Research Article

Amelioration of Skin cancer in mice by β-carotene and Phenolics of Carrot (*Daucus carota*)

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ABSTRACT

Carrot (*Daucus carota*) is a widely used nutritional source and is rich in β-carotene. Our previous work highlights the potential anticancer properties of phenolics from various dietary sources. The current study is designed to understand the relative levels of phenolics and β-carotene and their contribution to antioxidant (AOX), tyrosinase inhibitory (TI) and antiproliferative (AP) properties in UV-DMBA induced skin cancer in mice. Phenolic fractions of carrot - free (CRFP) and bound (CRBP) and β-carotene were extracted and quantitated by HPLC. AOX, TI and AP capacities of each of the phenolic acids of CRFP / CRBP and β-carotene were determined. DMBA followed by UV treatment was employed to induce skin cancer in mice. Different doses of CRFP, CRBP and β-carotene were evaluated for anticancer potency using tumor index, biochemical parameters and tumor markers in various groups of animals. Although same levels of β-carotene were present in CRFP and CRBP, a higher reduction in tumor formation (~ 2 folds), tyrosinase (~5 folds), galectin-3 (~18 folds) and increased antioxidant levels (~1-3 folds) in CRFP rather than in CRBP suggests that, in addition to β-carotene, the nature of other phenolic acids in CRFP do play a key role in anticancer property. Thus carrot with enriched levels of phenolics and β-carotene may be efficient in the prevention of skin cancer as evidenced by *in vitro* and *in vivo*.

KEYWORDS: β-carotene, deferoxamine, galectin-3, skin cancer, tyrosinase, UV-DMBA.

ABBREVIATIONS: DMBA, 7, 12-dimethylbenz (a) anthracene; CAT, catalase; SOD, superoxide dismutase; GSH, glutathione; SGPT, serum glutamate pyruvate transaminase; SGOT, serum glutamate oxaloacetate transaminase; SALP, serum alkaline phosphatase; TBARS, thiobarbituric acid reactive substances.

1. INTRODUCTION

Skin cancer and melanomas are on raise due to environmental change where liberated UV radiations are more deleterious while dysregulated tyrosinase levels have been attributed to enormous perturbation in tyrosinase levels, a key player in the control of melanocytes growth and function. Melanomas, in addition to causing cancer specific difficulties, limit the patient to avail chemotherapeutic treatments due to chemo resistance properties of melanomas [1]. It is thus warranted to identify and validate the use of alternative sources for tyrosinase inhibition in addition to inhibition of various steps of melanoma pathogenicity. Recently papers published from our laboratory have revealed that phenolic acid pools present in greater abundance in carrot and other dietary sources appear to contribute significantly to the antiulcer, anti-*H.pylori*, anti-proliferative and anticancer properties. They have been reported to possess antioxidant activity that can scavenge both reactive oxygen species and electrophiles, to inhibit nitrosation and to chelate metal ions to modulate cellular enzyme activities [2]. In addition, phenolic acids are not only powerful antioxidants they also have been reported to demonstrate antibacterial, antiviral, anti-carcinogenic, anti-inflammatory and vasodilatory actions.

Carrot has been ranked sixth in per capita consumption among 22 popular vegetables [3] worldwide. Traditional uses indicate that carrot is one of the major sources of provitamin A, providing 17% of the total vitamin A that can contribute to antioxidant status, in addition to β-carotenes [4] that are known to function as anticancer agents. Apart from this, carrot has been identified as a potent nutritional/nutraceutical source, since they contain phytochemicals like, glutathione, calcium, potassium and vitamins that act as antioxidants.

Despite the availability of a plethora of information on carrot from cosmoceutical world, the role of phenolics and phenolic acids of carrot in contributing to skin protection had to be clarified in order to explore their knowledge of skin care to skin cancer. The current study therefore focuses on the careful evaluation of the profile of phenolics and phenolic acids and their contribution to protection against skin cancer induced by UV-DMBA in experimental mice model - in comparison with the already known components of carrot β-carotene. Our studies also addressed the role of both free (CRFP) and bound (CRBP) phenolic fractions of carrot in inhibiting cancer and metastasis on UV-DMBA induced skin cancer, in *in vitro* and *in vivo* models.

2. MATERIALS AND METHODS

2.1 Chemicals

Agarose, calf thymus DNA, gallic, tannic, caffeic, p-coumaric, ferulic, gentisic, protocatechuic, syringic and vanillic acids, butylatedhydroxyanisole (BHA), 2-thiobarbituric acid (TBA), 1,1-diphenyl-2-

picryl hydrazyl (DPPH) and DMBA were obtained from Sigma (St. Louis, MO). Folin-Ciocalteu reagent, ferric chloride, trichloroacetic acid, sodium carbonate, ferrous sulphate and ascorbic acid were purchased from Qualigens Fine Chemicals (Mumbai, India). HPLC grade solvents employed for HPLC analysis were obtained from Spectrochem Bio chemicals (Mumbai, India).

2.2 Plant material

Fresh carrot (*Daucus carota*) was purchased from the local market (Devaraja market, Mysuru, Karnataka, India). The Carrots (10 kg) were cleaned, washed under running tap water, diced, air dried, powdered for particle size of 20 meshes, and isolated into free and bound phenolics.

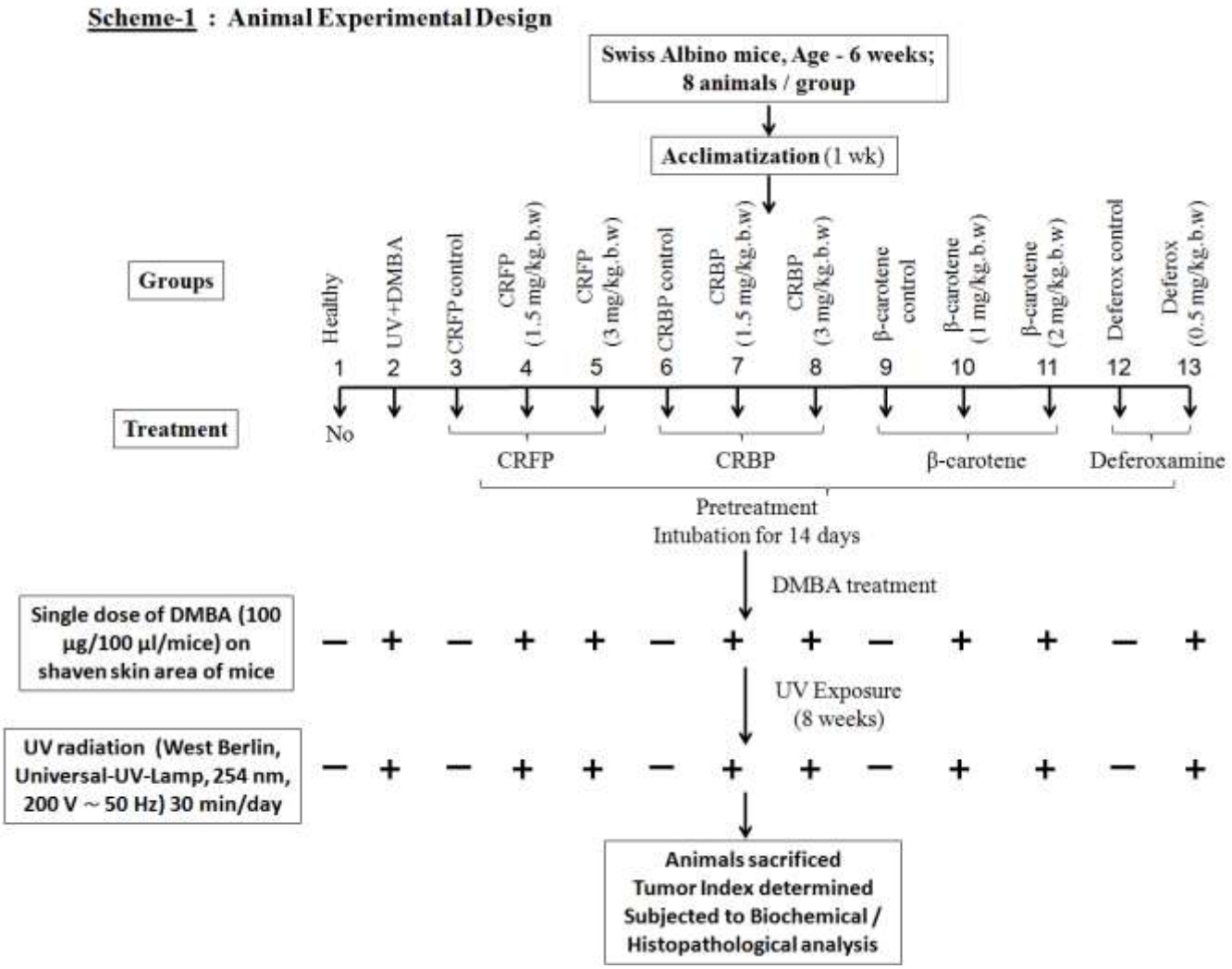
2.3 Isolation of Free and Bound Phenolic fractions of carrot and Estimation of Total Phenolic content and HPLC analysis

