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 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 (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.

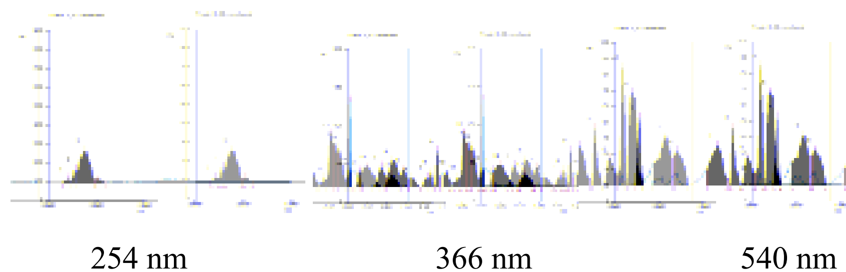
There was increase in phagocytic index in the treatment groups indicating phagocytosis. Methanol extract at 200 and 500mg/kg were found to be

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 .01and 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.

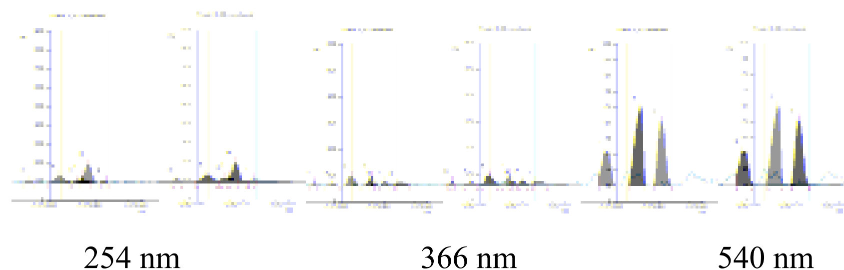
In case of forced swim model in rats different biochemical parameters, organ weigh and blood count were evaluated. There was no change in the organ weight but biochemical parameters like serum glucose, triglycerides, cholesterol and BUN were altered in the treatment groups. Blood count and DLC were significantly 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 phagocytosis 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.)

Table 4 - Effect of methanol extract of *Hedychium spicatum* on stress mediated changes in rats

No	Groups	Dose (mg/Kg)	Swimming survival time(min)	Biochemical parameters				Organ weight		
				Glucose (mg/ dl)	Cholesterol (mg/dl)	Triglyceride (mg/dl)	BUN (mg/ dl)	Liver (g)	Spleen (mg)	Adrenals (mg)
1	Group I	Control	96.65 ±1.56	150.7 ±2.81	115.16 ±2.04	55.22 ±2.35	52.19 ±2.48	4.983 ±0.18	934.0 ±0.041	0.044 ±0.0046
2	Group II	Methanol 100	118.63 ±1.25*	139.5 ±3.39	82.38 ±6.38	43.29 ±3.41*	42.46 ±2.35	4.867 ±0.13	884.0 ±0.39	0.036 ±0.007
3	Group III	Methanol 200	125.35 ±2.36**	121.3 ±3.67**	61.41 ±3.59**	41.77 ±1.23**	32.22 ±1.07**	4.750 ±0.22	838.2 ±0.30	0.043 ±0.0046
4	Group IV	Methanol 500	136.32 ±2.65**	103.2 ±2.70***	51.66 ±2.35***	43.27 ±2.39**	33.74 ±1.39**	4.300 ±0.23*	1237.0 ±0.75*	0.039 ±0.0059

*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 5 - Effect of methanol extract of *Hedychium spicatum* on stress mediated changes blood cell count

No	Groups	Dose (mg/Kg)	RBC	WBC	DLC			
					L	N	E	M
1	Group I	Control	11.07 ±0.97	11.69 ±0.64	7478 ±26.52	3037 ±29.83	12.50 ±0.83	12.50 ±0.83
2	Group II	Methanol 100	10.67 ± 1.14	10.33 ±1.01	7055 ±37.49	3021 ±25.59	14.00 ±1.09	12.00 ±1.78
\2	Group II	Methanol 200	8.550 ±1.14**	9.58 ±0.67**	6938 ±23.16***	2667 ±41.00***	10.83 ±0.98***	9.833 ±0.75**
3	Group III	Methanol 500	7.783 ±1.47***	7.789 ±0.56***	6281 ±7.02***	2108 ±25.19***	9.333 ±1.03***	9.667 ±0.51**

L-Lymphocytes , N- Neutrophils, E- Eosinophils, M- Monocytes

*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.)

