RESEARCH COMMUNICATION

Immunomodulatory and Antitumor Activity of *Biophytum sensitivum* Extract

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Abstract

An alcoholic extract of *Biophytum sensitivum* was studied for its immunomodulatory and antitumor activity. The extract was 100% toxic at a concentration of 0.5 mg/ml to Dalton's lymphoma ascites (DLA) and Ehrlich ascites carcinoma (EAC) cells. *B. sensitivum* extract was also found to be cytotoxic towards L929 cells in culture at a concentration of 0.1 mg/ml. Administration of *B. sensitivum* extract (500μg/dose/animal) could inhibit the solid tumor development in mice induced with DLA cells and increase the lifespan of mice bearing Ehrlich ascites carcinoma tumors by 93.3%. *B. sensitivum* treatment significantly ($p<0.001$) reduced the tumor cell glutathione (GSH) levels as well as serum gamma glutamyl transpeptidase (GGT) and nitric oxide (NO) levels in ascites tumor bearing animals. The total WBC count was also increased to 14,087 cells/mm^3 on the 12th day in BALB/c mice. The number of plaque forming cells also enhanced significantly ($p<0.001$), and bone marrow cellularity and β-esterase positive cells were also increased by the administration of *B. sensitivum* extract.

Key Words: Antitumor - antibody titre - *Biophytum sensitivum* – immunomodulation - plaque forming cells - nitric oxide

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Introduction

One of the main reasons for the rapid progression of human cancers is the ability of tumor cells to escape from the immune surveillance mechanism of the body. Cancer cells may secrete immunosuppressive factors to modify the hosts' immune responses. These factors can suppress immune responses, thereby impairing the inflammatory responses, chemotaxis of macrophages, and the complementary cascade. Some of these factors seem to be non-specific and lead to a generalized decline of immunity.

One of the major drawbacks of the current cancer therapeutic practices such as chemotherapy and radiation therapy is the suppression of immune system (Devasagayam and Sainis, 2002). Side effects produced by both of these conventional therapies of cancer are nausea, vomiting, mucosal ulceration, alopecia, pulmonary fibrosis, cardiac and hepatic toxicity etc. Drugs that could alleviate these side effects will be useful in cancer therapy.

Immunomodulators are substances, which can modify the activity of the immune system. Plant and plant products have been the basis of treatment for human diseases since time immemorial. There are several medicinal plants that are considered to possess immunomodulatory properties. They have shown to augment specific cellular and humoral immune response (Duke, 1985). Some of the plants with known immunomodulatory activity are *Viscum album* (Kuttan and Kuttan, 1992), *Tinospora cordifolia* (Mathew and Kuttan, 1999), *Withania somnifera* (Davis and Kuttan, 2000) and *Piper longum* (Sunila and Kuttan, 2004).

Biophytum sensitivum (L.) DC. (Syn. *Biophytum petersianum* Klotzch), an important medicinal plant is used in traditional medicine by many people in Asia, Africa and Pacific islands especially in Indian medicine (Jirovetz et al., 2004; Inngjerdingen et al., 2006). The reported beneficial effects of *Biophytum sensitivum* include anti-inflammatory (Jachak et al., 1994) and antidiabetic (Puri, 2001) effects. A polysaccharide isolated from *Biophytum sensitivum* has been found to enhance complement fixation (Inngjerdingen et al., 2006). Amentoflavone, one of the constituents of *Biophytum sensitivum* has been shown to inhibit Cyclooxygenase-1 (COX-1) and Cyclooxygenase-2 (COX-2) catalyzed prostaglandin biosynthesis (Bucar et al., 1998). However, no study on the immunomodulatory and antitumor activity of *Biophytum sensitivum* has been reported. In order to verify the anecdotal claims that *Biophytum sensitivum* has numerous phytochemical benefits, we have investigated the immunomodulatory and antitumor activities of this plant.

Materials and Methods

Animals

BALB/c and Swiss albino mice were taken from the Breeding section, Amala Cancer Research Centre, Thrissur.

