CHAPTER 6

IMMUNOSTIMULATION ACTIVITY

6.1. Introduction

Ayurveda, the Indian traditional system of medicine lays emphasis on promotion of health as a concept of prevention of diseases and strengthening of both physical and mental health [1]. It was recognized in the Ayurveda that the immune system was involved in the etiology and pathophysiologic mechanisms of various inflammatory diseases of the skin, gut, respiratory tract, joints and central organs as well as in infectious diseases. It held the doctrine that modulation of the immune response would alleviate the diseases and the concept of Rasayana in Ayurveda was based on related principles [2].

Immunostimulants or immunopotentiators are drugs leading predominantly to a non-specific stimulation of immunological defense mechanisms. Most of them are not real antigens but antigenomimetics or so-called mitogens. Non-specific and non-antigen dependent stimulants do not affect immunological memory cells and, since their pharmacological efficacy fades comparatively quickly, they have to be administered either in intervals or continuously. Some immunostimulants may also stimulate T-suppressor cells and thereby reduce immune resistance, hence the term immunomodulation or immunoregulation, denoting any effect on, or change of, immune responsiveness [3, 4].

The human body is continuously exposed to a series of stress factors, which more or less weaken the function of the immune system and hereby generate immunosupression. Immunosupression can be generated by severe bacterial and viral infections, cancer, environmental agents such as pesticides or allergens, excessive long-term chemo- or radiotherapy, malnutrition, psychic stress or endogenic autoimmune reactions [5]. Day by day dramatic increase in microorganisms resistant to antibiotics and chemotherapeutic agents are being recorded. Also the emerge of new plagues such as AIDS and return of the old diseases like tuberculosis has been observed in the modern world. We have no effective vaccines against some severe infections or parasitic diseases, and many chronic diseases are the consequences of an unbalanced or impaired immune response. Among them, the recurrent opportunistic infections, skin and intestine inflammations are the most

important ones, and we should realize that the incidence of some severe infections such as AIDS might be positively influenced by a restoration of the chronically suppressed immune system. In view of the above, the present investigation was undertaken to evaluate the immunostimulatory potentiality of *H. nepalense* and *C.oppositifolia* both by *in vitro* and *in vivo* model system based on the evidence of ethnomedicinal use of these plants in such diseases of immunosupression state in Sikkim.

6.2. Materials and Methods

6.2.1. Plant materials

Methanol extracts of *C.oppositifolia* leaf and *H.nepalense* root as well as their isolated compounds (described in **Chapter 3**) were used as test drug in these experiments.

6.2.2. Test compound formulations

Oral suspensions of the leaf & root extract and isolated compounds were prepared by suspending them separately in 1% solution of sodium carboxy methylcellulose to obtain suitable dosage forms.

6.2.3. Animal used

Swiss Albino mice of either sex, weighing 17–25 g each, were used. They were housed under standard conditions of temperature (23 ±10°C) and relative humidity (55±10%); 12h/12h light/dark cycle and fed with standard pellet diet and water *ad libitum*.

6.2.4. Drugs and Chemicals

EDTA, RPMI- 1640, Hank's balanced salt solution (HBSS), Dextran, Phosphate buffered saline, Fetal calf serum, Streptomycin, Penicillin, Amphotericin, Trypan blue were purchased from Himedia Laboratories Pvt. Ltd, Mumbai. Phythaemagglutinin, Ficoll Hypaque, L-glutamine were purchased from Sigma diagnostic, USA. Interferon α-2b and levamisole were obtained as gift samples from Fulford (I) Ltd and Khandelwal Laboratories Ltd, Mumbai respectively.

6.2.5. Antigen

Fresh blood of a healthy sheep was collected from the local slaughterhouse. Sheep red blood cells (SRBCs) were washed three times with normal saline and adjusted to a concentration of 1×10^8 cells in 0.1 ml for immunization and challenge.

