

## ***In vitro* immunomodulatory study of different parts of *Prunus cerasus* L. (sour cherry) Plant**

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### **ABSTRACT**

Five different parts (fruit, seed, root, leaf and stem-bark) of sour cherry *Prunus cerasus* L. were extracted with methanol, water-methanol 1:1 and water. Total fifteen extracts were prepared and screened for *In vitro* immunomodulatory potential employing Nitroblue Tetrazolium Reduction test (NBT), Inducible Nitric Oxide Synthase test (iNOS), Bactericidal activity of macrophages (Phagocytosis) and T and B cell proliferation by MTT assay. Results showed a significant difference in the immunomodulatory activities according to the parts of sour cherry plant. Among all tested extracts, the methanolic fruit extract (Pc-MeOH-F-Ext.) showed the maximum potential for NBT, iNOS and Phagocytosis as well as splenocyte proliferation, whereas the aqueous seed extract (Pc-W-S-Ext.) showed the minimum effect. Further a preliminary analysis of the most immunopotent extract (Pc-MeOH-F-Ext.) showed the presence of bioactive phytochemicals like flavonoids, anthocyanins, etc. The results of this study demonstrate the immunostimulatory effect of *Prunus cerasus* L. in a concentration-dependent manner. The results suggest the fruits of sour cherry could be applied as an immunomodulator.

**Key words:** Sour cherry (*Prunus cerasus* L.); Immunomodulation; Phagocytosis; Nitro Blue Tetrazolium, splenocytes; Nitric oxide.

### **INTRODUCTION**

The immune system is known to be involved in the etiology as well as pathophysiologic mechanism of various diseases. This problem can be overcome by boosting the immune system by the use of immunomodulatory drugs of natural or synthetic origin. Only a few chemotherapeutic agents as immunopotentiators are available today and most of them are cytotoxic and exert a variety of side effects [1]. This has given rise to stimulation in the search for investigating natural resources showing immunomodulatory activity. Immunotherapy using plants can provide an alternative to conventional chemotherapy for a variety of diseases. Several types of immunomodulators have been identified, including substances isolated and purified from plants [2]. It has become a global concern to treat and manage the course of the debilitated diseases such as cancer, HIV infections and mycobacterial tuberculosis/leprosy etc where patients among others, suffer drastically from a state of immune dysfunction often associated with suppression of immune cells and molecules. Immunology is probably one of the most rapidly developing areas of biomedical research and has great promises with regard to prevention and treatment of wide range of disorders [3].

Commonly consumed raw materials of plant origin are the most important sources of bioactive phenolic compounds. The combination of vitamins, fibre, phenolics and other antioxidants are probably reliable for these effects [4]. The importance of such phenolic antioxidants of fruits in maintenance of health and in protection from degenerative diseases is of growing interest among scientists, food manufacturers, consumers and health organizations [5].

A number of fruits used in the traditional medical system of remedies in India, have been shown to possess immunostimulating activity acting at different levels of the immune system [6]. Few fruits fall in this category and emerging science shows sour cherries is one among them. *Sour cherry* is a medicinal fruit claimed to possess number of therapeutic uses including anti-inflammatory as well as antioxidant [7]. *Prunus cerasus* or sour cherries is a species of genus *Prunus* in the sub-genus *Cerasus* (cherries) belong to Rosacea family, native to much of Europe and Southwest of Asia. It is closely related to wild cherry or sweet cherry but has a fruit that is more acidic. Cherry been used as a medicinal plant for a long time in Asia. Red cherry fruits are used in a traditional herbal remedy for various diseases such as heart failure, beriberi, dropsy, mastitis. The stalk from sour cherry has been used medicinally as an astringent. Bark and stem of the cherry tree are used for detoxification and relaxation. The bark of sour cherry is astringent, bitter and an infusion of this bark has been used in the treatment of fevers, coughs and colds. The seed (pit) is nervine and an edible drying oil obtained from these seeds is also used in cosmetics. A green dye can be obtained from the leaves and can be used as a natural colouring agent [8].

