

Immunopotentiating Effect of an Ayurvedic Preparation from Medicinal Plants

Dnyaneshwar Umrao Bawankule,^{*,a}
 Dayanandan Mani,^a Anirban Pal,^a
 Karuna Shanker,^b Narayan Prasad Yadav,^a
 Sachidanand Yadav,^a
 Amit Kumar Srivastava,^a Jyoti Agarwal,^a
 Ajit Kumar Shasany,^a
 Mahendra Pandurang Darokar,^a
 Madan Mohan Gupta,^b
 and Suman Preet Singh Khanuja^a

^aGenetic Resources and Biotechnology Division and
^bAnalytical Chemistry Divisions, Central Institute of Medicinal and Aromatic Plants (CIMAP), Council of Scientific and Industrial Research (CSIR), Kukrail Picnic Spot Road, Lucknow-226015, India

(Received October 7, 2008; Accepted January 19, 2009)

A formulation of *Ocimum sanctum*, *Phyllanthus emblica*, and *Glycyrrhiza glabra* in a defined ratio termed CIM-Candy was prepared based on Ayurvedic methodology. To study the humoral immune response, rabbit red blood corpuscles (rRBCs) were used as an antigen. Mice were immunized on day 7 and 14 days the start of the experiment using an intraperitoneal injection of 200 μ l of rRBCs (2×10^8 cells/ml). Humoral immune response, body weight variation, and hematology parameters were studied. CIM-Candy at doses of 10 and 100 mg/kg body weight enhanced the immune function by significantly increasing ($p < 0.05$) the hemagglutination antibody titer response against the rRBCs. Significant changes were not observed in body weight and hematology parameters when comparing the normal vehicle control and treatment groups. As the biological activity of the plant-derived formulation was not assigned to a single chemical, high performance thin layer chromatography (HPTLC) fingerprinting was also performed to confirm the chemical characteristic of the formulation. The results of this study suggest that the standardized formulation CIM-Candy exhibited significant humoral immune response in

Swiss albino mice.

Key words—CIM-Candy, *Ocimum sanctum*, *Phyllanthus emblica*, *Glycyrrhiza glabra*, immunopotentiation, humoral response

INTRODUCTION

Ayurveda, a traditional Indian medical system has a long history and is one of the great living traditions. Many medicinal plants in ayurvedic medicine are believed to be useful in strengthening the human immune system,¹⁾ of and formulations based on such plants play an important role in modern health-care, particularly where effective as well safe treatment is not available.²⁾ A formulation termed CIM-Candy containing *Ocimum sanctum* (*O. sanctum*, Tulsi), *Phyllanthus emblica* (*P. emblica*, Awla), and *Glycyrrhiza glabra* (*G. glabra*, Mulethi) was prepared following the methodology described in the Ayurvedic Formulary of India for avaleha preparation. The selection criteria for these medicinal plants were based on availability in abundance, traditionally well proven in various disease conditions, and being considered for scientific validation as nutraceutical preparations. *Ocimum sanctum* has also been recognized by the Rishis (scholars of Veda) for thousands of years as a prime herb in Ayurvedic treatment. The herb has been traditionally used by Hindus, and now others, for its diverse healing properties. *Ocimum sanctum* extracts are used in Ayurvedic remedies for common colds, headaches, stomach disorders, inflammation, heart disease, various forms of poisoning, and malaria. Traditionally, *O. sanctum* is taken in many forms: as an herbal tea, dried powder, fresh leaf, or mixed with ghee. The dried leaves of *O. sanctum* have been mixed with stored grains to repel insects. Recent scientific studies have suggested that *O. sanctum* inhibits the expression of cyclooxygenase,³⁾ reduces blood glucose levels, and reduces total cholesterol levels due to its antioxidant properties.⁴⁾ *Phyllanthus emblica* fruit strengthens the lungs, helping to fight chronic lung problems as well as upper respiratory tract infections.⁵⁾ Microbiological studies on *P. emblica* showed that it exhibits antibacterial activity against gram-negative urinary pathogens,⁶⁾ protects against radiation-induced hematology biochemical alterations in mice⁷⁾ and attenuates age-related renal dysfunction.⁸⁾ *Glycyrrhiza glabra* is used traditionally to relieve coughs, sore throats, and gastric in-

