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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 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.

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 25^{th} day from the day of start of treatment; all groups received a single dose cyclophosphamide 250mg/kg orally. On the 26^{th} 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)		WBC (106/ mm3)		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 (l0.1% 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***

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