

## ORIGINAL ARTICLE

# EFFECTS OF *SAUSSUREA LAPPA* ROOTS EXTRACT IN ETHANOL ON LEUKOCYTE PHAGOCYtic ACTIVITY, LYMPHOCYTE PROLIFERATION AND INTERFERON – GAMMA (IFN- $\gamma$ )

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### ABSTRACT

Effects of *Saussurea lappa* root extracts prepared in ethanol according to the homeopathic principles were assessed on leukocyte phagocytic activity, lymphocyte transformation and mitogen-induced interferon-gamma (IFN- $\gamma$ ) in the cultures of peripheral blood mononuclear cells of goats (PBMC) in vitro. Leukocyte phagocytic activity was measured by flow cytometry, lymphocyte proliferation by MTT and IFN- $\gamma$  level in cell culture supernatants was determined by ELISA. The results obtained demonstrated that all test dilutions (D4, D6, D8) of *Saussurea lappa* in ethanol have exerted a stimulating effect on leukocyte phagocytic activity in dose-dependent manner. A 10 $\mu$ l dose of *Saussurea lappa* of each dilution markedly enhanced phagocytic activity, while other doses tested made only a feeble stimulating effect. The increases with 10 $\mu$ l dose were found significantly ( $P < 0.01$ ) different between each dilution, maximal stimulation was observed by D8 dilution. Different doses (10 $\mu$ l, 2 $\mu$ l, 1 $\mu$ l, 0.5 $\mu$ l) of all test dilutions (D4, D6, D8) of *Saussurea lappa* in sterile 0.9% NaCl solution inhibited lymphocyte proliferation. Maximal inhibitory effect was observed with the 2  $\mu$ l dose. Similarly, *Saussurea lappa* suppressed the secretion of IFN- $\gamma$  by mitogen-activated (PHA; 2.5 $\mu$ g/ml) of peripheral mononuclear cells in dose-dependent manner. In conclusion these findings suggest that enhanced leukocyte phagocytic activity may be helpful to clear the soluble immune complexes produced during a sustained immune response against self antigens which causes chronic inflammatory injury of tissue. On the other hand, inhibition of lymphocyte proliferation and IFN- $\gamma$  by *Saussurea lappa* may contribute to suppress immune-mediated inflammatory reactions possibly through a cell-mediated cytokine pathway. Thus it is conceivable that ethanolic extracts of *Saussurea lappa* roots in homeopathic dilutions may be considered as a potential candidate for therapeutic support in autoimmune and chronic inflammatory disorders.

**Keywords:** *Saussurea lappa*, phagocytic activity, lymphocyte proliferation, interferon – gamma, goats.

### INTRODUCTION

Besides diverse pharmaceutical applications, *Saussurea lappa* roots (Family: Compositae) have been widely recommended in inflammation-related diseases characterized by rheumatoid arthritis, chronic gastritis, asthma and bronchitis in traditional medicine (Chopra, 1982, Ikram *et al.*, 1978; Jain, 1968). The scientific evidences of their significance are inadequate. Akhtar and Farah (1987) reported chemical contents exerting anthelmintic effects in animals. Recently, a small number of *in vitro* studies have been published describing effects of the methanolic root extracts of *Saussurea lappa* on cell mediated immunity in rats (Lee *et al.*, 1995; Taniguchi, *et al.*, 1995; Cho *et al.*, 1998; Jung *et al.*, 1998; Lee *et al.* 1999). However, the toxicological effects of these preparations on individual's general health remain yet to be ascertained.

The homeopathic preparations are known to have no side effects. Therefore, the present studies have been undertaken to evaluate the effects of ethanolic effects of *Saussurea lappa* roots prepared according to the homeopathic principles on the immunological parameters like leukocyte phagocytic activity, lymphocyte proliferation and IFN- $\gamma$  in the mitogen-induced peripheral blood mononuclear cell goats of goats in vitro (PMBC).

### MATERIALS AND METHODS

#### *Animals used*

Sixteen healthy goats, aged between 2-10 years, reared in the stalls of Department of Animal Breeding and Genetics, University of Bonn, Germany were used in this study.

#### *Preparation of tested extracts*

The roots of *Saussurea lappa* were purchased from herbal

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dealer of Faisalabad, Pakistan. They were got duly authenticated by the experts of Botany Department, University of Agriculture, Faisalabad. All raw extracts and individual decimal dilutions were prepared according to the Homeopathic Arzneibuch (HAB)<sup>1</sup> (1985), following instruction 4a. Briefly, the roots of *Saussurea lappa* were sliced and raw material was extracted with ethanol (62%). Decimal dilutions until D3 were prepared with 62% ethanol. Further dilutions were prepared using 43% ethanol. Different dilutions tested in lymphocyte transformation assay were based in sterile 0.9% NaCl in order to minimise the ethanol level. These dilutions were prepared following instruction 11 in Homeopathic Arzneibuch I(1985).