*To whom correspondence should be addressed: Genetic Resources and Biotechnology Division, Central Institute of Medicinal and Aromatic Plants, Council of Scientific and Industrial Research, Kukrail Picnic Spot Road, Lucknow-226 015, India. Tel.: +91-522-236444; Fax: +91-522-2342666; E-mail: bawankuledu@gmail.com

flammation. One of the main active ingredients is glycyrrhizin, which has cortisone-like effects and, additionally, is 50-fold sweeter than sucrose.⁹⁾ Recent studies have found that *G. glabra* exhibits anti-inflammatory action,¹⁰⁾ *in-vitro* and *in-vivo* neuroprotective effects¹¹⁾ and antimicrobial potential.¹²⁾ The possibility of variation in the extraction of pharmacologically active constituents can not be ruled out during the process and product development stages, which can affect the desired bioactivity. To overcome this issue, high performance thin layer chromatography (HPTLC) fingerprinting is a practical solution to characterize complex herbal extracts. We have developed the HPTLC fingerprints of CIM-Candy by comparing the R_f , UV-Visible spectra and color of bands. The aim of the present study was to investigate study the immunological effects of the traditional knowledge-based CIM-Candy.

MATERIALS AND METHODS

Preparation of CIM-Candy Formulation — The formulation was prepared following the methodology described in the Ayurvedic Formulary of India for avaleha preparation.¹³⁾ Avaleha is a semisolid formulation prepared with the addition of sugar and boiled with the prescribed decoction. The leaves of *O. sanctum*, roots of *G. glabra*, and fruits of *P. emblica* were collected from the experimental farm of our institute. The plant material was dried under shade. The dried powder of *O. sanctum* and roots of *G. glabra* 100 g each were boiled in distilled water (1 l for 1 hr). In the same vessel, *P. emblica* fruit (100 gm) were wrapped in muslin cloth and dipped in the vessel. When the volume of the decoction had reduced to about 25% of the original volume the suspended *P. emblica* was removed and pulped using a pulping machine. The decoction was filtered and boiled with sugar until the preparation became sticky. The *P. emblica* pulp was then mixed into it with constant stirring and heated for 60 min. The preparation was then gradually cooled with stirring. When this semisolid preparation reached a temperature of about 40–45°C, it was molded into Candy. The representative formulation (CIM-Candy) is depicted in Fig 1.

***In-vivo* Immunomodulatory Activity in Mice** — A total of 36 female Swiss albino inbred mice, weighing 16–21 g, were obtained from Jeevanika, Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India for



Fig. 1. Ayurvedic Preparation in the Form of CIM-Candy

this experiment. The mice were acclimatized for one week in the room where the experiment were conducted. They were fed with a pelleted balanced diet (Dayal Feed Agency, Lucknow, India) and water *ad libitum* in the experimental room under a 12 hr light/dark cycle at a temperature of $22 \pm 1^\circ\text{C}$ and a humidity of $60 \pm 5\%$. The study was approved by Institute's Animal Ethical Committee and conformed to national guidelines (Committee for the purpose of Supervision and Control of Experimentation on Animals, CPSCEA) on the care and use of laboratory animals. The mice were divided into 6 groups each comprising 6 animals: group-1, vehicle control that received distilled water 10 ml/kg body weight; group-2, CIM-Candy 1 mg/kg body weight; group-3, CIM-Candy 10 mg/kg body weight; group-4, CIM-Candy 100 mg/kg body weight; group-5, positive control, levamisole hydrochloride 0.68 mg/kg body weight; group-6, negative control, cyclophosphamide 200 mg/kg body weight.

Body Weight and Hematology Parameters — To observe the effects of CIM-Candy on body weight variation and hematology parameters such as total red blood corpuscle (RBC) and white blood cell (WBC) counts using a hemocytometer (Rohem, New Delhi, India) and hemoglobin using the Drabkin method were examined.

