List of Publications:

1. Evaluation of Aqueous and Methanol extracts of *Pistacia integerrima* galls as potent immunomodulator. Joshi Uttara and Mishra S. H. Pharmacognosy magazine. 2008. 4(14): 126-131
2. Preliminary evaluation of immunomodulatory and antistress activity of methanol extract of *Hedychium spicatum*. Pharmacologyonline. 2009. 1(1): 1057-1071.
3. In vitro hepatoprotective activity of isolated diterpene from *Hedychium spicatum.* (Communicated)

List of Presentations:

1. Oral Presentation: Evaluation of immunomodulatory activity of Aqueous and Methanol extracts of *Pistacia integerrima*. Joshi Uttara and Mishra S. H. GUJCOAST, March 2007.
2. Poster presentation: Evaluation of immunomodulatoty activity of Aqueous and Methanol extract of *Hedychium spicatum*. Joshi Uttara and Mishra S. H. 60th IPC, Delhi, December 2008.
3. Oral Presentation: Evaluation of adaptogenic activity of Aqueous and Methanol extract of *Hedychium spicatum*. Joshi Uttara and Mishra S. H. GUJCOAST, January 2009.

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Evaluation of aqueous and methanol extracts of *Pistacia integerrima* galls as potential immunomodulator.

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ABSTRACT

The aqueous and methanol extracts of *Pistacia integerrima* (*Anacardiaceaea*) leaf-galls were evaluated for immunomodulatory and adaptogenic activities using *E. coli* induced abdominal sepsis, cyclophosphamide induced myelosuppression, carbon clearance test in mice and forced swim test in rats. Both the extracts showed the presence of phenolics, flavonoids, carbohydrates and volatile oils in preliminary phytochemical screening. The Co-TLC of extracts confirmed presence of Gallic acid, and Quercetine known to possess antioxidant activity. The immunomodulatory and adaptogenic activity of above extracts may be attributed to the presence of Gallic acid and Quercetine.

KEY WORDS: *Pistacia*, phagocytocis, immunomodulation, cyclophosphamide

INTRODUCTION

In recent years, usage of indigenous drugs has increased to a very great extent as alternative and complementary medicine. This has cropped immediate need of undertaking studies for development of parameters of assessing these drugs scientifically. One of the main concepts of Rasayana drugs of ayurvedic medicines is those drugs which increase the resistance of body as rejuvenating agents. Many plants are described under this class with a claim of promoting and restoration of health. In many cases these natural products are used as an alternative to the conventional chemotherapy against a variety of diseases. It is well-known that immune system plays an important role in biological adaptation contributing to maintenance of homeostasis and establishment of body's' integrity. In therapy immunomodulators are related to stimulation or suppression of immune response of the host. These are now recognized as an alternative to conventional chemotherapy in a variety of diseased conditions, especially when host's defense mechanisms have to be activated under the conditions of impaired immune response. A selective immunomodulator has to be induced in situations where it acts by stimulating specific and nonspecific responses and may be even useful for prevention and/ or treatment of immunodeficiency related disorders allergic reactions, organ transplantation. (2) and AIDS. (3)

The plants like *Picrorrhiza kurroa, Tylophora indica, Aconitum heterophyllum* and *Holarrhena antidyssentrica, Tinospora cordifolia Ocimmum gratissimum* are known to possess immunomodulatory activity through different mechanisms. Phytoconstituents like sesquiterpene glycosides, alkaloids, flavonoids are shown to possess immunomodulatory activity.

Pistacia integerrima leaf galls are one of the appendages of plant which are used as remedy for asthma. It is also used as tonic and stimulant (4).*Pistacia integerrima* showed the presence of Gallic acid, Quercetine, leuteolin and chebulenic acid. (5, 6, 7) The phenolics and flavonoids are reported as antioxidants found in nature which may also act as immunomodulator (8) and plants containing such constituents are in turn considered as immunomodulator. Since plants used in the Indian traditional medicines, are the potential source of such immunomodulatory medicines, the present study was aimed to evaluate aqueous and methanol extracts of *Pistacia integerrima* for immunomodulatory activity. The Galls are used in some of the ayurvedic formulations like *'Chvyanprash avaleha', 'KumariAsava', 'KumariKalp'* (9) etc. prescribed in weakness as rejuvenating agent and tonic.

In the present study, the aqueous and methanol extracts were evaluated for their effect as immunomodulator using different models like *E. coli* induced abdominal sepsis, cyclophosphamide induced myelosuppression, Phagocytic index by carbon

clearance test in mice and adaptogenic activity by forced swim model in rats.

