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RESEARCH ARTICLE

Evaluation and comparison of immunomodulatory potential of natural and *in vitro* cultivated high altitude medicinal plant *Eupatorium cannabinum* L.

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ABSTRACT

The increase in incidence of immunosuppression diseases demands alternative ways to combat the infections. Keeping this in view, the present study was undertaken to explore one of the plants *i.e. Eupatorium cannabinum* from our natural resources for its immunomodulatory potential. Animals treated with aqueous extracts prepared from native, *in vitro* shoot organ cultures and callus cultures were immunized with SRBC and their blood samples and spleen were collected to carry out tests for cell mediated and humoral immune responses *viz*., CRLB cell enumeration, nitroblue tetrazolium reduction (NBT), inducible nitric oxide synthase (iNOS), phagocytic activity and development of anti SRBC antibody titres. Results revealed that shoot organ cultures possessed the maximum immunomodulatory activity.

Key words : *Eupatorium cannabinum,* Immunomodulation, Plant tissue culture

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INTRODUCTION

India has rich biodiversity and well developed traditional system of medicine which exploit herbal medicines. The natural products, especially the plants used in the traditional system of medicine for curing of diseases by using powdered mixture of a variety of plant materials roots, stems, leaves, flowers etc. are potential source of immunomodulatory compounds. Though in recent years a number of plants have been screened for their immunomodulatory activity but the plants of high altitude area still remain unexplored.Presently with increase in allergies and side effects of chemicals, drugs and lack of medicines for some deadly diseases,and increase in the incidence of immunological disorders due to immunosuppressive diseases like AIDS, drug resistant, malaria, tuberculosis, cancer, gastrointestinal infections it becomes more important to pay attention to this field and develop new immunomodulators which are cost effective easily available and without side effects, have a wider range of curing and can be employed as anti immunosuppressants or immunopotentiators.

Keeping in view the above problem the present study was designed to study the immunomodulatory potential of *Eupatoriumcannabinum* L. (Asteraceae), a common wild plant growing in high altitude areas of Asia and Europe.This plant is used as a source of alternative medicine and febrifuge. Herbalists recognize its wound healing, cathartic, diuretic and anti-scorbutic properties and consider it a good remedy for purifying the blood, either used by itself, or in combination with other herbs. The leaves contain a volatile oil, which acts on the kidneys, and likewise some tannin and a bitter chemical principle which will cut short the chill of intermittent fever. Its chemical constituents have been investigated and pharmacological studies, which deals with the choleretic and hepatoprotective properties have been demonstrated in the

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rat (Lexa *et al.,* 1989). Some of the important chemical constituents present in *Eupatorium cannabinum* are - caffeic acid, chlorogenic acid, hyperin, L-Malic acid, quercetin, rutin and eupatoriopicrin.

The aim of the present study is to evaluate the immunomodulatory potential of both natural and *in vitro* raised plants of *Eupatorium cannabinum*on various humoral and cell mediated parameters. The comparison of the natural and *in vitro* raised plants is being carried out to explore the possibility of exploiting the tissue culture raised plants of high altitude area for evaluating their medicinal value. In our earlier studies we have evaluated the immunomodulatory potential of different extracts of *Eupatorium cannabinum* prepared by soxhlet extraction in different solvents *i.e.* hexane, chloroform and ethanol, the water extracts were prepared separately. The results from these four extracts revealed that the water extracts gave the maximum immunomodulating activity, therefore the aqueous extract was selected for the comparison with tissue culture raised plants. The *in vitro* raised plants has an advantage that these can be used further for producing novel compounds and increasing the yield of the bioactive constituents.

MATERIALS AND METHODS

Plant material:

Plants were collected from the high altitude area ~ 2200 m (approx.) from Darjeeling, West Bengal, India.

Devolopment of tissue culture raised plants:

The sterilized shoot tips and internodes (1.5-2.5 cm) were cultured under laminar flow for initiation of shoot organ cultures and callus in Murashige and Skoog (MS) medium supplemented with various plant growth regulators(IAA, IBA, NAA, 2,4-D, BAP, Kn) in different combinations. The cultures were maintained at 25±2ºC temperature under a 16h/8h darkness cycle.

Preparation of plant extract:

Shade dried leaves were powdered and dried powder was suspended in distilled water in a flask. Suspension was stirred for 24hrs on the shaker and filtered. Finally filtrate was filtered through 0.45µm Millipore filter and stored at 4º C.

Animals:

Swiss albino mice 6-8 weeks old weighing 20-25 g obtained from Disease free Animal House, Hissar and maintained on standard laboratory diet and water were used for the study.

Inoculation of the extract:

Six to eight weeks old swiss albino mice were inoculated with ten non-lethal equal doses of extracts over a span of 30 days. Each dose was of the volume 0.2ml so that each mouse received a total dose of 2ml and the dose inoculated was 800 mg /kg body weight.