The principal nutrients thought to provide the protection afforded by fruits and vegetables are antioxidants such as vitamins, and flavonoids (including flavones, isoflavones, and anthocyanins). Sour cherries are considered as good sources of both flavonoids and phenolic acids like anthocyanins etc. [9, 10] providing health-promising effects in humans [11]. Sour cherries have been ranked 14 in the top 50 foods for highest antioxidant content per serving size-surpassing well known leaders such as red wine, prunes, dark chocolate and orange juice [12]. Sour cherry is used for conditions involving Inflammation and pain, such as: arthritis, gout, muscle pain, back pain, diabetes, and neurodegenerative diseases; however the immunostimulatory potential of *Prunus cerasus* L. plant on immune system has not yet been explored [13]. Therefore, the objective of the present study was to study the immunomodulatory activity of different extracts prepared from various parts of *Prunus cerasus*.

## MATERIALS AND METHODS

### 2.1. Reagents

All chemicals and reagents used in the current study were of analytical grade and mostly purchased from Sigma chemicals (India)

### 2.2. Collection of plant material

Different parts of *Prunus cerasus* L. or sour cherry plant (Seed, Leaf, Root, Bark and Fruit) were collected at their appropriate growth and maturity season from the field research centre of pomology division of the Sheri-Kashmir University of Agricultural Sciences and Technology (SKUAST-K) Srinagar, Jammu and Kashmir India. The collected plant parts (leaf, shoot- bark, root, seed and fruit) were carefully shade dried and ground to coarse powder except fruit-pulp which was lyophilised.

### 2.3. Preparation of extracts

Methanolic, hydro-methanolic and aqueous extracts were prepared from each selected part of the plant.

#### 2.3.1. Preparation of methanolic extract

Dried and powdered plant parts were placed in the beakers and sufficient quantity of methanol was added so as to submerge the plant material. The material was stirred mechanically for four hours and then double filtered through muslin cloth. The extraction process was repeated similarly three times more and the crude methanolic extract was evaporated to dryness under reduced pressure at 50°C on a rotavapour. Final drying was done in a vacuum desiccator. The methanolic extracts of all selected parts were prepared in the manner

#### 2.3.2. Preparation of 50% hydro-methanolic extract

The selected dried plant parts were extracted in the same manner as described above using 50% aqueous methanol (1: 1) solvent system.

### 2.3.3. Preparation of aqueous extract

The dried and powdered plant material was extracted in the same manner as described above using distilled water. The crude aqueous extracts were lyophilized. All the prepared extracts were weighted and stored at 4 °C.

## 2.4. Immunomodulatory potential of different solvent extracts prepared from various parts of *Prunus cerasus* L. (*In vitro*):

### 2.4.1. Lymphocyte isolation from the spleen

Spleen was excised aseptically in HBSS, as lymphocytes were isolated by teasing the tissue and passed through a fine steel mesh to obtain a homogeneous cell suspension. The erythrocytes were lysed with ammonium chloride (0.8%, w/v). After centrifugation ( $380 \times g$  at 4 °C for 10 min), the pelleted cells obtained were washed three times with PBS and adjusted to desired concentration in complete medium [RPMI 1640 supplemented with 12 mM HEPES (pH 7.1), 0.05 mM 2-mercaptoethanol, 100 IU/mL penicillin, 100 µg/mL streptomycin and 10% FCS] for further use. The cell number was counted with a haemocytometer by the trypan blue dye exclusion technique. Cell viability exceeded 95%.

### 2.4.2. Incubation of lymphocytes with plant extracts

The sample extracts (dissolved in PBS) at different concentrations, were incubated with  $2 \times 10^6$  cells lymphocytes/well at 37 °C for 24 hrs. in humid saturated atmosphere containing 5% CO<sub>2</sub> chamber.

