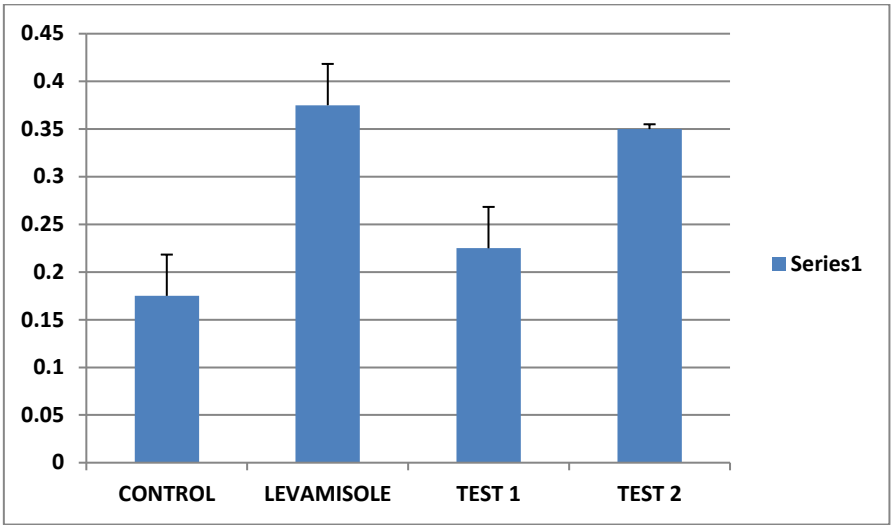
Table-4: Effect of Methanolic preparation of Phyllanthus Acidus on CCT and DTH Response

Group	Treatment	Dose(mg/kg) for 7 Days	CCT (Mean ± SD)	DTH Response (Mean ± SD)
1	CONTROL	-----	0.0061±0.00015	0.175±0.043301
2	LEVAMISOLE	50	0.0137±0.000232	0.375±0.043301
3	TEST 1	100	0.0095±0.000318	0.225±0.043301
4	TEST 2	200	0.0105±5E-05	0.350±0.05

n=4 for group, ***p<0.0001 very significant, **p<0.01 very significant, ***p<0.05 significant



Carbon clearance of Phyllanthus Acidus



Delayed hypersensitivity activity of Phyllanthus Acidus

4.DISCUSSION:

Methanolic extract of pedalium murex and phyllanthus acidus has been found to stimulate macrophages as evidenced by increase in phagocytic index in carbon clearance test on albino mice. Methanolic extracts in the doses of 100 and 200mg/kg body weight have shown a significant increase in phagocytic index when compared with control. In presnt study the methanolic extracts shown a significant increase in phagocytic index.

Stimulation of phagocytosis is influenced by the activation of macrophages these activated macrophages secrete a number of cytokines such as CSF and IL-1 which in turn stimulate other immunocytokinins like neutrophils. Finding of experiment indicate that the activated macrophages are significantly influenced by the methanolic extract.

Pedalium murex and phyllanthus acidus have shown a significant activity on cell mediated immune system in delayed type hypersensitivity response results also reveals that pedalium murex and phyllanthus acidus influences the secretions of various cytokines which activate the T-cell and activated T-cell stimulate the secretions of various enzymes and harmones which results in increase in delayed type hypersensitivity response.

Studies on humoral immune system of pedalium murex and phyllanthus acidus with sheep erythrocytic agglutination test suggest that the methanolic extracts capable to increase the agglutination. Studies reveals that the drug is capable to activate the proliferation of B lymphocyte significantly. It is the possibility that there is an enhancement in IgM and IgG levels because antibody titre against SRBC was raised. It could be stated that the potentiating in the humoral immune response may be activated by T lymphocyte.

These studies conform the stimulatory effect of pedalium murex and phyllanthus acidus on both the cell mediated and humoral immune response in experimental animal models. Italso observed the drug is capable to stimulate the cell mediated and humoral immune response in dose dependent manner.

5.CONCLUSION:

The study demonstrates that phyllanthus acidus and pedalium murex triggers both specific and non-specific responses to a greater extent. The study comprised the acute toxicity and preliminary phytochemical screening of both plant extracts. From the results obtained and phytochemical studies the immunostimulant effect of both plants could be attributed to the flavonoid content.

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CONFLICT OF INTEREST:

We declare that there was no conflict of interest in this research work.

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