Humoral Immune Response — Rabbit red blood corpuscles (rRBCs) isolated from New Zealand white rabbits were used as an antigen. Blood was collected from the central artery of the ear and mixed with the anticoagulant heparin. Blood was immediately centrifuged at 2000 rpm at 4°C for 10 min and the supernatant containing the plasma was discarded. Pellets containing the rRBCs were re-suspended in an equal volume of Alsever's solution consisting dextrose (2.05 g), sodium citrate (0.80 g), and sodium chloride (0.42 g) in 100 ml of distilled water, and centrifuged again after discarding the supernatant. The process of washing

was repeated 3 times before suspending the rRBCs in sterile normal saline to make a 10% suspension. Two schedules of immunization were used. In the first, mice were injected intraperitoneally with 200 μ l (2×10^8 cells/ml) of rRBCs on day 7 from the start of the experiment. A booster immunization was given 1 week later (day 14) and blood samples were collected on day 28 to detect the presence of antibodies. About 0.5 ml of blood was collected from the retro-orbital plexus of the mice using hematocrit capillaries (Hi-Media, Mumbai, India). The blood was allowed to clot at room temperature for 1 hr and then kept at 4°C for 60 min, followed by centrifugation at 2500 rpm for 10 min. The serum was collected and stored at -20°C until further use. To quantify the antibody titer, the hemagglutination test was performed using serial two-fold dilutions of serum samples in Alsever's solution to which 100 μ l of 10% rRBCs was added in U-bottom microtiter plates (Greiner, Mumbai, Germany). The plates were incubated for 1–2 hr at 25°C before rRBC patterns were read. The hemagglutination titer was expressed as the reciprocal of the highest dilution of the serum showing a definite positive pattern (flat sediment or shield formation) compared with the negative pattern (smooth dot in the center of the well). The respective antibody titer was expressed as the serial dilution of the serum per well, following the method previously described.¹⁴⁾

Standardization of CIM-Candy through Chemical Fingerprinting—CIM-Candy 2.0 g was extracted with mixture of solvent (chloroform : BuOH : MeOH–25 : 3 : 0.4; 3 \times 25 ml) for 30 min at 60°C over water-bath. The pooled extracts were filtered and evaporated to dryness at 40°C under vacuum in a rotary evaporator. The residue was dissolved with 1.0 ml of methanol and used as test solution. TLC silica gel 60 F₂₅₄ plates (10 cm \times 10 cm, Merck, Mumbai, India) were activated by heating for 15 min at 55°C before use. Five microliters of test solutions was applied to the plates with Linomat IV (Camag, Muttenz, Switzerland) as 5 mm bands, with space between the bands of 10 mm, start position 15 mm, speed 5 s/ μ l, 15 mm from lower edge. The plates were then developed in a twin trough chamber (10 cm \times 10 cm) with a stainless steel lid. The chamber was pre-equilibrated with the mobile phase (toluene : ethyl acetate : AcOH, 3 : 3 : 1 : 0.4 v/v/v). No spots were visualized under both short (254 nm) and long (366 nm) wavelength due to lack of chromophoric groups in the extracted and

separated compounds over TLC. Vanillin-sulphuric acid reagent (vanillin : sulfuric acid : ethanol, 1.0 g : 5 ml : 95 ml) was used as the derivatizing reagent. Plates were dipped in the derivatizing reagent with the help of Immersion Device-III (Camag) and heated at 110°C for 5 min and scanned at 540 nm using a slit size 6.0 \times 0.45 mm with WINCATS-3 (Camag) and the image was captured under ordinary light with Reprostar 3 (Camag).

RESULTS AND DISCUSSION

Immunopotentiating Effects of CIM-Candy

Ayurvedic medicines are largely based upon herbal and herbal-mineral preparations and have specific diagnostic and therapeutic principles.¹⁵⁾ Modulation of immune responses to alleviate diseases has been of interest for many years and the concept of Rasayana in Ayurveda is based on related principles. Specific stimulatory or suppressive agents can normalize or modulate the immunology process in pathophysiology conditions and are hence called immunomodulatory agents.¹⁶⁾ Mice that received oral CIM-Candy 10 and 100 mg/kg body weight for 28 days exhibits a significant increase in antibody titer after immunization with rRBCs compared with normal vehicle control ($p < 0.05$). The representative data are depicted Fig. 2. Previous reports suggested that agents that induce increases in hemagglutinin antibody titers act as immunostimulatory agents and that several Indian medicinal plants have immunostimulatory activity.^{17–21)} Significant changes were not observed in body weight gain, total RBC counts,

