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Immunomodulatory activity of phytoconstituent of *Melissa officinalis*

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ABSTRACT

Melissa officinalis, Labiateae has been used for the treatment of several disorders including diseases of the heart, liver, brain, insanity, giddiness, gripping, muscular pain, asthma, hydrophobia, tuberculous glands, headache, and scabies. In the present study immunomodulatory property of isolated compound obtained from ethanolic extract of whole plant of Melissa officinalis was investigated. The immunomodulatory activity of isolated compound was evaluated using various in vivo models including delayed type hypersensitivity (DTH) reaction, humoral antibody titer and carbon clearance test. Oral administration of isolated compound I (at 30 mg/kg) significantly inhibited sheep red blood cells induced delayed type hypersensitivity reactions and significantly increased the phagocyctic index. It also produced a significant dose related decrease in sheep erythrocyte specific haemagglutination antibody titer. The total leukocyte count (TLC), lymphocyte and neutrophil count increased significantly. These results clearly indicated that the isolated compound obtained from the whole plant ethanolic extract of Melissa officinalis presented a strong immunomodulatory potential.

Keywords: Immunomodulation, *Melissa officinalis*, DTH, TLC, DLC, SRBC

INTRODUCTION

Modulatory response of immune system to alleviate the disease condition was the major interest in Ayurveda for development 'Rasayana' drugs. Many plants have been extensively used as 'Rasayana' drugs in 'Ayurveda' for the management of neurodegenerative diseases, as well as rejuvenators, immunomodulators, aphrodisiac and nutritional supplements [1, 2].

Melissa officinalis, Labiatae has been used for the treatment of several disorders including diseases of the heart, liver, brain, insanity, giddiness, gripping, muscular pain, asthma, hydrophobia, tuberculous glands, headache, and scabies. In the present study immunomodulatory potential of isolated compound obtained from ethanolic extract of whole plant was investigated [3, 4, 5].

MATERIALS AND METHODS

Plant material

The whole plant of *Melissa officinalis* was collected in June 2010 from Tirupathi, Andhra Pradesh, India. The plant material was taxonomically identified and authenticated by Dr K Madhav Chetty, Department of Botany, Sri Venkateswara University, Tirupathi, India. A voucher specimen (BIT/03/10) has been deposited in the Herbarium of the School of Pharmacy, Bharat Institute of Technology, India, for future reference. The plant was dried under controlled temperature, powdered and passed through a 40 mesh sieve and stored in an air tight container**.**

Chemicals
Analytical grade solvents and reagents were obtained from S.D. Fine Chem. Ltd., Mumbai, and Himedia laboratories Pvt. Ltd., Mumbai. The commercial sample, Levamisole (Himalaya Drug) was purchased from medical store. Alsever's solution was purchased from Sigma-Aldrich, St. Louis, MO, USA.

Extraction procedure and isolation of crude ethanolic extract of *Melissa officinalis* whole plant
Whole air dried powdered plant material was defatted with petroleum ether and the marc was extracted using 95% ethanol. The extract obtained was evaporated under vacuum at the temperature of 50°C to yield a reddish brown residue. The residue showed a positive Shinoda test for flavanoids. It was dried and again extracted with *n*- BuOH in a 2000 ml separation funnel. The *n*-BuOH residue was dried and chromatographed over silica gel. The column was eluted with benzene: methanol (1:1). Eluates upon drying, furnished a residue which upon repeated crystallization from methanol yielded compound I [6, 7]. The pure compound isolated by this method was evaluated for its immunomodulatory potential and tested for its UV spectra, as well as its shifts (bathochromic and hypsochromic), that occur due to addition of specific agents: NaOMe, NaOAc, H_3BO_3 and$AlCl_3$. IR spectra were also made.

Animals
Male and female Wistar rats (150-250g) were used. Four groups were maintained for the experiment (n = 5). The animals were housed at standard conditions of temperature (25±1°C), relative humidity (55±10%) and 12/12 h light/dark cycles in the institute's animal house with standard food (Hindustan Lever Ltd., Kolkata, India) and water ad libitum. The experimental protocols of the study were approved by the institutional animal ethical committee (Registration no. 1147/ab/07/CPCSEA) and accordance with the guidelines of the CPCSEA, Government of India.

Antigen (immunization)
SRBC (Sheep Red Blood Cells) were used to immunize the animal, which were stored in a sterile Alsever's solution and washed thrice with pyrogen free normal saline and adjusted to 1×10^8 cells/ml. The animals were immunized by injecting 1ml of 20% SRBC, i.p. The day of immunization was considered as day 0.