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The animals were kept in air-controlled room, fed with normal mice chow (Sai Feeds, Bangalore, India) and water *ad libitum*. All the animal experiments were performed according to the rules and regulations of the Animal Ethical Committee, Govt. of India.

Cells

L929 cells were procured from National Facility for Animal Tissue and Cell Culture, Pune, India and maintained in Minimum Eagle's Medium supplemented with 10% fetal calf serum and antibiotics. Ehrlich ascites carcinoma (EAC) cells were procured from Adayar Cancer Institute, Chennai, India. Sheep red blood cells (SRBC) were collected from local slaughterhouse in Alsever's solution.

Chemicals

Minimum Eagle's Medium (MEM) was purchased from Hi-Media, Mumbai, India. Para-Rosaniline and a-napthyl acetate were obtained from Loba Chemie, Mumbai. Harri's hematoxylin was purchased from Glaxo, Mumbai, India. 5'-5' dithiobis (2-nitrobenzoic acid) (DTNB) was purchased from SRL Pvt Ltd. Mumbai. All other chemicals used were of analytical reagent grade.

Drug Preparation

Authenticated *Biophytum sensitivum* obtained from Amala Ayurvedic Centre was dried at 45˚C and powdered. Ten grams was stirred overnight in 70% methanol (100ml), centrifuged at 10,000 rpm at 4˚C for 10min, and supernatant was collected. Methanol was removed by evaporation, and yield was 12% (w/w). The extract was suspended in DMSO for in vitro studies. For animal experiments it was resuspended in 1% gum acacia and administered i.p. at a concentration of 0.5mg/dose/animal.

Determination of the Effect of Biophytum sensitivum on Hematological Parameters

Two groups (6 animals/group) of BALB/c mice were used for the study, of which one group was treated with *Biophytum sensitivum* for 5 days. The remaining group was kept as untreated control. Blood was collected from caudal vein and parameters such as total WBC count (haemocytometer), differential count (Leishman's stain)and hemoglobin level (Cyanmethemoglobin) was recorded prior to the extract administration and continued every third day for 30 days after the administration of the extract.

Determination of the Effect of Biophytum sensitivum on the Organ Weights

BALB/c mice were divided into two groups (6 animals/group).Group I : Normal untreated control, Group II : *B. sensitivum* treated (500µg/dose/animal) for 5 consecutive days. Body weights of the animals were recorded before sacrifice at 24h after the last dose of the drug and weight of the vital organs such as liver, spleen, thymus, lungs and kidneys were recorded and expressed as relative organ weights.

Determination of the Effect of Biophytum sensitivum on the Circulating Antibody Titer

Two groups (6 animals/group) of BALB/c mice were used in this study. Group I : Normal animals immunized with SRBC (0.1 ml, 20%) Group II : *B. sensitivum* ((500_g/dose/animal) for 5 consecutive days + SRBC. SRBC was administered along with the 5^{th} dose of the drug. Blood was collected from the caudal vein every third day after drug administration and continued for a period of 30 days. Serum was separated, heat inactivated at 56^0C for 30 min and used for the estimation of antibody titer (Singh et al., 1984) using SRBC as antigen.

Determination of the Effect of Biophytum sensitivum on the Antibody Producing Cells

BALB/c mice were divided into two groups (6 animals/group) Group I : Normal animals immunized with SRBC (0.1 ml, 20%) Group II : *B. sensitivum* (500_g/dose/animal) for 5 consecutive days + SRBC. SRBC was administered along with the 5th dose of the drug. The animals were sacrificed on different days starting from the third day after immunization up to the 9th day, spleen was processed to single cell suspension and the number of plaque forming cells (PFC) was determined by the Jerne's Plaque assay (Jerne and Nordin, 1963).