Free (CRFP) and Bound phenolic fractions (CRBP) were extracted as described earlier [5] in triplicate to evaluate the yield with statistical significance. The total phenolic content was determined by Folin-Ciocalteu reagent [6]. Phenolic acids of CRFP and CRBP were analyzed by HPLC (model LC-10A, Shimadzu) on an RP Shimpak C18 column (4.6 mm X 250 mm, Shimadzu) using a diode array UV-detector (operating at max 280 nm). A solvent system consisting of water/acetic acid/methanol (isocratic, 80:5:15 v/v/v) was used as mobile phase at a flow rate of 1 mL/min [7]. Phenolic acid standards were employed for identification of phenolic acids present in CRFP and CRBP by comparing the retention time under similar experimental conditions.

2.4 UV-DMBA induced skin carcinogenesis *in vivo*

Swiss albino mice of 6 weeks weighing around 25-30g were maintained under standard conditions of temperature (23 ± 5 ºC), humidity (30-70%) with a 12h Light/ Dark cycle and were provided with standard rodent pellet diet (M/s. Sai Durga feeds, Bangalore, India) and tap water *ad libitum*. The basal composition of the diet according to the manufacturer is: protein, 21%; carbohydrates, 60%; fat, 7%; fibre, 6%; mineral mix, 6%; and moisture content <10%. The study was approved by the Institutional Animal Ethical Committee (IAEC No.116/08), which follows the guidelines of CPCSEA (Committee for the purpose of Control and Supervision of Experiments on Animals, Reg. No. 49, 1999), Government of India, New Delhi, India.

All animals were divided into 13 groups of 8 animals each; their body weights were recorded and their backs were shaved prior to the start of the experiments. Treatments, types of treatments and animal experimental designs are provided in Scheme-1.



Samples were given every day throughout the experimental period, which was about 60 days. Animal body weights were recorded and sacrificed under mild ether anesthesia; blood was collected from heart into heparinized tubes and serum was separated by centrifuging at 1000 x g for 15 min at 4° C. The Dorsal skin affected by tumors was quickly excised and a part of the skin tissue was fixed in 10% formalin and embedded in paraffin for histopathological studies while rest of the skin tissues were washed thoroughly with chilled 0.9% NaCl (pH 7.4) weighed and blot dried. A 10% tissue homogenate was prepared from part of the skin sample in 0.15 M Tris-HCL (pH 7.4), and the homogenate was then centrifuged at 12,000 x g for 15 min. The supernatant thus obtained was taken for estimation of enzyme activity. The Tumor Index was calculated as described earlier [8].

2.5 Histo-immunological and biochemical analysis

Skin samples were fixed in 10% buffered formalin for 24 h. The processed tissues were embedded in paraffin blocks and the redied sections were stained with Hematoxylin and Eosin

dye [9]. The sections were analyzed by observing under light microscope (Leitz, Germany) at 10X magnification. The expression of galectin-3 protein was confirmed by immunohistochemistry analysis using ani-galectin-3 antibodies (Abcam, US) [10]. The tyrosinase enzyme activity was measured in serum, and skin homogenate in healthy, cancer induced and sample treated groups using L-Dopa as substrate with slight modifications [11]. SOD, CAT and GSH levels and TBARS were measured as per the protocol described earlier by our group [12]. Activities of the enzymes SGOT, SALP, and SGPT in serum were estimated in all the groups using standard enzyme kits to evaluate toxicological parameters [13].

2.6 Measurement of, Tyrosinase inhibitory, Antiproliferative and Antioxidant activity in CRFP and CRBP *in vitro*

2.6.1 Skin tyrosinase inhibitory activity *in vitro*

The tyrosinase enzyme activity was measured using L-Dopa as substrate as described above. The protective ability of phenolic fractions of carrot was compared along with the known phenolic standards on the tyrosinase activity.

2.6.2 Antiproliferative activity *in vitro*

For antiproliferative activity determination, RAW 264.7 cells (obtained from a tumor, induced by the Abelson murine leukemia virus) were plated in 96 well tissue culture plate ($5x10^4$ cells/well) and incubated in the presence and absence of CRFP, CRBP, β-carotene and deferoxamine for 24 h. At the end of the incubation, MTT (25µL, 5 mg/mL of media) was added to the plate, incubated at 37° C for 4 h. 100µL of ethyl alcohol and DMSO (1:1 v/v) was added to the washed pellet at the end of the incubation to dissolve the dark blue crystals obtained due to the action of mitochondrial reductase which converts yellow MTT, a tetrazole to purple formazan in living cells. Absorption of formazan solution was measured at A570 nm in a microplate reader [14].

2.6.3 Antioxidant ability by *In vitro* methods

In vitro radical scavenging, reducing power and lipid peroxidation activities of CRFP and CRBP were determined as described by our earlier group [5].

2.6.4 Statistical analysis

All experiments were done in triplicates and the data presented are the averages of the mean of three independent experiments with standard deviation. Statistical analyses were carried out to determine the significance between values of different extracts by one-way analysis variance (ANOVA) with post test followed by Tukey-Kramer multiple comparisons test using Instat statistical software. Values are expressed as the mean ± SD for each experimental group. Values sharing different superscript are statistically significant ($p < 0.05$) as determined by ANOVA.

3. RESULTS

3.1 Total Phenolic acids and Phenolic content in CRFP and CRBP

The total phenolic content in CRFP and CRBP fractions was found to be 12.08 µg GAE/g and 20.91 µg GAE/g respectively. Since β-carotene has been identified as the active antioxidant component of carrot, amount of β-carotene extracted along with free and bound phenolics as determined by HPLC. The Data indicated the presence of 7.87µg/g and 6.75µg/g in CRFP and CRBP respectively. Total β-carotene content was found to be 35µg/g. HPLC analysis indicated phenolic acid composition in both CRFP and CRBP fractions of carrot (Fig I).

Figure I. HPLC analysis of phenolic acids and β-carotene constituents in free (CRFP) and bound (CRBP) phenolic fractions of carrot