Delayed type hypersensitivity reaction
Animals were divided into three groups Group I (control) was given 1% SCMC in water (0.3 ml/mouse, orally), Group II (treated) and Group III standard were given the dose orally for seven days. The animals were immunized by injecting 0.1 ml of SRBCs suspension, containing 1×10^8 cells, i.p., on day 0. On Day 8, after immunization the thickness of the right hind footpad was measured using a vernier caliper. The rats were then challenged by injection of 1×10^8 SRBCs in the left hind footpad. The footpad thickness was measured again after 24 h of challenge. The difference between the pre- and post challenge footpad thickness, expressed in millimeter was taken as a measure of the DTH response. The following formula was used to measure the DTH response [8].

$$\frac{\text{(Left foot pad challenged with antigen-right foot pad control)} \times 100}{\text{Left foot pad challenged with antigen}}$$

Humoral antibody titer
Animals were divided into three groups and were then immunized with 20% SRBC (0.1 ml) i.p. Group I (control) was given 1% SCMC in water (0.3 ml/mouse, orally), Group II (treated) and Group III standard were given the dose orally for seven days. Blood samples were collected from individual animals by retro-orbital puncture on day 8 and were centrifuged at 2500 rpm for 10 min to separate the serum. Two-fold dilution of 50 µl sera (heat inactivated at 56°C for 30 min) was performed in RPMI-1640 medium. Serial dilution (taking 50 µl of the aliquot) was performed in 50 µl RPMI-1640 medium into 96 well micro-titre plates. The fresh, SRBC (1.0%; 25 µl) suspension was dispensed into each well and mixed thoroughly. The plates were then incubated at room temperature for 2 h and examined for button formation. The reciprocal of the dilution, just before the button formation, was observed and titre values were calculated. Group II, III (treated) and standard were given dose for seven days. The experiment was performed on day 7 as for the control group [9].

***In vivo* phagocytosis using carbon clearance method**
Animals were divided into three groups, each containing 10 animals. Group I (control) was given 1.0% SCMC in water (0.3 ml/mouse) for 5 days. Group II (treated) and Group III standard were given the dose orally for 5 days. At the end of five days, after 48 h, rats were injected via the tail vein with carbon ink suspension (10 µl/gm body wt.). Blood samples were drawn (in EDTA solution 5 µl) from the retro-orbital vein at 0 and 15 min, a 25-µl sample was

mixed with 0.1% sodium carbonate solution (2 ml) and its absorbance at 660 nm was determined. The phagocytic index K was calculated using the following equation: K= (Loge OD1-Loge OD2)/15, where OD1 and OD2 are the optical densities at 0 and 15 min, respectively [9].

RESULTS AND DISCUSSION

Isolation and Characterization of Compound I

Compound I was isolated from the butanol extract of the whole plant of *Melissa officinalis*. It crystallized from methanol as yellowish needles; m.p: 312-313°C. The compound gave a positive Shinoda test for flavanoids (pink colour). IR spectrum of compound I (KBr) showed absorption band at 3307cm^{-1} (-OH group) and 1608 cm^{-1} (Chromone carbonyl). (Figure 1) λ_{max} (MeOH) 267, 336; the bathochromic shift of band II by 8 nm in presence of NaOMe indicates 7-OH position; the bathochromic shift of 48 nm in band I in presence of $AlCl_3$ indicates 5-OH position in the molecule; 5, 7, 4' trioxygenated condition is confirmed by observing a bathochromic shift of band I by 40 nm in presence of NaOAc; with H_3BO_3 the spectrum is almost unchanged indicating that there is no catechol system in the molecule. As flavanoids show conspicuous patterns in their UV exposure upon treatment with certain shift reagents (NaoMe, $AlCl_3$, NaOAc, H_3BO_3) the same was performed on *Melissa officinalis*. It has been established as apigenin compared the data with that in literature. IR has also been recorded which further confirms its identity.

Delayed type hypersensitivity reaction

SRBC-induced delayed-type hypersensitivity was used to assess the effect of the isolated compound on cell-mediated immunity. Administration of compound I (30 mg/kg) produced a significant, dose-related decrease of DTH reactivity in terms of the paw thickness when compared to standard group. In the control animals response was 24.31±1.05 at 24 h taken as a parameter for evaluating the reaction. The response to compound I at 30 mg/kg of *Melissa officinalis* (23.97±0.65) was as compared to the standard drug Levamisole 21.24±0.97 (Table 2).

DTH reaction is characterized by an immune-inflammatory reaction, in which macrophages and these cells play major role. DTH reaction requires a specific antigenic substance which will release cytokines by activation with T-lymphocytes [10]. In this study, SRBC was used as the antigenic substance which elicits the hypersensitivity reaction in rats. Therefore, it is anticipated that increase in DTH reaction in mice in response to T-cell-dependent antigen evoked the stimulatory effect of compound I from ethanolic extract of *Melissa officinalis* whole plant.

