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SCREENING OF BEETROOT JUICE FOR ANTICANCER ACTIVITY

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ABSTRACT

Purpose: To assess the anticancer activity of juice of Beta vulgaris Linn by estimating antioxidant potential by DPPH and Nitric oxide scavenging assay, followed by effect on cell proliferation by MTT assay, tumor volume, survival time, change in body weight, hematological parameters and antioxidant enzyme in Ehrlich Ascites Carcinoma induced mice. **Method:** Antioxidant activity was determined, by measuring the reducing power of juice of Beta vulgaris by spectrometric DPPH scavenging assay followed by Nitric oxide scavenging activity using sodium nitroprusside as a NO donor. The cytotoxicity was evaluated by MTT assay. Swiss albino mice of 20±5 g of either sex were used for in vivo study. Animals were divided into 5 groups (n=12). Experimental tumor was induced by inoculation of $1x10^6$ Ehrlich ascites carcinoma (EAC) cell. Group-I (control) animals received vehicle, Group II was EAC control, Group III received 5-fluorouracil (20 mg/kg b.w,i.p.), Group IV, V received Beta vulgaris (200 and,400 ml/kg b.w.p,o), for 9 days. Post 9th day treatment, 6 mice were sacrificed, hematological parameters and antioxidant enzymes were estimated, remaining were utilized for analysis of body weight, survival time, tumor volume. **RESULTS:** Beta vulgaris showed DPPH and Nitric oxide scavenging activity, cytotoxic activity on EAC cell lineand reduced body weight, tumor volume and increased survival time, decreased WBC count, increased RBC count, Hb content and antioxidant enzymes. **CONCLUSION:** Beta vulgaris exhibit significant anticancer activity.

KEYWORDS: Beetroot, Antitumor activity, Ehrlich ascites carcinoma.

INTRODUCTION

Cancer, also known as a malignant tumor or malignant neoplasm, is a group of diseases characterized by abnormal proliferation of cell with the potential to invade other parts of the body through circulation.Cancer is caused by external factors, such as tobacco, infectious organisms and an unhealthy diet and internal factors, such as inherited genetic mutations, hormones and immune conditions. These factors may act together or in sequence to cause cancer.[1]

Treatment for cancer is frequently an assault on the immune system. The side effect of conventional anticancer modalities, whether through the process of radiation or chemical treatment is a general weakening of the body's immune system resulting in high toxicity such a bone marrow suppression, alopecia, nausea and vomiting that can significantly increase patient's risk for infection.Medicines derived from plants play pivotal role in health care. Ayurveda, Indian system of medicine mainly used plant based drugs or formulation to treat various ailments including cancer.[2]

Beetroot (Beta vulgaris L) is vegetable plant and belongs to family Amaranthaceae. It is grown widely in Germany and France and in lesser amount in other European countries, Africa, Asia and South America. It is grown all over the world in temperate areas. Betanine is the main component of red colorant extracted from Beta vulgaris. Besides Betanine, another pigment which is extracted from beetroot is Vulgaxanthine.[3] Beta Vulgaris extract (root) possess antihypertensive, hypoglycemic, anti-inflammatory, hepato protective activities and antioxidant which can contributes to the anticancer activity.[4]

The present study is taken up to evaluate anticancer activity of beetroot juice by in-vitro and in-vivo models. This will enable us to suggest increasing the frequency of it in diet for prophylactic/curative purpose in adjuvant to drugs in cancer patients.

METHODOLOGY

Betavulgaris L was obtained from the Bangalore local market, authenticated, cleaned, washed and ground and squeezed to get the juice of 20 mg/ml w/v concentration. Healthy adult Swiss albino mice weighing 25±5 g were used for present study. They were maintained under standard environmental conditions and were fed with standard pellet diet and water. Antioxidant activity of sample was estimated by DPPH and Nitric oxide scavenging activity.