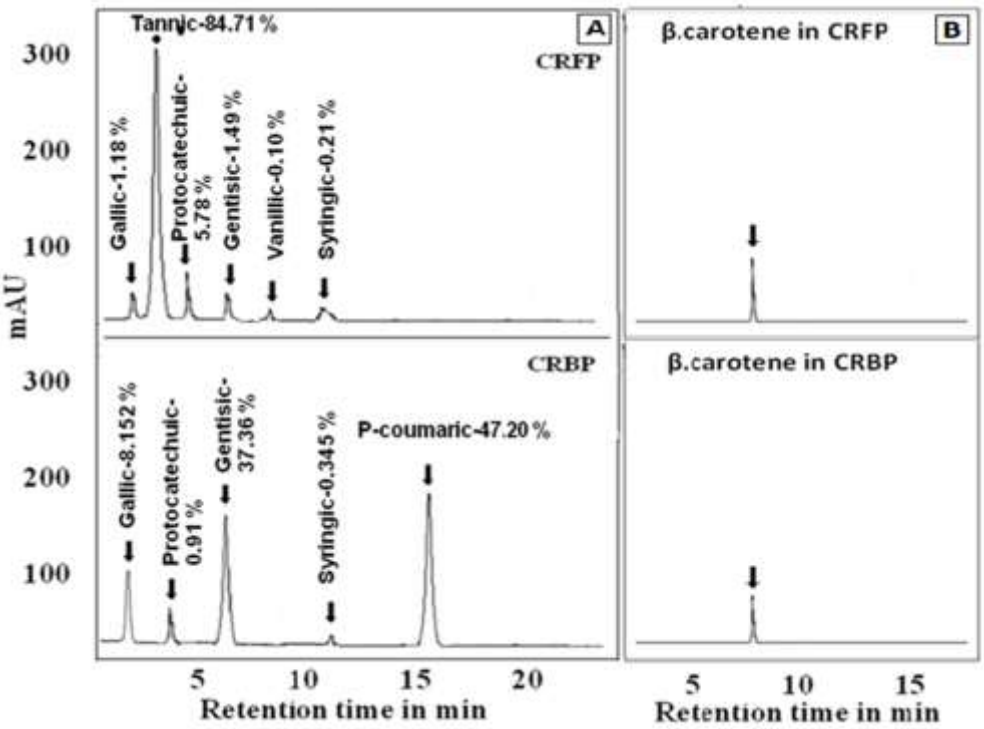


Figure I. HPLC analysis of A) Phenolic acids and B) β-carotene constituents in free (CRFP) and bound (CRBP) phenolic fractions of carrot. A) Phenolic acids were analyzed by HPLC Shimpak C18 column (4.6 mm x 250 mm, Shimadzu) with the mobile phase-water/acetic acid/methanol 80:5:15 v/v/v, isocratic. 20 µL of mg/mL standard phenolic acids were loaded independently and their specific retention time (min) was established. Phenolic acids in each fraction were identified comparing their retention time with known standards. B) β-carotene was analyzed by HPLC, C18 Shimpak column (4.6 mm x 250 mm, Shimadzu) with the mobile phase acetonitrile: methanol: dichloromethane (70:20:10, v/v/v) containing 0.1% Ammonium acetate. Injection volume was 20 µL. An isocratic analysis was performed at a flow rate of 1 mL/min by monitoring at 450 nm. β-carotene in each fraction was identified while comparing with the retention time of the standard.

3.2 Effect of CRFP, CRBP and β-carotene on UV-DMBA induced skin cancer *in vivo.*

3.2.1 Animal Body weight

A gradual increase in body weight was noted in all animal groups. However ~ 50% reduction in the weight was observed in cancer induced animals and they were recovered to different extent at different doses of test fractions – CRFP, CRBP and standards - β-carotene and deferoxamine (Table I). A significant reduction (~50%) in the weight of "cancer induced" animals suggested that weight loss of animals be due to induction of tumors and exploitation of nutrients by tumor cells, as observed in cancer patients [15]. In fact weight loss is one of the characteristic features in cancer patients [15]. CRFP, CRBP, and β-carotene treated controls showed body weight equivalent to that of healthy controls suggesting no or least toxicity induced by carrot fractions on health of animals. Apparently ~ 20% weight reduction in the deferoxamine treated controls may indicate toxicity of this drug on animals. Further there is also a direct correlation between reduction in tumor index (TI) and the body weight gain with R^2 range of 0.9996 -1.0 for CRFP, CRBP, β-carotene and deferoxamine respectively Vs TI. Data may suggest that body weight gain in CRFP, CRBP and β-carotene treated animals could be due to reduction in tumor index. The measurement of body weight thus confirms as one of the important parameter to evaluate the different degree of protection against UV-DMBA induced tumors by carrot fractions.

Table I. Effect of Healthy, Cancer induced, CRFP, CRBP, β-carotene and deferoxamine on body weight of the mice before and after the development of skin tumors by UV-DMBA treatment

Group	Initial Body Weight (g)	Final Body Weight (g)	Body weight gain (g)	% Weight gain	% Weight reduction
Healthy	25.20 ± 0.28	34.50 ± 0.70^{a}	9.30 ± 0.42	100^{a}	-
Cancer Induced (CI)	26.83 ± 1.65	31.66 ± 0.41^{e}	4.83 ± 1.24	51.93^{d}	49.07
CRFP control	25.05 ± 0.19	34.15 ± 0.91^{a}	9.10 ± 0.72	97.85^{a}	2.15
CRFP(1.5mg/kg b.w) + CI	25.50 ± 0.32	34.06 ± 0.93^{ab}	8.56 ± 0.61	92.04^{ab}	7.96
CRFP (3mg/kg b.w) + CI	24.83 ± 0.25	33.93 ± 0.50^{b}	9.10 ± 0.25	97.85^{a}	2.15
CRBP control	25.16 ± 0.04	34.20 ± 0.70^{a}	9.04 ± 0.66	97.20^{a}	2.8
CRBP (1.5mg/kg b.w) + CI	25.80 ± 0.60	32.16 ± 0.24^{d}	6.36 ± 0.36	68.38^{c}	31.62
CRBP (3mg/kg b.w) + CI	25.70 ± 0.21	32.83 ± 0.11^{cd}	7.13 ± 0.10	76.66^{b}	23.34
β-carotene control	25.30 ± 0.30	34.14 ± 0.50^{a}	8.84 ± 0.20	95.05^{a}	4.95
β-carotene (1mg/kg b.w) + CI	26.00 ± 0.53	33.23 ± 0.50^{c}	7.23 ± 0.03	77.74^{b}	22.26
β-carotene (2mg/kg b.w) + CI	26.94 ± 0.89	34.30 ± 0.30^{a}	7.36 ± 0.59	79.14^{b}	20.86
deferoxamine control	26.50 ± 0.50	34.02 ± 0.77^{ab}	7.52 ± 0.27	80.86^{b}	19.14
deferoxamine (0.5mg /kg b.w) + CI	25.40 ± 0.01	33.96 ± 0.15^{c}	8.56 ± 0.14	92.04^{ab}	8

Table I. This table represents the initial and final body weight of the mice during the experimental period. A gradual increase in body weight was noted in all animal groups. However in cancer induced animals ~ 50% reduction in the weight suggested the weight loss of animals due to induction of tumors and they were recovered to different extent at indicated doses of test fractions – CRFP, CRBP, β-carotene and deferoxamine. Suggesting that weight gain in CRFP, CRBP, β-carotene and deferoxamine treatment improved the body weight of the animal by reducing the tumor index. Differences in the weight gain is indicated as alphabets as superscript. Values are expressed as the mean ± SD. Values not sharing a similar superscript within the same column are significantly different ($p < 0.05$) as determined by ANOVA.

3.2.2 Determination of tumor index

In UV-DMBA induced group of animals 100% cancerous condition with many skin papillomas were observed. 89%, 47%, 68% and 69% reduction in tumor incidence were observed (Table II) upon treatment with CRFP, CRBP, β-carotene and deferoxamine (standard antiproliferative drug). Results were substantiated by histological analysis (Fig II).

3.2.3 Histopathological and immunohistological analysis for metastatic marker galectin-3

Histopathological investigations showed a normal histological pattern in the skin of control as well as CRFP, β-carotene and deferoxamine treated groups of animals, while irregular distribution with finger like papilloma indicative of cancerous growth were found in the skin sections of UV-DMBA and CRBP administered mice group. Penetrations of tumor cells from epidermal layer to dermal layer clearly suggest the infiltering and invading tumor types. Total disruption of skin architecture in UV-DMBA treated animals were resolved in CRFP, β-carotene and deferoxamine treated groups suggests the prevention of skin damage that occurred during cancer condition (Fig II).

Thus results point towards the chemopreventive effects of CRFP, β-carotene & deferoxamine on UV-DMBA induced skin carcinogenesis**.** Further, the extent of inhibition of tumor progression was also assessed by estimating galectin-3 in the serum as well as in the skin tissue that has been identified as a marker of metastasis. Increased galectin-3 concentration in the serum of UV-DMBA treated groups was decreased by 23.61, 1.28, 4.77 and 17.45 folds upon treatment with CRFP, CRBP, β-carotene and deferoxamine respectively. Immunostained results showed lower galectin-3 expression in Healthy, CRFP, β-carotene and deferoxamine treated groups in the epidermal layer, whereas in UV-DMBA treated group increased galectin-3 concentrations were observed around the tumor colonies in the epidermal and dermal layer. Elevated galectin-3 expression is associated with increased homotypic aggregation, and tumor cell colonization.