### 2.4.3. Nitroblue Tetrazolium Reduction assay

NBT reduction test, (a measure of respiratory burst in the leucocytes) was carried out by employing the method described by [14]. Briefly, the lymphocyte suspension was incubated with NBT and formazon formed was extracted in dioxan. The reduction in NBT was measured spectrophotometrically at 520 nm (Shimadzu, UV-1650 PC) against dioxan as blank. The results were expressed as mean  $\pm$  S.E.M. of percentage dye reduced to formazon.

### 2.4.4. Inducible Nitric Oxide Synthase iNOS activity

Inducible nitric oxide synthase activity of lymphocyte suspension was assessed spectrophotometrically by employing the method mentioned [14] using arginine. Briefly the splenocytes were incubated with arginine at 37 °C for 24 hours in CO<sub>2</sub> chamber. The colour developed (indicating citrulline formation from arginine) was measured spectrophotometrically at 540 nm against RPMI and Griess reagent as blank and the results were expressed as mean  $\pm$  S.E.M. of percentage enzyme produced.

### 2.4.5. Bactericidal activity (Phagocytosis)

The macrophage function was evaluated by phagocytosis of microorganism (Bactericidal activity) using the method given [14]. Briefly, the lymphocyte suspension was incubated with bacterial suspension (*Escherichia coli*) at 37 °C for 60 min. The lymphocytes were lysed with sterile distilled water spread on agar plate and incubated at 37 °C for 24 h. Bacterial suspension was spread in the control plate. Number of colony forming units (CFU) developed in control and test plates were counted and results were expressed as mean  $\pm$  S.E.M. of bactericidal activity.

### 2.4.6. T and B cell proliferation assay by MTT assay (*In vitro*).

To evaluate the effect of different extracts prepared from sour cherry plant on the proliferation of splenic lymphocytes, the spleen cell suspension containing ( $1 \times 10^7$  cells/mL) were seeded in a 96-well culture plates (200 µL/well) in triplicates. Splenocytes were treated with 10 µg/ml of each test sample and sub-optimal concentrations of Con-A (2.5 µg/mL) and LPS (2.5 µg/mL) were added to each well separately for priming T cells and B cells respectively. Plates were incubated at 37 °C for 72 h in a humid saturated atmosphere containing 5% CO<sub>2</sub>. After 72 h, 20 µL of MTT solution (5 mg/mL) was added to each well and the plates were incubated for 4 h. Thereafter, plates were centrifuged ( $1400 \times g$ , 5 min) and the untransformed MTT was removed carefully by pipetting. To each well, 100 µL of a DMSO working solution (192 µL DMSO with 8 µL 1 M HCl) was added and the absorbance was evaluated in an ELISA plate reader at 570 nm after 15 min [15].

## 2.5. Preliminary analyses of phenol, flavonoid, and anthocyanin contents of the most immunopotent extract (Pc MeOH-F-Ext.)

[i]. Total Phenols: The amount of total phenols in Pc-MeOH-F-Ext. was determined by using Folin-Ciocalteu reagent according to the method of [16]. The total phenols of the sample are expressed as milligrams of gallic acid equivalents (GAE) per 100 g of fresh material and reported as phenol milligrams per 100 g of fresh product.

[ii]. Total Flavonoids: The flavonoid content of *Pc*-MeOH-F-Ext. was measured using a colorimetric assay developed by [17]. The flavonoid content is expressed as milligrams of catechin equivalents (CA) per 100 g of fresh material and reported as flavonoid milligrams per 100 g of fresh product.

[iii]. Total Anthocyanins: The total anthocyanin content of *Pc*-MeOH-F-Ext. was measured by using a colorimetric assay [18]. Anthocyanin content is expressed as milligrams of kouroumanin (cyanidin-3-*O*-glucoside) equivalents (KO) per 100 g of fresh material and reported as anthocyanin milligrams per 100 g of fresh product.

## 2.6. Statistical analysis

Data are expressed as Mean  $\pm$  S.E.M. Statistical analysis of the data was performed on the original data by one-way analysis of variance ANOVA (Bonferroni correction multiple comparison test). Differences at  $P < 0.05$  were considered to be statistically significant.