6.2.6. Immunostimulant activity of *C.oppositifolia* leaf

6.2.6.1. *In vivo* carbon clearance test

Mice were divided into eight groups, each containing ten animals. Group I (Control) was given 1% Sodium carboxy methyl cellulose in water (0.3 ml/mouse) for seven days, Group II- V were administered different concentration of methanol extract (250- 1000 mg/kg, p.o.), Group VI was administered standard drug (Levamisole 50 mg/kg, p.o.) and Group VII & VIII were givén compound I (25 and 50 mg/kg, p.o.) for seven days. At the end of seven days, mice of all the groups were injected via the tail vein the carbon ink suspension (10 µl/gm body weight). Blood samples were drawn (in EDTA solution 5µl) from the retro-orbital vein at intervals of 0 and 15 min, a 25 µl sample was mixed with 0.1% sodium carbonate solution (2 ml) and their absorbance were measured at 660 nm. The carbon clearance was calculated using the equation: $(\mathrm{Log_e}\ OD_1 - \mathrm{Log_e}\ OD_2)/\ 15$, where OD_1 and OD_2 are the optical densities at 0 and 15 min, respectively [6].

6.2.6.2. *In vivo* humoral antibody titer and delayed type hypersensitivity response

6.2.6.2.1. Humoral antibody titer test (HA)

Mice were divided into eight groups, each group containing six mice. Group I (Control) was given 1% Sodium carboxy methylcellulose in water (0.3 ml/mouse) for seven days, Group II- V were given different concentration of methanol extract (250- 1000 mg/kg, p.o.). Group VI was administered standard drug (Levamisole 50 mg/kg, p.o.) and Group VII & VIII were given compound I (25 and 50 mg/kg, p.o.) for seven days.

The animals of all the groups were immunized by injecting 0.1 ml of SRBCs suspension containing 1×10^8 cells intraperitoneally on day 0. Blood samples were collected in microcentrifuge tubes from individual animal of all the groups by retro-orbital vein

puncture on day 8. The blood samples were centrifuged and serum was separated. Antibody levels were determined by the haemagglutination technique [(6)]. Briefly, equal volumes of (50 μl) individual serum samples of each group were polled. To serial two fold dilutions of pooled serum samples made in 50 μl volumes of RPMI-1640 in microtitration plates, 50 μl of 1% suspension of SRBC in RPMI-1640 was added. After mixing, the plates were incubated at 37°C for 1 h and examined for haemagglutination under microscope (button formation). The reciprocal of highest dilution, just before the button formation, was observed as the titre values of the test samples.

6.2.6.2.2. Delayed type hypersensitivity test (DTH)

The experiment of DTH was commenced after the Humoral Antibody titre model in the same animals. On day 8, the thickness of the right hind footpad was measured using vernier calliper. The mice were then challenged by injection of 1×10^8 SRBCs in right hind footpad. The footpad thickness was measured again after 24 h of the challenge. The difference between the pre and post challenge footpad thickness expressed in mm was taken as a measure of DTH response [(7)].

6.2.6.3. *In vitro* phagocytic index determination

6.2.6.3.1. Preparation of microorganism

Escherichia coli 832 *(E. coli)* was grown and kept on a slope of solid agar medium. Before use, microorganism is cultured in 100 ml of 2.5% nutrient broth (Oxoid) for about 18 h at 37°C. The culture was then washed twice with phosphate buffer saline and re-suspended in gelatin- Hank's balanced salt solution (HBSS) to a concentration of (1×10^7 cells/ml). To determine the exact number of microbes used during each time the number of viable microorganism was determined microbiologically by counting colony forming units (cfu) using nutrient agar plates followed by incubation at 37°C for 18h [(8)].

6.2.6.3.2. Preparation of human polymorphonuclear leukocyte (PMNCs)

Human blood was collected from local blood bank and the red blood cells were removed by sedimentation in 5% (w/v) solution of dextran in buffered saline (m.w 200,000; 3 ml

of solution to 10 ml of blood) for 30 min at 37°C. The PMNCs rich supernatant layer was washed twice with heparin-saline, concentrated by centrifugation (10 min at 110 g), counted with a haemocytometer, and suspended in gelatin in HBSS to a concentration of (1×10^7 cells/ml).