MATERIALS AND METHODS

Pistacia integerrima leaf galls were purchased from local market of Pune, India and authenticated at Regional Research Institute (Ayurveda), Pune.

Animals

Mice of either sex (CD_1 strain), weighing between 20-30 g and Albino rats of either sex (100-150 g) were obtained from M/S Zydus Research Center, Ahmedabad and were housed under standard environmental conditions with free access to food and water. The experiments were done after obtaining necessary approval from Institutional Animal Ethics Committee, Baroda (404/01/9/CPCSEA).

Preparation of extracts

Course powder (500 g) of the leaf galls was macerated for overnight in distilled water (1.5 L) and methanol (1.5 L) separately. The extracts so obtained were concentrated in a rotary vacuum evaporator and then placed in a desiccator. The Yield was 29.6 %w/w and 21.5% w/w of aqueous and methanol extract respectively.

Acute Toxicity Study

Acute toxicity study was carried out as per stair case method. (10) The animals were divided into 5 groups of six animals each (n= 6). The test samples were prepared by suspending Methanol extract in a known concentration in 0.1 % sodium carboxy methyl cellulose in water as vehicle. The aqueous extract was dissolved in distilled water. The animals of different groups were administered both the test samples in the doses of 100, 200, 300, 400 and 500 mg/kg p.o. in an increasing manner of concentration, while animals of a group treated as control were administered only vehicle. The initial dose was fixed based on the utility of the drug in many internal formulations used traditionally. The dose of 2000mg/ kg body wt was found safe as no mortality was observed.

Preliminary phytochemical screening

The powdered crude drug (100 gm) was subjected to successive solvent extraction using soxhelt apparatus. The different successive extracts so obtained were then subjected to preliminary phytochemical screening by applying different qualitative testes for phytoconstituents. (11) The petroleum ether extract showed presence of volatile oil. The ethyl acetate and methanol showed presence of phenolics and flavonoids and aqueous extract showed presence of carbohydrates, phenolics and flavonoids. The constituents like Alkaloids, proteins and fats were, however not detected.

TLC profile of Aqueous and Methanol extracts (12)

Since the galls were reported to contain Gallic acid and Quercetine as one of the constituents the extracts were subjected to co-TLC studies to reveal their presence. The mobile phase used were, toluene: ethyl acetate: formic acid (1: 3.5:0.3) and ethyl acetate: methanol: water (10: 1.35: 1) and NP-PEG reagent as spraying agent. The authentic samples of Gallic acid and Quercetine were procured from M/s Hi Media, India.

Immunomodulatory Activity

The extracts were then subjected to evaluation of Immunomodulatory Activity using following models:

***E. coli* induced abdominal sepsis Model (13, 14)**

Animals were divided into seven groups of six animals in each group. Each animal of group I was administered, 0.1% sodium CMC as vehicle only, and that of groups II, III and IV were administered, aqueous extract of *P. integerrima* in a range of 100mg/kg, 200mg/kg ,and 500mg/kg. body wt. p.o. respectively. The animals of group V, VI and VII were administered, methanol extract of *P. integerrima* in similar manner of 100mg/kg, 200mg/kg and 500mg/kg body wt. p.o.

All the animals were treated with extracts for15 days prior to bacterial challenge. On the 16th day, the animals were injected with 0.2 ml suspension of *E. coli* (1x 10^8 cells) i.p.

Animals were then observed for 16 hours to find mortality if any. Blood samples were then withdrawn from retro-orbital plexuses using heprarinized capillary tubes and by cardiac puncture in case of dead animals. Blood samples were analyzed for total and differential WBC count.

Cyclophosphamide induced myelosuppression (15, 16)

The animals of all the seven groups were administered the extracts in similar manner as described earlier, for 15 days. After 15 days of treatment blood was collected from retro orbital plexus of each animal. The haematological parameters such as Hemoglobin content (HB), Haematocrit value (HCT), Leukocytes count, Erythrocyte count, and Mean corpuscles volume (MCV) were determined using reported methods.(17) The treatment was continued for further 10 days and on the 25th day from the day of start of treatment; all groups received a single dose cyclophosphamide 250mg/kg orally. On the 26th day, blood was collected from retro orbital plexus of each animal and the

haematological parameters such as HB, HCT, Leukocytes count, Erythrocyte count and MCV were determined.