Table II. Quantitative difference in tumor incidence and galectin-3 concentrations in Healthy, Cancer induced, CRFP, CRBP, β-carotene and deferoxamine treated groups

Group	Tumor incidence (%)	Mean tumor volume mm3	% Reduction in mean tumor burden	Serum galectin-3 concentration (µg/mg protein)
Healthy	-	-	-	-
Cancer Induced (CI)	100	438.11 ± 0.12^{e}	0	8.03 ± 0.06^{e}
CRFP control	-	-	-	-
CRFP (1.5mg/kg b.w) + CI	16.62	72.81 ± 0.2^{b}	83.38	1.26 ± 0.11^{b}
CRFP (3mg/kg b.w) + CI	10.42	45.68 ± 0.14^{a}	89.58	0.34 ± 0.03^{a}
CRBP control	-	-	-	-
CRBP (1.5mg/kg b.w) + CI	69.32	303.72 ± 0.18^{d}	30.68	6.26 ± 0.14^{de}
CRBP (3mg/kg b.w) + CI	52.55	230.24 ± 0.22^{cd}	47.45	5.50 ± 0.10^{d}
β-carotene control	-	-	-	-
β-carotene (1mg/kg b.w) + CI	36.72	145.43 ± 0.27^{c}	63.28	3.92 ± 0.08^{c}
β-carotene (2mg/kg b.w) + CI	31.7	125.56 ± 0.11^{c}	68.3	1.68 ± 0.05^{b}
deferoxamine control	-	-	-	-
deferoxamine (0.5mg/kg b.w) + CI	31.12	136.34 ± 0.15^{c}	68.88	0.46 ± 0.02^{a}

Table II. Results indicated 90%, 47%, 68% and 69% decreased tumor incidence in, Carrot Free Phenolics (CRFP), Carrot Bound Phenolics (CRBP), β-carotene and deferoxamine receiving groups respectively. Galectin-3 concentration was estimated in the serum of mice. UV-DMBA induced groups showed 8 folds increased galectin-3 concentrations. Galectin-3 concentration was decreased by 23.61, 1.28, 4.78, and 17.45 folds by CRFP, CRBP, β-carotene, and deferoxamine respectively. Values are expressed as the mean ± SD. Values not sharing a similar superscript within the same column are significantly different ($p < 0.05$) as determined by ANOVA.

Figure II. Skin tumours, Angiogenesis, Histopathology and Immuno-staining

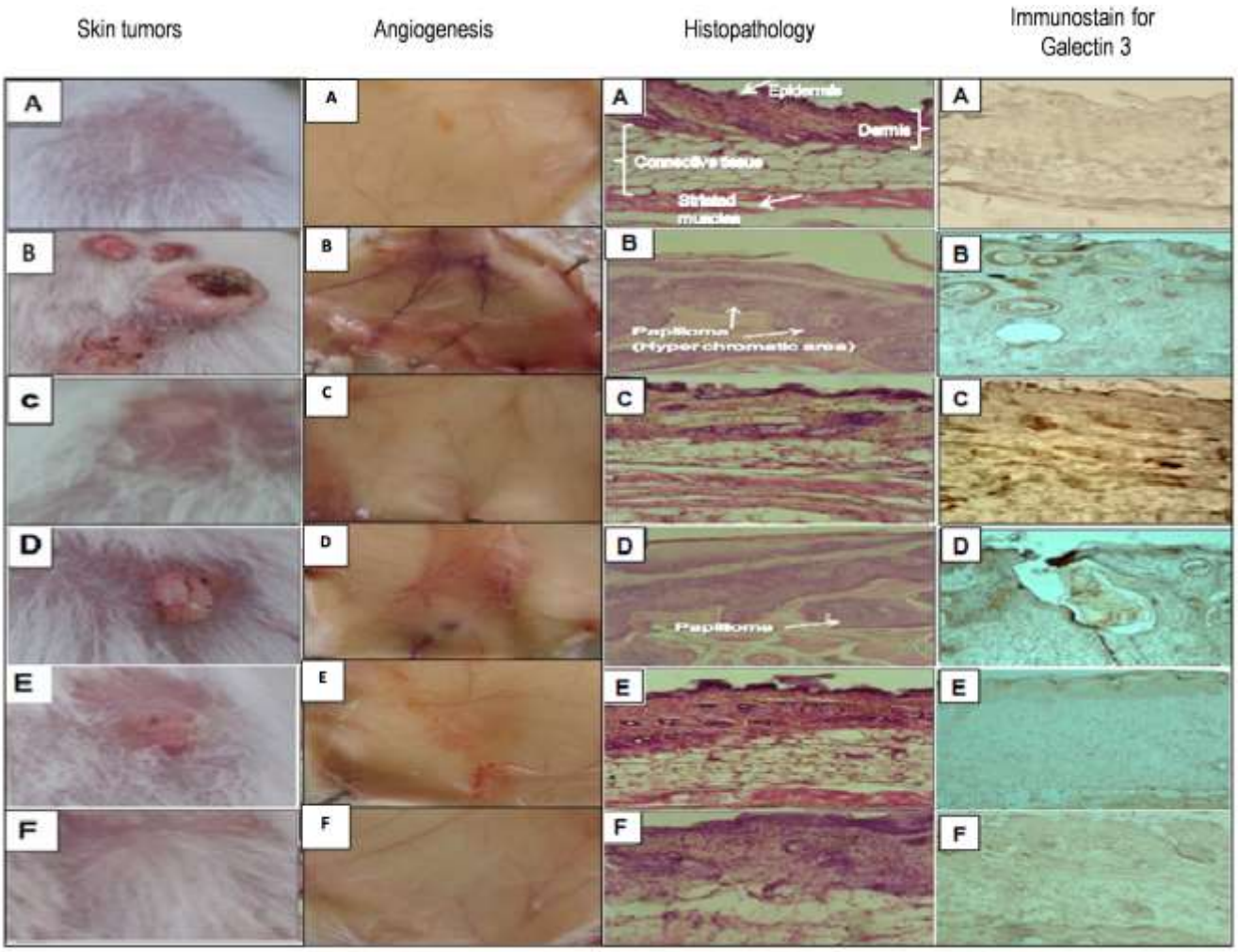


Figure II. Large numbers of lesions in the form of hardened and thickened skin were observed in mice which are treated with UV-DMBA (Group B) and very few numbers of lesions were observed in the groups receiving CRBP (D) and β-carotene (E). Such lesions were not observed in Healthy group (A) as well as CRFP (C) and deferoxamine (F) receiving groups. Histological investigations also showed a normal histological pattern in the skin of Healthy (A) as well as CRFP (C) and deferoxamine (F) receiving groups; while irregular distribution with finger like papilloma indicative of cancerous growth were found in the skin sections of UV-DMBA (B) induced group and also in CRBP (D) and β-carotene (E) receiving groups. Immunostaining of differentially treated skin sections showed no galectin-3 expression in healthy and reduced levels in CRFP, β-carotene and deferoxamine treated groups in the epidermal layer. In UV-DMBA and CRBP treated groups increased galectin-3 levels were observed around the tumor colonies in the epidermal and dermal layer as evidenced by intense staining.

