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Research Article

EVALUATION OF *IN-VITRO* ANTI-PROLIFERATIVE ACTIVITY AND *IN-VIVO* IMMUNOMODULATORY ACTIVITY OF *BETA VULGARIS*

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ABSTRACT

The present study was carried out to evaluate the *in-vitro* anti-proliferative activity and *in-vivo* immunomodulatory activity of *Beta vulgaris* against MCF7 breast cancer cell line. Different concentrations of the methanolic extracts of roots of the plant were subjected to anti-proliferative study against MCF7 breast cancer cell lines by Trypan blue dye exclusion technique and MTT Assay. *In-vitro* immunomodulatory activity was studied by assessment of Humoral antibody response and Delayed type hypersensitivity response. The maximum tumor cell growth inhibition was observed with 1000 mcg/ml. The immunomodulatory effect of the extract on specific antibody production at these concentrations was comparable to the effect of Levamisol (2 mg/kg) as a positive control at both primary (10.9±0.4) and secondary (11.6±0.4) response. The methanolic extract of *Beta vulgaris* inhibited tumor cell growth *in-vitro* and showed immunomodulatory effects *in-vivo*.

Keywords:

INTRODUCTION

Beta vulgaris, also known as red beet (Family: Chenopodiaceae), is a small sized plant, cultivated in many parts of India. It is popularly known as 'chukandar' or 'beet root', is an erect annual herb with tuberous root stocks. It is native to Mediterranean region and widely cultivated in America, Europe and throughout India[1]. The leaves of *Beta vulgaris* possess diuretic, purgative and anti-inflammatory activity, seeds known to possess expectorant and carminative properties, roots possess sedative and emenogogue effects[2]. It is also used as a natural food colour in dairy and meat products[3]. The presence of phytochemicals such as betalains i.e., betacyanins (red-violet pigments) and betaxanthines (yellow pigments), flavonoids, polyphenols, vitamins and minerals has been shown in roots[4]. Different beet root compounds, e.g., betalains, became especially important for phytomedicine: betalains (betacyanins and betaxanthis) have been detected only in red-violet-, orange-and yellow-pigmented botanical species belonging to closely related families of the order caryophyllalles[4]. Betalain pigments have specifically been shown to possess various antioxidant functions[5]. The betalaine group contains about 50 red pigments and 20 yellow pigments. Betanine accounts for 75-90 % of total betacyanin content and betaxanthnin comprises vulgaxanthine I and vulgaxanthine II.

Breast cancer is one of the main life-threatening diseases that a woman may have to face during her lifetime[6]. The increasing incidence of breast neoplasia reported over the last a few decades has led to development of new anticancer drugs, drug combinations, and chemotherapy strategies by methodical and scientific exploration of enormous pool of synthetic, biological, and natural products[7]. In light of the continuing need for effective anticancer agents, and the association of fruit and vegetable consumption with reduced cancer risk, edible plants are increasingly being considered as sources of anticancer drugs[8]; there is a large amount of scientific evidence showing that fruits and vegetables lower the risk of cancer[9], and medicinal plants constitute the main source of new pharmaceuticals and healthcare products, including medications for ethnoveterinary medicine[10]. Recently, cancer chemoprevention with strategies using foods and medicinal herbs has been regarded as one of the most visible fields for cancer control[11]. However, whether fruit, vegetable, and antioxidant micronutrient consumption is associated with a reduction in breast cancer incidence remains unresolved[12]. Most of the anti-tumor drugs currently used in chemotherapy are toxic to normal cells and cause toxicity for immune cells. Therefore, the identification of new anti-cancer drug with low side effects on immune system or which boosts it has become an essential goal in many studies of immunopharmacology[13]. In the present study, we investigated the *in vitro* anti-cancer effect and *in vivo* immunomodulatory activities of methanolic *Beta vulgaris* extract.