Phagocytic index by carbon clearance test (18)

Animals were divided in seven groups as stated before. The animals in group I were given 0.1% sodium CMC for 5 days, whereas the animals of group II to group VII were given test extracts for 5 days orally in similar manner described above. The animals were then injected carbon ink suspension (Pelican ink, Germany) via the tail vein, 48 hrs after 5th day administration. Blood samples were withdrawn from the retro orbital plexuses at 0 and 15 min. The blood (25µl) was dissolved in 0.1% sodium carbonate (2 ml) and absorbance was determined at 660 nm. The Phagocytic index K was calculated.

Adaptogenic activity (Forced swim model) (13)

Albino rats of either sex (100-150 g) were divided in seven groups and administered the test extracts in similar manner as stated above. Stress was exerted by keeping rats in cylindrical vessels (length 48 cm and diameter 30 cm) filled with water to a height of 25 cm over period for two hours daily for seven days. Blood was collected from retro orbital plexus of each animal and the biochemical parameters such as SGPT, SGOT, serum glucose, cholesterol and triglycerides were determined.

RESULTS

The different successive extracts showed presence of volatile oil, carbohydrate, phenolics and flavonoids when subjected to preliminary phytochemical screening. The presence of Gallic acid and Quercetine was confirmed using co-TLC with authentic samples. In the E. coli induced abdominal sepsis model, 100% mortality was observed in group treated with vehicle only within 16 hours whereas 33.3% mortality was observed in treated group. (Table 1). A reduction in WBC, RBC and Heamoglobin count in Cyclophosphamide induced myelosuppression was observed case of animals of control group while in those of treated groups there was no decrease in RBC, hemoglobin and WBC values was observed. It shows that the drug offers protection against cyclophosphamide induced myelosuppression. MCV and haematocrit values were also not altered. (Table2) Although increase in Phagocytic index was observed in both the test extracts, a significant increase was observed in the dose ranges of 200 and 500 mg/kg body weight p.o.(Table 4).In the present study, on stress induced forced swimming model for adaptogenic activity the values of biochemical parameters like glucose, cholesterol, triglycerides, GPT in the serum were found lower when compared with that of the values of control group (Table 3).

DISCUSSION

The reports on the galls of *P.integerrima* stated its usage in many ayurvedic formulations prescribed as tonic and rejuvenating agents. It has prompted a need for evaluating the extracts showing the presence of Gallic acid and Quercetine being active adaptogenic compounds for immunomodulatory activity using reported parameters. The common gram negative bacterial pathogen *E. coli*. induced abdominal sepsis model was used to observe effect on the infected animals. Acute bacterial peritonitis is a life threatening condition characterized by the presence of bacteria in germ free peritoneal cavity. The innate immune system enables the host to mount an immediate response to invading pathogens. The innate immune system is the central element of host defense in peritonitis. Lipopolysaccharide (LPS) is the major constituent of outer cell wall of pathogen which is the mediator of immune response. Lipopolysaccharide binding protein enhances release of LPS and produces abdominal sepsis. (19) In E. coli induced abdominal sepsis, protection offered by the extract could be attributed to secretion of IL-1 and GM-CSF from activated macrophages. Activated macrophages secrete number of cytokines like IL-1 and GM-CSF which in turn stimulate other immunocytes like neutrophils (13). Both the extracts showed an increase in WBC and % neutrophils. The aqueous extract at 500mg/kg body weight was more significant indicating the protection.

Table 1- Effect of Aqueous and Methanol extracts in E. coli induced abdominal sepsis in mice

No	Groups	Dose (mg/Kg)	WBC (10^3 / mm^3)	Neutrophils (%)
1	Group I	Control	1.800 ±0.17	9.33 ±1.16
2	Group II	Aqueous 100	2.337±0.07*	13.83 ±1.7*
3	Group III	Aqueous 200	2.40 ± 0.18**	14.00± 1.2*
4	Group IV	Aqueous 500	3.65_±0.28***	23.67± 1.22***
5	Group V	Methanol 100	2.11_±0.14	11.17 ±1.6
6	Group VI	Methanol 200	1.95 ±0.14	12.83 _±2.04
7	Group VII	Methanol 500	2.350 ±0.17*	13.50 ± 2.07*

**p< 0.5, ** p<0 .01, *** p< 0.001; Six animals were used. (Statistical analysis is done by applying One way ANOVA followed by Bonferronis multiple column test.)*

Table 2 – Effect of Aqueous and methanol extracts on haematological parameters after 15 days of treatment with extracts and on 26th day in cyclophosphamide induced myelosuppression in mice.