Discussion

The reports on the rhizomes of *H. spicatum* stated its usage in ayurveda prescribed as tonic, stimulant and antistress agents. It has prompted a need for evaluating the extracts showing the presence of flavonoids, diterpenes and terpenoids being active compounds for immunomodulatory activity using reported parameters.

Acute bacterial peritonitis is a life threatening condition characterized by presence of bacteria in the germfree peritoneal cavity (20).Host defense is a classical domain of the innate immune system as a rapid response to pathogens is essential for the host to survive (21).Treatment of this condition has focused on surgery, antibiotics and nutritional support. But in spite of this, fatal complications have been reported. A factor which influences the recovery is host defense mechanism. In *E. coli* induced abdominal sepsis, protection offered by the extracts 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 (22).The methanol extract of *H. spicatum* significantly protected the animals against peritonitis. Extract also showed significant increase in WBC and neutrophils thus it may have humoral immune response potentiating effect.

Phagocytosis provides the first line defense to the host against infectious microorganisms. Polymorphnuclear leukocytes (neutrophils and eosinophils) and mononuclear phagocytes (Monocytes and macrophages) are most commonly recognized 'professional' phagocytes. The primary target of most of the immunomodulators is believed to be macrophages which play a major role by engulfing pathogens or foreign substances and initiating innate immune response. This may enhance the defense ability to counter the infection stress. It suggests enhancement in phagocytic function of macrophages and thus nonspecific immunity. Phagocytosis by macrophages is important against the smaller parasites and its effectiveness is markedly enhanced by opsonization of the parasites with the antibody and complement C3b, leading to more rapid clearance of parasites fm the blood. Extract showed significantly high phagocytic index which indicates marked increase in the rate of carbon clearance(23).

Cyclophosphamide is an alkylating agent resulting in cross linking of DNA and causes inhibition of DNA synthesis. Major drawback of this drug is myelosuppression. An attempt to overcome this problem has been made by pro-host therapy (24).In case of cyclophosphamide induced myelosuppression, methanol extract of *H. spicatum* was able to

bring back the levels of WBC to normal. There was no significant change in other haematological parameters suggesting these drugs can be used for long term treatment.

In case of anoxia stress tolerance test, a significant Adaptogenic activity was observed with methanol the extract indicated by the increase in the duration of anoxic stress tolerance time.

Forced swim involves physical exercise and physiological stress which leads to increased serum cholesterol and protein levels. Blood sugar levels in response to stress in rats shows fluctuation in blood sugar level ranging slight decrease, relative increase or no change. In the present study, hyperglycemia was observed. Under stressful conditions adrenal cortex secretes cortisol in man and corticosterone in rats. Hyper secretion of cortisol helps maintenance of internal homeostasis through the process of gluconeogenesis and lipogenesis (25).The stress raises serum cholesterol level through enhanced activity of hypothalamus hypophyseal axis resulting in increased liberation of catecholamines and chorticosteroidsThe effect of triglycerides is probably due to mobilization of lipids from adipose tissues by catecholamine (26,27). There was increase in BUN level in stress control group as these are the end products of protein metabolism and in excess adrenocorticoid activity, urea excretion increases. The decreased BUN level in the treated groups indicates diminished amount of protein catabolism in extract treated groups. Stress induced adrenomedullary response in man leading to increased production of corticotropic hormone that leads to increase in weight adrenal gland and liver and reduction in spleen weight (28).But in this study, there was no alteration in organ weight. Stress induces adrenomedullary response in man causing release of adrenalin which in turn stimulates β_2 receptors by pituitary gland causing greater release of ACTH. This stimulates the adrenal medulla and cortex leading to weight increased weight of adrenal gland. Cortisol increases m-RNA levels in the liver cells and facilitate metabolic anabolic effect. This leads to weight of liver. Spleen contracts to release more blood cells during stress leading to decrease in weight of spleen. During stress, heart rate, blood pressure and flow rate increase. To meet the extra demands, RBC and WBC increase (29).

The phytochemical screening revealed presence of flavonoids, triterpenoids, diteprnes and steroids. These types of components are reported to exhibit immunomodulatory activity in various experimental models. Potent immunomodulatory activity has been seen in plants like *Clerodandrum phlomisis* and *Premna integrifolia* which showed presence of flavonoids and diterpenes. Methanol extract of *H. spicatum* was found to be rich in diterpenoids. It was observed that andrographaloid, a diterpene stimulates both specific and nonspecific immune response. A study on azadirachtin- a triterpenoid from *Azadirachta indica* on the immune

response of Oreochromis mossambicus has been undertaken. It was found to be effective in both specific and nonspecific immunity.[30]

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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