DPPH scavenging assay

Various concentration of sample solutions were mixed with 1 mL of DPPH solution. Ascorbic acid was used as standard. The mixture was shaken vigorously and left to stand for 30 min in the dark. The reduction of DPPH radical was determined by measuring the absorbance at 517nm, against blank without sample. Scavenging percentage of different concentration of the samples were determined by following formula:[5]

$$\% \text{ scavenging} = \frac{\text{Control (OD)} - \text{Test (OD)}}{\text{Control (OD)}} \times 100$$

Nitric oxide scavenging assay

1ml of Sodium nitroprusside solution of 10mM was mixed with sample and standard in phosphate buffer (pH7.4). The mixture was incubated at 25ºC for 150 min. To incubated solution, Griess reagent was added. The absorbance was recorded at 546nm by UV visible spectrophotometer against blank without sample. Scavenging percentage of different concentrations of sample was determined by following[6]

$$\% \text{ scavenging} = \frac{\text{Control (OD)} - \text{Test (OD)}}{\text{Control (OD)}} \times 100$$

MTT assay[7]

In vitro cytotoxic activity was carried out using trypan blue cytotoxic assay.Ehrlich Ascites Carcinoma (EAC) cells were obtained from Amla Cancer Research Center, Trissur, Kerala, India.Cells were harvested by centrifugation. Adherent cells were released from their substrate by trypsinization. Cells resuspended at $1x10^6$ per ml. Dilutions of cells were prepared in culture medium from $1x10^6$ to $1x10^3$ cells per ml.100 µg of dilutions into wells of a microtiter plate were plated out, in triplicate. Three control wells of medium alone were included to provide the blank for absorbance reading. Cells were incubated under conditions appropriate for the cell line for 6-48 hours. 10µl of MTT reagent was added to each well, included control. Plate was returned to cell culture incubator for 2 to 4 hours. The cells were viewed periodically under the microscope and added 100µl of detergent reagent to all wells, included control. Plates were left with cover in the dark for 2 to 4 hours at room temperature. Plates cover were removed and absorbance was measured in each wells at 570nm in microtiter reader. Cell survival was determined by following formula:

$$(\text{Absorbance of treated group} / \text{Absorbance of control}) \times 10$$

In vivo study

Animals were divided into 5 groups (n=12). Experimental tumor was induced by inoculation of $1x10^6$ Ehrlich ascites carcinoma (EAC) cell. Group-I (control) animals received vehicle, Group II was EAC control, Group III received 5-fluorouracil (20 mg/kg b.w,i.p), Group IV, V received Beta vulgaris (200 and,400 ml/kg b.w.p,o), for 9 days. Post 9^{th} day treatment, 6 mice were sacrificed, hematological parameters and antioxidant enzymes were estimated, remaining were utilized for analysis of body weight, survival time, tumor volume.

Determination of survival time[8]

At termination surviving EAC tumour bearing mice were counted and Mean Survival Time (MST) and percentage Increase in Life Span (%ILS) were calculated by the formula.

%ILS= (MST of treated group – MST of control group)/MST of control group ×100

Body weight analysis:[8]

All mice were weighed on the day of tumour inoculation and weekly intervals. Average gain in body weight and percentage decrease in body weight was calculated by the formula.

$$\%\text{decrease in body weight} = \frac{(\text{gain in body weight of control} - \text{gain in body weight of treated}) \times 100}{\text{Gain in body weight of control}}$$

Estimation of total ascites fluid[9]

Ascitic fluid of EAC tumor bearing mice was collected on 9^{th} day in graduated centrifuge tube. The total volume of fluid was measured.

Hematological parameters[8]

Blood was drawn from each mouse by the retro orbital plexus method and the white blood cell (WBC), red blood cell (RBC) and hemoglobin were determined.

Measurement of lipid peroxidation[10]

Lipid peroxidation in the liver was estimatedcolorimetrically by thiobarbituric acid reactive substances. The reaction of tissue homogenate with thiobarbituric acid were extracted with n-butanol and absorbance was estimated at 532 nm by U V visible spectrophotometer against an appropriate blank without the sample.

Measurement of glutathione level[11]

Supernatant layer of centrifuged liver homogenate was treated with Ellman's reagent (4 mg of DTNB in 100 ml of 0.2 m phosphate buffer with 1% sodium citrate) and phosphate buffer (3.56gms of Na_2HPO_4 in 100 ml deionized water, 2.76 gm of NaH_2PO_4**.** $2H_20$ in 100 ml deionized water. These two solutions was taken in the equal proportion and mixed).After completion of the total reaction, solutions were measured at 412 nm by U V visible spectrophotometeragainst blank.