6.2.6.3.3. Microbiological assay for the phagocytosis

For assessing phagocytosis, different concentrations of methanol extract (250-1000 μg/ml), compound I (25 and 50 μg/ml) and standard drug Interferon α-2b (0.5 million IU) in the final volume of 0.1ml were incubated respectively with 2ml of the PMNCs suspension (1×10^7 cells/ml), 2ml of the suspended microorganisms (1×10^7 cells/ml) and 0.4 ml of fetal calf serum at 37°C for 1 h in 5% CO_2 atmosphere in slanting position. At 30 min intervals up to 120 min, 0.5 ml aliquot of the suspension was removed and added to 1.5 ml of the ice-cooled gelatin-HBSS to stop phagocytosis. The control was run using gelatin-HBSS in place of the test compounds. These samples were centrifuged at 110 g for 4 min. Under this condition the non-ingested microorganisms were remained in the supernatant fluid. The viable count of the microorganisms was done by using the colony counter [8]. Phagocytosis was expressed as the percentage decrease in the initial number of viable extra cellular bacteria according to the following formula: $P(t) = (1 - N_t/N_0) \times 100$, where P (t) is the phagocytic index at time t=t, N_0 and N_t are the number of viable extra cellular bacteria at time t = 0 and t = t, respectively [9].

6.2.6.4. *In vitro* Cell proliferation assay

This test was performed with total peripheral mononuclear blood cells, following their separation from the blood by using ficoll-hypaque gradient centrifugation, according to manufacture's instructions (Sigma diagnostic, USA). The rate of proliferation of mononuclear cells under the influence of mitogens was measured by the method of Sriwanthana [10] with minor modification. Briefly, under sterile conditions, the cells were diluted to 1×10^7 cells/ml with RPMI-1640 (supplemented with 20% fetal calf serum). The suspension of cells (2 ml) was transferred into sterile culture tube and to each sample different concentration of the plant extract (250-1000 μg/ml, filtered through 0.22 μ pore

size filter), compound I (25 and 50 μg/ml) and standard drug Interferon α-2b (0.5 million IU) in the final volume of 0.1 ml were added respectively. Proliferation of cells was induced by 50 μl phythaemagglutinin (PHA, 0.1 mg/ml). The prepared samples were incubated for 72 h at 37°C in a CO_2 atmosphere, supplemented with 2 mM L- Glutamine solution, streptomycin 100 μg/ml of sample, 100 units/ml samples of penicillin and 0.25 μg/ml sample of amphotericin. The control was kept for incubation with cells without the plant extract. The viability of the cells was assessed after incubation with test compounds with the help of trypan blue dye exclusion method [11]. Briefly, 20 μl of the incubation mixture was mixed with 20 μl of 10% w/v solution of Trypan blue dye. The total number of mononuclear cells and mononuclear dead cells (stained blue) were counted under inverted microscope (Olympus, Japan), using haemocytometer. The percentage of cell viability was taken as a measure of cell proliferation and calculated as per the following formula [12].

$$\% \text{ of viability} = \frac{\text{Total number of cells} - \text{Total number of dead cells} \times 100}{\text{Total number of cells.}}$$

Similarly, the percentage of cell stimulation was calculated as per the following formula[5].

$$\% \text{ of cell stimulation} = \frac{\% \text{ Viability of test compound} - \% \text{ Viability of control} \times 100}{\% \text{ Viability of control}}$$

6.2.7. Immunostimulant activity of *H.nepalense* root.

The immunostimulation activity of methanol extract of *H.nepalense* root and its isolated compound II was determined as per the methods described above in **6.2.6** for *C.oppositifolia* leaf.