Determination of the Effect of Biophytum sensitivum on the Bone Marrow Cellularity and a-Esterase activity

BALB/c mice were dived into two groups (6 animals/group).Group I : Normal untreated control Group II : *B. sensitivum* treated (500µg/dose/animal) for 5 consecutive days. Animals were sacrificed 24h after the drug treatment and the bone marrow cells were collected from the femur and made into single cell suspension and number determined using haemocytometer. Bone marrow cells from the above preparation was smeared on clean glass slides and stained with Harri's Hematoxylin to determine the non-specific a-esterase activity by the azodye coupling method (Bancroft and Cook, 1984).

Determination of Antitumor Activities

The antitumor activities of *Biophytum sensitivum* extract was determined by both in vitro as well as in vivo methods.

Determination of the in vitro Cytotoxic Activity of Biophytum sensitivum to DLA and EAC cells

DLA or EAC cells (1×10^6 cells) were incubated with various concentrations (0.1, 0.25 and 0.5 mg/ml) of *Biophytum sensitivum* extract in a final volume of 1ml for 3h at 37^0C. After incubation the viability of the cells were determined by the trypan blue dye exclusion method (Talwar, 1974).

Determination of the Cytotoxicity of Biophytum sensitivum to L929 cells in culture

Cytotoxicity of the extract of *Biophytum sensitivum* was determined using L929 cells. Cells were seeded in 96-well

flat-bottom plates (5000 cells/well) and allowed to adhere for 24h at 37^0C with 5% CO2 atmosphere. Different concentrations of *Biophytum sensitivum* extract (10-100 mg/ml) were added and incubated further for 48h. Before 4h of the completion of incubation, 20ml of MTT (5mg/ml) was added (Cole, 1986; Campling et al., 1991). Percentage of dead cells was determined using an ELISA plate reader set to record absorbance at 570 nm.

Determination of the effect of Biophytum sensitivum on solid tumor development

Solid tumor was induced by injecting DLA cells (1 x 10^6 cells/animal) subcutaneously to the right hind limbs of two groups (6 animals/group) of Swiss albino mice. Group II was treated with five consecutive doses of *Biophytum sensitivum* extract (0.5mg/dose/animal). The radii of developing tumors were measured using vernier calipers at 3 days intervals for one month and tumor volume was calculated using the formula $V = 4/3\ \pi r1^2\ r2$, where 'r1' and 'r2' represent the major and minor diameter, respectively (Atia and Weiss, 1966). This was compared with untreated control (Group I).

Determination of the effect of Biophytum sensitivum on the survival of ascites tumor bearing animals

Two groups (6 animals/group) of Swiss albino mice were induced ascites tumor by injecting 1 x 10^6 cells/animal to the peritoneal cavity.Group I : EAC alone (Control)Group II : EAC + *B. sensitivum* treated (500_g/dose/animal) for 5 consecutive days. The death pattern of animals due to tumor burden was noted and the percentage of increase in lifespan was calculated using the formula ((T-C)/T) x 100, where 'T' and 'C' represent the number of days that treated and control animals survived, respectively.

Determination of the effect of Biophytum sensitivum on Serum Gamma Glutamyl Transpeptidase (GGT) and Nitric Oxide (NO) levels

Two groups (6 animals/group) of Swiss albino mice were induced ascites tumor by injecting EAC cells (1 x 10^6 cells/animal) to the peritoneal cavity. Group I : EAC alone (Control) Group II : EAC + *B. sensitivum* treated (500_g/dose/animal) for 5 consecutive days. Blood was collected at different time points (5, 10 and 15th day), and the serum was used for the estimation of GGT (Szasz, 1976) and NO (Green et al., 1982).

Determination of the effect of Biophytum sensitivum on Cellular Glutathione (GSH) and Nitric Oxide (NO) levels

BALB/c mice were dived into two groups (6 animals/group). Ascites tumor was induced by injecting EAC cells (1 x 10^6 cells/animal) to the peritoneal cavity. Group I: EAC alone (Control) Group II: EAC + *B. sensitivum* treated (500_g/dose/animal) for 5 consecutive days.EAC cells were collected at different time points (5, 10 and 15^{th} day), and the cells (1 x 10^6 cells/ml) were sonicated for 30 seconds and used for the estimation of GSH (Moron et al., 1979) and NO (Green et al., 1982).

