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Effective Inhibition of Skin Cancer, Tyrosinase, and Antioxidative Properties by Astaxanthin and Astaxanthin Esters from the Green Alga *Haematococcus pluvialis*

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ABSTRACT: Astaxanthin mono- (AXME) and diesters (AXDE) were characterized and examined for anticancer potency with total carotenoids (TC) and astaxanthin (AX) against UV−7,12-dimethylbenz(*a*)anthracene (DMBA)-induced skin cancer model in rat. At 200 μg/kg bw, AXDE and AXME reduced UV-DMBA-induced tumor incidences up to 96 and 88%, respectively, when compared to AX (66%) and TC (85%). UV-DMBA has been known to generate high levels of free radicals and tyrosinase enzyme, leading to characteristic symptoms of skin pigmentation and tumor initiation. Intriguingly, ∼7-fold increase in tyrosinase and 10-fold decrease in antioxidant levels were normalized by AXDE and AXME as opposed to only ∼1.4−2.2-fold by AX and TC, respectively. This result together with the appearance of 72 and 58 ng/mL of retinol in the serum of respective AXE-treated (AXDE + AXME) and AX-treated animals suggested that better anticancer potency of AXEs could be due to increased bioavailability.

KEYWORDS: *microalgae, AX, AXME, AXDE, UV-DMBA, skin cancer, retinol*

INTRODUCTION

Developing novel strategies to prevent skin cancer represents a desirable goal due to the rise in the incidence of skin cancer patients throughout the world.[1] According to the World Cancer Report, skin cancer constitutes ∼30% of all newly diagnosed cancers in the world.[2] This rise in incidence has been attributed to overexposure of skin to sun/UV light, due to reduction in the ozone in the atmosphere.[3]

Skin cancer thus is a disease in which malignant cells are found in the outer layer of the skin. Melanoma is one of the most serious consequences of skin cancer where melanocytes proliferate actively with enhanced accumulation of melanin pigment leading to pigmentation and discoloration of the skin in addition to tumor formation. Up-regulated levels of tyrosinase enzyme appear to contribute significantly to the enhanced synthesis and accumulation of melanin in melanocytes.

Like most cancers, melanoma is best treated when it is diagnosed early. Melanoma can metastasize quickly to other parts of the body through the lymph system or through the blood. Most of the cytotoxic drugs used presently in cancer therapy are highly toxic to a wide spectrum of tissues such as the gastrointestinal tract, bone marrow, heart, lungs, kidney, and brain. Latrogenic failure of these organs has been observed frequently as a cause of death from cancer.[4] Melanomas are difficult to eradicate by chemotherapy because they exhibit a well-known phenomenon, "chemoresistance". Expression of survivin molecules in the cells appears to cause drug resistance, resulting in very little option for curing the disease. Attempts are underway with the use of tyrosinase inhibitors,[5] particularly from natural sources, to overcome chemoresistance and to avoid side effects.[6] Indeed, much progress is being made in the direction of pharmacological evaluation of various plant products and dietary sources with the hope of achieving effective chemoprevention.[7]

Extensive research has been done on *Haematococcus pluvialis*, a unicellular green alga, in our laboratory including its biotechnological production, characterization of type of astaxanthin (AX), astaxanthin esters (AXEs), etc. Astaxanthin esters are unique, constituted by 70% of monoesters, 15−20% of diesters, and 4−5% of free forms, indicating the predominance of esterified astaxanthin forms in *H. pluvialis* as opposed to free forms in other plant sources.[8] Antioxidant activities 100 and 10 times greater than those of vitamin E and β-carotene have been reported in AX.[9,10] Recently developed downstream processing for the large-scale production of AX and AXEs may potentiate their uses as anticancer alternatives.[11] Furthermore, intriguing studies by Camera et al.[12] and Savouré et al.[13] implied that different carotenoids exhibit varied potential to offer protection against UV-induced skin cancer. Among the three important carotenoids, AX, canthaxanthin (CX), and β-carotene (BC), AX, which is an oxocarotenoid, has a superior preventive effect toward photo-oxidative changes in

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cell culture. Results of the present investigation add to the previous observation that AXEs are more protective than AX in UV−7,12-dimethylbenz(a)anthracene (DMBA)-induced skin cancer in rats. Studies also address two possible mechanisms of increased potency of AXEs, such as free radical scavenging activity per se, as well as vitamin A activity in terms of observing the generation of relative levels of retinol from AX and AXEs. The data revealed for the first time that AXEs are more potent than AX and have an impact on their uses as anticancer alternatives, because they are available in great abundance in *H. pluvialis* with the defined protocol for their optimization.

