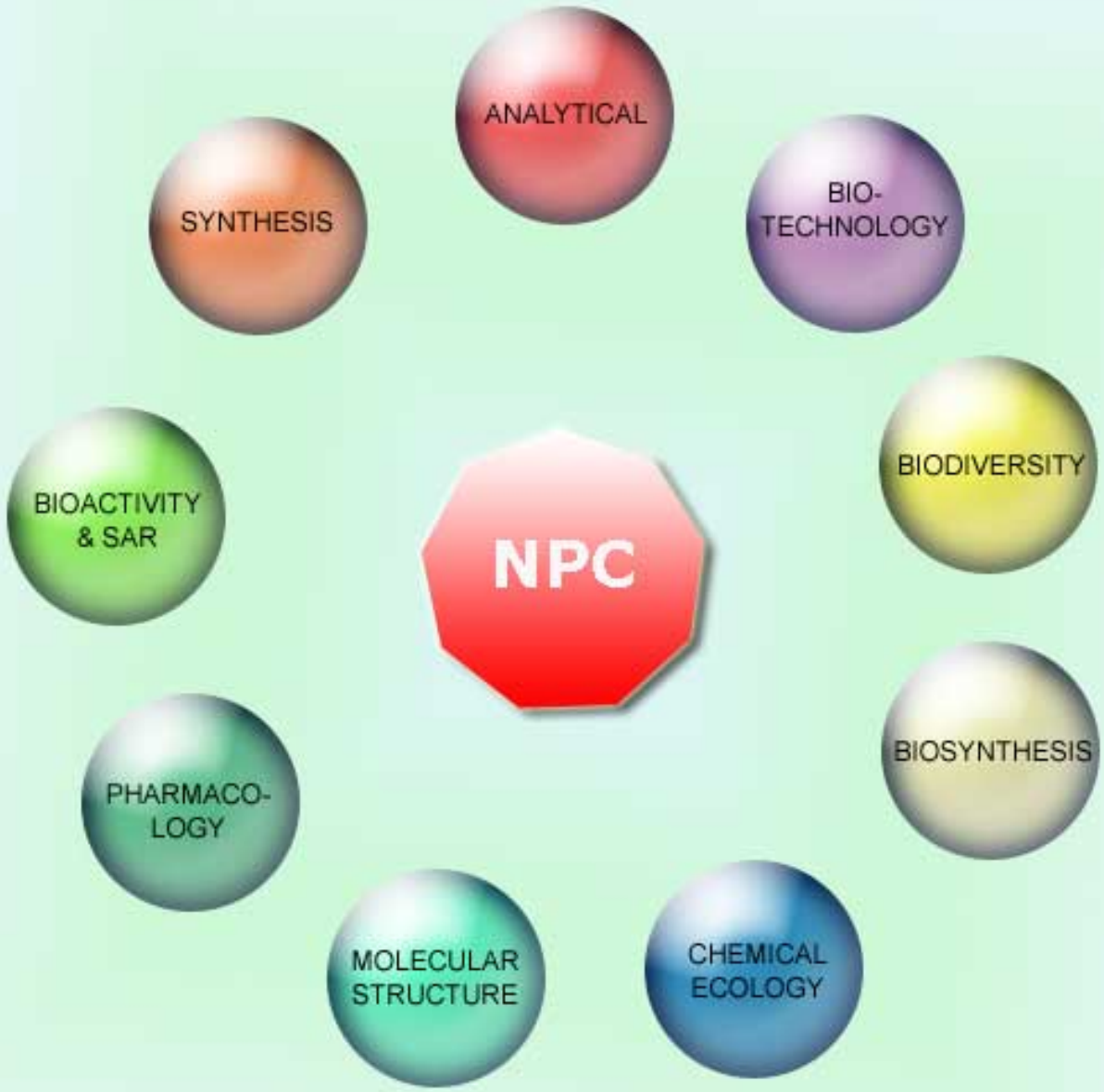
NATURAL PRODUCT COMMUNICATIONS

An International Journal for Communications and Reviews Covering all Aspects of Natural Products Research