No	Groups	Dose (mg/Kg)	RBC (106/ mm3) 15th day	RBC (106/ mm3) 26th day	WBC (106/ mm3) 15th day	WBC (106/ mm3) 26th day	HB 15th day	HB 26th day	MCV 15th day	MCV 26th day	HCT 15th day	HCT 26th day
1	Group I	Control (10.1% Sod CMC)	7.95±0.15	5.05± 0.22	2.26±0.19	0.86± 0.21	10.0±1.63	8.53± 0.36	49.70±1.33	49.34± 1.2	34.72±1.33	33.31± 0.21
2	Group II	Aqueous 100	7.32±0.41	7.10 ± 0.28	2.20±0.20	1.18± 0.65*	9.22±0.14	9.38± 0.29	52.38±1.00	52.00± 1.23	37.80±0.64	35.21± 0.40
3	Group III	Aqueous 100	6.27±0.25	6.14± 0.20	2.35±0.47	1.18± 0.30*	9.26±0.18	9.80± 0.38	47.12±0.83	46.85± 0.33	36.54±0.66	36.84± 1.02
4	Group IV	Aqueous 100	8.88±0.25	8.85± 0.25	1.48±0.14	1.95± 1.96***	10.18±0.15	9.75± 0.22	51.65±1.9	52.00± 0.91	34.53±0.57	35.11± 0.92
5	Group V	Methanol 100	7.62±0.13	6.76± 0.36	2.23±0.12	1.13± 1.56	10.12±0.20	9.66± 0.20	51.00±1.44	51.98± 0.87	40.53±1.62	39.15± 1.14
6	Group VI	Methanol 200	7.78±0.25	6.24± 0.22	2.26±0.17	1.20± 1.00**	10.11±0.27	9.66± 0.57	52.43±1.10	50.62± 0.91	39.30±0.83	40.02± 0.83
7	Group VII	Methanol 500	6.00±0.25	5.87± 0.20	2.23±0.12	1.86± 0.81***	9.02±0.18	9.06± 0.22	44.23±1.07	44.18± 0.81	34.53±0.58	35.98± 0.44

**p< 0.5, ** p<0 .01, *** p< 0.001 Six animals were used. (Statistical analysis is done by applying One way ANOVA followed by Bonferronis multiple column test)*

Table 3- Effect of Aqueous and methanol extracts on stress mediated changes in biochemical parameters in rats.

No	Groups	Dose (mg/Kg)	Day	Glucose (mg/ dl)	Cholesterol (mg/dl)	Triglyceride (mg/dl)	SGPT (IU/ml)	SGOT (IU/ml)
1	Group I	Control (0.1% Sod CMC)	1	181.2±6.5	144.5±1.86	56.65±0.63	78.33±1.50	84.70±2.16
			7	137.7±4.96	42.29±4.96*	56.65±0.25	75.00±1.82	83.19±1.32
2	Group II	Aqueous 100	1	133.2±9.69	138.7±4.12	53.53±0.70	65.21±3.74	85.69±2.16
			7	108.0±2.7*	47.05±0.86**	44.77±1.97*	54.00±1.36***	78.65±1.36
3	Group III	Aqueous 200	1	106.4± 1.31	136.2±2.9	52.21±1.2	61.67±1.89	85.16±2.30
			7	103.4± 8.33**	96.34±0.58***	44.26±1.56*	52.67±1.90***	80.36±2.12
4	Group IV	Aqueous 500	1	106.4±1.31	122.6±4.30	52.23±2.07	62.83±2.08	81.4±1.22
			7	110.3±6.71***	83.87±0.65**	37.26±0.65 ***	52.67±1.90***	78.25±2.50
5	Group V	Methanol 100	1	121.0±0.63	142.0±3.86	53.96±1.47	68.50±3.4	85.25±1.25
			7	113.2±1.78*	47.88±3.4 NS	46.19±3.46 NS	60.83±1.51 **	74.25±1.05
6	Group VI	Methanol 200	1	117.0±0.91	137.6±1.08	55.62±1.23	66.67±3.60	78.43±2.74
			7	113.5±1.31*	27.59±1.88 NS	46.30±1.38 NS	60.83±2.35**	75.25±1.50
7	Group VII	Methanol 500	1	108.3±0.65	127.5±6.44	55.53±2.61	79.63±1.11	79.25±2.15
			7	115.4±1.04*	31.58±2.44 NS	46.30±1.38 NS	64.83±3.7**	75.36±1.50

**p<0.5, **p<0.01, ***p<0.001. Six rats were used. (Statistical analysis was done by One way ANOVA followed by Bonferonnis multiple column test)*

Table 4: Effect of Aqueous and methanol extracts on Phagocytic index in mice.