MATERIALS AND METHODS

H. pluvialis. *H. pluvialis* was obtained from Sammlung von Algenkulturen, Pflanzenphysiologisches Institute, Universitat Gottingen, Gottingen, Germany, and was maintained on modified autotrophic bold basal medium and agar slants.[8]

Extraction, Isolation, and Characterization of AX and AXEs. Total carotenoid (TC) from *H. pluvialis* biomass was extracted and characterized as described previously.[14,15] Briefly, TC was subjected to preparative thin layer chromatography (TLC) using the solvent system acetone/hexane (3:7, v/v) and separated astaxanthin (AX), astaxanthin monoester (AXME), and astaxanthin diester (AXDE) bands from TLC plates and characterized by mass spectra.

HPLC Analysis of AX, AXEs, and Retinol. Isolated AX and AXEs from *H. pluvialis* and retinol in serum and liver were analyzed using a HPLC (Shimadzu 10AS, Kyoto, Japan) reverse phase 25 cm × 4.6 mm, 5 μm, C_{18} column (Wakosil 11 5C 18RS) with an isocratic solvent system consisting of dichloromethane/acetonitrile/methanol (20:70:10, v/v/v) at a flow rate of 1.0 mL/min.[16,17] AX, AXEs, and retinol were monitored at 476 and 325 nm with a UV−visible detector (Shimadzu). Peak identification and λ_{max} values of these components were confirmed by their retention times and characteristic spectra of standard chromatograms recorded with a Shimadzu model LC-10AVP series equipped with an SPD-10AVP photodiode array detector. They were quantified from their peak areas in relation to the respective reference standards.

Characterization of AX and AXEs by Liquid Chromatography−Mass Spectroscopy (LC-MS) in Atmospheric Pressure Chemical Ionization (APCI). Isolated AX and AXEs from *H. pluvialis* biomass were characterized by using the Waters 2996 modular HPLC system (autosampler, gradient pump, thermoregulator, and DAD), coupled to a Q-Tof Ultima (UK) mass spectrometer. In brief, the APCI source was heated at 130 °C, and the probe was kept at 500 °C. The corona (5 kV), HV lens (0.5 kV), and cone (30 V) voltages were optimized. Nitrogen was used as sheath and drying gas at 100 and 300 L/h, respectively. The spectrometer was calibrated in the positive mode, and $[M + H]^+$ ions were recorded. Mass spectra of AX and AXEs were acquired with m/z 400−2000 scan range.

Effect of AX and AXEs on UV-DMBA-Induced Skin Carcinogenesis in Vivo. Healthy albino Wistar rats (220 ± 5 g) used for the experiments were maintained under standard conditions of temperature, humidity, and light and were provided with standard rodent pellet diet (M/s. Sai Durga Feeds, Bangalore, India) and tap water ad libitum. Animals for the study were approved by the Institutional Animal Ethical Committee (IAEC No. 116/08), which follows the guidelines of the 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 14 groups (n = 6 for each group), their body weights were recorded, and their backs were shaved prior to the start of experiment. TC, AX, and AXEs dissolved in ground nut oil were intubated to groups 3, 4, 7, 10, and 13 at 100 μg/kg body weight (bw) and groups 6, 9, and 12 at 200 μg/kg bw, respectively. Group I is the healthy group, whereas group II animals were exposed to UV and DMBA and hence served as the "cancer-induced" group. Groups 5, 8, 11, and 14 served as sample control groups and were treated with only TC, AX, AXME, and AXDE at μg/kg bw, respectively. The samples/standard was intubated prior to cancer induction for 14 days. From the 15th day onward UV radiation (West Berlin, Universal-UV-Lamp, 254 nm, 200 V ~ 50 Hz) daily (30 min/day) followed by DMBA (100 μg in 100 μL of acetone/rat, weekly twice applied on skin) was given. Samples were given everyday throughout the experimental period, which was about 60 days.