### **Phagocytic Activity**

A commercial test kit, Phagotest<sup>®1</sup>, was used for the tests. This test avoids cell preparation by using technique of lysed whole blood. Briefly, 100µl of heparinized blood mixed with 100µl NaCl containing test doses of drug (10µl, 5µl and 1µl) based in ethanol (43%). The controls received equal quantity of ethanol (43%) in 100µl NaCl for each dose of drug. Both experimental and control tubes were incubated at 37°C for half an hour in continuously shaking water bath.

Control/drug mixtures were incubated with FITC-conjugated *E. coli* (opsonized with pooled serum) for 10 minutes at 37°C, afterwards the phagocytosis was stopped at 0°C and a quenching solution (15% trypan blue in PBS) was added to suppress the fluorescence of the bacteria that were bound at the surface of the cells. Cells were washed twice (250g, 8min, 4°C). Thereafter, erythrocytes were lysed and fixed (diethylene glycol formaldehyde), washed again and propidium iodide (PI) was added to stain the DNA of the cells and bacteria. The incubation of 100µl blood with the same amount of *E. coli* at 0°C served as a negative control. All tests were performed in duplicate and means were taken for statistical analysis.

The evaluation of the results were done with the help of flow cytometer<sup>2</sup>. 15,000 were acquired flow-cytometrically (FACScan) using a first live gate in the red fluorescence histogram (propidium iodide) on the leucocytes and a second live gate in the FSC versus SSC displayed around the granulocytes. The analysis of the green fluorescence histogram (FITC) showed the intracellular, FITC-conjugated *E. coli*. All events with a higher fluorescence intensity (FI) than that of the negative control were defined as phagocytosing cells and expressed as percentages of the total granulocytes, the mean FI of the positive peak (phagocytosing granulocytes) correlates

with the number of the phagocytosed bacteria per cell and is expressed in FI units. The results are presented as percent relative index.

### **Lymphocyte Proliferation Assay**

Whole blood was collected into heparinized monovette tubes by jugular venipuncture from adult goats. The blood was diluted 1:1 with phosphate buffer saline (PBS) and 21 ml of the blood/PBS mixture was layered over 12 ml of Histopaque 1077 (Sigma Labs., USA) in 50 ml conical bottom tubes. Following centrifugation (550 x g for 30 minutes), the interface cells were collected and washed twice with serum free RPMI 1640. The cell pellet was resuspended in complete medium (RPMI 1640 + 20% autologous serum + 100IU of Penicillin/ml, 100µg of streptomycin/ml, and 2% Glutamine). Cells were counted on a hemocytometer, and cell suspensions were diluted with RPMI to 5 x 10<sup>5</sup> cells/ml. Viability of cells (>95%) was determined by trypan blue dye exclusion.

Lymphocyte cultures were carried out in triplicate in 96-well flat bottomed microtiter plates (Nunc, Denmark). All experimental wells received 5 x 10<sup>4</sup> cells (100µl) and 100µl of test substance solution containing 90 µl RPMI<sup>+</sup> and respective volume of drug dissolved in 0.9% NaCl. All control wells received 90µl of RPMI<sup>+</sup> and 10 µl NaCl. Total volume in all wells was 200 µl. Parallel, a reference test was carried out with 2.5µg PHA along each culture. All cultures were carried out simultaneously using the same cell suspensions, and cultures were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for 72 hours.

Following incubation, 20µl WST-1 solution (Boehringer Mannheim, Germany) was added in each well, and plates were incubated for an additional 4 hours. Measurements were done with the help of multiwell spectrophotometer using a test filter of 450 nm and reference filter of 690 nm. Data were presented in SI (%), which was calculated using following formula:

$$SI (\%) = SI - 1 \times 100\%$$

### **Interferon-gamma**

Cell cultures were prepared in the same way as described for lymphocyte proliferation test. Culture plates were incubated for 24 hours at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. IFN-γ levels in the supernatants were determined by ELISA kit (Diaclone Research, France). Briefly, the supernatant or a standard IFN-γ was added to a microtiter plate coated with a monoclonal antibody (mob) for IFN-γ. Following the addition of a biotinylated monoclonal antibody which was coupled to enzyme, streptavidine peroxidase (pod), the plate was incubated at room temperature for 4 hours. Unbound enzyme removed by a washing step and the amount of bound peroxidase was removed by a short incubation

<sup>1</sup> Orpegan pharma, Germany.

<sup>2</sup> Becton Dickinson, USA.