## RESULTS

### 3.1. Extractions yield using three solvent systems.

The results of extraction process showed that the yield of different extracts obtained from the selected parts of *Prunus cerasus* L. plant using three solvent systems varied according to part of plant. Table-1, shows the details of extract yields, collection time, fresh weight as well as dry weight for three separate solvents systems (methanol, water-methanol 1:1 and water) respectively.

### 3.2. Effect of sample extracts on NBT reduction

Results of NBT reduction potential revealed that incubation of splenic lymphocytes with different extracts of *Prunus cerasus* L at concentrations (10, 30, & 100  $\mu$ g) resulted in significant ( $p < 0.05$ ) rise in bioactivity as compared to control. Of all the tested sample extracts, *Pc*-methanolic fruit extract (*Pc*-MeOH-F-Ext.) showed maximum reduction potential while as the *Pc*-aqueous-seed extract (*Pc*-W-S-Ext.) produced minimum effect (Figure 1).

Table 1. Preparation of (a) Methanol, (b) Hydro-methanol and (c) Aqueous extracts using various parts of sour cherry (*Prunus cerasus* L.) plant.

Plant part	Collection period	Fresh weight	Dry weight	Extractive yield / grams		
				MeOH	Hydro-MeOH	Aqueous
Leaf	July	500 g	170 g	9	7	5.5
Shoot-bark	October	400 g	190 g	16	12.5	10
Root	November	350 g	160 g	15	11	10
Seed	June	400 g	180 g	13	9	8
Fruit	June	3 g	337 g	45	40	33

### 3.3. Effect of sample extracts on iNOS activity

The study revealed that different extracts of *Prunus cerasus* L at concentrations (10, 30, & 100  $\mu$ g) significantly ( $p < 0.001$ ) enhanced the iNOS activity as compared to untreated control. Among all the sample extracts, *Pc*-methanolic fruit extract (*Pc*-MeOH-F-Ext.) showed maximum immune-potential while as the *Pc*-aqueous-seed extract (*Pc*-W-S-Ext.) produced minimum effect (Figure 2).

### 3.4. Effect of sample extracts on phagocytosis.

Like iNOS and NBT parameters, bactericidal activity also followed the same trend. The maximum potential was produced by (*Pc*-MeOH-F-Ext.) and (*Pc*-W-S-Ext.) resulted in least effect as depicted in (Figure 3).

### 3.5. Effect of sample extracts on *In vitro* lymphocyte proliferation by MTT assay.

The effect of various extracts prepared from different parts of *Prunus cerasus* L. on Con-A and LPS-stimulated spleenocyte proliferation is shown in (Table 2) was studied by MTT assay. Different extracts of *Prunus cerasus* caused profound lymphocyte activation and triggered a significant ( $p < 0.01$ ) proliferation of spleenocytes which was observed maximum in Con-A (2.5  $\mu$ g/ml) and LPS (2.5  $\mu$ g/ml) stimulated spleenocyte by *Pc*-MeOH-F-Ext. compared to the control whereas minimum in (*Pc*-W-S-Ext.) stimulated spleenocyte.

Table - 2. Influence of different extracts of P-cerasus plant on proliferation of T and B lymphocytes (*In vitro*). One hundred microlitres (100 µl) of this cell suspension were pipetted in triplicates into each well of a 96 well flat bottom titration plates and 10µg/ml of each test sample. Con A (2.5 µg/ml) to stimulate T-cell mitogenesis or LPS (2.5 µg/ml) to stimulate B cell mitogenesis. The plates were incubated at 37°C in 95% humidity at 5 % CO<sub>2</sub> in a CO<sub>2</sub> incubator for 72 hours. The proliferation was calculated based on MTT assay. Absorbance was recorded at 570 nm

Values are expressed as Mean ± S.E. of three observations.  
(Percent potentiation or suppression as calculated against control)