Measurement of catalase activity[12]

Catalase activity was determined spectrophotometrically by the method of Aebi et al (1984). Tissue homogenate supernatant layer was added to cuvette containing phosphate buffer (pH 7.0). Reaction was started by the addition of freshly prepared H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically at 240 nm.

$$\text{Catalase activity} = \frac{\text{absorbance}}{0.0436} \times \frac{\text{total volume (ml)}}{\text{sample volume (ml)}} \times 1 \times 10(\mu\text{mol/min/g liver})$$

Measurement of superoxide dismutase activity[12]

Assay mixture contained tissue sample, Sodium pyrophosphate buffer (pH 8.3, 0.052 M), Phenazine methosulphate (186 µM), 300 µM Nitroblue tetrazolium, NADH (750 µM). Reaction was started by addition of NADH. After incubation at 30°C for 90 s, the reaction was stopped by the addition of glacial acetic acid. Reaction mixture was stirred vigorously with n-butanol.Mixture was allowed to stand for 10 min, centrifuge and butanol layer was separated. Color intensity of the chromogen in the butanol layer was measured at 560 nm spectrophotometrically. Following formula was used for measuring SOD activity.

$$\text{Percentage inhibition} = \frac{\text{absorbance of the test}}{\text{Absorbance of the control}} \times 10$$

SOD activity (units/g Liver) = % of inhibition/50 × dilution factor × 10

[50% inhibition = 1 U]

Statistical analysis

The results were expressed as mean± S.E.M (n=6). The statistical analysis involving 7 groups was performed by means of analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison test.

RESULTS

As shown in table 1, free radical scavenging activity by beetroot juice was less in lower concentration and more in higher concentration which is comparable with standard ascorbic acid. Nitric oxide scavenging activity by beetroot juice was dose dependent and is comparable with standard ascorbic acid (table 1).

TABLE 1: Free radical scavenging activity by beetroot juice by DPPH and Nitric oxide scavenging assay

Concentration µg/ml	%inhibition by Standard Ascorbic acid by DPPH assay	%inhibition by Beetroot by DPPH assay	%inhibition by Standard Ascorbic acid by Nitric oxide scavenging assay	%inhibition by Beetroot by Nitric oxide scavenging assay
05	19.60%	16.66%	13.77%	8.98%
10	41.17%	27.45%	23.35%	18.56%
25	55.80%	40.19%	39.52%	31.13%
50	68.62%	52.94%	50.89%	43.71%
100	82.35%	68.62%	64.07%	56.88%

Values are mean ± S.E.M, one way ANOVA followed by Dunnett's multiple test.

Table 2 shows percentage of cell viability of EAC cells in presence of beetroot juice, which was least at highest concentration of beetroot juice and increased with decrease in concentration.

TABLE 2: Cytotoxic activity of beetroot juice by MTT assay

Dilution no.	Concentration µg	% viability of EAC in presence of Beetroot juice
1	500	2.13%
2	250	4.86%
3	125	23.36%
4	62.5	36.90%
5	31.25	40.56%
6	15.62	65.07%
7	7.81	68.94%
8	3.90	73.85%
9	1.953	82.42%

Values are mean ± S.E.M, one way ANOVA followed by Dunnett's multiple test

Figure 1 shows the effect of beetroot juice on tumor volume, mean survival time and change in body weight. In EAC induced cancerous mice tumor volume and body weight was increased and mean survival time was decreased. The pretreatment with beetroot juice

reversed this in dose dependent manner. The results shown in 400ml/kg beetroot juice treated mice is statistically highly significant and is comparable with the results of standard 5-fluoruracil treatment.

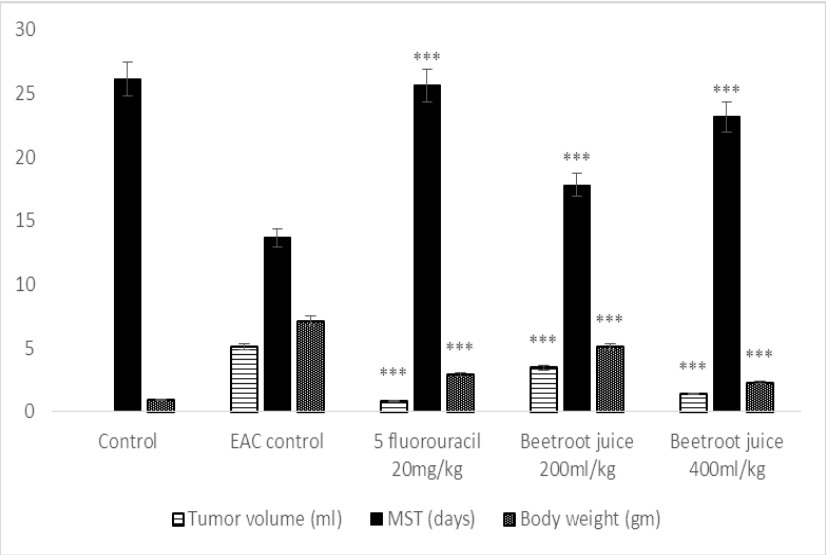

Fig 1: Effect of beetroot juice on Tumor volume, Mean survival time and change in body weight.