3.2.4 Effect of CRFP, CRBP and β-carotene on Tyrosinase enzyme activity *in vivo* and *in vitro*

Exposure of skin to UV and DMBA has been shown to rise in tyrosinase enzyme levels significantly. Level of the enzyme increased from 15.77 ± 3.10 to 192.29 ± 11.41 μmoles of DOPA/mg protein. Similarly ~ 12 folds increase in tyrosinase activity was observed in the serum also. Interestingly in groups of animals treated with CRFP, CRBP and β-carotene, 11.3, 2 and 4.7 folds inhibition of tyrosinase activity was observed suggesting the ability of carrot phenolics and β-carotene to inhibit tyrosinase enzyme levels; Phenolics (CRFP) inhibited much better than β-carotene (2.4 folds) (Table III). Differential level of inhibition could be attributed to the ability of individual phenolic acids to inhibit tyrosinase enzyme levels. Tyrosinase inhibitory ability of pure phenolic acids *in vitro* assays were undertaken in addition to better efficacy with anticancer property, to understand whether differences in the phenolic profile in CRFP and CRBP were responsible for increased inhibition of tyrosinase in CRFP treated group as compared to that of CRBP treated animals. As presented in table III, CRFP inhibited tyrosinase enzyme activity 2 folds better than CRBP. Vanillic acid showed potent tyrosinase inhibitory (TI) activity with IC_{50} of 0.14μg/mL followed by syringic acid (IC_{50} - 0.63 ug/mL) and β-carotene (IC_{50} - 0.68 ug/mL). ~ 3 folds poorer activity was observed with gallic acid (IC_{50} – 2.27 ug/mL) although it is a better antioxidant. The data thus may also suggest that antioxidant potency may not be the prerequisite for TI activity. Further results are substantiated by calculating the antioxidant efficiency of phenolic and β-carotene constituents. It is imperative to understand the precise contribution of each of these phenolic acids present in CRFP, CRBP and β-carotene to TI and antioxidant activity per se, since both are implicated in anticancer properties. Results suggest that major contribution to TI activity is offered by β-carotene (~ 42%), followed by tannic acid (36%) and vanillic acid (12%) in CRFP. Although similar contribution to TI activity is from β-carotene in CRBP, p–coumaric (24%) followed by gentisic acid (21%) contributes to TI activity (Fig III). Antioxidant activity however in both CRFP and CRBP is contributed by gallic/tannic acid. β-carotene plays a minimal role in contributing to antioxidant activity, which is apparently due to their respective abundance. Antioxidant activity was also higher (4 to 6 folds) in all the three assays such as free radical scavenging, reducing power and inhibition of lipid peroxidation in CRFP when compared to that of CRBP (Fig IV). The data thus may indicate that both phenolic fractions and β-carotene may contribute to inhibiting UV-DMBA induced skin damage.

Table III. Effect of Healthy, Cancer induced, CRFP, CRBP, β-carotene and deferoxamine on tyrosinase activity in Serum and Skin of UV-DMBA induced experimental mice

Group	Serum (oxidation of Dopa in µmoles/mg protein)	Skin (oxidation of Dopa in µmoles/mg protein)
Healthy	03.22 ± 0.56^{a}	15.77 ± 3.10^{a}
Cancer Induced (CI)	36.87 ± 1.62^{e}	192.29 ± 11.41^{e}
CRFP control	04.45 ± 0.64^{a}	19.83 ± 0.20^{a}
CRFP(1.5mg/kg b.w) + CI	06.21 ± 1.19^{b}	29.65 ± 4.03^{b}
CRFP (3mg/kg b.w) + CI	03.98 ± 0.77^{a}	17.00 ± 2.32^{a}
CRBP control	05.19 ± 0.54^{b}	18.85 ± 2.84^{a}
CRBP (1.5mg/kg b.w) + CI	26.09 ± 1.09^{d}	99.17 ± 2.34^{d}
CRBP (3mg/kg b.w) + CI	17.73 ± 3.01^{c}	85.24 ± 9.36^{d}
β-carotene control	05.24 ± 0.89^{b}	20.10 ± 1.30^{a}
β-carotene (1mg/kg b.w) + CI	16.00 ± 0.50^{c}	40.93 ± 1.50^{c}
β-carotene (2mg/kg b.w) + CI	12.30 ± 1.30^{bc}	31.14 ± 5.50^{bc}
deferoxamine control	04.50 ± 0.54^{a}	21.10 ± 1.17^{a}
deferoxamine (0.5mg /kg b.w) + CI	07.21 ± 0.3^{b}	25.37 ± 6.06^{ab}

Figure III. Relative percentage contribution of individual phenolic acids towards antioxidant, tyrosinase inhibition and antiproliferative activity

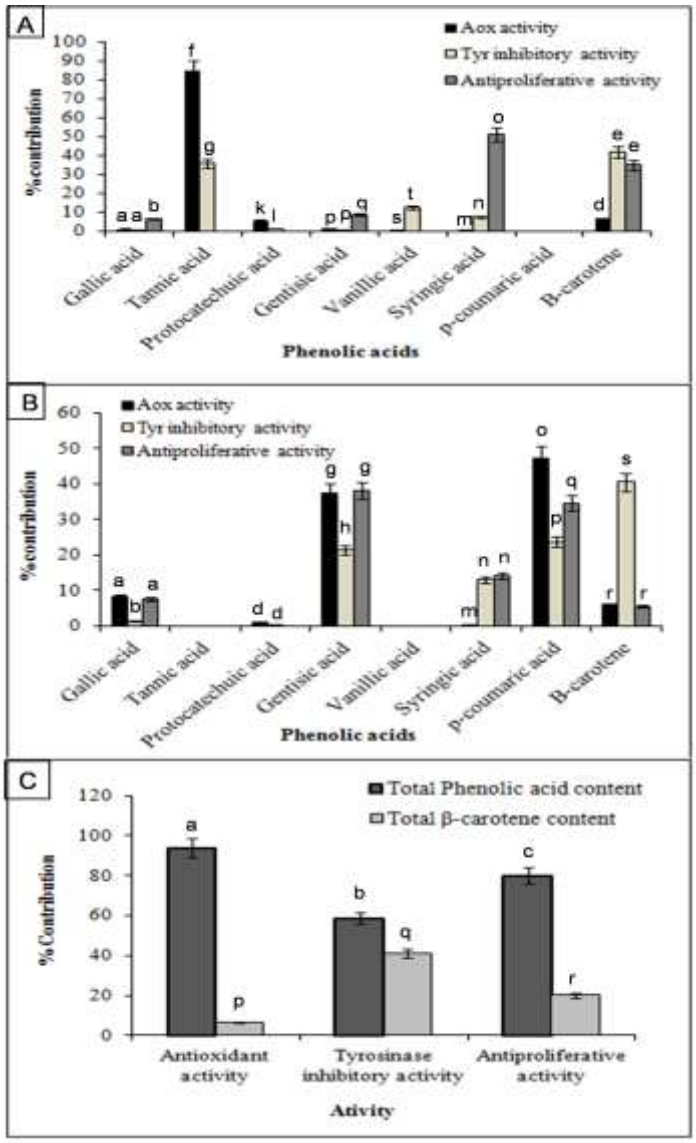


Figure III. The graph depicts the relative percent contribution of each phenolic acid and β-carotene in CRFP (A), CRBP (B), total phenolic acids and β-carotene (C) against antioxidant activity, tyrosinase inhibition, and antiproliferative activity. In CRFP (A) antioxidant activity was mainly contributed by tannic acid; tyrosinase inhibitory activity by tannic acid and β-carotene and; antiproliferative activity to an equal extent by syringic acid and β-carotene. In CRBP (B) however, contribution to all these activities is more from gentisic acid, p-coumaric acid and β-carotene. The combined role of both phenolic acids and β-carotene in carrot is therefore evident. It is also evident from Figure IIIC that 15, 2 and 4 folds increased activity was contributed from total phenolic acids than β-carotene for antioxidant, tyrosinase inhibitory and antiproliferative activity respectively. Values are expressed as the mean ± SD. Values not sharing a similar superscript are significantly different ($p < 0.05$) as determined by ANOVA.