Treatment Dose 10 µg/ml	% Stimulation(↑) /Suppression(↓) Con A	% Stimulation(↑) /Suppression(↓) LPS
Control	-	-
Bark – Methanol	30.61 ↑	24.90 ↓
Bark - water-methanolic	32.05 ↑	25.90 ↑
Bark – Aqueous	28.88 ↑	17.18 ↓
<b>Fruit - Methanol</b>	<b>48.82</b> ↑	<b>37.00</b> ↑
Fruit - water-methanolic	30.12 ↑	26.09 ↑
Fruit – Aqueous	30.76 ↑	20.36 ↓
Root – Methanol	30% ↑	28.39 ↑
Root - water-methanolic	27.03% ↑	24.11 ↓
Root - Aqueous	29.31% ↑	16.12 ↓
eed- Methanol	6.01 ↓	22.01 ↓
Seed - water-methanolic	5.89 ↓	17.63 ↓
Seed - <b>Aqueous</b>	<b>4.45</b> ↓	<b>14.33</b> ↓
Leaf Methanol	26.21 ↑	57.81 ↑
Leaf - water-methanolic	15.23 ↓	44.03 ↑
Leaf - Aqueous	10.07 ↓	18.10 ↓

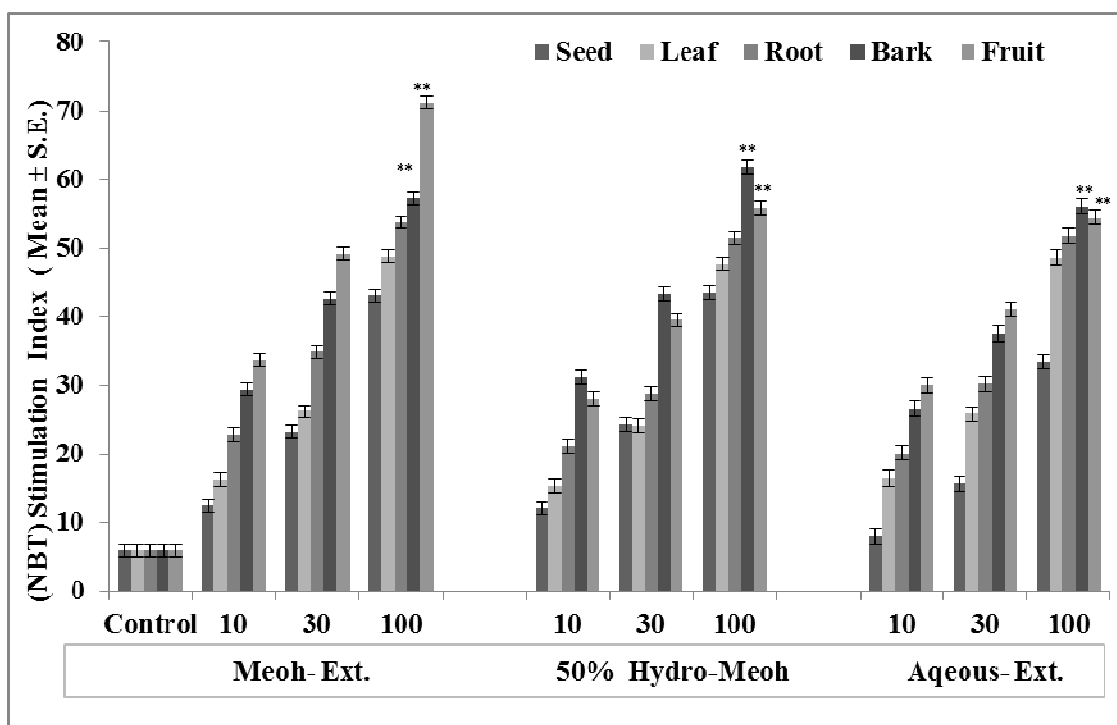


Figure- 1. Influence of MeOH, Hydro-MeOH and Aqueous extracts of Seed, Leaf, Root, Bark and Fruit of *Prunus cerasus* L. at (10, 30 & 100 µg) on function of macrophages evaluated by studying the NBT reduction potential, NBT reduction test, (a measure of respiratory burst in the leucocytes) was carried out by employing

Data are expressed as Mean ± S.E.M. Statistical analysis of the data was performed on the original data by one-way analysis of variance ANOVA (Bonferroni correction multiple comparison test). Differences at P\*\* < 0.05 were considered to be statistically significant.