Measurement of Tumor Index. Tumors and skin lesions were detected in UV-DMBA-treated rats. The tumor index was calculated as described by Koul et al.[18] Tumor volume and burden were calculated using the following formulas: mean tumor volume = $4/3\pi r^3$ (r = mean tumor radius in mm); mean tumor burden = mean tumor volume × mean number of tumors. The intensity of tumor was also calculated by histopathological analysis using Image J software.[19]

Assay for Tyrosinase, Protein, and Antioxidant Enzymes in the Skin Homogenate and Serum. Tyrosinase enzyme activity was measured in serum and skin homogenate in control and UV-DMBA-treated groups following the standardized protocols employed earlier.[20,21] Tyrosinase enzyme activity was measured in the serum and skin homogenates of all groups of animals using L-Dopa as substrate with slight modifications. L-Dopa (0.1 mL of a 1 mg/mL solution) was mixed with 0.8 mL of 0.1 M phosphate buffer (pH 6.0) and incubated with 0.1 mL of serum/skin homogenates at 37 °C for 15 min. Dopachrome formation was measured fluorometircally (excitation, 360 nm; and emission peak, 720 nm). The increased tyrosinase activity was determined by the increase in the absorbance at excitation of 360 nm and emission of 720 nm. The protective ability of treated groups with astaxanthin and its esters on the tyrosinase activity was determined and quantitated.[17]

Protein content was determined using the method described.[22] Superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) levels and TBARS were measured as per the protocol described earlier by our group.[23−26]

Hematological and Histopathological Analysis. EDTA anticoagulated blood samples were used to obtain a complete blood count with a Hemavet Mascot Multispecies Hematology System Counter 1500R (Ravi Diagnostic Laboratory, Mysore, Karnataka, India). Lymphocytes, mean cell hemoglobin count, platelets, mean cell volume, packed cell volume, red blood cells, mean cell hemoglobin, hemoglobin, and neutrophils were analyzed. For histopathological studies, skin tissue samples were fixed in 10% buffered formalin for 24 h. The processed tissues were embedded in paraffin blocks, and sections made were stained with hematoxylin and eosin dye. The sections were analyzed by observation under light microscope (Leitz, Germany) at 10× magnification. Tumor areas in untreated and sample-treated sections were localized using Image J software. Results were compared between the groups using SPSS Statistics 17.0. OA one-way ANOVA test followed by a post hoc Tukey test was performed.

Determination of Bioavailability of AX and AXEs in Different Animal Groups. AX is known to convert into retinol. Hence, to determine whether the observed bioactive potential of AX and AXEs in prevention of UV-DMBA-induced rats is due to the generation of retinol, AX and retinol were measured in the serum and liver by HPLC using our earlier procedures.[16,17]

Toxicological Studies. Activities of the enzymes serum glutamate oxaloacetate transaminase (SGOT), serum alkaline phosphatase (SALP), and serum glutamate pyruvate transaminase (SGPT) in serum and skin were estimated in healthy control, sample control, and UV-DMBA-treated groups using standard enzyme kits.[27−29]

Statistical Analysis. Results were expressed the mean ± standard deviation (SD) of six. The data were analyzed by ANOVA using Microsoft Excel XP (Microsoft Corp., Redmond, WA, USA), and the post hoc mean separations were performed by Duncan's multiple-range test at $p < 0.05$.