n=6, values are represented as mean ± SEM, and ***P<0.001 V/S EAC control.

Table 3 reveals the effect of beetroot juice on hematological parameters on 10th day of treatment. The total WBC count was found to be increased in EAC control group when compared with normal control. Beetroot juice has significantly decreased the number of WBC. RBC count and Hb content was decreased in EAC induced animals, which was increased significantly in animal treated with beetroot juice.

TABLE 3: Effect of beetroot on hematological parameters

Treatment	Hb (gm %)	RBC ($\times10^{-6}$/ml)	WBC($\times10^{-3}$/ml)
Control	15.61 ± 0.12	5.35 ± 0.02	6.5 ± 0.006
EAC control	7.29 ± 0.02	2.14 ± 0.02	18.57 ± 0.09
5 fluorouracil 20mg/kg	13.55±0.14***	5.19 ± 0.16***	6.12 ± 0.02***
Beetroot juice 200ml/kg	9.38 ±0.03***	3.88 ± 0.04***	13.47 ± 0.06***
Beetroot juice 400ml/kg	10.52±0.03***	4.55 ± 0.15***	8.32 ± 0.03***

n=6, values are represented as mean ± SEM. ***P<0.001 V/S EAC control.

As shown in figure 2, LPO level is increased and SOD, GSH, CAT decreased in EAC induced cancerous animals. All antioxidant enzymes increased significantly and LPO level decreased in beetroot juice treated animals, which is comparable with results of 5-fluorouracil treated animals.

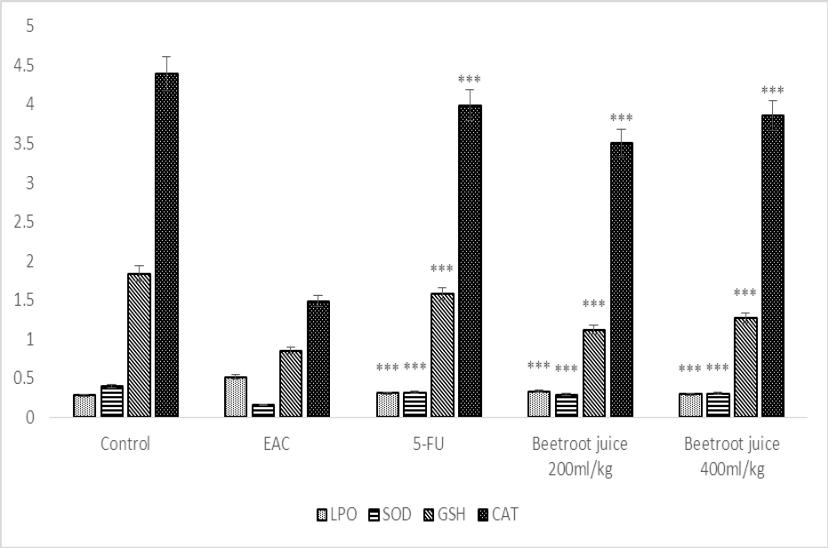

Fig 2: Effect of beetroot juice on antioxidant enzyme activity

n=6, values are represented as mean ± SEM, and ***P<0.001 V/S EAC control.