Statistical analysis

Values are expressed as mean ± S.D. The statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett's test. p-values less than 0.05 was considered to be significant.

Results

Effect of Biophytum sensitivum on the Hematological Parameters

Administration of the methanolic extract of *Biophytum sensitivum* increased the total WBC count in normal BALB/c mice (Fig.1). The maximum WBC counts obtained in the *Biophytum sensitivum* treated animals was 14, 087 cells/mm^3 on 12^{th} day after drug administration. There was no appreciable change in the differential count and haemoglobin content after the administration of *Biophytum sensitivum*.

Effect of Biophytum sensitivum on the organ weight

Effect of *Biophytum sensitivum* on the organ weight is given in Table 1. There was an increase in the weight of thymus after the administration of *Biophytum sensitivum* (0.17 ± 0.01 g/100g body wt.) compared to normal (0.12 ± 0.06 g/100g body wt.). The size and weight of spleen was also enhanced significantly by the administration of *Biophytum sensitivum* (0.44 ± 0.01g/100g body wt.) compared to normal (0.38 ± 0.01 g/100g body wt.). The kidney weight was slightly increased by the treatment of *Biophytum sensitivum* (1.8 ± 0.02 g/100g body wt.) compared to normal (1.3 ± 0.08 g/100g body wt.). There was no significant change in the weight of other vital organs such as liver and lung.

Effect of Biophytum sensitivum on the Circulating Antibody Titer

The enhancement of total antibody production by the administration of methanolic extract of *Biophytum sensitivum* is shown in Fig.2. The maximum antibody titer

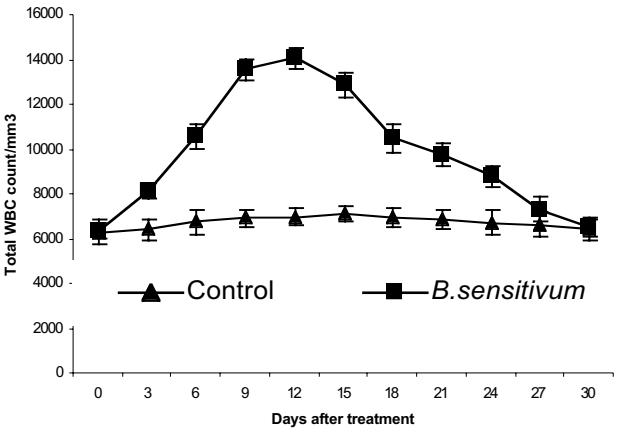

Figure 1. Effect of *Biophytum sensitivum* on total WBC Count

Table 1. Effect of *Biophytum sensitivum* on Relative Organ Weights[a]

Treatment	Relative Organ Weight (g/100g body wt.)				
	Spleen	Thymus	Liver	Kidney	Lungs
Normal	0.38 ± 0.01	0.17 ± 0.01	3.28 ± 0.13	1.3 ± 0.08	0.48 ± 0.04
B. sensitivum	0.44 ± 0.01	0.12 ± 0.06	3.33 ± 0.25	1.8 ± 0.02**	0.50 ± 0.01

[a]Animals were treated with five doses of *B. sensitivum* (500_g/dose/animal)** p<0.001

Table 2. Effect of *Biophytum sensitivum* on Bone Marrow Cellularity and β-Esterase Activity[a]

Treatment	Bone marrow cellularity (Cells/femur)	β-Esterase activity (No. of β-esterase positive cells /4000 cells)
Normal	$17.3 \times 10^6 \pm 0.28$	905 ± 35
B. sensitivum	$28.3 \times 10^6 \pm 0.85$**	1421 ± 52**

[a]Animals were treated with five doses of *B. sensitivum* (500 μg/dose/animal).** p<0.001

value of 256 was observed on 12th day in *Biophytum sensitivum* treated animals. The control animals showed the maximum antibody titer value of only 128 on the 12th day.