Figure IV. Antioxidant capacities *in vitro* of CRFP and CRBP

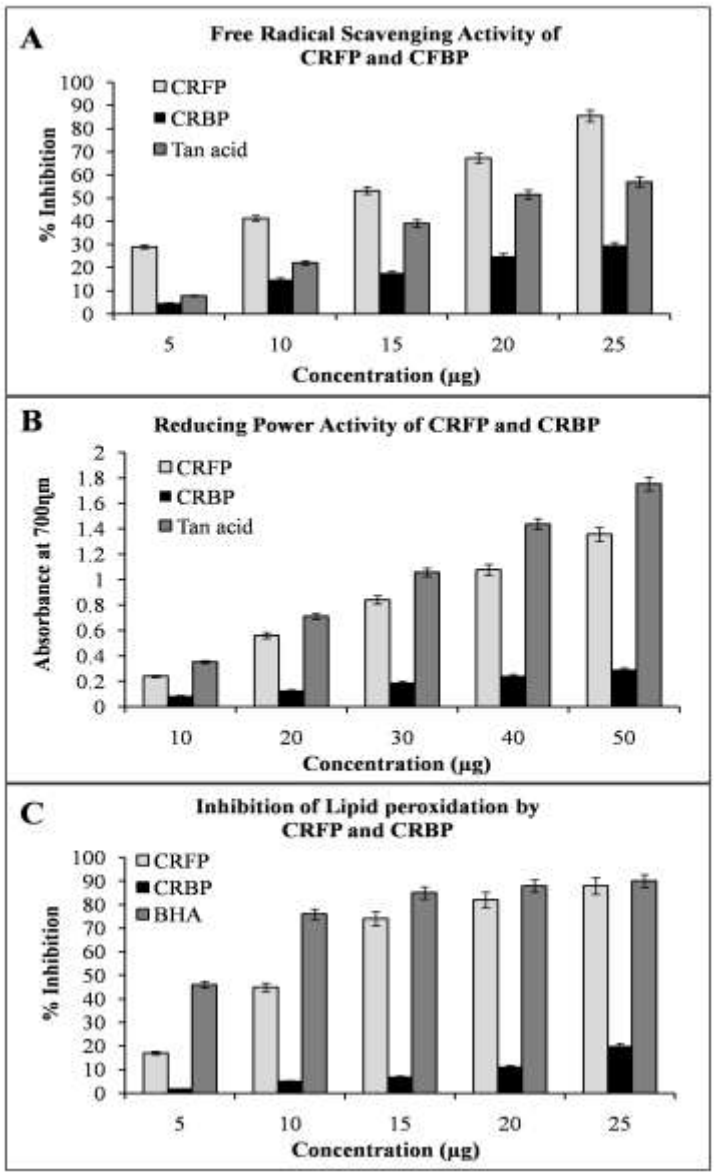


Figure IV. Antioxidant Potency of CRFP and CRBP. Concentration of 2-10 µg of GAE/mL of CRFP and CRBP was examined for Free Radical Scavenging (A), Reducing Power (B), and Inhibition of Lipid Peroxidation (C) as per the protocol described under Materials and Methods. All data are the mean ± SD of three replicates.

Table IV. Effect of Healthy, Cancer induced, CRFP, CRBP and deferoxamine on antioxidant and antioxidant enzymes

Group	SOD (U/mg protein)	Catalase (nmol H_2O_2/ mg protein)	GSH (µg GSH / mg protein	TBARS (µmols/MDA/ mg protein)
Serum				
Healthy	27.40 ± 0.43^{a}	0.48 ± 0.0^{a}	3.89 ± 0.10^{a}	0.10 ± 0.03^{a}
Cancer Induced (CI)	40.46 ± 0.40^{d}	0.11 ± 0.09^{e}	1.86 ± 0.09^{d}	2.17 ± 0.06^{d}
CRFP control	28.11 ± 0.04^{a}	0.45 ± 0.07^{a}	3.80 ± 0.39^{a}	0.10 ± 0.05^{a}
CRFP (1.5mg/kg b.w) + CI	33.99 ± 0.44^{b}	0.28 ± 0.05^{c}	3.58 ± 0.12^{b}	0.83 ± 0.03^{b}
CRFP (3mg/kg b.w) +CI	28.43 ± 0.34^{a}	0.36 ± 0.03^{b}	3.71 ± 0.22^{a}	0.55 ± 0.04^{b}
CRBP control	29.70 ± 0.80^{a}	0.46 ± 0.03^{a}	3.75 ± 0.24^{a}	0.10 ± 0.01^{a}
CRBP (1.5mg/kg b.w) + CI	38.69 ± 0.59^{c}	0.21 ± 0.07^{d}	2.70 ± 0.26^{c}	1.64 ± 0.05^{c}
CRBP (3mg/kg b.w) + CI	35.93 ± 1.85^{bc}	0.26 ± 0.08^{cd}	2.91 ± 0.13^{c}	1.55 ± 0.03^{c}
deferoxamine control	28.80 ± 1.11^{a}	0.42 ± 0.10^{a}	3.74 ± 0.10^{a}	0.10 ± 0.08^{a}
deferoxamine (0.5mg / kg b.w) + CI	32.25 ± 0.74^{b}	0.39 ± 0.01^{b}	3.35 ± 0.23^{b}	0.80 ± 0.05^{b}
Skin				
Healthy	142.53 ± 1.62^{a}	0.14 ± 0.03^{a}	23.32 ± 0.02^{a}	0.01 ± 0.00^{a}
Cancer Induced (CI)	245.44 ± 8.55^{e}	0.07 ± 0.04^{d}	13.39 ± 1.28^{d}	0.23 ± 0.03^{e}
CRFP control	145.22 ± 0.49^{a}	0.13 ± 0.09^{a}	23.20 ± 0.63^{a}	0.01 ± 0.02^{a}
CRFP (1.5mg/kg b.w) +CI	167.89 ± 7.69^{b}	0.12 ± 0.06^{b}	21.42 ± 1.72^{ab}	0.08 ± 0.03^{bc}
CRFP (3mg/kg b.w) + CI	154.52 ± 5.32^{b}	0.13 ± 0.07^{a}	22.62 ± 0.19^{a}	0.05 ± 0.02^{b}
CRBP control	143.69 ± 2.32^{a}	0.14 ± 0.05^{a}	23.09 ± 0.47^{a}	0.01 ± 0.01^{a}
CRBP (1.5mg/kg b.w) + CI	210.10 ± 3.86^{c}	0.08 ± 0.01^{d}	16.38 ± 4.25^{c}	0.19 ± 0.08^{d}
CRBP (3mg/kg b.w) + CI	191.72 ± 8.85^{cd}	0.10 ± 0.02^{c}	18.62 ± 1.70^{c}	0.16 ± 0.02^{d}
deferoxamine control	143.08 ± 6.09^{a}	0.14 ± 0.01^{a}	22.31 ± 1.16^{a}	0.01 ± 0.01^{a}
deferoxamine (0.5mg / kg b.w) + CI	165.57 ±1.49^{b}	0.11 ± 0.04^{bc}	20.63 ± 2.24^{b}	0.10 ± 0.06^{c}

Table IV. Effect of Healthy, Cancer induced**,** CRFP, CRBP and deferoxamine on Antioxidant enzymes in skin cancer induced mice serum and skin: Values are expressed as the mean ± SD. Values not sharing a similar superscript within the same column are significantly different ($p < 0.05$) as determined by ANOVA.

3.2.5 Changes in the antioxidant enzymes and lipid peroxidation levels in serum, and skin homogenate

In serum of UV-DMBA treated group of animals TBARS and SOD levels increased by 22 and 1.5 folds respectively and CAT and GSH levels decreased by 4.3 folds and 2 folds, their levels were normalized upon treatment with CRFP, CRBP and deferoxamine in a dose dependent manner. Whereas in skin homogenate of UV-DMBA treated group of animals SOD and TBARS levels were increased by 1.7 and 23 folds, CAT and GSH levels decreased by 2 folds and 1.7 folds, levels were normalized upon treatment with CRFP, CRBP and deferoxamine (Table IV).

3.2.6 SGPT, SGOT and SALP levels in serum

SGPT and SALP showed enhancement of activities in serum of UV-DMBA treated groups by 1.4 folds and 1.2 folds respectively, whereas SGOT level decreased by 0.72 folds. No significant differences between control and other groups were observed suggesting no toxic effect of CRFP and CRBP on liver damage (Table V).

Table V. Effect of Healthy, Cancer induced, CRFP, CRBP and deferoxamine on SGOT, SGPT and SALP in serum of UV-DMBA induced experimental mice.