DISCUSSION

Cancer is often associated with increased risk of death and the toxic side effects caused by the modern medicine. Many cancer patients seek alternative and complementary methods of treatment such as usage of phytomedicine. Natural dietary agents have drawn a great deal of attention because of their potential to suppress cancers and to reduce risk of cancer development by decreasing oxidative stress, which plays a significant role in the pathogenesis and pathophysiological process of cancer.[13] Studies have shown that Beetroot (Beta vulgaris) has an excellent antioxidant property which can contributes to the anticancer activity.[14,15]

The in vitro cytotoxic study on Beetroot juice against EAC cells indicates cytotoxicity increased in higher concentration of beetroot juice. Previous phytochemical studies of Beta vulgaris indicate presence of phenolic groups, flavonoids, betaxanthins and betacyanins.[15] Polyphenolic compounds might inhibit cancer cells by xenobiotic metabolizing enzymes that alter metabolic activation of potential carcinogens, while some flavonoids could also alter hormone production and inhibit aromatase to prevent the cancer cells. The mechanism of

action of anticancer activity of phenols could be by disturbing the cellular division during mitosis at the telophase stage. It was also reported that phenols reduce the amount of cellular protein and mitotic index and colony formation during cell proliferation of cancer cells.[9]

Present study indicates, percentage (%) scavenging of DDPH free radical by beetroot juice. The effect of antioxidants on DPPH radical scavenging is due to their hydrogen donating ability. DPPH is a stable free radical and accept an electron or hydrogen radical to become stable diamagnetic molecule. Beetroot juice possess considerable amount of phenolic compound, flavonoids and anthocyanin and betaxanthins which may contribute to its free radical scavenging activity.[15]

Nitric oxide is an essential bio regulatory molecule required for several physiological processes like neural signal transmission, immune response, control vasodilation and control of blood pressure.[16] However the elevation of NO results in several pathological condition including cancer. The present study shows percentage inhibition of NO by beetroot juice in concentration dependent manner, which can be due to presence of flavonoids and phenolic compounds present in beetroot.

Results of present study show a significant ($P<0.001$) decrease in tumor volume and body weight in all EAC induced animal treated with beetroot juice. Ascitic tumor implantation promotes local inflammatory reactions leading to increase in vascular permeability and results in intense edema formation, cellular migration and progressive ascites fluid formation, which is essential for tumor growth since it provides direct nutritional source for tumor cells. The reduced tumor volume in EAC induced mice treated with beetroot juice suggest that plant might have exerted a delay in vascular permeability to the cells.[9] The effect of beetroot juice on tumor volume might be due to the phytoconstituents and flavonoids present in Beta vulgaris.

The study also reveals significant increase in mean survival time of EAC induced mice treated with beetroot juice. The reliable criteria for evaluating the potential of any anti-cancer agent is life span of animal. The previous phytochemical analysis of Beta vulgaris reported the presence of flavonoids.[15,16] Flavonoids and phenolic compounds were reported to possess antioxidant as well as free radical scavenging property. Thus increase in life span can be attribute to antioxidant activity.

Anemia and myelosuppression have been frequently observed in ascites carcinoma. Anemia encountered in ascites carcinoma due to iron deficiency either by hemolytic or myelopathic condition, finally lead to reduced RBC count.[9] Hematological parameters of tumor bearing mice on 10th day was significantly altered compared to untreated group. The total WBC count was found to be increased with reduction of Hb content and RBC. Treatment with beetroot juice brought back the hemoglobin content (Hb), RBC and WBC count more or less to normal level. This indicates that phytoconstituents present in Beta vulgaris possess hemopoietic activity without inducing myelotoxicity, which is most common side effect of cancer therapy.

Lipid peroxidation mediated by free radicals is considered as a primary mechanism of cell membrane destruction and cell damage. Malondialdehyde (MDA) is formed during oxidative degeneration as a product of free oxygen radicals, which is accepted as an indicator of lipid peroxidation. MDA, the end product of lipid peroxidation, was reported to be higher in cancer tissues than in non-diseased organ. The present study indicates Beta vulgaris phytoconstituents are able to decrease significantly MDA level in liver tissue.

Superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) are present in all oxygen metabolizing cells. GSH plays an important role in the endogenous antioxidant system. It is found in particularly high concentration in the liver and is known to have key function in the protective process and clearance of superoxide and hydrogen peroxide and excesses of free radicals in oxidative stress which leads to damage to macromolecules. It was also reported that the presences of tumors in human body or experimental animal is known to affect many functions of the vital organs especially liver. The SOD, CAT and GSH level was decreased in EAC bearing mice due to the liver damage. The inhibition of GSH, SOD and CAT activity results in tumor growth.[10,16] The administration of beetroot juice increased significantly CAT, GSH and SODantioxidant which can be due to free radical scavenging property of phytoconstituents present in the Beta vulgaris.

CONCLUSION

Based on the results of experimental studies and discussion thereafter, it can be concluded that beetroot has anticancer activity.

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