6.3. Results

6.3.1. Immunostimulant activity of *C.oppositifolia* leaf

The results presented in Table 6.1 showed the immunostimulatory activity of methanol extract of *C.oppositifolia* leaf and compound I in mice. The results revealed that animals

treated with lower doses of methanol extract i.e. 250 – 750 mg/kg did not show much increase rate of in carbon clearance from blood. However at higher dose (1000 mg/kg) increase in the rate of carbon clearance was evident though the values were not statistically significant. Administration of isolated compound I at 50mg/kg resulted in significant increase in the rate of carbon clearance compared with control group. On the other hand the rate of carbon clearance value of compound I was found to be slightly lower than that of the standard compound levimasole at the same dose.

Next the ability of methanol extract and compound I to induce the HA titer and DTH response has been determined. The results of HA titer is presented in Figure 6.1. The HA titer and DTH response are believed to be related with humoral mediated and cell mediated immunity. Treatment of mice with methanol extract at 250 – 1000 mg/kg dose did not show much increase in HA titer value as evident from haemagglutination after incubation of serum with SRBCs. While the compound I and levimasole at 50 mg/kg showed 228.6 ± 3.23 and 432.02 ± 6.5 HA titer value respectively. In DTH response test the methanol extract at higher dose (1000 mg/kg) showed statistically significant increase in mean paw edema (0.38 ± 0.34) in mice, as compared with (0.42 ± 0.21) the standard drug levamisole at a concentration of 50 mg/kg. The isolated compound I at 50 mg/kg dose showed significant DTH response (0.40 ± 0.32) which is considered to be almost equal to the standard drug.

The effect of methanol extract (250 – 1000 μg/ml) and compound I (25 – 50 μg/ml) on phagocytic index is presented in Table 6.2. The phagocytosis of microorganism was assessed at 30 min interval up to 120 min incubation with the test substances. The results showed that the methanol extract at 1000 μg/ml possesses moderate phagocytic activity (94.34 ± 1.24) compared with the control (89.78 ± 1.84) after 120 min incubation. Maximum phagocytic index was observed at 50 μg/ml concentration of isolated compound (96.24 ± 2.14), which is slightly lower than that of the standard drug Interferon α-2b (99.54 ± 1.62) after 120 min of incubation.

Results concerning the proliferative response of the test compound on the viability of mononuclear cells in presence of the PHA mitogen were presented in Table 6.3. Percentage of viability of PHA activated mononuclear cells has found to be not increased at all the concentrations of the methanol extract tested and hence there was stimulation of cells viability. However, the compound I at 50 μg/ml concentrations showed 56.2 ± 6.54 % cell viability compared to the control (51.5 ± 4.62 %). Further, the standard drug Interferon α-2b at 0.5 millions IU and compound I at 50 μg/ml concentration showed 22.26 % and 9.70 % cell stimulation respectively.

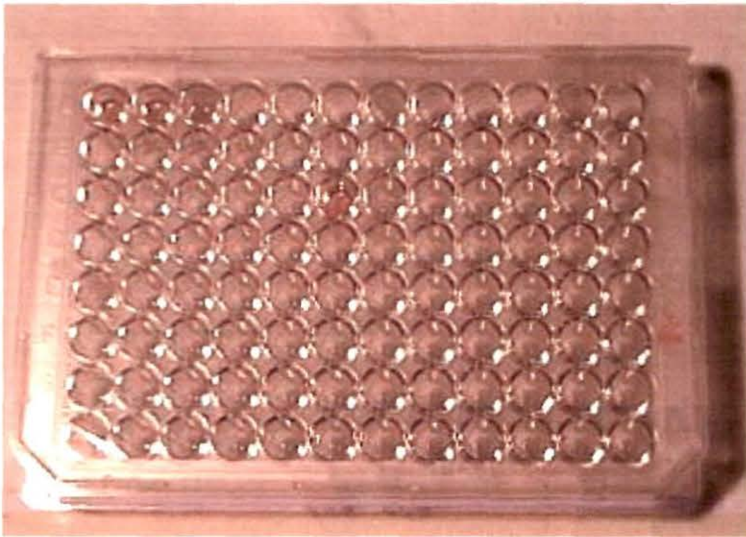

Figure 6.1. Photograph showing effect of methanol extract of *C.oppositifolia* (1000 mg/kg) on haemagglutination antibody titer in mice.

