International Journal of Phytomedicine 6 (2014) 316-321
http://www.arjournals.org/index.php/ijpm/index

Original Research Article

ISSN: 0975-0185

Comparative study of methanolic and aqueous extracts of *Cocculus hirsutus* leaves on specific and non specific immune responses

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Abstract

The aim of the present study was to find out comparatively about methanolic and aqueous extract of *Cocculus hirsutus* on specific and non specific immune responses in experimental mice. Oral administration of the methanolic (200 and 400 mg/kg) and aqueous extract (250 and 500 mg/kg) were studied on various immune paradigm like determination of antibody titer, delayed type hypersensitivity reaction using SRBC as an antigen, carbon clearance assay as a measure of phagocytic index and total leukocyte count in cyclophosphamide induce immunosuppresed animals. Methanolic extract of *Cocculus hirsutus* to a large extent enhanced specific and non-specific activity in dose 200 and 400 mg/kg body weight. Aqueous extract at 250 mg/kg dose level failed to show appreciably immunomodulatory activity but 500 mg/kg of aqueous extract potentiated the activity however less significantly compared with both dose of methanolic extract.

Keywords: Immunomodulator, *Cocculus hirsutus* , Humoral immune responses, Delayed type hypersensitivity reaction, Phagocytosis

Introduction

Immunomodulation is the regulation and modulation of immunity either by enhancing or by reducing the immune response. Modulation of immune response may involve expression, induction or amplification of resistant reaction [1]. Immunomodulatory agents may selectively activate either cell mediated or humoral immunity. Number of plants reputed in traditional Indian medicine literature to promote physical and mental health, improve defence mechanisms of the body and enhances longevity [2]. Traditional Indian system of medicine like Siddha and Ayurveda has suggested means to increase the body's natural resistance to disease. Medicinal plants used for immunomodulation can provide potential alternatives to conventional chemotherapies for a variety of diseases, especially when the host defence mechanism has to be activated under the conditions of impaired immune response. The use of plant products in the indigenous system of medicines as immunomodulators indeed can modulate the body's immune system, as a variety of plant derivatives such as polysaccharides, lectins, peptides, flavonoids and tannins have been reported to modulate the immune system in various *in vivo* models [3]. A number of leaves used in the traditional medical system of remedies in India. They have been shown to possess immunostimulating activity acting at different levels of the immune system [4,5].

Cocculus hirsutus (L.) Diels is an important medicinal plant belonging to the family Menispermaceae. It is commonly known as Jal-jamni. Traditionally the decoction of the leaves is used for treatment of gonorrhea, spermatorrhoea and diarrhoea. Leaves and stem are used for treating eye disease and root extracts showed analgesic and anti-inflammatory activity [6,7] .

Phytochemical analysis of the leaves reported for the presence of alkaloids, phenolic compounds, flavonoids, glycosides, and carbohydrates [8] . The present work aims at comparative evaluating the scientific validity as immunomodulatory properties of methanolic and aqueous extracts *Cocculus hirsutus* leaves in different experimental models of specific and non specific immune response.

Materials and Methods

Plant Materials

The leaves of the plant *Cocculus hirsutus* were collected from the local field, Bhopal. The plant was identified and authenticated as *Cocculus hirsutus* (L.)Diels (Family: Menispermaceae) from Department of Botany, M.V.M. College, Bhopal by Prof. Madhuri Modak, Botanist. A voucher specimen of the plant has been deposited in the Herbarium-cum-museum of kept at the Department of Botany.

Preparation of the extract

The leaves of *Cocculus hirsutus* were collected and shade dried. The dried leaves were coarse powdered and the powder was packed in to soxhlet column and extracted successively with Petroleum ether (60 - 80 C), chloroform, methanol and water for 24 hrs. The dried extracts were stored in airtight container in refrigerator below 10°C.

Drugs and chemicals

Accurately weighed quantities of the methanolic and aqueous extracts were suspended in 1% sodium carboxy methylcellulose (SCMC) to prepare suitable forms of the dosages. Cyclophosphamide 30 mg/kg was used as a standard immunosuppressant. Sheep red blood cells (SRBCs) for immunization and for challenge as antigen.

Preliminary phytochemical screening

To identify the essential constituents of the methanolic and aqueous extract of *Cocculus hirsutus* leaves such as alkaloids, terpenes and steroids, saponins, flavonoids, polysaccharides and tannins, a preliminary phytochemical screening was carried out using various test methods of Draggendorff's and Mayer's test, Liebermann–Burchard test, foam formation test, lead acetate test, Molish's and Fehling's test and ferric chloride test, respectively [9].