RESULTS AND DISCUSSION

Characterization of AX and AXEs by HPLC and LC-MS (APCI). The total carotenoid (TC) constituted about 2−3% of *H. pluvialis* total biomass. Of TC, AX and its esters (AXME + AXDE) constituted ~2 and 80%, respectively. They could be

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separated from total carotenoid extract of *H. pluvialis* with acetone/hexane (3:7, v/v) mobile phase on silica-based thin layer chromatography with different R_f values. AX had an R_f of ~0.54, whereas astaxanthin monoesters (AXME, R_f = 0.77) and diesters (AXDE, R_f = 0.82) showed characteristic mobility. AX and AXEs were identified by absorption spectra at 470–476 nm by HPLC, and both were obtained in 98% purity. Because there was a good resolution between AX, AXME, and AXDE on TLC, respective spots were scraped and reconfirmed their mobility with the same chromatographic system, and such pure components isolated by preparative thin layer chromatography were identified as AX and AXEs by LC-MS. LC-MS (APCI) positive mode and the MS data used for the identification of AX, AXME, and AXDE are summarized in Table 1. The mass

Table 1. LC-MS (APCI) Data for Astaxanthin and Astaxanthin Esters

	m/z			
no.	[M]	$[M + 2H - FA_1]^{+1}$	MS_2	compound[a]
1	597	$[M - H_2O]$	579	free astaxanthin
2	835	579	836	ME $C_{16:0}$
3	845	579	846	ME $C_{17:2}$
4	847	579	848	ME $C_{17:1}$
5	849	579	850	ME $C_{17:0}$
6	855	579	854	ME $C_{18:4}$
7	857	579	856	ME $C_{18:3}$
8	859	579	858	ME $C_{18:2}$
9	861	579	859	ME $C_{18:1}$
10	1072	579	1071	DE $C_{16:0}/C_{16:0}$
11	1096	579	1095	DE $C_{16:0}/C_{18:2}$
12	1120	579	1119	DE $C_{18:1}/C_{18:3}$
13	1122	579	1121	DE $C_{18:1}/C_{18:2}$
14	1124	579	1123	DE $C_{18:1}/C_{18:1}$

[a]ME, monoesters; DE, diesters.

spectrum was obtained from an AXME (ME $C_{16:0}$, ME $C_{17:2}$, ME $C_{17:1}$, ME $C_{17:0}$, ME $C_{18:4}$, ME $C_{18:3}$, ME $C_{18:2}$, ME $C_{18:1}$). Because only mass differences between quasimolecular and fragment ions were used for assignment of acyl chains, the location of double bonds could not be determined by the mass spectrum. Thus, many isomers of astaxanthin esters in *H. pluvialis* could not be identified unequivocally. We have observed that the fragmentation pattern of astaxanthin esters was dominated by the loss of fatty acid and water. Protonated $[M + H]^+$ resulted from the positive ion mode. A total of eight AXME have been identified. The mass spectrum was obtained from an AXDE (DE $C_{16:0}/C_{16:0}$, DE $C_{16:0}/C_{18:2}$, DE $C_{18:1}/C_{18:3}$, DE $C_{18:1}/C_{18:2}$, DE $C_{18:1}/C_{18:1}$) in *H. pluvialis*. The basic peaks of other astaxanthin diesters showed characteristic fragment ions of losing one fatty acid, but their fragment ions of losing the second fatty acid had relatively weaker intensity.

Effect of TC, AX, and AXEs on UV-DMBA-Induced Skin Carcinogenesis in in Vivo Model. Having identified uniquely modified carotenoids such as esterified AX in a natural source, *H. pluvialis*, and taking the fact that carotenoids exhibit anticancer property differentially, through different mechanisms, the following were hypothesized in this study: (1) the comparative anticancer efficacy of differentially esterified AX (AX, AXME, and AXDE) in UV-DMBA-induced skin cancer model; (2) the probable mechanism of action such as free radical scavenging or vitamin A activity; and (3) differences in the ability of AX and AXEs on retinol and TBARS in the plasma of experimental animals. These questions were addressed because AX has been reported to exhibit multiple potencies of protecting skin during the physiological conditions of growth and development, when they are exposed to photo-oxidation. Also, evidence has been accumulated in the literature regarding the accumulation of carotenoids in the epidermal cells of skin, attributing the skin protective abilities. The aim of the current work indeed is to understand the role of AXEs under the above-mentioned conditions. Accordingly, experiments were designed with the purified fractions of AX and AXEs from *H. pluvialis* with the standardized protocol of our laboratory against the skin during UV exposure condition that is known to cause carcinogenic conditions in humans. Because UV is known to induce reactive oxygen species (ROS), the ability of these AX and AXEs to inhibit TBARS and the subsequent induction of tyrosinase that happens due to depletion of antioxidant GSH and antioxidant enzymes that are known to defend the animals against oxy radicals were studied. Retinol content was also measured in both the serum and liver of animals, which indicates the efficiency of conversion of AX and AXEs toward vitamin A activity. Results were compiled with appropriate statistical methods and interpreted to arrive at the role of AX and AXEs against skin cancer.