No	Groups	Dose (mg/Kg)	Phagocytic index
1	Group I	Control (l0.1% Sod CMC)	0.1056 ± 0.03
2	Group II	Aqueous 100	0.1066 ± 0.02
3	Group III	Aqueous 200	0.1362 ± 0.05***
4	Group IV	Aqueous 500	0.1362 ± 0.05***
5	Group V	Methanol 100	0.1056 ± 0.02
6	Group VI	Methanol 200	0.1322 ± 0.01***
7	Group VII	Methanol 500	0.1558 ± 0.01***

*$p < 0.05$, **$p<0.01$, *** $p< 0.001$ six animals were used. (Statistical analysis is done by applying One way ANOVA followed by Bonferronis multiple column test.)*

Cyclophosphamide is one of the therapeutic agents having suppressive and cytotoxic activity. It has number of side effects in long term treatment. Cyclophosphamide is an alkylating agent resulting in cross linking of DNA and causes inhibition of DNA synthesis. The major drawback of this drug is myelosuppression. An attempt to overcome this problem has been made by introducing Pro- Host therapy. (20) In the present study WBC counts in control animals were reduced to a low value while incase of treated animal with the extracts of *Pistacia integerrima* the reduction in the values were restricted. Hence the test extracts hold promise as candidates to overcome this problem. In case of *Withania somnifera* also similar results are reported. (21)

The Phagocytic activity is measured by the rate of removal of carbon particles from blood stream. Aqueous and methanol extracts of *Pistacia integerrima* have macrophage stimulatory activity as evidence by increased Phagocytic index in carbon clearance test. (22)Chronic fatigue syndrome (CSF) is a condition of unknown etiology characterized by extreme fatigue lasting over for 6 months. It is accomplished by syndromes like feverishness, headache, difficulty to concentrate and disordered cell mediated immunity. The studies have reported abnormalities of Hypothalamic- Pituitary Adrenal axis in CSF. (23) An increase in glucose level on day 1was observed, but the increase was not sustained on subsequent days. It may be due to homeostasis mechanism which regulates blood glucose level. The mechanism by which stress raises serum cholesterol is likely to be due to enhanced activity of hypothalamus- hypophyseal axis resulting in increased liberation of catecholamines and corticosteroids. The change in serum triglyceride is possibly mediated via adrenal medullary secretions and through activation of sympathetic nervous system. (24) In present study the test extracts when subjected to forced swim model for adaptogenic activity in rats showed An increase in serum cholesterol and serum triglyceride level on day 1 was observed, but the increase was not sustained on subsequent days. The test extracts could restrict the increase in the level of these markers during stress.

CONCLUSION

The studies indicate that the aqueous and methanol extracts of *Pistacia integerrima* possess a potential of significant immunomodulatory and adaptogenic activity. The results are encouraging to pursue further studies on the other bioactivity guided fractionation of these extracts to isolate and characterize probable bio active molecules.

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PRELIMINARY EVALUATION OF IMMUNOMODULATORY AND ANTISTRESS ACTIVITY OF METHANOL EXTRACT OF *HEDYCHIUM SPICATUM*

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Summary

Hedychium spicatum (Zinziberaceae) commonly known as Kapurkachari, is wildely recognized in Ayurvedic system of Indian medicine for anti-inflammatory and antistress activities. It is also reported to be tonic and stimulant. The methanol extract of rhizomes of *Hedychium spicatum* was evaluated for immunomodulatory activity using *E. coli* induced abdominal sepsis, cyclophosphamide induced myelosuppression, carbon clearance test in mice and forced swim test in rats and anoxia stress tolerance in mice. The extract showed pronounced immunoprophylactic activity in *E. coli* induced abdominal sepsis by increasing the WBC count and% neutophils. The immunostimulant effect was observed in carbon clearance test by increased Phagocytosis. In cyclophosphamide induced myelosuppression, there was increase in number of WBC in the treatment groups indicating immunostimulant potential of the extract. The extract was found to be effective in forced swim model and anoxia stress tolerance in rats. The extract at dose level 200 and 500 mg/kg was found to be more effective.

Keywords: Cyclophosphamide, Immunoprophylactic, Phagocytosis, Immunoprotective, antistress

Introduction

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 immunomodulation 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 effector 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. (1, 2)

An immunomodulator can be defined as a substance, biological or synthetic, which can stimulate or modulate any of the components of the immune system including both innate and adaptive arms of the immune responses. (3) These substances have been described to possess pharmacological properties like immunostimulant, tonic, antiaging, antistress, antirheumatic, adaptogenic anticancer, antibacterial etc. These immunomodulatory agents are of plant origin which is claimed induce paraimmunity, the nonspecific immunomodulation of essentially granulocytes, macrophages, natural killer cells and complement function. In general immunomodulators are biological response modifiers that affect the immune response in either positive or negative fashion(4, 5).