Effect of Biophytum sensitivum on Plaque Forming Cells (PFC) in Spleen

The effect of *Biophytum sensitivum* on the number of Plaque Forming Cells (PFC) is shown in Fig.3. The maximum number of plaque forming cells in the *Biophytum sensitivum* treated group (295 PFC/10^6 spleen cells) was observed on the 6th day while control animals had a maximum of 161.67 PFC/10^6 spleen cells.

Effect of Biophytum sensitivum on the Bone Marrow cellularity and a-Esterase Positive Cells

The effect of *Biophytum sensitivum* on the bone marrow cellularity and a-esterase positive cells is given in Table 2. Administration of the methanolic extract of *Biophytum sensitivum* showed a significant (p<0.001) enhancement in the bone marrow cellularity (28.3×10^6 cells/femur) compared to the normal control (17.3×10^6 cells /femur) animals.

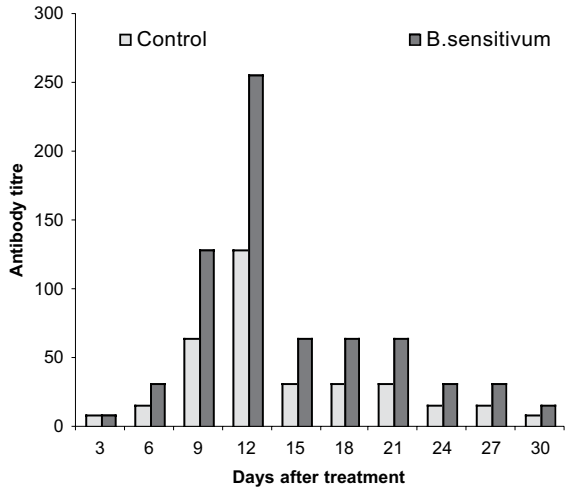

Figure 2. Effects of *Biophytum sensitivum* on Antibody Titre

Moreover the number of a-esterase positive cells was also found to be increased significantly (p<0.001) in the *Biophytum sensitivum* treated animals (1421 cells/4000 bone marrow cells) compared to the normal animals (905 cells/4000 bone marrow cells).

Cytotoxicity of Biophytum sensitivum towards DLA and EAC Cells

Methanolic extract of *Biophytum sensitivum* was found to be toxic at a concentration of 0.5 mg/ml to DLA and EAC cells. *Biophytum sensitivum* at a concentration of 0.25 and 0.1 mg/ml produced 45% and 12% cytotoxicity to DLA cells and 55% and 14% cytotoxicity to EAC respectively (Table 3).

Cytotoxicity of Biophytum sensitivum towards L929 cells in culture

Extract of *Biophytum sensitivum* was found to be cytotoxic towards L929 cells in culture. Methanolic extract of *Biophytum sensitivum* was found to be 100% toxic at a concentration of 0.1mg/ml (Table 4).

Effect of Biophytum sensitivum on Solid Tumor Development

There was a significant reduction of tumor volume in *Biophytum sensitivum* treated animals (Fig. 4). Tumor volume of control animals was 2.32 mm3 on 30th day while that of *Biophytum sensitivum* treated animals was only 0.338 mm^3, on the same day.

Effect of Biophytum sensitivum on the Survival of Ascites Tumor Bearing Animals

Life span of ascites tumor bearing mice treated with methanolic extract of *Biophytum sensitivum* was found to be significantly (p<0.001) increased (Table 5). Control animals survived only 15 days after the tumor induction