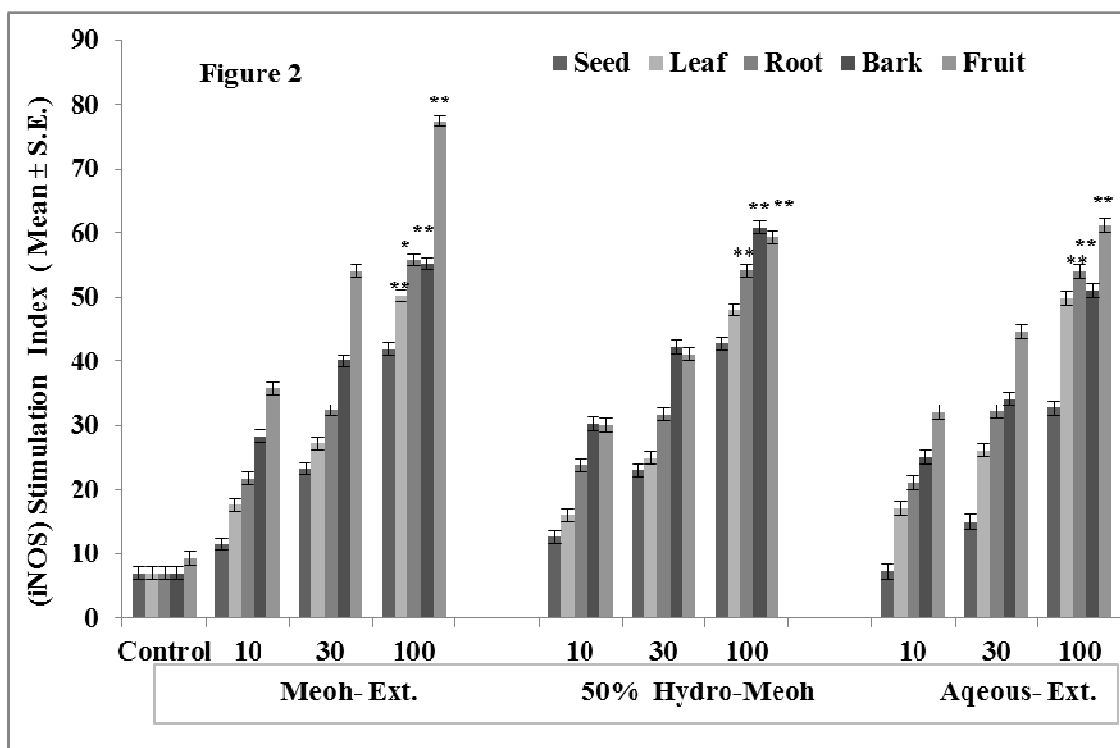
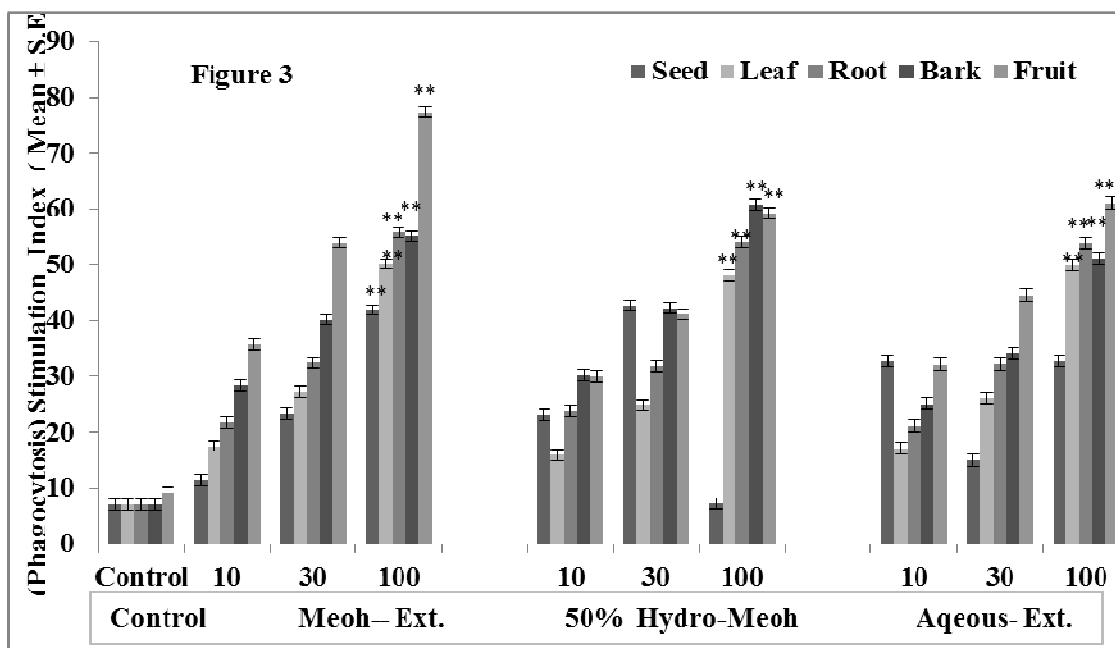