Hedychium spicatum (Zingiberaceae) which is reported to be a bitter tonic and stimulant was evaluated for immunomodulatory activity (6). It is also used in treatment of inflammation, liver complaints and vomiting. It is also used as brain tonic (7). The plant showed presence of sesqueterpene alcohols, furanoid diterpene like hedechnone, (8) glycosides, carbohydrates, steroids etc (9).

However, so far no systematic study has been reported to evaluate immunomodulatory potency of *Hedychium spicatum*. In the present study, the aqueous and methanol extracts were evaluated for their effect as

immunomodulator using different models like *E. coli* induced abdominal sepsis, cyclophosphamide induced myelosuppression, Phagocytic index by carbon clearance test in mice and as adaptogen by forced swim model in rats and anoxia stress tolerance in mice.

Materials and methods

Animals:

Swiss albino mice of either sex (20-25 g) and albino rats of either sex (100-150 g) were obtained from M/S Zydus Research Center, Ahmedabad and were housed under standard environmental conditions with free access to food and water. The experiments were done after obtaining necessary approval from Institutional Animal Ethics Committee, Baroda (404/01/9/CPCSEA).

Plant materials:

Authenticated *Hedychium spicatum* rhizomes were obtained as gift samples from Indian Herbs, Saharanpur. A voucher specimen is preserved in Pharmacy Department, Baroda (MS/ PH/UJ/07).

Extraction procedure

500 Gms of course powder of the rhizomes was macerated for overnight in 1.5 ml methanol. The extract was concentrated in a rotary vacuum evaporator and then placed in a desiccator. The Yield was 5.2% w/w of methanol extract.

Preliminary phytochemical screening

Methanol extract was subjected to preliminary phytochemical screening by applying different qualitative testes for phytoconstituents (10).

HPTLC profile for methanol extract for *Hedychium spicatum*

The methanol extract was found to be rich in terpenes and diterpenes, the extract was subjected to HPTLC for confirming presence of these phytoconstituents. A stock solution (1 mg/ ml) was prepared in methanol. Suitably diluted stock solution was spotted on pre-coated silica gel G 60 F 254 TLC plates using CAMAG Linomat V Automatic sample spotter and the plates were developed in solvent systems of different polarities to resolve

polar and nonpolar components of the bioactive fraction. The plates were scanned using TLC scanner 3 (CAMAG) at 254 nm (absorbance/ reflectance mode) and 366 nm (fluorescence/ reflectance mode) and R_f values, spectra, λ max and peak areas of resolved bands were recorded. Relative percentage area of each band was calculated from peak areas. The solvent system selected for terpenes was toluene: chloroform: ethanol (4:4:1), spraying reagent AS reagent and for diterpenoids hexane: ethyl acetate (17:3), spraying reagent 10% sulphuric acid (11). The results are represented in Figure 1 and 2.

Acute toxicity

Acute toxicity study was performed as per OECD guidelines No 423.

Immunomodulatory Activity

The methanol extract was subjected to evaluation of Immunomodulatory Activity using following models

***E. coli* induced abdominal sepsis Model**

Animals were divided into four groups of six animals in each group. The animals of group I were administered 1% sodium CMC as vehicle only. Animals of groups II, III and IV were administered methanol extract of *H. spicatum* in dose100mg/kg, 200mg/kg and 500mg/kg body wt. p.o. All the animals were treated with extracts for15 days prior to bacterial challenge. On the 16^{th} day, the animals were injected with 0.2 ml suspension of *E. coli* (1x 10^8 cells) i.p. Animals were then observed for 16 hours to find mortality if any. Blood samples were then withdrawn from retro-orbital plexuses using heprarinized capillary tubes and by cardiac puncture in case of dead animals. Blood samples were analyzed for total and differential WBC count (12,13)

Phagocytic index by carbon clearance test (14)

Animals were divided in four groups as stated earlier. The animals in group I were given 1% sodium CMC for 5 days, whereas the animals of group II to group IV were given test extracts for 5 days orally in similar manner described above. The animals were then injected carbon ink suspension (Pelican ink, Germany) via the tail vein, 48 hrs after 5th day administration. Blood

samples were withdrawn from the retro orbital plexuses at 0 and 15 min. The blood (25µl) was dissolved in 0.1% sodium carbonate (2 ml) and absorbance was determined at 660 nm. The phagocytic index K was calculated by using following equation:

$K = (\ln OD_1 - \ln OD_2) / (t_2 - t_1)$

Where OD_1 and OD_2 are the optical densities at times t_1 and t_2 respectively.