Selection and maintenance of animals

Swiss albino mice (DRDO, Gwalior, India) weighing between 18 to 25 g of either sex were used. They were housed in polypropylene cages and maintained at 27°C ± 2°C, relative humidity 65 ± 10% under 12 hour's light / dark cycle. The animals were acclimatized for ten days under laboratory conditions. Animal ethical clearance for performing the experiments on animals was obtained from the Institutional Animal Ethical Committee (IAEC).

Preparation of SRBC suspension

The blood was collected from a healthy sheep from the local slaughterhouse in Alsever's solution, Bhopal, India. It was preserved at a temperature of 2–8 C. On the day of immunization, the blood sample was centrifuged at 5000 rpm for 10 min and then washed three times to remove plasma with 0.9% sodium chloride solution. The SRBC (20% v/v) suspension was then prepared in 0.9% sodium chloride solution.

Preparation of Alsever's solution

Formula of Alsever's Solution

Citric acid 0.055gm
Sodium citrate 0.8gm
Glucose 2.05gm
Sodium chloride 0.42gm
Distilled water to make volume up to 100 ml

Acute toxicity study in mice

Acute toxicity test was performed according to the Organization of Economic Co-operation and Development (OECD) guideline for testing of chemicals (OECD, 423). Three healthy female albino mice weighing 25-30g, maintained under controlled conditions were administered a single oral dose of 2,000 mg/kg body weight .All animals were observed at the first, second, fourth and sixth hours and clinical signs of toxicity such as respiratory pattern, color of body surfaces, frequency and nature of movement, marked involuntary contraction or seizures of contraction of voluntary muscle, and loss of reflex etc. If mortality was observed in 2 or 3 animals among 3 animals then the dose administered was assigned as a toxic dose. If mortality was observed in one animal, then same dose was repeated again to confirm the toxic dose.

Immunomodulatory protocols

SRBC specific humoral immune responses

The mice were divided into 5 groups, each consisting of 6 animals. Mice in group I (Control) were given 0.5 mL 1% SCMC for 14 days .Mice in group II-III were given methanolic extract at dose 200 and 400 mg/kg/ b.w. respectively for 14 days. Animals in group IV-V were administered aqueous extract at dose 250 and 500 mg/kg b.w. for 14 days.

The animals were immunized by injecting 20 µl of fresh sheep red blood cells suspension intraperitoneally on day 0. Seven days later they were challenged by injecting 20 µl of SRBC suspension. Blood samples were collected in micro centrifuge tubes from individual animals by retro-orbital plexus on *DAY 7* for primary antibody titer and for secondary antibody titer on *DAY 15.* Antibody levels were determined by the haemagglutination technique, this is performed by using 96 wells (12x8) V bottomed titre plate. The wells were marked from 1 to 12. In the first and last well 25 µl of serum collected from treated animals is added. Afterwards 25 µl of normal saline was added to all the wells except well number 12 and mixed well. Then 25 µl of sample from first well was taken and added to 2nd well, again 25 µl from second well was taken and added it to third well and continued the same procedure up to well number 10. After this 25 µl of sample from well number 10 was discarded. Finally 25 µl of 1% SRBC was added to all the wells and kept at room temperature for two hours. Each well was examined for haemagglutination. The reciprocal of highest of the test serum giving agglutination was taken as the antibody titer. The mean titer values of the drug and test extracts treated groups were compared of the control (Sensitized) [11,12].

SRBC –induced delayed type hypersensitivity reaction

The mice were divided into 5 groups, each consisting of 6 animals. Mice in group I (Control) were given 0.5mL 1% SCMC for 14 days .Mice in group II-III were given methanolic extract at dose 200 and 400 mg/kg/ b.w. respectively for 14 days. Animals in group IV-V were administered aqueous extract at dose 250 and 500 mg/kg b.w for 14 days.

The mice were primed with injecting 20 µl of SRBC suspension intraperitoneally, on day 7 and challenged on day 14 with same amount of SRBC suspension intradermally in the right hind foot pad. The contra lateral paw received equal volume of saline, served as control. The thickness of the foot pad was measured at 24 h after challenge using speromicrometer [13].