Figure-2. Influence of MeOH, Hydro-MeOH and Aqueous extracts of Seed, Leaf, Root, Bark and Fruit of *Prunus cerasus* L. at (10, 30 & 100 µg) on function of macrophages evaluated by iNOS activity. Inducible nitric oxide synthase activity of lymphocyte suspension was assessed spectrophotometrically using arginine. Briefly the splenocytes were incubated with arginine at 37 °C for 24 hours in CO<sub>2</sub> chamber. The colour developed (indicating citrulline formation from arginine) was measured spectrophotometrically at 540nm against RPMI and Griess reagent as blank

Data are expressed as Mean ± S.E.M. Statistical analysis of the data was performed on the original data by one-way analysis of variance ANOVA (Bonferroni correction multiple comparison test). Differences at  $P^{**} < 0.05$  were considered to be statistically significant.

### 3.6. Determination of total phenols, total anthocyanins and total flavonoids. Contents of the most potent extract (Pc-MeOH-F-Ext.)

A preliminary analysis of total phenol, flavonoid, and anthocyanin contents was spectrophotometrically carried out. The amount of phenolic substances was equal to 137.5 mg of gallic acid equivalents in 100 g of fresh product. Comparison of these data to the apple phenol content (about 77.0-84.0 mg) emphasized the sour cherry phenol wealth. Total flavonoid content was estimated to be equal to 3.3 mg of catechin equivalents in 100 g of fresh product, whereas the amount of anthocyanin substances was 1.2 mg of kouroumanin in 100 g of fresh product.



**Figure-3.** Influence of MeOH, Hydro-MeOH and Aqueous extracts of Seed, Leaf, Root, Bark and Fruit of *Prunus cerasus* L. at (10, 30 & 100 µg) on macrophage bactericidal activity. The macrophage function was evaluated by phagocytosis of microorganism (Bactericidal activity). Briefly, the lymphocyte suspension was incubated with bacterial suspension (*Escherichia coli*) at 37°C for 60 min. Data are expressed as Mean ± S.E.M. Statistical analysis of the data was performed on the original data by one-way analysis of variance ANOVA (Bonferroni correction multiple comparison test). Differences at  $P < 0.05$  were considered to be statistically significant.