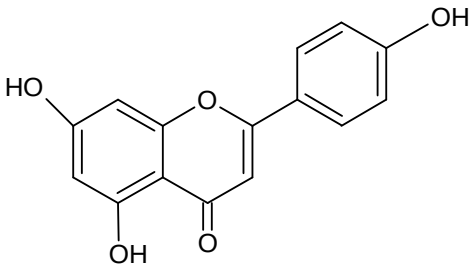
Heamagglutination antibody titre

A dose-related increase in humoral antibody titer (Heamagglutination antibody titre) was observed in rats treated with the Compound I in a dose of 30 mg/kg of *Melissa officinalis* as compared to standard group (Table 2). Compound I was found to significantly enhance the production of circulating antibody titer. This indicates the enhanced responsiveness of macrophages and T and B lymphocyte subsets involved in antibody synthesis [10].

Carbon clearance assay

Administration of compound I (30 mg/kg) increased the rate of elimination of carbon particles from blood as indicated by a significant increase in phagocytic index 0.0385±0.002 respectively when compared with control group 0.0225±0.00. Levamisole (50 mg/kg) showed a significant effect on the phagocytic index 0.062±0.02 (Table 2). The role of phagocytosis is primarily the removal of microorganisms and foreign bodies, but also the elimination of dead or injured cells. Phagocytic defects are associated with varied pathological conditions in humans [9]. In view of the pivotal role played by the macrophages in coordinating the processing and presentation of antigen to B-cells, compound I was evaluated for its effect on macrophage phagocytic activity and showed a promising clearance of the particulate matter from blood.

Immune activation is an effective and protective approach for treating infectious diseases. Among the leukocytes, only antigen specific lymphocytes possess the diversity, specificity, memory and self-reorganization indicating an adaptive immune response [11].



Compound I (Apigenin)

Table 1 UV spectrum for compound I in MeOH and shift solvents.

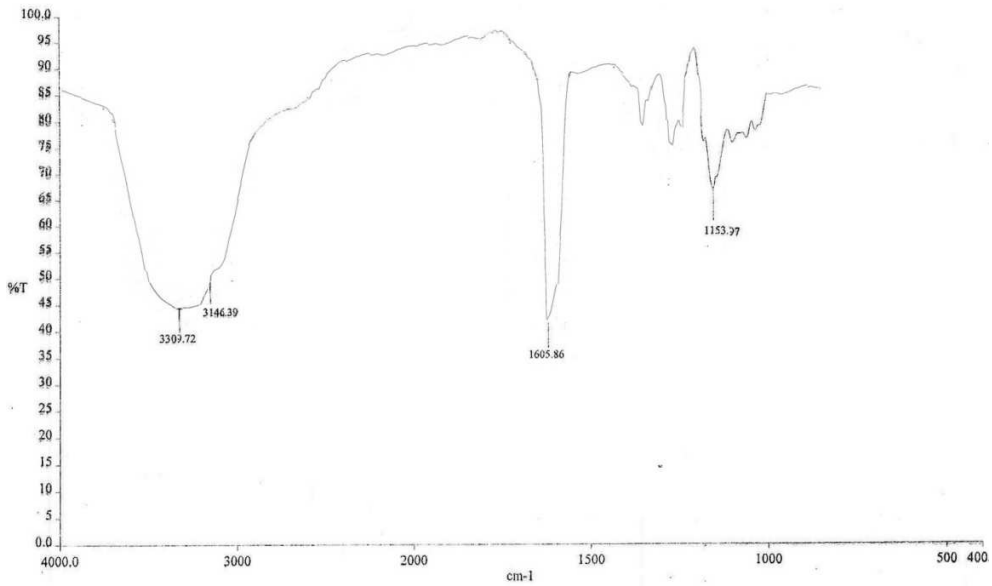
Shift reagent	λ_{max}				
MeoH	267	336	-	-	-
NaOMe	275	324	392	-	-
$AlCl_3$	276	299	348	384	
NaoAc	274	301	376	-	-
H_3BO_3	268	338	-	-	-

Table-2 Effect of isolated compound I on immunomodulation

Group	Treatment/ Dose	Phagocytic Index ± SEM (n=5)	HA Titre ± SEM (n=5)	DTH response in (mm) mean paw edema ± SEM (n=5)
1	Control	0.0225±0.00 1	2.17±0.22	24.31±1.05
2	CompoundI (30mg/kg)	0.0385±0.002**	2.49±0.21**	23.97±0.65**
3	Levamisole (50mg/kg)	0.062±0.02*	2.07±0.33*	21.24±0.97**

*DUNNETT t test and p values as significant * if p < 0.05, highly significant** if p < 0.01, and extremely highly significant*** if p < 0.001 as compared to control.*

Figure 1- IR spectrum of isolated compound 1



CONCLUSION

In conclusion, this result provided primary evidence that compound I identified as apigenin from whole plant ethanol extract of *Melissa officinalis* altered the total and differential WBCs count, potentiated the effect on DTH response, phagocytic index and increased circulating antibodies. Thus the isolated compound showed stimulation of defense system by modulating the immunological parameters and holds the promising therapeutic benefits of the plant parts on immunomodulation. These preliminary results support to the use of this plant in folk medicine

alleviating various diseases, mainly because of low toxicity. Further, investigations are required to clarify the exact mechanism of action responsible for immunomodulatory effect.

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