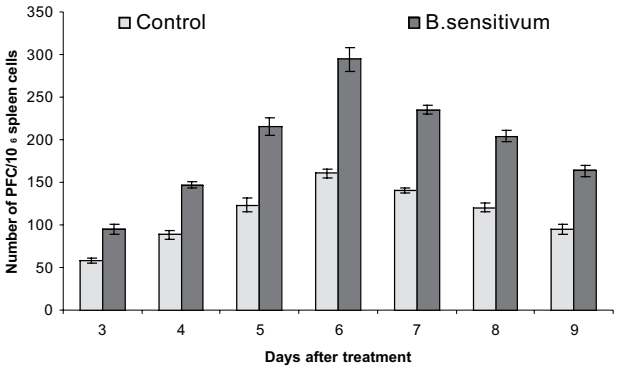

Figure 3. Effects of *Biophytum sensitivum* on Plaque Forming Cells

Table 3. Cytotoxicity of *Biophytum sensitivum* to Dalton's Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) Cells

Concentration (mg/ml)	Percentage cytotoxicity	
	DLA	EAC
0.1	12	14
0.25	45	55
0.5	100	100

DLA or EAC (10^6 cells) were incubated with different concentrations (0.1-0.5 mg/ml) of extract. Percentage of dead cells was determined by trypan blue exclusion method.

Table 4. Cytotoxicity of *Biophytum sensitivum* Extract to L929 cells in Culture

Concentration (_g/ml)	Percentage cytotoxicity
10	0
50	52
100	100

L929 cells were incubated with different concentrations (10-100μg/ml) of alcoholic extract. Percentage of cytotoxicity was determined using MTT assay.

Table 5. Effects of *B. sensitivum* on the Lifespan of Ascites Tumor-bearing Animals

Treatment	Mean survival day	Percentage of increase in life span (%ILS)
Control	15 ± 1.6	—
B. sensitivum	29 ± 2.6**	93.3

Table 6. Effects of *Biophytum sensitivum* on the Serum GGT and NO Levels of EAC-Bearing Animals

Treatment	Days	GGT (nmol p-nitroaniline/ml)	NO (μM)
Normal		-	24.3 ± 0.2
Control	5	43.6 ± 3.2	21.9 ± 1.2
	10	128.0 ± 5.0	25.4 ± 1.1
	15	151.8 ± 11.4	33.5 ± 4.2
Treated	5	27.7 ± 3.2**	18.3 ± 0.7**
	10	39.6 ± 2.8**	21.1 ± 1.6**
	15	48.8 ± 10.6**	22.2 ± 1.6**

The serum was collected from tail vein on 5, 10 and 15th day after tumor and assayed for biochemical parameters. Values are mean ± S.D.** p<0.001

Table 7. Effects of *Biophytum sensitivum* on the Cellular GSH and NO Levels of EAC cells at Different Stages of Tumor Growth in vivo

Treatment	Days	GSH (nmol /mg protein)	NO (μM)
Control	5	6.0 ± 0.6	7.3 ± 1.0
	10	13.2 ± 0.5	10.8 ± 0.6
	15	11.8 ± 2.0	12.0 ± 0.8
Treated	5	5.1 ± 1.0	5.6 ± 0.6*
	10	7.4 ± 0.4**	7.6 ± 0.3**
	15	7.8 ± 0.5**	7.6 ± 0.4**

EAC cells were collected on 5, 10 and 15th day after tumor challenge, sonicated and assayed for biochemical parameters. Values are mean ± S.D.* p< 0.01, ** p<0.001

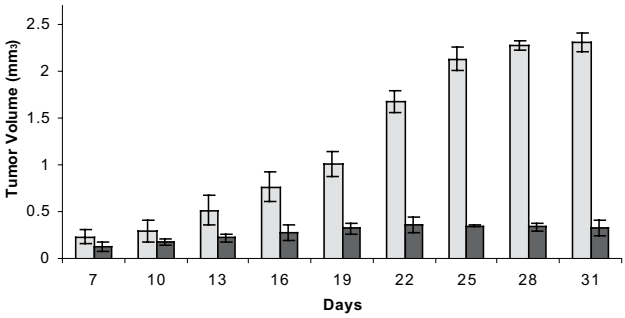

Figure 4. Effects of *Biophytum sensitivum* on Solid Tumor Reduction

while the *Biophytum sensitivum* treated animals survived up to 29 days with an increase in life span of 93.3%.