Group of animals:

Animals were divided into following four groups:

Group I(n =10): Control to group II, III and IV(distilled water treated animals)

Group II (n=10):Animals treated with aqueous extract of native *Eupatorium cannabinum*

GroupIII(n=10):Animal streated with aqueous extract of *in vitro* shoot organ culture of *Eupatorium cannabinum*

Group IV (n=10): Animalstreated with aqueous extract of callus extract of *Eupatorium cannabinum*

Immunization:Animals were immunized with single dose of 1 x 10^8 sheep red blood cells (SRBC) after 6-7 doses of extract

Follow up of the study:

Animals were bled on 5th day of SRBC inoculation to check humoral response and their spleen was collected to study the cell mediated immunological parameters. The sacrificed mice were dissected from ventral side and their spleen was taken out, it was perfused with MEM and then teased to get splenocytes. Volume of splenocytes suspension was noted and cells were enumerated using haemocytometer.

Assessment of immune status of the animals:

Following parameters were used to assess the immune status of the animals-

CRLB cell enumeration, nitroblue tetrazolium reduction (NBT), inducible nitric oxide synthase (iNOS), phagocytic activity and development of anti SRBC antibody titres.

Complement receptor bearing B-lymphocytes (CRLB):

CRLB were enumerated by the rossette method givenin the manual by Bhatia (2001). Briefly, SRBCs collected in Alsever's solution were suspended in MEM to a concentration of 5 per cent (E) and incubated with equal volume of amboceptor (A) at 37ºC (EA).Incubated equal volume of EA with complement (C) *i.e.*, 10 per cent fresh mouse serum to give EAC. EAC suspension was incubated with lymphocytes (2x10^6cells/ml). The rossetting and non rossetting lymphocytes were counted and per cent CRLB was calculated as follows:

$$\text{Perc ent of CRLB-lymphocytes} = \frac{\text{Rosetting lymphocytes}}{\text{Rosetting lymphocytes + Non-rosetting lymphocytes}} \times 100$$

Nitro blue tetrazolium reduction test (NBT):

NBTreduction was carried out by the method given in Hudson and Hay (1989). Briefly,splenocyte suspension from each mouse was incubated in the presence (test) or absence of NBT. The formazon formed was extracted in dioxan and the

absorbance was measured at 520nm using dioxan as blank.

Calculation:

$$\text{NBT reduction} = \frac{\text{O.D. in presence of NBT} - \text{O.D. in absence of NBT}}{\text{O.D. absence of NBT}}$$

Bactericidal activity:

Bactericidal activity test was carried out by the methods given by Raghuramulu *et al*. (1983). Briefly, the splenocytes and bacterial suspension were mixed in 1:2 ratio and incubated at 37^{0}C for 60 min. Then added 100 µl sterile distilled water to above suspension. 100 µl of above suspension was spread on nutrient agar plates using a spreader. The plates were incubated at 37^{0}C for 24 hr. and the colonies were counted the next day. Plated bacterial suspension was used as control.

Determination of iNOS activity in macrophage:

The iNOS activity was measured using the method of Stuehr and Marletta (1987) using Greiss reagents splenocytes were diluted to 1× 10^6 cells/ ml in MEM and 800 ul of this was taken in test tubes in duplicate. Only 800µl of MEM was taken in test tube marked control and to each test tube marked test 200µl of aqueous extracts were added and incubated at 37^{0}C for 2 hr. in 5 per cent CO_2 atmosphere. Then tubes were inverted on filter paper and to the adherent cells were added 1 ml fresh MEM and 20µl of 0.2M arginine solution. Incubated at 37^{0}C for24 hrs in 5 per cent CO_2 atmosphere.To the supernatant added 1 ml of freshly prepared Griess reagent and kept the tubes in dark for 10 min. O.D. was measured at 540 nm against control.

Anti SRBC antibody direct haemagglutination test:

Thedevelopment of anti SRBC antibody was studied by direct haemagglutination testgiven by Bhatia (2001). Serial dilutions of serum samples were incubated with SRBC for 2 hr in microtitre plates. The wells were noticed for mat formation. The wells showing mat formation were positive for agglutination; wells showing button formation were negative for agglutination. The last well which showed positive agglutination was recorded as the abtitre of the serum

RESULTS AND DISCUSSION

The findings of the study have been discussed in detail as under:

Plant tissue culture :

The optimum medium for the shoot organ culture was found to be the MS medium supplemented with BAP 1.0mg/l and IAA 1.0 mg/l and for raising callus cultures the optimum medium was MS medium supplemented with Kn 1.0 mg/l and NAA 1.0 mg/l (Table 1).

Immunological assays:

The results of various immunological assays carried out are given in Table 2. Values of all the parameters studied in both the humoral as well as cell mediated were more in the treated animals as compared to those in control animals. The spleen weight increased invariably in all the treated groups. There was no difference in CRLB observed in Group I, II, III and IV. However, the antibody titre were significantly higher in all the treated groups as compared to 1:64 in the control group. The anti-SRBC antibody titre were 1:1024, *i.e.* 16 times more in animals treated with natural and *in vitro* raised shoot organ cultures extracts and it was 1:128, *i.e.* 2 times more in callus culture extracts. The weight of spleen was found to be higher in all the treated groups, in comparison to control. The weight of spleen was 53.84 per cent higher in the animals treated with

Table 1 : Morphogenetic responses of nodal explants of *Eupatorium cannabinum* to various media treatments