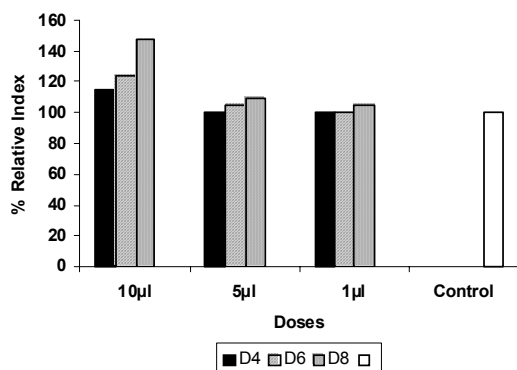
tetramethyle benzidine (TMB) On stopping the reaction with sulphuric acid the colour changed to yellow and it's intensity was determined at 450 nm by a multiwell photometer. The amount of IFN- $\gamma$  was determined from a standard curve. This ELISA had an assay range from 12.5 to 400 pg/ml.

## STATISTICS

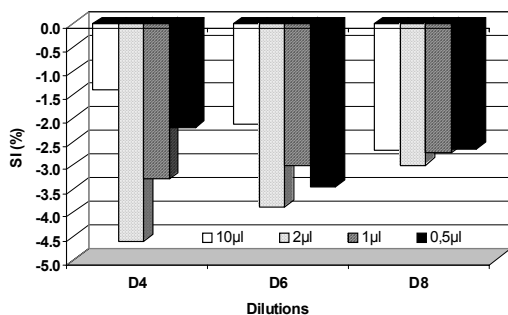
The results are presented in median. Wilcoxon's-test for paired differences was applied to compare the values. All computations were carried out with SPSS computer programme. Differences at  $P < 0.01\%$  level were accepted as significant.

## RESULTS

The effects of different dilutions (D4, D6 D8) of *S. lappa* based in ethanol (43%) were tested on leukocyte phagocytic activity. Among different doses (10 $\mu$ l, 5 $\mu$ l, 1 $\mu$ l), a dose of 10 $\mu$ l of each dilution markedly enhanced leukocyte phagocytic activity, while other doses of each dilution made only a slight stimulating effect (fig. 1). The differences with 10  $\mu$ l dose between different dilutions were found statistically significant ( $P < 0.01$ ).



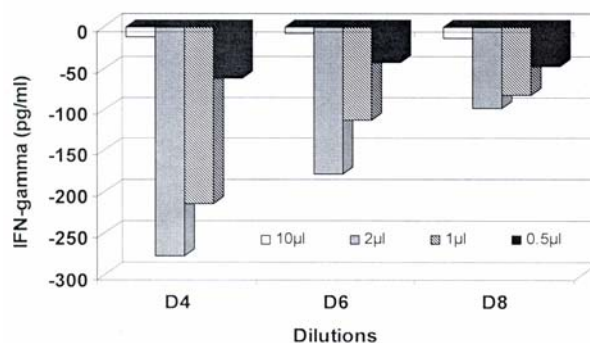
**Fig. 1:** Effect of different doses (1 $\mu$ l-10 $\mu$ l) of various dilutions of homeopathic preparations of *Saussurea lappa* root extract on phagocytic activity in goats. Results are expressed as relative index.



**Fig. 2:** Effects of different doses (0.5 $\mu$ l and 10 $\mu$ l) of various dilutions of homeopathic preparations of *Saussurea lappa* root extract on lymphocyte proliferation in goats.

Effects of different homeopathic dilutions (D4, D6 D8) of *Saussurea lappa* based in sterile 0.9% NaCl solution for four tested doses (10 $\mu$ l, 2 $\mu$ l, 1 $\mu$ l, 0.5 $\mu$ l) on lymphocyte proliferation are presented in fig 2. All dilutions and doses of *S. lappa* inhibited lymphocyte proliferation. The 2 $\mu$ l dose of *S. lappa* of each dilution exerted a maximal inhibitory effect on lymphocyte activity, whereas 10 $\mu$ l dose induced least inhibition. However, this difference was statistically not found significant.

Mitogen-induced (PHA, 2.5  $\mu$ g/ml) secretion of IFN- $\gamma$  in cultures of peripheral blood mononuclear cell (PBMC) supernatants was suppressed by *Saussurea lappa* (fig. 3).



**Fig. 3:** Influences of various dilutions of homeopathic preparations of *Saussurea lappa* roots extract in ethanol on the mitogen (PHA: 2.5 mg/ml) induced secretion of interferon gamma (IFN- $\gamma$ ) in the cultures of peripheral blood mononuclear cells (PBMC) supernatants of goats.

## DISCUSSION

All doses (1 $\mu$ l, 5 $\mu$ l, 10 $\mu$ l) of various dilutions (D4, D6, D8) of *Saussurea lappa* tested enhanced leukocyte phagocytic activity, maximal rise was observed by 10 $\mu$ l dose of each dilution. To date, there is no literature available to discuss the effects of homeopathic dilutions of *Saussurea lappa* on immun parameters reported here.