Phagocytic response

The mice were divided into 5 groups, each consisting of 6 animals. Mice in group I (Control) were given 1 mL 1% SCMC for 5 days. Mice in group II-III were given methanolic extract at dose 200 and 400 mg/kg/ b.w. respectively for 5 days. Animals in group IV-V were administered aqueous extract at dose 250 and 500 mg/kg b.w for 5 days.

At the end of five days, after 48 hours, mice were injected via tail vein with carbon ink suspension (10 µl /gm body weight).Blood samples were drawn (in EDTA solution, 5 µl) from the retro orbital vein at 0 and 15 minutes.; a 25 µl sample was mixed with 0.1% sodium carbonate solution (2 ml) and its optical density was measured at 680 nm.

The phagocytic index (K) was calculated using the equation: K= (logOD1-logOD2)/ 15 where OD1 and OD2 are optical densities at 0 and 15 minutes respectively [14].

Cyclophosphamide-induced myelosuppression

The mice were divided into 6 groups, each consisting of 6 animals. Mice in Group I (Control) were given 0.5 mL 1% SCMC for 13 days .Mice in group II were given cyclophosphamide (30 mg/kg b.w) for 11,12 and 13 days. Mice in group II-III were given methanolic extract at dose 200 and 400 mg/kg/ b.w. respectively for 13 days. Animals in group IV-V were administered aqueous extract at dose 250 and 500 mg/kg b.w for 13 days.

On 11th, 12th and 13th day, all the animals of each group except control were given cyclophosphamide (30mg/kg i.p.), one hour after administration of extract. On 14th day blood samples were then withdrawn from retro-orbital plexus lysed in sodium carbonate solution from all the groups and total leucocytes count was determined [15].

Statistical analysis

Data were expressed as standard error of the means (S.E.M) of and statistical analysis was carried out employing one-way ANOVA followed by Dunnett test, which compares the test groups with the control groups.

Results and Discussion

Phytochemical screening

The preliminary phytochemical screening of *Cocculus hirsutus* leaves revealed the presence of alkaloids, saponins, terpenoids, phenolics, flavonoids and polysaccharides as essential phytochemical constituents of the methanolic and aqueous leaves extract.

Acute toxicity study

The LD_{50} of methanolic and aqueous extract of *Cocculus hirsutus* leaves was determined. Since no mortality was observed at 2000 and 5000 mg/kg respectively.

Effect of *Cocculus hirsutus* on in- vivo SRBC specific humoral immune responses

Methanolic and aqueous extract of *Cocculus hirsutus* leaves on primary and secondary antibody response on H A titre are shown in is shown in Figure No-1.

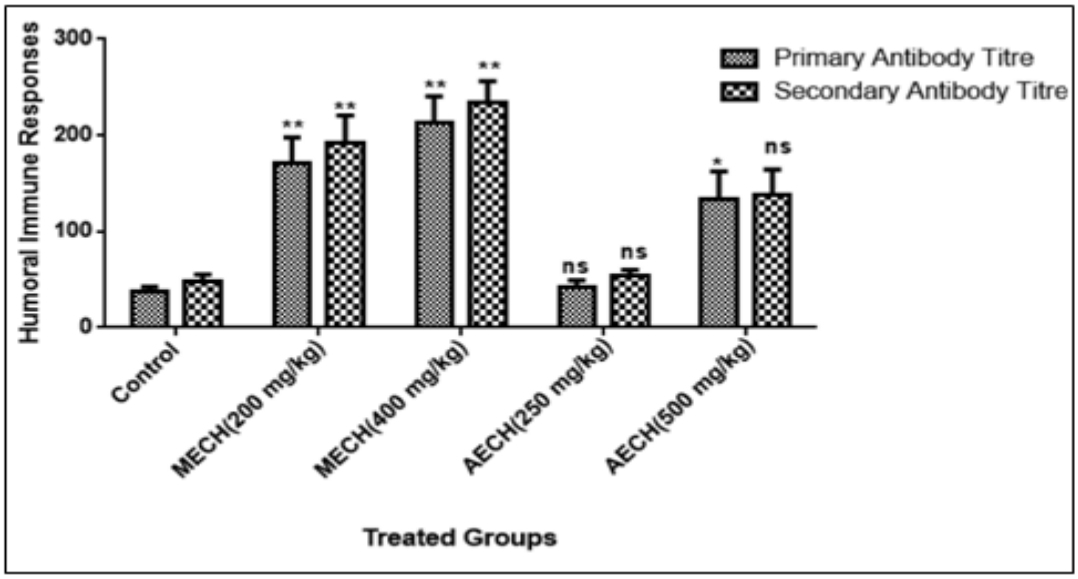

Figure No-1 Effect of *Cocculus hirsutus* on in- vivo SRBC specific humoral immune responses