MATERIAL AND METHODS

Collection of plant material and preparation of extract

The fresh roots of *Beta vulgaris* were collected from the outskirts of the city, Bhubaneswar and authenticated by the taxonomist, Dr. P.K.Sahoo, Professor, Department of Botany, Utkal University. Initially these roots were washed with fresh water to remove adhering dirt and foreign particles. Excess of water was shaken off and dried at 35 - 40°C in an oven for 24 hours. The dried roots were crushed, ground and weighed. It was stored in an air-tight, hard polyethylene container with silica pouch up to 10-12 days. One kg of powdered plant (40 mesh size) was extracted by cold percolation with 3 liters of 70% methanol in a percolator for 72 h, at room temperature. The residue was removed by filtration. The solvent was then evaporated to dryness, under reduced pressure, in a rotary evaporator at 42-45°C. The percent yield of *Beta vulgaris* roots extract is 23.26%. The concentrated methanol extract was kept in a dessicator for further use.

Animals

Swiss albino mice (6 to 8 weeks old) were purchased from Jena Broiler Rabbit Farm (Cuttack, Odisha). They were housed in microlon boxes in a controlled environment (temperature 25±2 °C) and 12 hr dark/light cycle) with standard laboratory diet and water *ad libitum*. The animals were acclimatized to the laboratory conditions for a week prior to the experimentation and randomly divided into six groups of each six animals. Principles of animal handling were strictly adhered to the guidelines and handling of animals was made under the supervision of animal ethics committee of the institute. The experimental protocol was approved by Institutional Animal Ethical Committee (IAEC) of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals).

IAEC Reference Number-(990/UDPS/2005)

Cell line and Culture

Human breast cancer MCF-7 cell line was obtained from Sigma-Aldrich, Bangalore. The cells were maintained in RPMI-1640 supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37 °C.

Estimation of *In-vitro* Anti-proliferative activity

Trypan Blue Exclusion Assay

Trypan Blue is a blue acid dye that has two azo chromophores group. Trypan blue will not enter into the cell wall of plant cells grown in

culture. Trypan Blue is an essential dye, use in estimating the number of viable cells present in a population.

The Methanolic extract was studied for short term *in-vitro* cytotoxicity using MCF-7 cell line. 10mg of the extract was taken in an Eppeandorf vial of capacity 1ml and dilute to six different concentrations with its duplicate and control (50%) using DMSO as a solvent and mixed with the help of a vortexing machine. The cell viability was checked by trypan blue dye (1%) [14,15].The cell suspension ($1x10^6$ cells in 0.1ml) was added to tubes containing various concentrations of the test compounds and standard. The volume was made up to1ml using phosphate buffered saline (PBS). The Control tube contained only cell suspension. These assay mixtures were incubated for 3 hour at 37^0C. After incubation 0.1 ml trypan blue was added and number of dead cells determined by using haemocytometer. The percent viability was calculated by using formula:

% Viability = (Live cell count/Total cell count)*100

Micro culture tetrazolium (MTT) assay

Cell viability was assessed by MTT assay (Micro culture tetrazolium/formazan assay) in the presence and absence of different concentrations of the plants extract. The assay detects the reduction of MTT by mitochondrial dehydrogenase to blue formazan product, which reflects the normal function of mitochondria and cell viability[16]. The cells were seeded in 96-well plates. Four wells for each concentration were seeded and triplicate plates were used the cell line. Then, the cells were incubated at 37°C. After 24 h the medium was replaced by fresh medium containing different concentrations of the plants extract and standard drug Levamisole. The control groups received DMSO. Then, the medium was changed by fresh medium containing MTT ([3-(4, 5-dimethylthiazol-2-yl)-2, 4-diphenyltetrazolium bromide]) with a final concentration of 0.5 mg/ml (after 24 h). The cells were incubated for another 4 h in a humidified atmosphere at 37°C and after that the medium containing MTT was removed and remaining MTT formazan crystals were dissolved in DMSO. The absorbance was measured at 570 nm immediately using an ELISA reader. IC_{50} was defined as the concentration of the extract that produced a 50% decrease in cell viability relative to the negative control which was wells exposed to the solvent without any extract[17,18].