Table 6.1. Effect of *C.oppositifolia* leaf extract and compound I on immunostimulant activity in mice.

Group number	Treatment (mg/kg)	Carbon clearance	HA titer	DTH response (mm)
I	Control	0.069 ± 0.014	82.23 ± 2.24	0.28 ± 0.12
II	Extráct (250)	0.073 ± 0.015[a]	86.4 ± 2.12[a]	0.29 ± 0.14
III	Extract (500)	0.076 ± 0.013[a]	96.8 ± 2.34[a]	0.29 ± 0.15
IV	Extract (750)	0.077 ± 0.016[a]	112.4 ± 6.34[a]	0.31 ± 0.12[a]
V	Extract (1000)	0.094 ± 0.014	132.2 ± 2.32[a]	0.38 ± 0.24[a]
VI	Levamisole (50)	0.158 ± 0.014[a]	432.02 ± 6.5[a]	0.42 ± 0.21[a]
VII	IC 25	0.116 ± 0.014[a]	218.4 ± 2.3	0.39 ± 2.23
VIII	50	0.124 ± 0.015[a]	228.6 ± 3.23[a]	0.40 ± 0.32[a]

Values are mean ± SEM; n=6 in each group. [a]: $P < 0.05$ in comparison with control.

IC: Isolated Compound I.

Table 6.2. Effect of *C.oppositifolia* leaf extract and compound I on phagocytic index of polymorphonuclear leukocyte.

Concentration (μg/ml)	Phagocytic index (%)			
	30 min	60 min	90 min	120 min
Control	61.38 ± 2.21	64.18 ± 1.32	78.18 ± 1.49	89.78 ± 1.84
250	62.24 ± 1.62[a]	66.54 ± 1.28[a]	79.54 ± 1.41[a]	90.14 ± 1.56[a]
500	63.62 ± 1.48[a]	69.15 ± 1.21[a]	84.67 ± 1.21[a]	92.28 ± 1.33[a]
750	64.54 ± 1.34[a]	72.23 ± 1.22[a]	86.23 ± 1.42[a]	93.14 ± 1.26[a]
1000	66.52 ± 1.22[a]	78.52 ± 1.17[a]	89.24 ± 1.14[a]	94.34 ± 1.24[a]
Interferon α-2b (0.5 millions IU)	71.54 ± 1.24[a]	82.92 ± 1.46[a]	93.38 ± 1.72[a]	98.54 ± 1.62[a]
IC 25	67.23 ± 1.42[a]	80.21 ± 1.44[a]	89.14 ± 1.62[a]	93.46 ± 2.12[a]
50	68.24 ± 1.14[a]	81.24 ± 1.52[a]	90.85 ± 1.52[a]	96.24 ± 2.14[a]

Values are mean ± SEM; n=6 in each group. [a]: $P<0.05$ in comparison with control. IC: Isolated Compound I.

Table 6.3. Effect of *C.oppositifolia* leaf extract and compound I on proliferation of mononuclear cell

Concentration (µg/ml)		Viability of cells (%)	Stimulation of cells (%)
Control		51.2 ± 4.62	0
250		42.2 ± 5.32[a]	(– 17.57)
500		46.4 ± 4.52[a]	(– 9.37)
750		49.5 ± 4.26[a]	(– 3.32)
1000		51.6 ± 3.64[a]	(+0.78)
Interferon α-2b (0.5 millions IU)		62.6 ± 4.58[a]	(+22.26)
IC	25	54.6 ±3.75[a]	(+6.64)
	50	56.2 ± 6.54[a]	(+9.76)

Values are mean ± SEM; n=6 in each group. [a]: P<0.05 in comparison with control. IC: compound I. "+": Indicates increase and "–": Indicates decrease of cell stimulation.