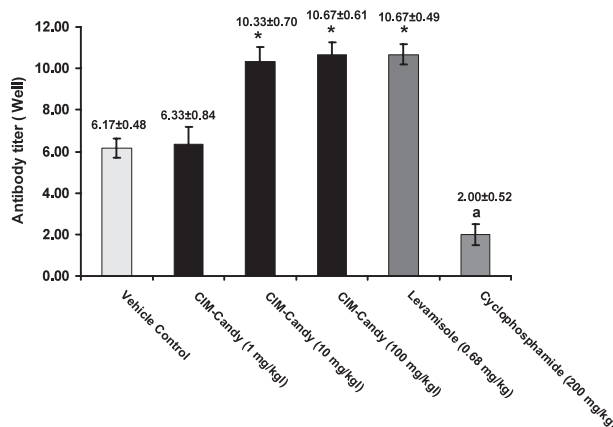


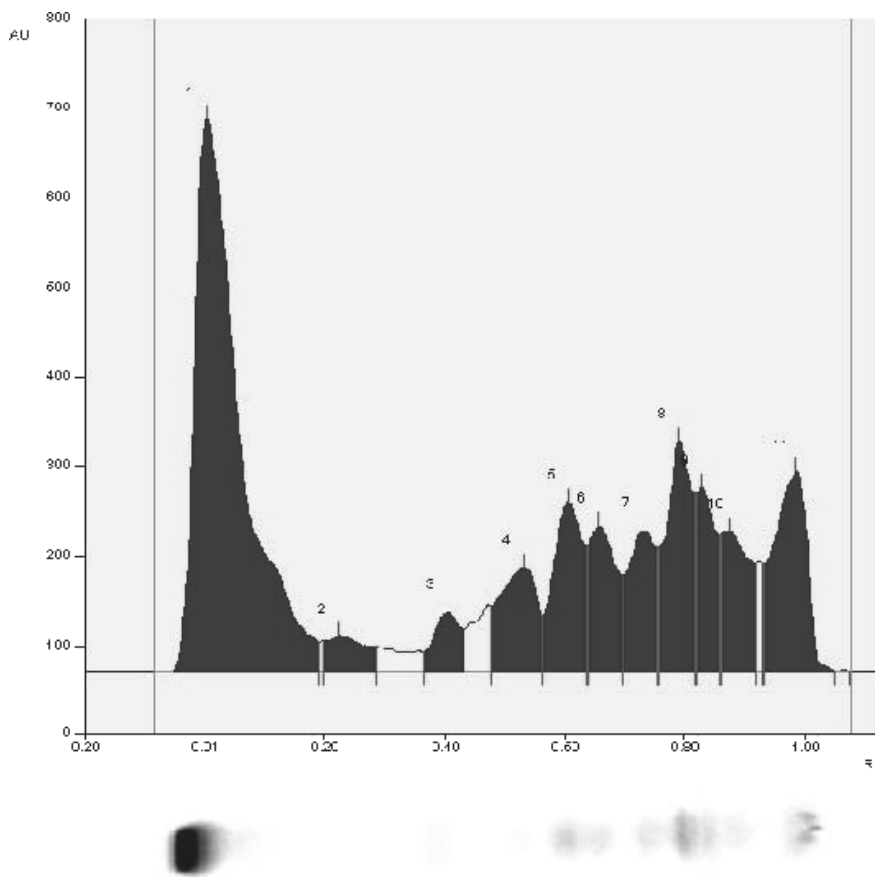
Fig. 2. Dose Response Relation of CIM-Candy preparation on Humoral Immune Response in Mice

*Significantly increased; a significantly decreased; $p < 0.05$, Student's t test (vehicle control vs. treatment); $n = 6$ mice in each group; data expressed as mean \pm S.E.M.