Cyclophosphamide induced myelosuppression

The animals of all the four groups were administered the extracts in similar manner as described earlier, for 15 days. After 15 days of treatment blood was collected from retro orbital plexus of each animal. The haematological parameters such as Hemoglobin content (HB), Haematocrit value (HCT), Leukocytes count, Erythrocyte count, and Mean corpuscles volume (MCV) were determined using reported methods (15,16))The treatment was continued for further 10 days and on the 25th day from the day of start of treatment; all groups received a single dose cyclophosphamide 250mg/kg orally. On the 26th day, blood was collected from retro orbital plexus of each animal and HB, HCT, Leukocytes count, Erythrocyte count and MCV were determined.

Adaptogenic activity:

Anoxia stress tolerance test

Albino mice of either sex were divided into four groups. Stress was induced in the animals by placing individual animal in hermetic vessel of 1 L capacity. Reaction to anoxia stress was recorded as anoxia tolerance time i.e. first sign of convulsion was considered as end point. The study was conducted for 21 days and at the end point of 1st, 2nd and 3rd week anoxia stress tolerance time was recorded with all animals (17, 18)

Forced swim model

Adaptogenic activity by forced swim method was performed by method described by Krupavaram *et al* (19)Albino rats of either sex (100-150 g) were divided groups of six animals each. The control group received 1% sodium carboxy methyl cellulose solution only as vehicle; where as the treatment

groups were given test extracts in 1% sodium carboxy methyl cellulose. The rats were subjected to swimming stress by keeping them in cylindrical vessels (48 × 30 cm) filled with water to a height of 25 cm and the total swim time for each rat was noted. Extracts were given to rats once daily for 7 days. On 8th day rats were allowed to swim till complete exhaustion. The animals were killed and blood was collected by cardiac puncture to estimate biochemical parameters like serum glucose (GOD/POD method), triglycerides (enzymatic method), cholesterol (CHOD-PAP method), BUN (enzymatic method) and blood cell count i.e. RBC, WBC (standard Neubauers chamber method) and DLC (standard Leishman's staining method). The weight of organs such as liver, spleen, adrenals was recorded after washing with alcohol.

Statistical analysis

Statistical analysis is done by applying One way ANOVA followed by Bonferronis test.

Results

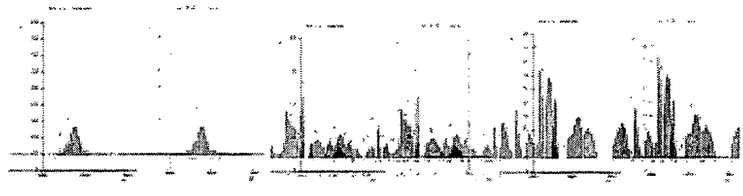
Methanol extract was subjected to phyrochemical screening. The extract showed presence of flavonoids, steroids, terpenoids and diterpenes. The presence of these constituents was confirmed by qualitative tests and TLC. Presence of furanoid diterpenes and labdane diterpenes was confirmed by Libermann Burchard test and orange color with acetone and sulfuric acid (Figs. 1-2). In *E.coli* induced abdominal sepsis, mortality due to peritonitis was evaluated in control and treatment groups. There was 100% mortality in control group. In the groups treated with 100 and 200mg/kg body wt of methanol extract, 50%and 33% mortality was observed respectively. In the group treated with 500mg/kg, 17 % mortality was observed. The effect of extract on WBC and % neutrophils was evaluated. There was dose dependent increase in WBC and % neutrophils when treated with 200 and 500mg/kg body wt.

There was increase in phagocytic index in the treatment groups indicating phagocytosis. Methanol extract at 200 and 500mg/kg were found to be

statistically significant (Tables 1-5). In case of cyclophosphamide induced myelosuppression, there was decrease in the WBC count in the control group. In treatment groups, the WBC count was found to be increased with $p<0.01$ and $p< 0.001$ at 200 and 500 mg/kg dose respectively. There was no alteration in other haematological parameters like RBC, HB, HCT and MCV. Methanol extract of *H. spicatum* has significantly enhanced anoxia stress tolerance time evident by delaying convulsion time.