Group	SGPT (U/mg protein)	SGOT (U/mg protein	SALP (U/mg protein)
Healthy	108.89 ± 1.61^{a}	111.72 ± 3.28^{a}	211.33 ± 5.10^{a}
Cancer Induced (CI)	158.33 ± 5.45^{d}	164.66 ± 3.21^{e}	452.34 ± 2.67^{e}
CRFP control	105.28 ± 4.87^{a}	111.54 ± 2.15^{a}	229.99 ± 4.72^{a}
CRFP (1.5mg/kg b.w) + (CI)	121.94 ± 2.64^{b}	128.69 ± 2.26^{b}	287.80 ± 5.42^{bc}
CRFP (3mg/kg b.w) + (CI)	113.35 ± 2.56^{a}	123.16 ±1.56^{a}	269.33 ± 1.90^{b}
CRBP control	101.98 ± 3.55^{a}	114.03 ± 4.17^{a}	227.89 ± 2.63^{a}
CRBP (1.5mg/kg b.w) + (CI)	154.90 ± 5.79^{d}	143.40 ± 1.01^{d}	377.51 ± 1.40^{d}
CRBP (3mg/kg b.w) + (CI)	136.91 ± 1.35^{bc}	134.39 ± 3.18^{c}	353.69 ± 3.13^{d}
deferoxamine control	104.35 ± 4.02^{a}	113.61 ± 1.05^{a}	230.16 ± 2.07^{a}
deferoxamine (0.5mg /kg b.w) +(CI)	131.63 ± 4.21^{b}	125.77 ± 2.17^{ab}	279.26 ± 1.18^{b}

Table V. Effect of Healthy, Cancer induced, CRFP, CRBP and deferoxamine on antioxidant enzymes in skin cancer induced mice serum : Values are expressed as the mean ± SD. Values not sharing a similar superscript within the same column are significantly different ($p < 0.05$) as determined by ANOVA.

Table VI. Antioxidant potency, Tyrosinase inhibitory and Antiproliferative activity of CRFP and CRBP.

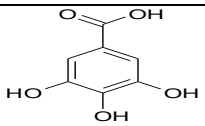
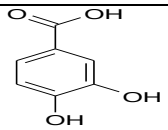
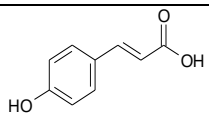
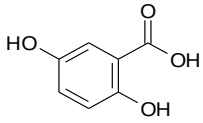
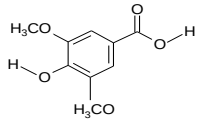
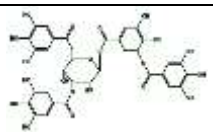
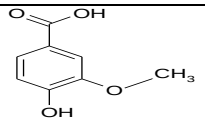
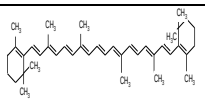
Standard Phenolic acids	Structures	Antioxidant activity IC_{50} (µg)	Tyrosinase inhibitory activity IC_{50} (µg)	Antiproliferative activity IC_{50} (µg)	Phenolic acid content in CRFP (µg/g)	Phenolic acid content in CRBP (µg/g)
Gallic acid (3,4,5 Trihydroxybenzoic acid)		1.1 ± 0.09[a]	2.27 ± 0.21[d] (16.21 folds ↓)	12.9 ± 0.31[a] (1)	0.125 ± 0.03[d]	0.8 ± 0.14[d]
P.catechuic acid (3,4 Dihydroxy Benzoicacid)		1.35 ± 0.16[a]	2.00 ± 0.14[d] (14.28 folds ↓)	-	0.75 ± 0.10[bc]	0.11 ± 0.03[d]
P.coumaric acid (p-Hydroxy cinnamic acid)		1.9 ± 0.20[a]	1.38 ± 0.08[c] (9.85 folds ↓)	28.58 ± 0.60[b] (2.21 folds ↓)	-	8.00 ± 0.28[a]
Gentisic acid (2,5 Dihydroxy benzoic acid)		3.0 ± 0.28[b]	1.92 ± 0.20[b] (13.71 folds ↓)	32.38 ± 0.70[b] (2.51 folds ↓)	0.43 ± 0.08[c]	10.0 ± 0.34[a]
Syringic acid (4-hydroxy-3,5 Dimetoxybenzoic acid)		64.90 ± 5.40[e]	0.63 ± 0.01[b] (4.5 folds ↓)	17.51 ± 0.41[a] (1.35 folds ↓)	1.35 ± 0.12[b]	2.00 ± 0.19[c]
Tannic acid (2,3-dihydroxy-5-phenyl 3,4,5-trihydroxybenzoate)		1.1 ± 0.09[a]	0.90 ± 0.10[bc] (6.42 folds ↓)	-	8.95 ± 0.24[a]	-
Vanillic acid (4 hydroxy-3-methoxybenzoic acid)		49.5 ± 2.4[d]	0.14 ± 0.04[a] (1)	-	0.478 ± 0.09[c]	-
β-carotene (1,3,3-Trimethyl-2-cyclohexene)		12.5 ± 0.1[c]	0.68 ± 0.09[b] (4.85 folds ↓)	150.00 ± 9.0[d] (11.62 folds ↓)	7.87 ± 0.60[a]	6.75 ± 0.41[b]
					* Total β-carotene content- 35µg/g	
CRFP	-	14.15 ± 0.3[c]	0.58 ± 0.06[b]	89.04 ± 3.60[c]	Total phenolic content (µg/g) 12.08	-
CRBP	-	16.80 ± 0.4[c]	1.03 ± 0.08[bc]	74.99 ± 2.52[c]	-	20.91

Table VI. CRFP and CRBP containing different phenolic acids and β-carotene are given with their yield (µg/g) and structure. Values are expressed as the mean ± SD. Values not sharing a similar superscript within the same column are significantly different ($p < 0.05$) as determined by ANOVA.* Represents total β-carotene content in carrot by HPLC. Parenthesis in brackets under tyrosinase inhibitory activity and antiproliferative activity columns; revealed decreased activity (in folds) of phenolic acids and β-carotene compared to vanillic acid and gallic acid respectively.

3.3 *In vitro* Antiproliferative activity of CRFP and CRBP

Treatment with CRFP, CRBP, β-carotene and deferoxamine showed differential antiproliferative activity (Table VI) with an IC_{50} of 89.04, 74.99, 150 and 10µg respectively. In CRFP antiproliferative activity was contributed to equal extent by syringic acid and β-carotene. Whereas in CRBP contribution to antiproliferative activity is more from gentisic acid, p-coumaric acid and β-carotene. Earlier literature had suggested the probable role of phenolic acids in possessing antiproliferative potential. However contribution of individual phenolic acids to antiproliferative potency was not clearly understood. Current study delineates the same by determining the antiproliferative potential of each of phenolic acids and β-carotene in pure form in carrot fractions. Data presented in Table VI revealed that phenolics in CRBP than CRFP contributed at least 2 folds better antiproliferative potency than β-carotene. Differences in the activity have been attributed to differences in phenolic acids composition. Also our study for the first time reveal that β-carotene (IC_{50} – 150 ± 9.0 µg/mL), although has been reported as anticancer agent it is ~ 1.62 folds less potent than gallic acid (IC_{50} – 12.9 ± 0.31 µg/mL). It is intriguing also to observe ~ 10 folds less antioxidant potency by β-carotene (IC_{50} – 12.5 ± 0.1 µg/mL) than gallic acid (IC_{50} 1.1 ± 0.09 µg/mL). Data thus justifies the significant role of phenolic acids in offering anticancer potency in carrot than β-carotene.

4. DISCUSSION

Skin carcinogenesis, the most common of all cancers, has been increasing in recent years all over the world [16]. Skin is the most common site of malignancy and represents 55% of all human cancers with tremendous impact on health and morbidity [17, 18]. In recent years, profound interest has been evinced in the identification of nontoxic natural products that are capable of reducing the tumorigenicity of the environmental carcinogen.