Table 1. Effects of CIM-Candy on Body weight Gain and Hematology Parameters after 28 Days Oral Administration

Group	Body weight gain (g)	RBCs (million/mm ³)	WBCs (thousands/mm ³)	Hemoglobin (mg/dl)
Vehicle control	3.17 ± 0.40	6.65 ± 0.49	10.90 ± 0.85	11.43 ± 0.42
CIM-Candy 1 mg/kg	3.08 ± 0.57	6.89 ± 0.63	10.68 ± 0.88	12.39 ± 0.60
CIM-Candy 10 mg/kg	3.92 ± 0.69	7.46 ± 0.36	12.05 ± 0.71	13.09 ± 0.62
CIM-Candy 100 mg/kg	3.58 ± 0.73	7.55 ± 0.26	12.22 ± 0.76	13.02 ± 0.91
Levamisole HCl 0.68 mg/kg	3.75 ± 0.53	7.55 ± 0.45	12.48 ± 0.70	13.15 ± 0.59
Cyclophosphamide monohydrate 200 mg/kg	1.42 ± 0.37 ^{a)}	5.31 ± 0.49	5.96 ± 0.63 ^{a)}	9.92 ± 0.22

n = 6 mice in each group, data expressed as mean ± S.E.M. *p* < 0.05, Student's *t* test (vehicle control vs. treatment). *a)* significantly decreased.

**Fig. 3.** HPTLC Densitogram of CIM-Candy after Derivatization with Vanillin-Sulphuric Acid and Scanning at 540 nm

total WBC counts and hemoglobin parameters between the vehicle control and treatment groups. The corresponding data are shown in Table 1. The results show that CIM-candy exhibits immunostimulatory activity and can be used to normalize or modulate the immune response during various pathophysiological conditions.

HPTLC Fingerprinting for Quality Assurance of CIM-Candy

Conventional TLC was used in many pharmacopoeia and official documents for the identification of herbal materials, but none of the dealt with herbal products. Recent advances in HPTLC have con-

firmed that this method has an advantage over conventional TLC due to the sorbent with a homogeneous, and narrow particle size (5 µm) distribution. Therefore, in this study, HPTLC plates were used to establish a TLC fingerprinting method. Sample preparation (extractability of active herbal constituents from the product) and the chromatographic conditions were carefully optimized. The densitogram observed at 540 nm after vanillin-sulphuric acid derivatization showed good separation of characteristic compounds (Fig. 3). The extract quality was identified by matching the colors and *R_f* values of bands in their fingerprints. For positive identification of compounds with steroidal skeleton, the sam-

ple exhibit bands with chromatographic characteristics, including colors and R_f values (0.40 ± 0.02 , 0.54 ± 0.03 , 0.59 ± 0.03 and 0.80 ± 0.02 at 540 nm after derivatization with vanillin sulphuric acid reagent). The area under each HPTLC peak profile of each batch was also compared using non-parametric statistical test considering two parameter peak R_f and area. The HPTLC profile showed that the data follow no special distribution assumption. Similarities ($p < 0.05$) between the HPTLC peak profile characteristics further confirming the reproducible quality of the CIM-Candy prepared and used for pharmacology studies.

Acknowledgement We are thankful to the Department of Biotechnology, Government of India, for providing funding for this work.