Exposure of UV-DMBA to rats showed alterations in the texture of the skin, in addition to inflammatory patches. Skin lesions with tumor nodules/tumor mass, angiogenesis, and inflammation was observed. No such skin tumor lesions and bleeding are noted in either healthy or sample control groups (Figure 1A,C,E,G,I). Animals pretreated with TC, AX, and AXEs showed different levels of tumor reduction (Figure 1B,D,F,H,J). AXEs, however, showed the best inhibition with 88–96% reduction in tumor index at 200 μg/kg bw (Table 2). AXDE was found to be better than AXME (1.1-fold) among AXEs. The differential effects of TC, AX, AXME, and AXDE appear to be due to their structural variations.

Quantitation of the data presented in Table 2 indicated that all fractions showed good protection; however, maximum protection was observed with AXDE followed by AXME, TC, and AX. It is interesting to also compare our data with those Choi et al.;[30] the efficacy of AX in humans would be better by 2.5-fold because the area under curve (AUC) of AX after its oral administration at a dose of 40 mg in human subjects, 80.8 μg min/mL, was close to 77.3 μg min/mL after its oral administration at a dose of 100 mg/kg in rats. In other words, it is possible to observe a 2.5-fold better efficacy of AX in offering anticancer property. However, because AXEs appear to be more potent, it would be interesting to compare their pharmacokinetics to understand their practical feasibility.[30]

Furthermore, it is also important to highlight here the significance of the slight modification in the methodology of UV-DMBA-induced cancer in rats. Conventionally, significant levels of tumors were induced only after 20–30 weeks[31−33] as opposed to 60 days in the current study, where we could observe the same between 8 and 9 weeks. This early induction of tumor could be due to exposure to UV radiation daily (30 min/day) and DMBA (weekly twice). The current study thus highlights the mode of early induction of skin tumors in an experimental animal model, which offers a desirable reduction of time in animal experiments.

Histopathological Changes. Histopathological observations revealed that skin lesions with normal histological features were observed for healthy controls (Figure 2A). Animals fed with just samples alone also showed normal structures (Figure

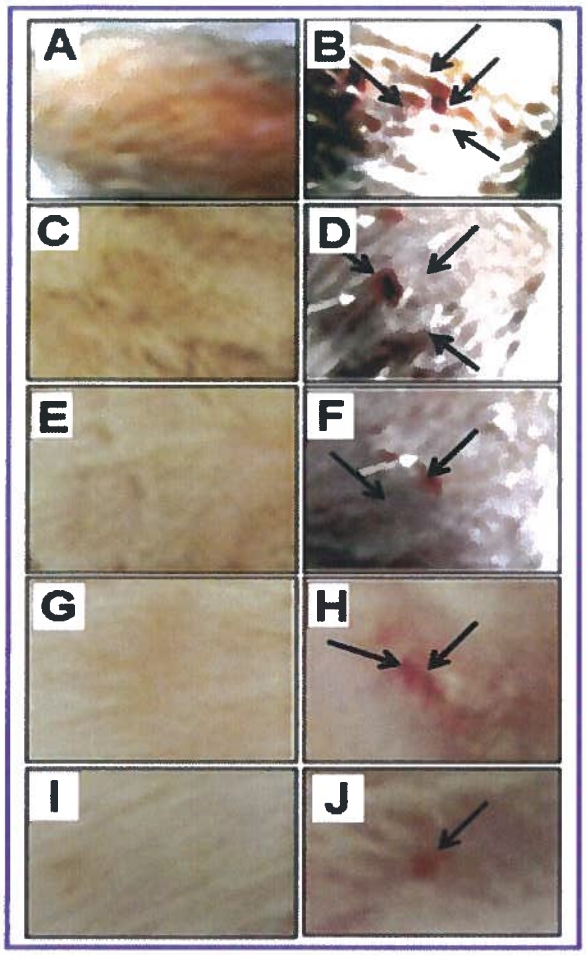

Figure 1. Photographs of rat skin tumors: (control groups) healthy (A), AX200* (C), TC200* (E), AXME200* (G), and AXDE200* (I); (UV-DBMA-treated groups) UV-DMBA (B), UV+AX200* (D), UV +TC200* (F), UV+AXME200* (H), and UV+AXDE200* (J). *, μg/kg bw.

