

## Evaluation of Immunomodulatory Activity of *Clerodendrum phlomidis* and *Premna integrifolia* Root

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**Abstract:** Roots of *Clerodendrum phlomidis* Linn. f. suppl. and *Premna integrifolia* Linn. Mant. (*Verbanacea*) are known under the common name Arni/Agnimantha. Roots of both the plants are important rasayana drugs and are considered to be useful in the treatment of variety of ailments. Roots of either of these two plants are incorporated as Arni/Agnimantha in many valued and popular Ayurvedic formulations. So the present study was aimed at evaluating the two roots for their immunomodulatory potential. Oral administration of methanol extracts of both the roots (300 mg kg<sup>-1</sup> × 7 days) in mice prior to immunization with Sheep Red Blood Cells (SRBC) resulted in a significant increase in haemagglutinating antibody titre, plaque forming cell assay and delayed type hypersensitivity to SRBC. *C. phlomidis* showed higher specific immune activity as compared to *P. integrifolia*, *C. phlomidis* and *P. integrifolia* enhanced the non specific immune response in carbon clearance test and showed significant immunoprophylactic effect, when tested on *E. coli* induced abdominal sepsis. In the present study *C. phlomidis* showed higher response to specific immune activity as compared to *P. integrifolia*, where as in case of non specific immune activity both the roots showed almost equal response.

**Key words:** *Clerodendrum phlomidis*, *Premna integrifolia*, humoral immunity, cell-mediated immunity, delayed type hypersensitivity

### INTRODUCTION

*Clerodendrum phlomidis*, Linn. f. suppl. and *Premna integrifolia*, Linn. Mant. are two different plants belonging to the family Verbanaceae, both of which are mentioned under the common names of Arni and/or Agnimantha in Ayurveda (Sharma, 1996; Bishnupriya *et al.*, 2003; Vaidya, 1965). Their roots are important ingredient of Ayurvedic preparations like, Dashmoola kwatha, Chyanprashavleh, Haritakiavleh, Ayushyavardhaak tel, Narayan tel etc., valued for the treatment of variety of affections (Anonymous, 2001).

*C. phlomidis* is a large bush or a small tree, growing throughout India. Roots are valued as tonic, diuretic, febrifuge, anti-diabetic, anti-inflammatory and antitussive (Nadkarni, 1982).

*P. integrifolia* is a large shrub or a small tree distributed on the western sea coast from Bombay to Molucca, Srilanka and the Andaman. The root is used in the treatment of diabetes, chyluria, inflammations, swellings, bronchitis, dyspepsia, liver disorders, piles, constipation and fever (Anonymous, 1997).

Previous pharmacological studies include reports of antidiabetic (Chaturvedi *et al.*, 1984) anti-inflammatory

(Surendrakumar, 1988) and antidiabetic (Rani *et al.*, 1999) activities for *C. phlomidis* roots. *P. integrifolia* is reported to possess hypolipidemic (Khanna *et al.*, 1991), anti-inflammatory (Barik *et al.*, 1992) and antidiabetic activity (Kar *et al.*, 1999). Phytochemical studies include reports of presence of  $\beta$ -sitosterol and  $\gamma$ -sitosterol, ceryl alcohol, clerodin, clerosterol, clerodendrin-A in root (Joshi *et al.*, 1979) and flavanoid pectolarigenin, hispidulin, apigenin and luteolin in flower (Seth *et al.*, 1982) of *C. phlomidis*. In *P. integrifolia*, alkaloids premnine (Basu and Dandiya, 1947) ganikarine (Basu and Joneja, 1949) and premnazole alkaloid (Barik *et al.*, 1992) are reported from roots; while flavanoid luteolin (Dasgupta *et al.*, 1984), sterols and triterpene (Debelmas *et al.*, 1973) are reported from the leaves.

In the present study methanol extracts of *C. phlomidis* (CPM) and *P. integrifolia* (PIM) roots were evaluated for specific immune response like haemagglutinating antibody titer (HA titer), Plaque Forming Cell (PFC) assay and delayed type hypersensitivity activity (DTH response) and nonspecific immune response like carbon clearance test and *E. coli* induced sepsis.