Phytotherapeutic studies have, however, indicated that total methanol extract of *Saussurea lappa* inhibited the production of pro-inflammatory cytokines such as interleukine – 8 (IL-8) and tumor necrosis factor (TNF- $\alpha$ ) in murine like macrophages (Cho *et al.*, 1998; Lee *et al.*, 1995). Recently, Lee *et al.* (1999) have reported that methanolic extract of sesquiterpene, a dehydrocostus lactone, isolated from *S. lappa* inhibited the production of nitric oxide in lipopolysaccharide (LPS) – activated macrophages by suppressing the inducible nitric oxide synthase (iNOS) enzyme expression (Lee *et al.*, 1995). Downregulation of pro-inflammatory cytokines diminish leukocyte recruitment and possible regulation of angiogenesis, while suppressed nitric oxide can contribute to reduce tissue injury. An enhanced leukocyte phagocytic activity observed in this study may be helpful

to clear the immune complexes produced during a sustained immune response against self-antigens which causes chronic inflammatory injury of tissue.

Current study indicated that all doses tested (0.5µl, 1µl, 2µl 10µl) of various dilutions (D4, D6, D8) of *Saussurea lappa* radix exhibited an inhibitory effect on proliferation of peripheral blood mononuclear cell (PBMC) cultures. Costunolide and dehydrocostus lactones isolated from an extract of *Saussurea lappa* inhibited the killing capacity of cytotoxic lymphocytes (Taniguchi *et al.*, 1995). Likewise, certain constituents of *Saussurea lappa* exhibited moderate cytotoxicities against the human tumour cell lines (Jung *et al.*, 1998). The suppression of proliferation of peripheral blood mononuclear cell (PBMC) cultures in the present study may be partly due to cardiac glycosides contained by *Saussurea lappa* roots. It is well known that cardiac glycoside (e.g., ouabain) is a specific inhibitor of membrane Na<sup>+</sup>K<sup>+</sup>-ATPase, which may inhibit the movement across the plasma membrane. It has also been shown to inhibit lymphocyte proliferation in mixed lymphocyte cultures or induced by various stimulating agents (Dormond *et al.*, 1984). Moreover, Bordie *et al.*, (1995) found that ouabain inhibit T-cell proliferation and this inhibition is similar to CD4 and CD8 T-cells. Ouabain also sets a block in the progression from G1 to S phase (Pires, 1997).

In this study, mitogen-induced (PHA, 2.5 µg/ml) secretion of IFN-γ in cultures of peripheral blood mononuclear cell (PBMC) supernatants was suppressed by *Saussurea lappa* in dose-dependent manner. IFN-γ is produced by NK cells and in large amount by Th1 cells. An inhibitory effect of *Saussurea lappa* on helper T-cells (CD4) and cytotoxic T-cells (CD8) might be responsible for reduced mitogen – activated secretion of IFN-γ in cultures of peripheral blood mononuclear cells (PBMC). It is believed that autoimmunity is initiated by responses involving T-cells. Cytotoxic T-cell responses can cause extensive tissue damage, while inappropriate T-cell help can cause harmful antibodies response to self antigens. In a variety of inflammatory autoimmune diseases, it appears that self antigens are presented to Th1 cells, but not to Th2 cells to cause disease on adoptive transfer For instance, rheumatoid arthritis may be caused by Th1 cells specific for an antigen present in joints, which triggers them to release lymphokines that initiate local inflammation within the joint. A naive CD4 effector T-cell becomes an armed Th1 cell in the presence of interleukin-12 (IL-12) and IFN-γ, which has a critical impact on the outcome of an adaptive immune response (Janeway and Travers, 1997). Inhibition of IFN-γ suggest that therapeutic effect of *Saussurea lappa* is achieved possibly by correcting Th1 and Th2 imbalance (a shift

from Th1 type to Th2 type), which may be involved in the pathogenesis of rheumatoid arthritis.

Taken as a whole, these findings suggest that inhibition of lymphocyte proliferation and IFN-γ by homeopathic dilutions of *Saussurea lappa* may contribute to suppress immune-mediated inflammatory reactions possibly through a cell-mediated cytokine pathway. On the other hand, enhanced leukocyte phagocytic activity may be helpful to clear the soluble immune complexes produced during a sustained immune response against self-antigens which causes chronic inflammatory injury of tissue. Thus, homeopathic preparations of *Saussurea lappa* may be considered as a possible therapeutic support in autoimmune and chronic inflammatory disorders.

This seems to be important because high dilutions of homeopathic drugs are considered not to block but to regulate the inflammatory process without any risk of the side effects. So this kind of therapy would be more biological.

#### ACKNOWLEDGEMENT

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