REFERENCES

- 1) Khan, S. and Balick, M. J. (2001) Therapeutic plants of Ayurveda: A review of selected clinical and other Studies for 166 Species. *J. Altern. Complement. Med.*, **7**, 405–515.
- 2) Patwardhan, B. and Chorghade, M. (2004) Ayurveda and natural products drug discovery. *Curr. Sci.*, **86**, 789–798.
- 3) Singh, S. (1999) Mechanism of action of anti-inflammatory effect of fixed oil of *Ocimum basilicum* Linn. *Indian J. Exp. Biol.*, **37**, 248–252.
- 4) Sethi, J., Sood, S., Seth, S. and Talwar, A. (2004) Evaluation of hypoglycemic and antioxidant effect of *Ocimum sanctum*. *Indian J. Clin. Biochem.*, **19**, 152–155.
- 5) Anonymous (2000) Bhavaprakashnighantu Harihityadiverg. In *Bhavaprakash*, Part-I (Misra, B. and Vaisya, R., Eds.), Chaukhambha press, Varanasi, India, pp. 10–12.
- 6) Saeed, S. and Tariq, P. (2007) Antibacterial activities of *Embllica officinalis* and *Coriandrum sativum* against Gram negative urinary pathogens. *Pak. J. Pharm. Sci.*, **20**, 32–35.
- 7) Singh, I., Soyal, D. and Goyal, P. K. (2006) *Embllica officinalis* (Linn.) fruit extract provides protection against radiation-induced hematological and biochemical alterations in mice. *J. Environ. Pathol. Toxicol. Oncol.*, **25**, 643–654.
- 8) Yokozawa, T., Kim, H. Y., Kim, H. J., Tanaka, T., Sugino, H., Okubo, T., Chu, D. C. and Juneja, L. R. (2007) Amla (*Embllica officinalis* Gaertn.) attenuates age-related renal dysfunction by oxidative stress. *J. Agric. Food. Chem.*, **55**, 7744–7752.
- 9) Bown, D. (1995) *Encyclopaedia of Herbs and their Uses*, Dorling Kindersley, London.
- 10) Rackova, L., Jancinova, V., Petrikova, M., Drabikova, K., Nosál, R., Stefek, M., Kostalova, D., Prónayová, N. and Kovacova, M. (2007) Mechanism of anti-inflammatory action of liquorice extract and glycyrrhizin. *Nat. Prod. Res.*, **21**, 1234–1241.
- 11) Yu, X. Q., Xue, C. C., Zhou, Z. W., Li, C. G., Du, Y. M., Liang, J. and Zhou, S. F. (2008) *In-vitro* and *in-vivo* neuroprotective effect and mechanisms of glabridin, a major active isoflavan from *Glycyrrhiza glabra* (licorice). *Life Sci.*, **82**, 68–78.
- 12) Gupta, V. K., Fatima, A., Faridi, U., Negi, A. S., Shanker, K., Kumar, J. K., Rahuja, N., Luqman, S., Sisodia, B. S., Saikia, D., Darokar, M. P. and Khanuja, S. P. S. (2008) Antimicrobial potential of *Glycyrrhiza glabra* roots. *J. Ethnopharmacol.*, **116**, 377–380.
- 13) The *Ayurvedic* Formulary of India, Part I (1976) Publisher: Ministry of Health and Family Welfare, Department of Indian System of Medicine and Homeopathy, Government of India. *Awleh and Paka*, pp. 31–48.
- 14) Ziauddin, M., Phansalkar, N., Patki, P., Diwanay, S. and Patwardhan, B. (1996) Studies on the immunomodulatory effects of Ashwagandha. *J. Ethnopharmacol.*, **50**, 69–76.
- 15) Patwardhan, B. and Hopper, B. (1992) Ayurveda and future drug development. *J. Altern. Complement. Med.*, **19**, 9–10.
- 16) Wagner, H. (1983) Immunomodulatory agents. *Proc. Alfred Benzon Symp.*, **20**, 559.
- 17) Atal, C. K. (1985) Role of some important ayurvedic drugs in modulating the immune system in human body. *Indian Drug Manufacturers Assoc. Bull.*, **16**, 17–30.
- 18) Atal, C. K., Sharma, M. L., Kaul, A. and Khajuria, A. (1986) Immunomodulatory agents of plant origin I: Preliminary screening. *J. Ethnopharmacol.*, **18**, 133–141.
- 19) Godhwani, S., Godhwani, J. L. and Vyas, D. S. (1988) *Ocimum sanctum*: a preliminary study evaluating its immunoregulatory profile in albino rats. *J. Ethnopharmacol.*, **24**, 193–198.
- 20) Dua, P. R., Shankar, G., Srimal, R. C. and Husian, A. (1989) Adaptogenic activity of Indian *Panax pseudoginseng*. *Indian J. Exp. Biol.*, **27**, 631–634.
- 21) Mungantiwar, A. A., Nair, A. M., Shinde, U. A., Dikshit, V. J., Saraf, M. N., Thakur, V. S. and Sainis, K. B. (1999) Studies on the immunomodulatory effects of *Boerhaavia diffusa* alkaloidal fraction. *J. Ethnopharmacol.*, **65**, 125–131.