## MATERIALS AND METHODS

**Plant material:** The fresh, well-developed plants of *C. phlomidis* were collected from the Ayurvedic garden, Gandhinagar in the month of October 2004 and plants of *P. integrifolia* were collected in the month of September 2004 from the Pharmacognosy Garden of Timba Ayurvedic Pharmacy College, Timba, Gujarat. The authenticity of plants was confirmed by a taxonomist of Gujarat Ayurveda University, Jamnagar, Gujarat. Voucher specimens LM108 and LM109 were deposited in the Department of Pharmacognosy, L.M. College of Pharmacy, Ahmedabad, Gujarat. Roots of both the plants after drying in the sun were reduced to (60 #) powder separately.

**Extraction:** Dried root powder of each root drug (200 g) was exhaustively extracted using methanol (3×500 mL) separately. The marc and filter paper was washed with methanol each time. Methanol extract of *C. phlomidis* (CPM) and *P. integrifolia* (PIM) obtained were concentrated, dried and used for the evaluation.

Clerodendrin-A was isolated from *C. phlomidis* and was estimated in both the roots by HPTLC method, data are published elsewhere.

**Animals:** BLAB/c albino mice of either sex (22-25 g) were used. The animals received a standard pellet diet (Lipton, Bombay), water *ad libitum* and were maintained in regulated temperature and humidity conditioned rooms.

In preliminary experiments both the extracts were found to induce significant response in the animals after oral administration for 5-7 days and the optimum response was served at 300 mg kg<sup>-1</sup> dose.

**Specific immune response:** BLAB/c albino mice were administered with CPM and PIM (300 mg kg<sup>-1</sup> b.wt.) in water for 7 days, on the 8th day mice were immunized by injecting 0.25 mL of fresh sheep red blood cell suspension (SRBCs) (10<sup>8</sup> cells mL<sup>-1</sup> suspended in normal saline) intraperitoneally. Four days later blood was collected from the retro-orbital plexus for the HA titre assay (Joharapurkar *et al.*, 2003; Puri *et al.*, 1994). The animals were then killed by cervical dislocation and spleen was taken out for PFC assay (Puri *et al.*, 1992; Jerne and Nordin, 1963). In another group, the DTH response to SRBC was determined (Joharapurkar *et al.*, 2003; Puri *et al.*, 1994).

**Haemagglutinating Antibody (HA) titre:** Serum was separated from the blood samples and antibody levels were determined by the microtitre hemagglutination

technique. Twenty five microliter aliquots of two-fold diluted sera were prepared in 0.15 M phosphate buffer saline (pH- 7.2) and dispensed in U bottom microtitre plates (Polylab); 1% SRBC suspension (25 µL) in PBS was dispensed into each well and mixed thoroughly. After 1 h of incubation at room temperature the highest dilution of test serum giving agglutination was observed and the reciprocal of the highest dilution observed was considered as antibody titre.

**Plaque Forming Cell (PFC) assay:** The assay was done according to technique of Jerne and Nordin (1963). Spleen cells were separated in RPMI-1640 medium; washed with the same and suspended in the same to a concentration of 1×10<sup>6</sup> cells mL<sup>-1</sup>. A bottom layer was prepared with 1.2% agarose in 0.15 M NaCl in glass petridishes. A mixture of 2 mL 0.6% agarose in RPMI-1640 medium (at 42°C), 0.1 mL suspension of 20% SRBC (in normal saline) and 1×10<sup>5</sup> spleen cells were poured over the bottom layer and petridishes were incubated at 37°C for 90 min. After that 2 mL of 1:10 diluted fresh Guinea pig serum in PBS was added as a source of complement in each petridish and further incubated for 60 min. The plaques were counted immediately and expressed as count per 10<sup>5</sup> spleen cells.

**Delayed type hypersensitivity (DTH) response:** On 11th day, the thickness of the right hind footpad was measured using vernier caliper. The mice were then challenged by injection of 20 µL of 1% SRBCs (suspended in normal saline) in right foot pad. Foot thickness was again measured after 24 h of this challenge (Saiki *et al.*, 1981). The difference between the pre and post challenge foot thickness expressed in cm was taken as a measure of delayed type hypersensitivity.