Effect of Biophytum sensitivum on Serum Gamma Glutamyl Transpeptidase (GGT) and Nitric Oxide (NO) levels

The effect of *Biophytum sensitivum* on the serum GGT and NO is presented in Table 6. On the 15th day after tumor challenge, elevated level of GGT in the serum of control tumor bearing animals (151.8 ± 11.4 nmol p-nitroaniline/ml serum) as compared to normal animals (24.3 ± 0.2 nmol p-nitroaniline/ml serum) was significantly ($p<0.001$) reduced to 48.8 ± 10.6 nmol p-nitroaniline/ml serum after the administration of *Biophytum sensitivum*. The serum NO level was found to be maximum (33.5 ± 4.2 _M) on the 15th day of tumor progression, where as the NO level in the BS treated group was only 22.2 ± 1.6 _M on the same day.

Effect of Biophytum sensitivum on Cellular GSH and NO

The effect of *Biophytum sensitivum* on cellular GSH and NO levels during various stages of tumor progression is shown in Table 7. The GSH content in EAC cells was found to be maximum (13.2 ± 0.5 nmol/mg protein) on the 10th day of tumor growth. A slight decrease in GSH level (11.8 ± 2.0 nmol/mg protein) was noted on the 15th day of tumor growth. In *Biophytum sensitivum* treated animals the cellular GSH level was found to be 5.1 ± 1.0, 7.4 ± 0.4 and 7.8 ± 0.5 nmol/mg protein on 5,10 and 15th day respectively.

The nitric oxide level in the EAC cells was found to be increasing gradually and was found to be maximum (12.0 ± 0.8 μM) on the 15th day of tumor progression. In *Biophytum sensitivum* treated animals the NO level was found to be 7.6 ± 0.4 μM on the 15th day of tumor progression.

Discussion

Immunoregulation is a complex balance between regulatory and effector cell and any imbalance in the immunological mechanism may lead to pathogenesis (Steven et al., 1985). Immunity has been shown to be suppressed in cancer. Chemotherapy and radiation therapy, useful in cancer treatment were found to deteriorate the immunity. Our laboratory has reported earlier that an extract from a plant *Viscum album* (Kuttan and Kuttan, 1992) could stimulate the immunity in normal and tumor bearing animals. Similar results have also been reported using herbal

immunostimulatory preparations, Rasayanas (Praveen kumar et al., 1994), which are used in the indigenous system of medicine.

In the present study, the immunomodulatory and antitumor activity of *B. sensitivum*, an important plant in indigenous medicinal practice was explored. Administration of *B. sensitivum* was found to increase total WBC and bone marrow cells significantly indicating that the extract could stimulate the haematopoetic system. Moreover there was increased presence of _-esterase positive bonemarrow cells indicating that extract treatment could also enhance the differentiation of stem cells. *B. sensitivum* extract was found to increase the circulating antibody titre and antibody forming cells indicating its stimulatory effect on the humoral arm of immune system. Moreover, the extract was found to stimulate the weight of spleen and thymus indicating that *B. sensitivum* stimulated the production of immune cells. Administration of *B. sensitivum* could also significantly inhibit the growth of solid tumor induced by DLA cells and ascites tumor induced by EAC cells.

GSH, a major non-protein thiol required for the proliferation and metabolism of tumor cells was reduced after the administration of *B. sensitivum*. Administration of *B. sensitivum* reduced the serum gamma glutamyl transpeptidase (GGT), an enzyme that catalyzes the transfer of gamma glutamyl moieties from glutathione to other aminoacids and dipeptides (Meister and Tate, 1976). Moreover, administration of *B. sensitivum* was found to inhibit nitric oxide production in tumor cells.

B. sensitivum has been shown to contain biflavones and flavanoids (Lin and Yang, 2003). At present we do not know whether these compounds are responsible for the immunostimulatory and antitumor activity produced by this extract. Further studies using isolated compounds are in progress.

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