Volume 2. Issue 9. Pages 883-958. 2007
ISSN 1934-578X (printed); ISSN 1555-9475 (online)
www.naturalproduct.us

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NPC Natural Product Communications

2007
Vol. 2
No. 9
923 - 926

An *in-vivo* Study of the Immunomodulatory Activity of Coumarinolignoids from *Cleome viscosa*

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Received: May 13th, 2007; Accepted: June 18th, 2007

Cleome viscosa (Capparidaceae) is used in fever, inflammation and liver diseases. Systematic investigation of the seeds of this species has resulted in the isolation of three coumarinolignoids, cleomiscosin A, B and C. The present study was undertaken to determine the immunomodulatory activity of these coumarinolignoids using inbred female Swiss albino mice as an *in-vivo* study. Experimental animals were divided into six groups, each comprised of six mice. These received oral treatment for a period of 28 days. Body weight variation, and hematological, humoral and cell mediated immune response related parameters were studied in which coumarinolignoids at a dose of 10 mg/kg body weight enhanced the body immune function by significantly increasing the white blood cell count, hemagglutination antibody titer responses, and reducing delayed type hypersensitivity response towards rabbit red blood cells.

Keywords: Coumarinolignoids, *Cleome viscosa*, immunomodulation, humoral, cell-mediated, immunity.

The immune system plays a vital role as the main line of defense against infections and medicinal plants have been extensively used as a source of medicine in traditional systems of medicine to promote health and to maintain the body's resistance against infection by potentiating immunity [1]. Immunomodulation using either medicinal plant extracts or plant derived pure molecules can provide an alternative to conventional chemotherapy for a variety of diseases, especially when the host defense mechanism has to be activated under the conditions of impaired immune response.

Cleome viscosa (Capparidaceae) is an annual herb with yellow flowers and strong penetrating odor, which occurs as a weed in rain fed soils from north east to northern parts of India. In the Ayurvedic system of medicine, this plant is used in the treatment of fever, inflammation, liver diseases, bronchitis, diarrhea and infantile convulsions [2]. The seeds of this plant are widely said to be anthelmintic [3]. The rural people use the fresh juice of the crushed seed for the treatment of infantile convulsions and in mental disorders [4]. Systematic investigation of the seeds of *C. viscosa* has resulted in the isolation of three coumarinolignoids, cleomiscosins A, B, and C [5]. *In-vivo* immunomodulatory activity of these coumarinolignoids is reported herein.

Body weight and hematological parameters: The effect of coumarinolignoids on the gain in body weights, along with hematological parameters (total RBCs and WBCs counts) were performed after 28 days oral administration of the test compounds in a dose-dependant manner. The rate of gain in body weight was found to be significantly higher in coumarinolignoids (30 and 100 mg/kg) and levamisole hydrochloride treated groups when compared with vehicle control. Significant difference was not observed in total RBC counts, but total WBC counts were significantly increased in the coumarinolignoids and levamisole hydrochloride treated groups and, conversely, significant decreases in total WBCs were observed in the cyclophosphamide monohydrate treated group when compared with vehicle control .The corresponding data are shown in Table 1.

Table 1: Effect of coumarinolignoids on rate of body weight gain and hematological parameters after 28 days oral administration.

Treatment	Humoral Immunity	Cell-mediated Immunity
	Antibody Titer (wells) (Mean±SE)	Foot Thickness (arbitrary unit) (Mean±SE)
Vehicle Control	3.71±0.57	0.028±0.003
Coumarinolignoids (10 mg/kg)	7.6±0.91*	0.023±0.007*
Coumarinolignoids (30 mg/kg)	7.14±0.57*	0.02±0.004*
Coumarinolignoids (100 mg/kg)	7.17±0.95*	0.018±0.005*
Levamisole (0.68 mg/kg)	7.17±0.47*	0.029±0.009*
Cyclophosphamide (200 mg/kg)	1±0.29[a]	0.019±0.005*

n=06, P<0.05, using student's *t* test (Vehicle Control vs Treatment)
* Significantly increased,[a]Significantly decreased

Rabbit red blood corpuscles (RRBCs) isolated from New Zealand white rabbits were used as antigen. Blood was collected from the central artery of the ear and mixed with an anticoagulant, heparin. Blood was immediately centrifuged at 2000 rpm at 4ºC for 10 minutes and the supernatant containing the plasma was discarded. The pellets containing the RRBCs were resuspended in an equal volume of Alsever's solution of the following composition, dextrose (2.05 g), sodium citrate (0.80 g), and sodium chloride (0.42 g) in 100 mL distilled water, and centrifuged again following the discarding of the supernatant. The process of washing was repeated 3 times before suspending the RRBCs in sterile normal saline to make it a 10% suspension.