### Non-specific immune response

**Carbon clearance test (phagocytic activity):** Phagocytic index was determined by a reported method (Gonda *et al.*, 1990; Bafna and Mishra, 2004). Swiss albino mice were given CPM and PIM (300 mg kg<sup>-1</sup> b.wt.) in water in treated groups and control group received vehicle (water) for 5 days orally. After 48 h of the last dose on 5th day, mice were injected via the tail vein with 0.1 mL Indian ink. Blood samples were drawn from orbital vein at 3, 7 and 15 min from each mouse. 25 µL of each blood sample was mixed with 2 mL of 0.1% w/v Na<sub>2</sub>CO<sub>3</sub>. The carbon clearance i.e., the rate of elimination of carbon from the blood was determined by turbidometric spectroscopy at 650 nm with UV spectrophotometer (Schimadzu 1601 Japan). The graph of absorbance vs. time was plotted. The rate of carbon clearance, termed as Phagocytic Index (PI), is the ratio of slope of time-concentration curve of sample and standard.

$$PI = \frac{K(\text{sample})}{K(\text{control})}$$

Where:

K = Slope of regression line.

**E. coli induced abdominal sepsis:** *E. coli* induced abdominal sepsis was done by a method reported by Pallable *et al.* (1998). The strength of *E. coli* ( $2.5 \times 10^8$  cells mL<sup>-1</sup>) was standardized to induce 100% mortality. Swiss albino mice were divided into two groups, treatment groups received CPM and PIM (300 mg kg<sup>-1</sup> b.wt.) and control group received water orally for 15 days. On 15th day, 3 h after the last dose of extracts, *E. coli* were injected intraperitoneally to all mice. Percentage mortality was observed for 24 h in the treated groups and control group.

**Statistical analysis:** All the data were statistically analysed by one way Analysis of Variance (multiple comparison procedure: Tukey's test).

## RESULTS

### Specific immune response:

**Haemagglutinating antibody titre:** Increase in the humoral antibody titer was evident on the 3rd day post immunization in treated mice in comparison with untreated mice (n = 8). Anti-SRBC-haemagglutination antibody titer with the use of the CPM and PIM treated mice were found to be 768±90.5 and 384±106.13, respectively, while the HA titre value for control animals was 104±11.711. The difference was statistically significant (F = 14.09, p = <0.05) (Table 1).

**Plaque forming cell assay:** Effect of CPM and PIM on PFC count (356±5.96, 252±5.36) was significantly higher than that of control animals (100.75±3.89) (F = 544.1, p = <0.001) (Table 1).

**Delayed type hypersensitivity response:** The mean value of the DTH response to SRBC was 0.118±0.0088 for the CPM and 0.107±0.0118 for the PIM treated animals as compared to 0.039±0.0024 for the control animals, which was significantly different (F = 27.77, p = <0.001) (Table 1).

### Non-specific immune response

**Carbon clearance test (Phagocytic activity):** CPM and PIM possessed macrophage stimulatory activity as evidenced by increased phagocytic index in carbon clearance test. The phagocytic activity of reticuloendothelial is generally measured by the rate of removal of carbon particles from the blood stream. CPM and PIM at the dose of 300 mg kg<sup>-1</sup> showed significant clearance of carbon particles. Phagocytic index and rate of clearance are shown in the Fig. 1 and Table 2.

**E. coli induced abdominal sepsis:** In this assay, after challenging animals with fatal dose of *E. coli* significant bacteraemia was found to be induced. All (n = 6) animals

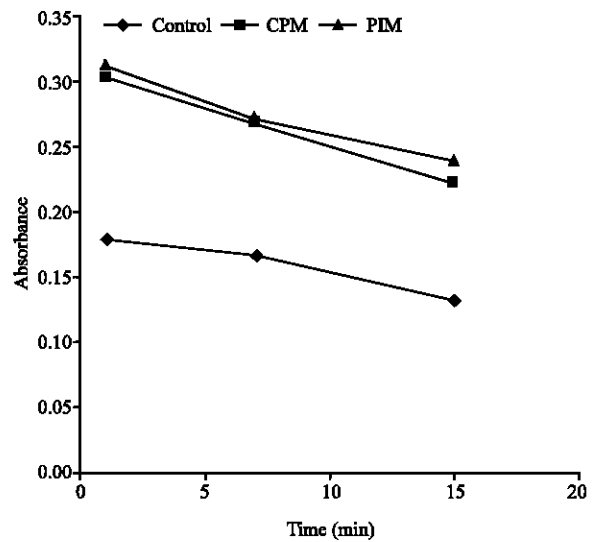


Fig. 1: Effect of *C. phlomidis* and *P. integrifolia* extract on rate of carbon clearance