Statistical analysis was carried out employing the ANOVA followed by Dunnett test *: $P<0.05$, **: $P<0.01$ comparing with the control;

Higher dose of methanolic extract (400 mg/kg b.w) produced maximum enhance with 192.00±28.62 and 234.67±21.33 primary and secondary antibody formation. Aqueous extract at the 500mg/kg does less significant augment in primary but same dose does not show any significant result in secondary antibody titre. Contrast with control, low dose of aqueous extract (250mg/kg) was not considerable enhanced in primary and secondary humoral immune responses.

It is composed of interacting B cell with antigens and subsequently proliferating and differentiating into antibody producing cells. Antibody molecules works by binding with antigen and involved in the complement activation, opsonization, neutralization of toxins, etc. as evidenced by increase in the antibody titre in mice. The result showed that levels of circulating antibodies are increased if the test animals are pretreated with Cocculus hirsutus leaves extracts. .The production of secondary antibodies was more prominent as compare to the primary antibodies. In case of aqueous extract the increase in haemagglutination titer is lower compared to methanolic extract showing that methanolic extract (400 mg/kg) may be superior compared to aqueous extract [16,17].

Effect of *Cocculus hirsutus* on SRBC –induced delayed type hypersensitivity reaction

Methanolic and aqueous extract of *Cocculus hirsutus* leaves on delayed type of hypersensitive activity is shown in Table No- 1.

Table No: 1 *Effect* of *Cocculus hirsutus* leaves on SRBC –induced delayed type hypersensitivity reaction:

S.No	Group	Dose (mg/kg b.w)	DTH Response	% Change
1	Control	1%SCMC	0.24±0.01	
2	Methanolic extract of *C. hirsutus*	200 mg/kg	0.35±0.02**	45.83↑
3	Methanolic extract of *C. hirsutus*	400 mg/kg	0.41±0.02**	70.83↑
4	Aqueous extract of *C. hirsutus*	250 mg/kg	0.26±0.01*	8.33↑
5	Aqueous extract of *C. hirsutus*	500 mg/kg	0.28±0.01ns	16.67↑

Statistical analysis was carried out employing the ANOVA followed by Dunnett test *: $P<0.05$, **: $P<0.01$ comparing with the control;

Methanolic extract of *Cocculus hirsutus* with the dose of 200 and 400 mg/kg increased paw volume as dose dependent manner after 24 hrs, paw volume in this group were increased by 45.83% and 70.83% i.e. most significantly ($p<0.01$) enhanced the delayed type of hypersensitive activity as compared to control (Sensitized) were observed at 24 hours after SRBC injection in the footpad. Whereas at dose 500 mg/kg b.w, aqueous extract of *Cocculus hirsutus* increased less significantly ($p<0.05$) in food pad thickness after 24 hours but at the dose 250 mg/kg b.w dose not show any significant result.

Cell-mediated immunity (CMI) involves effectors mechanisms carried out by T lymphocytes and their products (lymphokines). CMI responses are critical to defense against infectious organisms, infection of foreign grafts, tumor immunity and delayed-type hypersensitivity reactions. Therefore, increase in DTH reaction in mice in response to T cell dependent antigen revealed the stimulatory effect of methanolic leaves extract of *Cocculus hirsutus* on T cells [18].

Effect of *Cocculus hirsutus* on in vivo phagocytosis

Methanolic and aqueous extract of *Cocculus hirsutus* leaves on phagocytic activity is shown in Table No-2.

Table No- 2 Effect of *Cocculus hirsutus* leaves on Phagocytic index.

S.No	Group	Dose (mg/kg b.w)	Phagocytic index	% Change
1	Control	1%SCMC	0.062±0.002	
2	Methanolic extract of *C. hirsutus*	200 mg/kg	0.076±0.002**	22.58↑
3	Methanolic extract of *C. hirsutus*	400 mg/kg	0.086±0.002**	38.71↑
4	Aqueous extract of *C. hirsutus*	250 mg/kg	0.068±0.003ns	9.68↑
5	Aqueous extract of *C. hirsutus*	500 mg/kg	0.072±0.002*	16.13↑

Statistical analysis was carried out employing the ANOVA followed by Dunnett test *: $P<0.05$, **: $P<0.01$ comparing with the control;

Both dose 200 and 400 mg/kg b.w methanolic extract of *Cocculus hirsutus* significantly increased phagocytic activity as dose dependent manner. These groups were increased by 22.58% and 38.71% i.e. most significantly ($p<0.01$) enhanced the activity as compared to control.