Sr. No.	BM[a] + PGR mg/l	No. of shoots/ culture[b] ± S.E	Average length[b] in cm ± S.E.	Other morphogenetic responses
1.	Basal	1±0	3.98±0.97	Very few roots present
2.	NAA 1.0	1.5±0.34	3.3±0.90	Callus at base and few roots present
3.	2,4-D 1.0	-	-	Compact light brown callus
4.	IAA 1.0	1.5±0.42	7.41±0.82	Roots present
5.	BAP 1.0	3.66±1.28	1.41±0.15	No roots
6.	Kn 1.0	3.33±0.71	2.03±0.29	No roots
7.	BAP 1.0 + NAA 1.0	5.5±1.47	2.6±0.60	Swelling at base, no roots
8.	BAP 1.0 + 2,4-D 1.0	-	-	Compact light brown callus
9.	BAP 1.0 + IAA 1.0	7.66±1.59	3.06±0.33	Swelling at base, no roots
10.	Kn 1.0 + NAA 1.0	1.83±0.54	5.8±1.68	Callus at base and few roots present
11.	Kn1.0 +2, 4-D 1.0	-	-	Compact light brown callus
12.	Kn. 1.0 + IAA 1.0	2.5±0.34	7.75±1.62	Callus at base

a) BM[a] : All the treatments contained Murashige and Skoog (MS) basal salts with 3% (w/v) sucrose

b) [b] : Data recorded after eight weeks and represents an average of six replicates/ treatment with 2 parallel experiments

Table 2 : A comparison of immune status of animals

Test	Control Group I	Natural Group II	Shoot organ culture Group III	Callus cultures Group IV
Weight of spleen	0.13g	0.20g (53.84%)	0.19g (46.15%)	0.16g (23.07%)
NBT reduction	12.86%	24.78%	34.67%	14%
iNO's	.752	.821 (9.17%)	.900 (19.68%)	.896 (19.14%)
Phagocytosis	-	7.8%	19.2%	22%
CRLB	29%	27%	29%	28%
Antibody titre	1:64 (x)	1:1024 (16x)	1:1024 (16x)	1:128 (2x)

( ) =Per cent increase as compared to control
(x) =Number of times more than control

natural extracts, 46.15 per cent higher in shoot organ culture extract treated animals and 23.07 per cent higher in animals treated with callus extracts.

Similar to humoral immune response the per cent NBT, the iNO'S expression and the phagocytosis were significantly higher in all the three treated groups (II, III and IV) as compared to the control group. The NBT reduction was 12.86 per cent in control animals, 24.78 per cent in animals treated with natural plant extracts, 34.67 per cent in animals treated with shoot organ culture extracts and 14 per cent in animals treated with extracts of callus cultures. In comparison to control the iNO's activity was found to be 9.17 per cent higher in animals treated with natural plant extracts, 19.68 per cent in animals treated with shoot organ culture extracts and 19.14 per cent in animals treated with extracts of callus cultures. groups. In comparison to control animals the phagocytic activity was found to be 7.8 per cent higher in animals treated with natural plant extracts, 19.2 per cent higher in animals treated with shoot organ culture extracts and 22 per cent higher in animals treated with extracts of callus cultures.

The comparison of alteration in the immunomodulatory activity by natural and *in vitro* cultured plants revealed that maximum increase in these parameters was in group III, *i.e.* the group where animals were treated with extracts from the *in vitro* shoot organ cultures (Table 2) of *Eupatorium cannabinum.*

Effects of aqueous extracts of natural, shoot organ cultures and callus cultures of *Eupatorium cannabinum* have been studied on the numerical values and function of various immunocytes *i.e.* T cells, B cell and macrophage in albino mice using B cell enumeration, NBT, LAI, iNOS, bactericidal activity, haemagglutination tests.

Results revealed that both the*in vitro* and *in vivo* plantextracts of *Eupatorium cannabinum* modulate the immune functions of the immunocytes. Results of all the tests except B-cell enumeration showed an immunopotentiating effect of the extracts of *Eupatorium cannabinum*. None of the extracts showed immunosuppressive activity. Out of three extracts the maximum bioactivity was in shoot organ tissue culture extract. NBT, iNOS, bactericidal tests indicate the function of phagocytes. The development of anti SRBCantibody shows T dependent B cell function. In our results the increase in NBT, iNOS, per cent bactericidal activity in treated groups as compared to control showed the positive modulation of functions of macrophages, T as well as B cells. A similar potentiation / stimulation of macrophage for their function were reported earlier by Wagner *et al.* (1991) by using extracts of *Echinacea angustifolia, Batisiatintlors and Arnica monotrapa.* NBT reduction and iNOS production tests revealed that extracts could stimulate macrophages or phagocytes for their functions. A study in mice by Upadhayay *et al.* (1992) indicated selective activation of the cell mediated immune mechanism by neem oil.

Hence our results reveal that *Eupatorium cannabinum*extract especially from *in vitro* raised plants could be a potent stimulant and modulator of functions of immunocytes especially macrophage and hence can be employed in pharmaceutical industry to develop immunopotentiators.

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