Estimation of *In-vivo* Immunomodulatory activity

Humoral antibody synthesis

Animals (Five groups of six mice each) were immunized intraperitoneally (i.p.) with $5x10^9$ SRBC on days 0 and +7. In three groups, different doses of extract (1, 50 and 100 mg/kg of *B. vulgaris* extract) administrated on days -2, -1, 1 and 2 immunization. The mice in the fourth group were injected with Levamisol as positive control on the same day (2 mg/kg, i.p.). The fifth group was considered as non-treated control and injected only with equal amount of the vehicle. Blood samples were obtained from each mouse on day +7 for evaluating primary response and on day +14 for secondary response. Antibody titer was determined by hemagglutination test[19, 20]. 25 µl of 0.1% SRBC suspension was added to 25 µl of two-fold diluted serum samples in V-shape micro-titration plates. After 1 h of incubation, the last dilution of serum samples which caused hemagglutination was considered as antibody titer. To compare the results the mean Log2 of the titers was then calculated.

Delayed type hypersensitivity response

Sheep red blood cell (SRBC) was used as antigen for delayed type hypersensitivity reaction. SRBC collected in Alsever's solution, were washed three times in large volumes of pyrogen free 0.9% normal saline and standardized to 5×10^9 cells/ml for injection. Mice were divided into four groups, each group containing five mice. Different concentrations of the *B. vulgaris* extract immunized intraperitoneally in three groups at days -2, -1, 0, 1 and 2. The vehicle was injected at the same days in group four as the control. Mice were immunized subcutaneously by injecting (10^8 SRBC/100 µl) on day 0. The mice were then challenged by injection of SRBC suspension in right hind foot pad at day 7. The thickness of the right hind foot pad was measured using vernier caliper after 24 h[21].

STATISTICAL ANALYSIS

The results are presented as the means ± SD of at least three separate experiments. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Tukey Kramer multiple comparison test to express the difference among the groups. All analyses were performed using SPSS software16. P values < 0.001 were considered as highly significant and <0.05 were considered significant.

RESULTS

***Beta vulgaris* inhibited tumor cell growth.**

Percentage cell viability of cell lines was carried out by using Trypan blue dye Exclusion technique (Table 1). The *Beta vulgaris* extract exhibited growth inhibitory effect on MCF-7 cell line under experimental conditions in 72 h treatment. *In vitro* exposures of MCF-7 cells with various concentrations of *Beta vulgaris* extract (62.5, 125, 250, 500, 1000 Mcg/ml) significantly suppressed MCF-7 cancer cell growth in a dose-dependent manner (P<0.001). The maximum inhibition of MCF-7 cells due to exposure to *Beta vulgaris* was found at 1000 Mcg/ml of the extract (99.69% inhibition, Table-1, Figure 1). The results show dose dependent response against MCF-7. The cytotoxic activity may be due to the presence of flavonoids and betains present in the roots of *Beta vulgaris*.

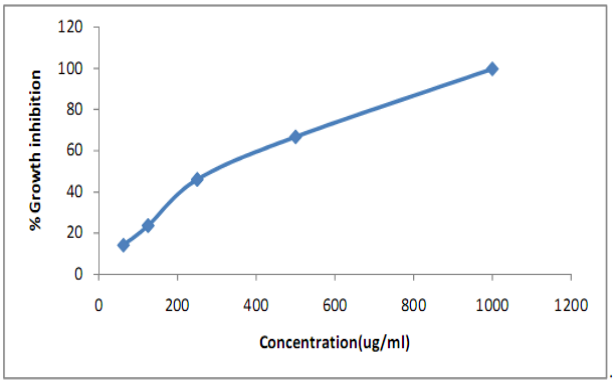

Figure 1: Percentage growth inhibition of methanolic extract of B*eta vulgaris* against MCF-7 cell line.

Table 1: Determination of Cytotoxicity

Plant extract	Conc (mcg/ml)	Absorbance	% inhibition	IC_{50} (mcg/ml)	R^2
Beta vulgaris	62.5	0.477	15.25		
	125	0.392	24.69		
	250	0.289	44.05	272.9	0.9628
	500	0.182	67.73		
	1000	0.00121	99.69		
	Standard	0.00021	99.91		
	Control	0.528			

Effect of *Beta vulgaris* on antibody response

The effect of the *Beta vulgaris* extract on specific antibody synthesis is showed in Figure 2. The mean antibody titer for 50 mg/kg of the extract was 9.6±0.4 versus 8.1±0.4 in non-treated mice at primary response and 10.8 ± 0.8 versus 8.4 ± 0.5 at secondary response ($p<0.001$). Moreover, the mean antibody titer for 100 mg/kg of the extract was 10.3±0.5 versus 8.1±0.4 in non- treated mice at primary response and 11.7 ± 0.6 versus 8.4 ± 0.5 at secondary response ($p<0.001$). The immunomodulatory effect of the extract on specific antibody production at these concentrations was comparable to the effect of Levamisol (2 mg/kg) as a positive control at both primary (10.9±0.4) and secondary (11.6±0.4) response.