Central theme of the current study was to understand whether there are compounds other than β-carotene that can contribute to the anticancer property. Highlighting of only β-carotene form Carrot for anticancer property from various

studies from other laboratories [19]; reporting of importance of phenolic acids in contributing to anticancer potential [20] makes us to undertake this investigation. Thus the finding of the present study is to report the role of various ingredients present in a food source; in this case carrot that contribute significantly to the anticancer property. Understanding of the same becomes the key part of the investigation, since it gives a handle to take care of these compounds during the processing of carrot as food.

Our previous experiences provided enough evidence for the fact that phenolics from varieties of dietary sources vary in their composition and it is the phenolic acid composition which offers a particular disease preventive property to the source [5, 21]. This study is an effort to understand the role of phenolic acids in carrot, in addition to reported β-carotene. *In vivo* experiments proved the anticancer property of CRFP, CRBP and β-carotene. It was interesting to observe 1.9 folds better anticancer activity by CRFP when compared to that of CRBP. Results were substantiated by histopathological analysis, from which it is evident that tumor regression is by controlling tyrosinase enzyme activity (~6 folds reduction), which otherwise has an impact in impairment of glutathione levels. Further, the extent of inhibition of tumor progression was also assessed by estimating galectin-3 that has been identified as a marker of metastasis from Dr. Raz group [22], as well as from our laboratory [23]. A correlation coefficient R^2 = .999 between reduction in tumor colonies and galectin-3 levels substantiates that, phenolic fractions of carrot and β-carotene could prevent the tumor size as well as its invasion (Fig. II). Results thus suggest the potent cancer preventive properties of phenolic fractions of Carrot. Histopathological analysis provided exclusive evidence for inhibiting tumor colonies from penetration to deeper layers of the skin as opposed to that observed in UV-DMBA treated groups of animals.

In order to understand the precise role of phenolic acids and β-carotene against cancer, phenolic acid composition in CRFP and CRBP was determined in addition to β-carotene and evaluated their efficacy on antioxidant, tyrosinase inhibition and antiproliferative properties that are required to be anticancerous. Based on the abundance of these compounds, relative percent contribution to each of these activities in CRFP, CRBP and β-carotene was calculated. In CRFP antioxidant activity was mainly contributed by tannic acid; tyrosinase inhibitory activity by tannic acid and β-carotene and; antiproliferative activity to an equal extent by syringic acid and β-carotene. In CRBP however, contribution to all these activities is more from gentisic acid, p-coumaric acid and β-carotene. The combined role of both phenolic acids and β-carotene in carrot is therefore evident (Fig III). It is interesting to observe that despite similar levels of β-carotene in CRFP and CRBP, a higher reduction in tumor formation (1.88 folds), tyrosinase (5 folds),

galectin-3 levels (18.4 folds) and modulation of antioxidant levels (1-3 folds) in CRFP than CRBP suggest that, in addition to β-carotene, the nature of other phenolic acids in CRFP play a key role in anticancer property. However, they may differ in their mechanism of action.

β-carotene has been known to contribute to anticancer property via direct antioxidative property. Our recently published data [8] also indicated that β-carotene may also be converted to retinol, where this can again serve as antioxidant; in other words, retinol can be an active metabolite form of β-carotene in exhibiting the anticancer property. Further it is also shown to be anti-cancerous by arresting cell cycle, inducing apoptosis [24]. With respect to phenolic acids versus UV-DMBA induced cancer, they may inhibit by quenching free radicals that are generated during UV exposure.

Inhibition of tyrosinase is a key player in UV-DMBA induced cancer and antiproliferative effect. All of these are substantiated by potent antioxidant, tyrosinase and antiproliferative effect in *in vitro* assays (Table VI). Since the anticancer potency of CRFP and not CRBP is on par with deferoxamine, a known antiproliferative drug, it is possible that CRFP may also inhibit cell proliferation by binding to iron which is necessary for active proliferation of cancer cells. Evidences also exist with high binding capacity or chelating of iron by tannic acid [25], as tannic acid is also enriched in CRFP. Further observed results are substantiated by metal/ Fe^{2+} chelating effect of phenolics [26]. In presence of phenolics as a chelating agent was also evidenced by reduction in Ferrozine complex (unpublished observation). CRFP thus may be a potential modulator of skin carcinogenesis by virtue of both β-carotene and phenolic acids. However, since phenolic acids show differential properties, the precise combination responsible for modulation can only be elicited by conducting *in vivo* efficacy studies. Current manuscript exclusively proves that the combination of phenolic acids in CRFP in presence of β-carotene may be an appropriate combination to combat UV-DMBA induced skin cancer.

Cancer preventive properties of exogenous antioxidants have been enumerated in number of epidemiological, intervention and biochemical studies [21, 27]. It is clear from the studies that type of phenolic acid influences the bioactive potential. Number of mechanisms may be involved in exhibiting enzyme inhibitory properties. Inhibition of important enzyme(s) or blockade of the receptor by binding to an active target site may be responsible for inhibition of enzyme(s) that participate in signaling cascade in cancer cells. It is clearly depicted by Alam et al., [28] that the inhibition of tyrosinase activity might depend on the hydroxyl groups on the phenolic acids via formation of hydrogen bonds with an enzyme or enzyme active site. In our current study atleast 5 to 16 folds better activity by vanillic acid – 4 hydroxy-3-

methoxybenzoic acid containing monohydroxy and methoxy group than a di hydroxy p-catechuic acid – 3,4 Dihydroxy Benzoic acid (14.28 folds less), p-coumaric acid - p-Hydroxy cinnamic acid (9.85 folds less), genitisic acid – 2,5 Dihydroxy benzoic acid (13.71 folds less) and tri-hydroxy gallic acid - 3,4,5 Trihydroxybenzoic acid (16.21 folds less) reveal the importance of OCH3 group in tyrosinase inhibition. 4 folds better activity in syringic acid - 4-hydroxy-3,5 Dimetoxybenzoic acid than gallic acid - 3,4,5 Trihydroxybenzoic acid (Table VI) further confirms this observation. The contribution for tyrosinase inhibitory activity in both CRFP and CRBP due to high abundance of β-carotene may not be ruled out. It is interesting to observe the contradictory data of β-carotene with respect to antiproliferative activity. Correlation coefficient of R2 between antiproliferative activities with antioxidant activity of 0.68 clearly indicates that the antiproliferative activity atleast partially is due to antioxidant potency of phenolic acids of carrot fraction. Tyrosinase inhibition however could be via binding of these phenolic acids to the enzyme or enzyme active site. *In vitro* protein binding studies where depiction of stronger binding constant for vanillic acid than other phenolic acids may substantiate our results [29]

Current investigation is important in terms of understanding the scope of carrot in cancer preventive properties. Although β-carotene for which carrot is known as a good anticancer source of β-carotene was debated, when clinical trials yielded a contradictory result. β-carotene fed human group developed higher cancer incidences than that of untreated groups [30]. Our report on the presence of enriched amount of phenolics especially gallic and syringic acids may enable the thinking to reorient towards the understanding on chemopreventive strategies from dietary sources. Further it is important to emphasize the doses of 1.8 – 2.0 mg requirement of phenolics for anticancer potential as that of grapes [31]; when other investigators reported the requirement of 3-18 mg. Our observed results thus caution the researcher not to conclude the anticancer potential of a food source, by performing anticancer potency with limited biomarker and phytochemical analysis.

5. CONCLUSION

The present study highlighting the role of both phenolic acids and β-carotene has an impact for the following reason. Although β-carotene has been identified earlier as an anticancer agent from several studies; results of human trial studies carried out at National Institute of Health, USA, indicated rather enhanced tumor incidences [32]. Pro-oxidant effect of β-carotene was attributed to such increased tumor incidences. Continuation of the study indeed indicated that a combination of β-carotene with vitamin E, offered significant reduction in tumor incidence suggesting the neutralization of prooxidant effect of β-carotene. It is therefore a generally accepted concept to use

multi-mechanistic antioxidants to avail benefit during cancer prevention/management. In this scenario, phenolic fraction containing β-carotene may be efficient in prevention of skin cancer due to the combined action of phenolic acids as well as β-carotene as evidenced in the current study.

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