6.3.2. Immunostimulant activity of *H.nepalense* root

The results presented in Table 6.4 showed that the methanol extract at 250 – 1000 mg/kg, p.o. and its isolated compound at 25 and 50 mg/kg, p.o. doses exhibited significant increase in carbon clearance from the blood in a dose dependent manner. The methanol extract at 1000 mg/kg exhibited ($P<0.05$) maximum carbon clearance (0.158 ± 0.018) and its isolated compound at 50 mg/kg dose showed (0.160 ± 0.018) carbon clearance ($P<0.05$); while levamisole showed (0.164 ± 0.016) clearance value. The results presented in Table 6.4 indicated that animals treated with doses of 250, 750 and 1000 mg/kg produced significant increase in HA titre (Humoral mediated immunity) as evident from haemagglutination after incubation of serum with SRBCs (Figure 6.2). While the isolated compound at 50 mg/kg and levamisole at the same dose showed 328.6 ± 10.4 and 430.06 ± 8.3 HA titer respectively, which is evident from the Figure 6.2. In DTH response (Cell mediated immunity) test the methanol extract at higher doses (750 and 1000 mg/kg) showed statistically significant increase in mean paw edema in mice. The isolated compound at a dose of 50 mg/kg on the other hand exhibited maximum DTH response of 0.50 ± 0.22 which is almost comparable to the result obtained for the standard drug levamisole (0.57 ± 0.21) at the same dose.

The effect of methanol extract (250 –1000 µg/ml) and its isolated compound (25 – 50 µg/ml) on phagocytic index model is presented in Table 6.5. The results showed that phagocytic index was increased ($P< 0.05$) on dose dependent manner after 30, 60, 90 and 120 min intervals in the presence of methanol extract and isolated compound. Maximum phagocytic index was observed at 1000 µg/ml of methanol extract (97.86 ± 1.67) and 50 µg/ml of isolated compound (97.24 ± 1.23) after 120 min of incubation; whereas the standard compound Interferon α–2b at 0.5 million IU concentration exhibited maximum phagocytic index (99.23 ± 1.11) after 120 min. Results obtained for the proliferative response of the test samples on basis of the viability of mononuclear cells to the PHA mitogen were presented in Table 6.6. Percentage of viability of PHA activated mononuclear cells was significantly increased by 56.3 ± 8.94 % at 1000 µg/ml concentration of root extract, compared with the control, in which it was observed to be 50.7 ± 10.42 %. The maximum viability (60.4 ± 4.58 %) was noticed with the standard

drug Interferon α-2b. The isolated compound at 50 μg/ml concentration has showed 58.2 ± 4.53 % viability, which is also a comparable value to the standard compound Interferon, α–2b. Further, the methanol extract at 1000 μg/ml and isolated compound at 50 μg/ml concentration showed 11.04 % and 14.79% cell stimulations respectively as compared with Interferon α-2b, for which it was observed to be 19.13%.

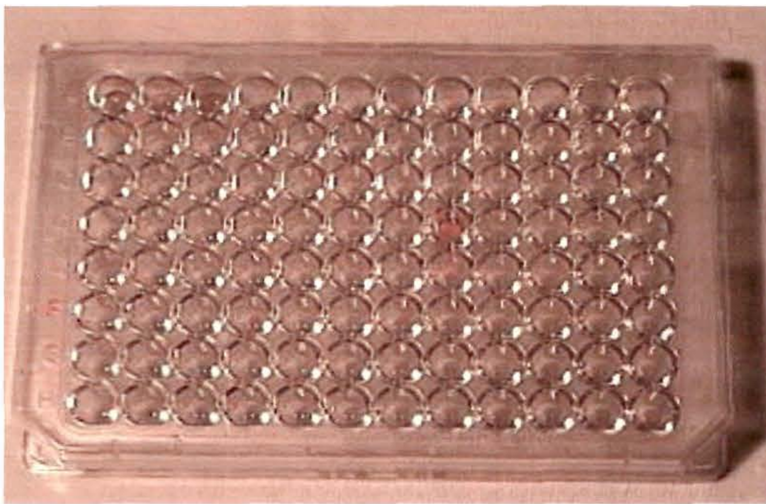

Figure 6.2. Photograph showing effect of methanol extract of *H.nepalense* (1000 mg/kg) on haemagglutination antibody titer in mice.