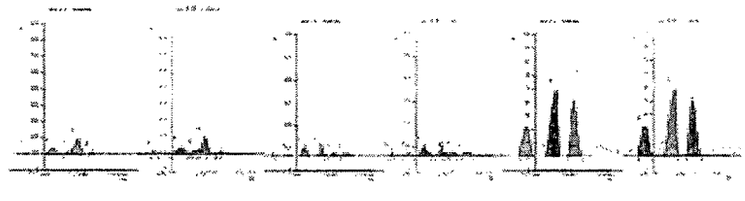
In case of forced swim model in rats different biochemical parameters, organ weigh and blood count were evaluated. There was no change in the organ weight but biochemical parameters like serum glucose, triglycerides, cholesterol and BUN were altered in the treatment groups. Blood count and DLC were significantly lowered in the treatment groups. Methanol extract at 200 and 500 mg/kg were found to be more effective.

Fig 1: HPTLC fingerprint of terpenes in methanol extract at different wavelengths



254 nm 366 nm 540 nm

Fig 2: HPTLC fingerprint of diterpenes in methanol extract at different wavelengths



254 nm 366 nm 540 nm

Table 1: Effect of methanol extracts of *H. spicatum* on WBC and neutrophils in *E. coli* induced abdominal sepsis and on phagoctytosis in carbon clearance test in mice.

No	Groups	Dose (mg/Kg)	WBC 10^3 / mm^3)	Neutrophils (%)	Phagocytic index
1	Group I	Control	2.167 ±0.40	11.17±2.04	0.1056±0.0003
2	Group II	Methanol 100	2.817±0.67	14.00±0.63	0.1170±0.015
3	Group III	Methanol 200	3.117±0.11**	14.67±1.03*	0.1322±0.014**
4	Group IV	Methanol 500	3.383±0.19***	15.50 ±2.07**	0.1558±0.0012***

*p< 0.5, ** p<0 .01, *** p< 0.001 Six animals were used. (Statistical analysis is done by applying One way ANOVA followed by Bonferronis test.)

Table 2: Effect of methanol extract of *H. spicatum* on Anoxia stress tolerance test in mice.

No	Groups	Dose (mg/Kg)	Mean duration of tolerance(in min) after treatment		
			1st Week	2nd Week	3rd Week
1	Group I	Control	23.66±1.25	30.89±1.65	31.32±0.98
2	Group II	Methanol 100	31.98±0.75	32.25±1.26	35.25±2.36
3	Group III	Methanol 200	45.24±0.58*	50.21±2.56*	58.45±1.25**
4	Group IV	Methanol 500	61.21±1.98*	69.54±2.01**	72.12±0.96***

*p< 0.5, ** p<0 .01, *** p< 0.001 Six animals were used. (Statistical analysis is done by applying One way ANOVA followed by Bonferronis test.)

Table 3: Effect of methanol extract of *H. spicatum* on haematological parameters after 15 days of treatment with extracts and on 26th day in cyclophosphamide induced myelosuppression in mice.

No	Groups	Dose (mg/Kg)	RBC (10^6/ mm^3)		WBC (10^3/ mm^3)		HB		MCV		HCT	
			15th day	26th day	15th day	26th day	15th day	26th day	15th day	26th day	15th day	26th day
1	Group I	Control	7.95 ±0.15	5.05 ± 0.22	2.26 ±0.19	0.86 ± 0.21	10.0 ±1.63	8.53 ± 0.36	49.70 ±1.33	49.34 ± 1.2	34.72 ±1.33	33.31 ±0.21
2	Group III	Methanol 100	7.62 ±0.13	6.76 ± 0.36	2.23 ±0.12	1.13 ± 1.56*	10.12 ±0.20	9.66 ± 0.20	51.00 ±1.44	51.98 ± .87	40.53 ±1.62	39.15 ± .14
3	Group IV	Methanol 200	7.78 ±0.25	6.24 ± 0.22	2.26 ±0.17	1.20 ± 1.00**	10.11 ±0.27	9.66 ± 0.57	52.43 ±1.10	50.62 ± 0.9	39.30 ±0.83	40.02 ± 0.9
4	Group V	Methanol 500	6.00 ±0.25	5.87 ± 0.20	2.23 ±0.12	1.86 ± 0.81***	9.02 ±0.18	9.0 6± 0.22	44.23 ±1.07	44.18 ± 0.8	34.53 ±0.58	35.98 ± 0.5

*p< 0.5, ** p<0 .01, *** p< 0.001 Six animals were used. (Statistical analysis is done by applying One way ANOVA followed by Bonferronis test.)

response of Oreochromis mossambicus has been undertaken. It was found to be effective in both specific and nonspecific immunity.

Conclusion

The studies indicate that the methanol extract of *H .spicatum* possess a potential of significant immunomodulatory activity. The results are encouraging to pursue further studies on the other bioactivity guided fractionation of these extracts to isolate and characterize probable bio active molecules.

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