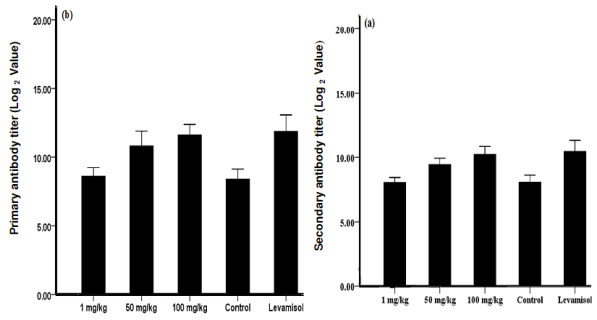

Figure2:Effect of different doses of methanolic extract of *Beta vulgaris* on antibody synthesis in mice (b) primary antibody titer and (a) secondary antibody titer.

Effect of *Beta vulgaris* on delayed type hypersensitivity response

Mice immunized with SRBC as antigen for delayed hypersensitivity reaction. The mean footpad thickness of all mice groups treated with 1, 50 and 100 mg/kg of the *Beta vulgaris* extract at 24 h after immunization of extract-treated mice with SRBC was measured. The mean footpad thickness of the mice groups treated with *Beta vulgaris* extract had not significantly any change compared to non-treated mice.

DISCUSSION

In the present study, the immunomodulatory effects of *Beta vulgaris* extract on immune response and tumor cell growth inhibition were investigated. *Beta vulgaris* is a traditional herb in India and has been used to treat as antioxidant, in diabetes, hypertensives etc. Beet roots have also been reported to be rich in antioxidant compounds[18]. Its juice has been found to counteract the xenibiotic-oxidative stress in rats by rejuvenating the activity of the majority of antioxidant enzymes in liver[22]. The active constituents present in Beet root, like betacyanins and betaxanthines are free radical scavengers and prevent active oxygen-induced and free radical-mediated oxidation of biological molecules[23]. The high content of folic acid amounting to 15.8 mg/g dry matter is another nutritional feature of the beets which may account for its anti-proliferative and immunomodulatory activity[24]. In this study, the methanolic extract of *Beta vulgaris* was studied for its probable anti-proliferative activity against MCF-7 cell line which is a kind of human breast cancer cell line and immunomodulatory effects *in vivo*. According to our results which were seen *in-vivo*, *Beta vulgaris* extract has potential immunomodulatory effect for specific humoral response to SRBC. This extract had not significant effect on delayed hyper-sensitivity reaction in mice. Finding of the present study showed stimulatory effect of *Beta vulgaris* on humoral immunity in mice. However, no anti-cancer effect of this species has ever been reported. In the past few years, a number of Indian herbal medicines with potent anti-proliferative activity were reported, such as *Dionysia termeana, Linum persicum and Euphorbia cheiradenia* [25,26,27]. *Beta vulgaris* is a promising anti-tumor herb whose mechanism of action is mostly unclear. Inhibition of proliferation has been a continuous effort in tumor treatment. Suppression in cell growth and induction of cell death are two major means to inhibit cancer growth[28]. In this study, we showed that methanolic extract of *Beta vulgaris* could cause significant growth inhibition of MCF-7 cell line in a dose-dependent manner. The methanolic extract of *Beta vulgaris* inhibited tumor cell growth *in-vitro* and showed immunomodulatory effects *in-vivo*. In conclusion, the enhancement of antibody synthesis and MCF-7 growth inhibition indicated that extract contains bioactive components that stimulate immune response and have anti-tumor effect. However, further studies are needed for their elucidation.

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