Humoral Immune Response

Two schedules of immunization were used. In the first, mice were injected, ip, with 200µL (2×10^8 cells/mL) of RRBCs on the seventh day from the start of the experiment. A booster immunization was given 1 week later (day 14) and the animals were bled on day 28 to detect the presence of antibodies. About 0.5 mL blood was collected from the retro orbital plexus of the mice using hematocrit capillaries (HiMedia, India). The blood was allowed to clot at room temperature for one hour and then kept at 4°C for 60 minutes, followed by centrifugation at 2500 rpm for 10 minutes. The serum was collected and stored at -20°C till further use.

To quantify the antibodies, hemagglutination was performed. Briefly, this involved serial two-fold dilutions of serum samples in Alsever's solution, to which 100 µL of 10% RRBCs was added to 100 µL of the diluted test samples in U-bottom microtiter plates (Greiner, Germany). The plates were incubated for 1 to 2 hours at 25°C before RRBC setting patterns were read. The HA titer was expressed as the reciprocal of the highest dilution of the serum showing definite positive pattern (flat sediment or shield formation) as compared to the negative pattern (smooth dot in the centre of the well).The respective antibody titer was expressed as the serial dilution of the serum per well, as per the method described [6] .

Mice treated with coumarinolignoids and levamisole hydrochloride exhibited a significant increase in hemagglutinin antibody titer, and cyclophosphamide significantly decreased the antibody titer when compared with vehicle control. The data are depicted in Table 2.

Table 2: Effect of orally administered coumarinolignoids on humoral and cell mediated immune response in mice.

Treatment	Body wt. gain (g)	RBCs (Millions/mm^3)	WB C (Thousands/mm^3)
Vehicle Control	4.17±0.59	8.51±0.34	8.71±0.88
Coumarinolignoids (10mg/kg B.wt.)	4.25±0.69	8.58±0.28	11.46±0.37*
Coumarinolignoids (30mg/kg B.wt.)	5.00±0.93	7.69±0.29	12.02±1.23*
Coumarinolignoids (100mg/kg B.wt.)	5.50±0.45	8.74±0.26	10.89±2.34*
Levamisole (0.68mg/kg B.wt.)	4.75±0.6	8.42±0.14	10.77±0.84*
Cyclophosphamide (200mg/kg B.wt.)	0.83±0.27[a]	6.17±0.33[a]	3.94±0.49[a]

n=06, P<0.05, using student's *t* test (Vehicle Control vs Treatment)
* Significantly increased ;[a] Significantly decreased

Cell Mediated Immune Response

Delayed type hypersensitivity test (DTH)/footpad thickness test in mice: The mice were immunized in the same way as that described for the humoral immune response, but in addition, on day 28 all mice were challenged with RRBCs (50 µL; 2 x 10^8 cells/mL) in the intra-plantar region of the hind right paw. The differences in the footpad thickness of the two paws were measured 24 hours later by the fluid displacement method using a phlethysmometer (Ugo Basile, Itlay). The data are expressed in arbitrary units, as in the method described [7]. The footpad thickness of coumarinolignoid treated mice was significantly decreased in a dose dependent manner when compared with vehicle control. The data are depicted in Table 2.