Table 1: Effects of *C. phlomidis* and *P. integrifolia* on antigenic specific responses on BLAB/c albino mice

Immune responses	Untreated/Control	<i>C. phlomidis</i>	<i>P. integrifolia</i>
HA Titre	104.000±11.711	780.000±101.29***	387.000±15.890*
DTH response(cm)	0.039±0.0024	0.118±0.0088*	0.107±0.0118*
Plaque forming cells	100.750±2.8140	356.000±6.3758**	252.000±5.7320**

The values are mean±SEM (n = 8) \*: p < 0.05; \*\*: p < 0.001

Table 2: Effects of *C. phlomidis* (CPM) and *P. integrifolia* (PIM) extracts on macrophage phagocytic activity *in vivo* on BLAB/c albino mice

Time (min)	Untreated/Control	<i>C. phlomidis</i>	<i>P. integrifolia</i>
3	0.177883±0.01440	0.300183±0.01230*	0.314233±0.00683*
7	0.166350±0.01960	0.267783±0.00933*	0.273617±0.00708*
15	0.132267±0.00631	0.221850±0.02480*	0.240533±0.00523*
Phagocytic Index (PI)	--	1.696	1.5757

The values are mean±SEM (n = 6), \*: p < 0.001

of control group died within 15 h while CPM and PIM treated animals showed 20 and 25% mortality, respectively in 24 h and remaining animals showed survival without any symptoms of peritonitis.

## DISCUSSION

The results of the present study provide an evidence of the immunostimulant activity in *C. phlomidis* and *P. integrifolia*, capable of inducing both antigen specific and nonspecific immune responses. In specific immune system, lymphocyte derived cells like T and B lymphocytes play active role (Pallabe *et al.*, 1998). It has become evident from the present study that CPM and PIM produced significant enhancement of the antigen specific response against humoral as well as cell mediated immune response. CPM and PIM induced about nine-fold and four-fold increase respectively in the antibody formation as compared to that of the control group as evident from respective HA titre values. This result is also supported by the *in vitro* PFC assay, where nearly three-fold and two-fold increase in the IgM antibody plaque formation was observed in the spleen cells of the CPM and PIM treated mice as compared to the control animals.

Furthermore, in DTH assay, the cell-mediated immune response via helper T cells was found to be augmented under the influence of the extracts (Sainis *et al.*, 1983). CPM and PIM treated animals showed similar responsiveness towards SRBC, which were higher than the control animals.

Since PI is greater than one, stimulation of reticulo endothelial system and activity of macrophages is evident from carbon clearance assay (Ponkshe and Indap, 2002). In this, *in vivo* assay macrophages are known to secrete a number of cytokines, which in turn stimulates other immunocytes. This may enhance the defense ability to counter the infectious stress. CPM showed higher PI than PIM, which reflects a marked increase in the rate of carbon clearance suggests a significant enhancement in the phagocytic function of the macrophages and thus, non-specific 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 from the blood (Sagle *et al.*, 2004).

In *E. coli* induced sepsis, mice of treated and untreated group when subjected to the fatal dose of *E. coli*, treated groups showed significantly lesser percentage of mortality as compared to untreated group, when observed for 24 h after *E. coli* challenge. Though the difference between the two treated groups was minor, CPM showed somewhat better survival pattern the PIM.

These observations are suggestive of immunomodulatory activity of *C. phlomidis* and *P. integrifolia* in different immunomodulatory modules of specific and nonspecific immune responses. It would also be interesting to notice that *C. phlomidis* gave marginally higher response in most of the models. The methanol extracts (CPM and PIM) were found to be rich in diterpenoids and flavonoids. Both the types of components are reported to exhibit Immunomodulatory activity in various experimental models (Puri *et al.*, 1993; Patwardhan *et al.*, 1990; Chiang *et al.*, 2003). Presence of these compounds in both the extracts might be contributing towards the Immunomodulatory activity.

Further, present study substantiates the claims made in Ayurveda regarding the use of both the roots as rasayana drugs under the common name of Arni or Agnimantha.

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