## DISCUSSION

The human immune system evolved to protect the host from potentially pathogenic agents, to eliminate neoplastic cells; and to reject non-self-components. It is a complex and highly interactive network of cells and their products which can be modulated by certain agents resulting in immunopotential or immunosuppression, resulting in modulation of diseases [19]. Immunomodulation using fruits and medicinal plants can provide an alternative to conventional chemotherapy for a variety of diseases, especially when host defense mechanism has to be activated under the conditions of impaired immune response or when a selective immunosuppression is desired in situations like autoimmune disorders. There are a number of diseases where immunostimulant drugs are required to overcome the immunosuppression induced by drugs or environmental factors and immunosuppressants are required where there is undesired immunopotential. There is strong requirement of the drugs which can boost immune system to combat the immunosuppressive consequences caused by stress, chronic diseases like tuberculosis, conditions of impaired immune responsiveness (e.g. AIDS) etc. [20]. In the past a number of drugs with plant or mineral origin have been advocated as means of immunomodulation for various diseased conditions in humans. Most of the plants so far reported with immunostimulatory action have major effect on the non-specific arm of immunity especially on macrophage functions. Nutrients and other constituents of fruit and vegetables have the potential to affect almost all aspects of the immune system. The benefits of “superfruits”, a unique group nutrient-rich fruits containing natural compounds are shown to have potential disease-fighting properties. Some of fruits fall in this category and emerging science shows sour cherries (*Prunus cerasus*) are one among them. Although fruit plants have been investigated for varied pharmacologic activities [21, 22], the immunomodulatory potential of *Prunus cerasus* still remains unexplored. This investigation deals with the immunomodulatory screening of different extracts prepared from various parts of *P-cerasus* plant based on the *In vitro* immunological potential using assays like Nitroblue tetrazolium (NBT reduction), inducible Nitric Oxide Synthase (iNOS), Bactericidal activities and on the modulation of T and B-cell proliferation. Total 15 extracts were prepared from various parts of *Prunus cerasus* L. (sour cherry) plant using three different solvent systems and screened for NBT reduction, iNOS, Bactericidal activities and mitogenic potential as well. The sample extracts stimulated macrophage activity (NBT, iNOS & phagocytosis) and augmented Con-A and LPS induced splenocyte proliferation. NBT reduction test is an indirect

marker of the oxygen dependent bactericidal activity of the phagocytes and metabolic activity of granulocytes or monocytes [23].

Present results indicate that most extracts of sour cherry plant are capable of stimulating the immune function of macrophages as evidenced by an increase in NBT reduction but out of all samples, *Pc*-MeOH-F-Ext. showed maximum effect. The functional ability of macrophages was evident from increased expression of iNOS that oxidizes L-arginine to citrulline and nitric oxide. The iNOS activity is correlated to bactericidal activity of macrophages and has been documented as a measure of immunomodulatory potential. The sample extracts produced a significant influence on this parameter also, as seen in case of NBT test, with *Pc*-MeOH-F-Ext. showing the maximum bioactivity.

Macrophages are important cells for the immune system which play an important role in host defense mechanisms for protection from microbial invaders and viral infected cells. When macrophages are stimulated with foreign substances, a variety of cytokines and chemicals are released to induce fundamental defense systems [24]. The augmentation of T and B cells with most of the sample extracts prepared from sour cherry plant may be due to a cytokine-mediated mechanism. The mitogenic influence of different extracts varied according to the plant part and solvent used. The *Pc*-MeOH-f-Ext. elicited significant showing relatively more lymphocyte proliferation potential ( $p < 0.01$ ).

### CONCLUSION

The results obtained suggested that a variation in the bioactivity was observed with respect to the part of the plant and type of solvent extract as well. Out of 15 different extracts screened, the methanolic fruit extract of the *P*-*cerasus* fruit (*Pc*-MeOH-f-Ext.) showed the maximum potency and was found to be the best immune-potent even at lower concentrations. It is clear from this study that *Pc*-MeOH-F-Ext. played an important role in the modulation of the immune response and thus may have applications as an immunomodulatory agent or a drug of choice, effective in treating the diseases where the underlying defect is a T-cell and B-cell deficiency or phagocytic dysfunction. Moreover basic immunomodulatory studies of *Pc*-MeOH-F-Ext. have been demonstrated under *In vitro* conditions, however the sample needs to go through a battery of various other animal and clinical studies.

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