Results of the present study demonstrate that coumarinolignoids produce a significant increase in the white blood cell count and hemagglutinin antibody titer when compared with the vehicle control mice. Previous reports [8] suggested that agents that increase white blood cell counts and

hemagglutinin antibody titers serve as immuno-stimulatory agents for the immune system. Immunostimulatory activity has been reported in a number of plants [9-12], including Indian Panax, *Panax heterophyllum*, *Tylophora indica*, *Ocimum gratissimum*, *O. sanctum* and *Picrorhiza kurroa* [13]. The foot pad thickness (local inflammation) induced by RRBCs was significantly reduced in coumarinolignoid treated mice when compared with the vehicle treated group. During the cell-mediated DTH response, the sensitized cells that are being challenged with the antigen secrete lymphokines [14]. Cutaneous DTH reactions are initiated when CD4 memory T cells are activated by antigen-presenting cells in the skin. The magnitude of the response to the antigen is measured as an increase in swelling at the site of challenge [15,16] Cutaneous DTH reactions induced by the antigen are a local inflammatory response [17,18] and so it may be hypothesized that the compounds could also be useful as a local anti-inflammatory agent.

This present study gives a clear indication that oral administration of cleomiscosins A, B, and C isolated from *C. viscosa* seeds enhance the body immune function by significantly increasing the white blood cell count, antibody responses and reducing the delayed type of hypersensitivity response towards rabbit red blood cells in female Swiss albino mice. Hence, the cleomiscosins have the potential to be used as an immunomodulatory agent.

Experimental

General: IR spectra were recorded on a PerkinElmer FTIR BX spectrophotometer. NMR spectra were recorded on a Bruker AVANCE 300 instrument. Mass spectra (electro spray ionization in positive mode) were recorded on an API 3000 (Applied Biosystem) spectrometer.

Seed material: The seeds were collected from the National Gene Bank for Medicinal and Aromatic Plants, CIMAP, Lucknow, India and the voucher specimens are available for authentication.

Isolation: Air dried pulverized seeds of *Cleome viscosa* (1 kg) were defatted with light petroleum (1L x 3) for 72 h. The defatted material was then exhaustively extracted with methanol (1L x 3) and concentrated to a small volume (50 mL). It was adsorbed onto celite, dried at room temperature for 24 h and then packed in a cheese cloth and extracted with toluene, followed by ethyl acetate and methanol. The toluene and ethyl acetate fractions were mixed together, concentrated, and chromatographed over silica gel (60-120 mesh) using light petroleum. The column was eluted with mixtures of light petroleum–ethyl acetate in the ratio of 1:1 and 1:3, successively. From the above two eluants, on concentration, crystals precipitated, which were removed by filtration and washed with light petroleum–ethyl acetate (1:1) to give a mixture of cleomiscosins A, B and C in the ratio 21:25:4. From this mixture, by repeated column chromatography over silica gel (60-120 mesh) using light petroleum: ethyl acetate (1:1) as eluant, the three compounds were separated and characterized by comparison with reported spectral data (UV, IR, NMR, MS) [8].

Immunomodulatory studies: The *in-vivo* immunomodulatory study was approved by the Institute's Animal Ethical Committee and conformed to national guidelines on the care and use of laboratory animals. Female Swiss albino mice weighing 18-23 g were obtained from the laboratory animal house, CIMAP, Lucknow. These animals were used for the study and were maintained at a room temperature of 22±23°C with 50-70% relative humidity and cycles of 12:12 h of light and dark with *ad libitum* food and water. Animals were divided into six groups each comprising six animals. The first group served as vehicle control, fed with distilled water, the second to fourth groups served as tests, which were fed with the mixture of coumarinolignoids at doses of 1, 10 and 100 mg/kg body weight; the fifth group served as a positive control, which was given levamisole hydrochloride (Sigma-Aldrich, USA) as a immunopotentiating agent at a dose of 0.68 mg/kg body weight, and the sixth group served as a negative control, which was given cyclophosphamide monohydrate (Sigma-Aldrich, USA) as an immunosuppressive agent at 200 mg/kg body weight. These mice were treated orally for a period of 28 days by delivery directly into the stomach using flexible newborn-sized intragastric cannulae.

Acknowledgments – The authors are thankful to DBT, CSIR, New Delhi, for funding, and the

National Gene Bank of the Medicinal and Aromatic Plants, and to the Faculty, technical staff and students of the GRB Division, CIMAP, Lucknow, India for providing seed material, and technical support during this work.

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Volume 2, Number 9

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