Whereas at dose 500 mg/kg b.w, aqueous extract of *Cocculus hirsutus* less significantly ($p<0.05$) increased phagocytic activity by 16.13% as compared to control. Low dose of aqueous extract (250 mg/kg) does not show any significant result.

The carbon clearance test was done to evaluate the effect of drugs on the reticulo endothelial system. The RES is a diffuse system consisting of phagocytic cells. For the clearance of carbon particles from the bloodstream the cells of RES play a major job. Since both extract of *Cocculus hirsutus* capable to enhance the phagocytic index but methanolic extracts are play superior role to increase the activity of the RES [19] .

Effect of *Cocculus hirsutus* on cyclophosphamide-induced myelosuppression

Methanolic and aqueous extract of *Cocculus hirsutus* on cyclophosphamide induced myelosuppression is shown in Figure No-2.

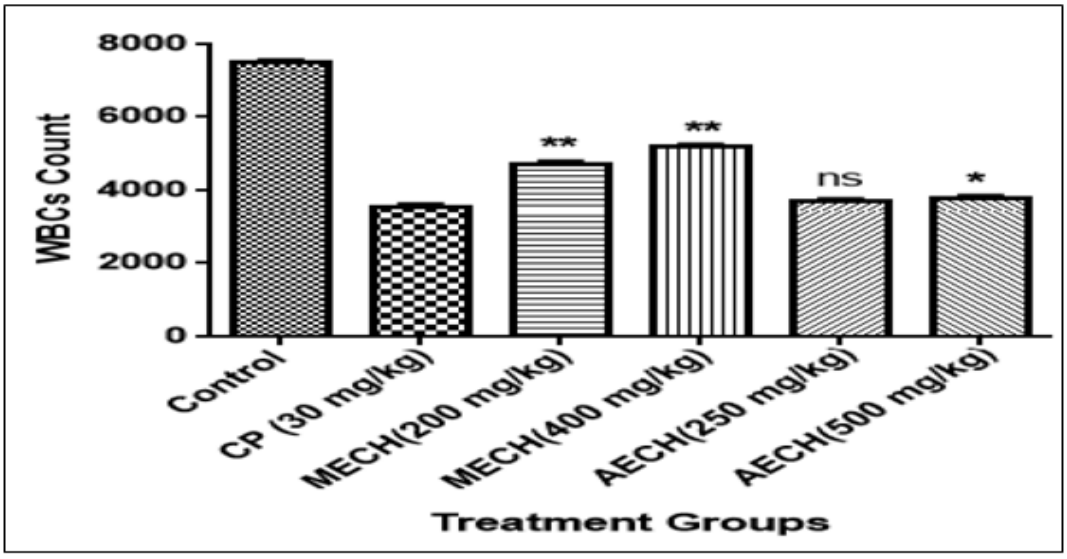

Figure No-2 Effect of *Cocculus hirsutus* on cyclophosphamide-induced myelosuppression

Statistical analysis was carried out employing the ANOVA followed by Dunnett test *: $P<0.05$, **: $P<0.01$ comparing with the control

Cyclophosphamide at the dose of 30 mg/kg, i.p. caused a major reduction in the WBCs count. Combined treatment of methanolic extract of *Cocculus hirsutus* (200 and 400 mg/kg) and cyclophosphamide resulted in a restoration of bone marrow activity as compared with cyclophosphamide treatment alone with 4738.33±50.72 and 5217.67±32.51, but aqueous extracts 250

mg/kg does not show significant result in white blood cell count with cyclophosphamide treat group.

Cyclophosphamide induced immunesuppressive mice model was used because the dyanamic and complex nature of the immune system in which a drug elicits its effect can be detected more reliably after immune challenge. The study avow that methanolic extract of the leaves was found to increase the total WBC count compare to aqueous extract which was lowered by cyclophosphamide, a cytotoxic drug, indicating that the test drug is effective immunomodulatory agent [19,20].

Conclusion

Finding of present study it can be concluded that statistically significant rise in HA titre, DTH response, phagocytic effect and WBCs count of the extracts of *Cocculus hirsutus* suggest that active principles of leaves which are responsible for stimulation of humoral and cell mediated response but the alcoholic extract is more potent than aqueous extract in producing immunomodulatory activity.

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