Table 6.4. Effect of methanol root extract of *H.nepalense* and compound II on immunostimulant activity in mice.

Group number	Treatment (mg/kg)	Carbon clearance	HA titer	DTH response (mm)
I	Control	0.068 ± 0.012	80.43 ± 2.21	0.28 ± 0.10
II	Extract (250)	0.122 ± 0.016[a]	185.3 ± 3.31[a]	0.39 ± 0.13
III	Extract (500)	0.132 ± 0.014[a]	172.7 ± 0.1[a]	0.28 ± 0.13
IV	Extract (750)	0.146 ± 0.016[a]	282.6 ± 8.1[a]	0.43 ± 0.10[a]
V	Extract (1000)	0.158 ± 0.018[a]	320.8 ± 10.6[a]	0.48 ± 0.21[a]
VI	Levamisole (50)	0.164 ± 0.016[a]	430.06 ± 8.3[a]	0.57 ± 0.21[a]
VII	IC 25	0.156 ± 0.016[a]	324.2 ± 8.3	0.48 ± 0.22
VIII	50	0.160 ± 0.018[a]	328.6 ± 10.4[a]	0.50 ± 0.22[a]

Values are mean ± SEM; n=6 in each group. [a]: P< 0.05 in comparison with control.

IC: Isolated compound II.

Table 6.5. Effect of methanol root extract of *H.nepalense* and compound II on phagocytic index of polymorphonuclear leukocyte.

Concentration (μg/ml)	Phagocytic index (%)			
	30 min	60 min	90 min	120 min
Control	61.14 ± 1.26	67.26 ± 1.52	84.32 ± 1.69	92.25 ± 1.83
250	63.53 ± 1.52[a]	78.54 ± 1.18[a]	85.74 ± 1.43[a]	94.27 ± 1.86[a]
500	64.12 ± 1.18[a]	79.23 ± 1.21[a]	86.23 ± 1.24[a]	95.67 ± 2.33[a]
750	67.38 ± 1.24[a]	81.52 ± 1.42[a]	88.15 ± 1.86[a]	96.55 ± 1.83[a]
1000	69.54 ± 1.12[a]	83.26 ± 1.37[a]	91.18 ± 2.14[a]	97.86 ± 1.67[a]
Interferon α-2b (0.5 millions IU)	70.27 ± 1.64[a]	84.14 ± 1.32[a]	94.52 ± 1.65[a]	99.23 ± 1.11[a]
IC 25	69.54 ± 1.12[a]	82.86 ± 1.45[a]	90.96 ± 1.14[a]	96.42 ± 1.42[a]
50	70.27 ± 1.64[a]	83.09 ± 1.51[a]	91.14 ± 1.62[a]	97.24 ± 1.23[a]

Values are mean ± SEM; n=6 in each group. [a]: $P<0.05$ in comparison with control.
IC: Isolated compound.

Table 6.6. Effect of methanol root extract of *H.nepalense* and compound II on proliferation of mononuclear cell

Concentration (μg/ml)		Viability of cells (%)	Stimulation of cells (%)
Control		50.7 ± 10.42	0
250		47.6 ± 4.42[a]	(– 6.11)
500		49.8 ± 5.42[a]	(– 1.77)
750		49.0 ± 4.45[a]	(– 3.35)
1000		56.3 ± 8.94[a]	(+11.04)
Interferon α-2b (0.5 millions IU)		60.4 ± 4.58[a]	(+19.13)
IC	25	56.9 ±6.62[a]	(+12.22)
	50	58.2 ± 4.53[a]	(+14.79)

Values are mean ± SEM; n=6 in each group. [a]: P<0.05 in comparison with control. IC: Isolated compound. "+": Indicates increase and "–": Indicates decrease in cell stimulation.

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