Table 2. Quantitative Differences in Tumor Incidences in Healthy, UV-DMBA, and UV-DMBA+AX/AXE Samples[a]

group	tumor incidence (%)	mean tumor burden (mm^3)	reduction in mean tumor burden (%)
healthy c			
UV-DMBA	100 a	748.84 ± 89.13 a	0
AX200 c	–[b]	–[b]	–[b]
TC200 c	–[b]	–[b]	–[b]
AXME200 c	–[b]	–[b]	–[b]
AXDE200 c	–[b]	–[b]	–[b]
AX200*	44.17 b	258.34 ± 38.47 b	65.51 c
TC200*	19.8 c	115.73 ± 12.70 c	84.54 b
AXME200*	15.85 d	87.38 ± 9.85 d	88.33 b
AXDE200*	6.96 e	24.34 ± 4.33 e	96.74 a

[a]Each value represents the mean ± SD (n = 6). Values not sharing a similar letter within the same column are significantly different (p < 0.05) as determined by ANOVA. *, μg/kg bw. AX, astaxanthin; TC, total carotenoid; AXME, monoester of astaxanthin; AXDE, diester of astaxanthin from *H. pluvialis*. [b]–, not found.

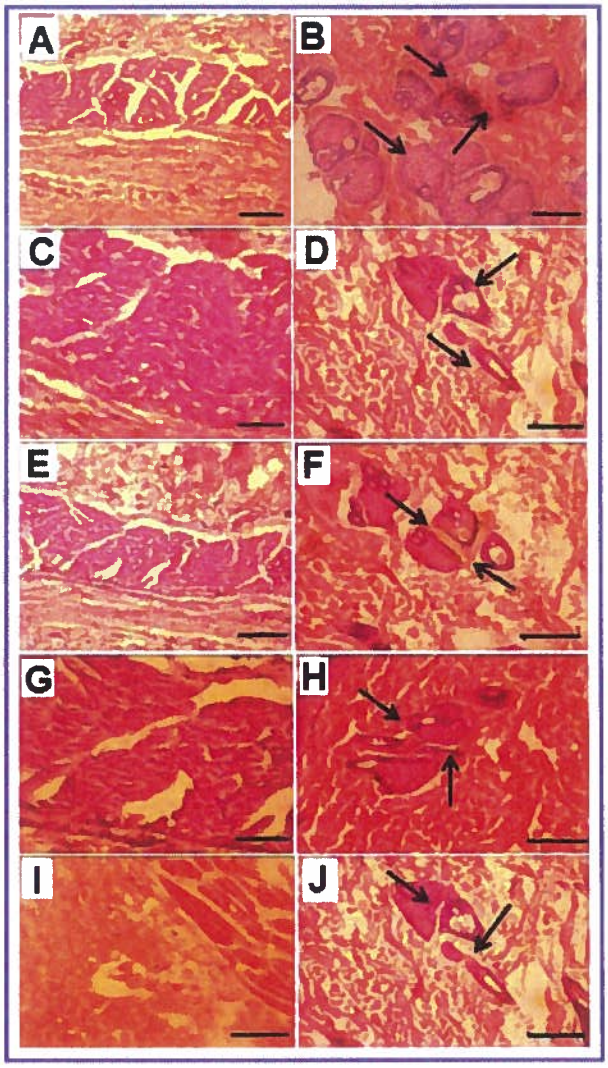

Figure 2. Histopathological control groups: healthy (A), AX200* (C), TC200* (E), AXME200* (G), and AXDE200* (I). UV-DBMA +samples-treated groups: UV-DMBA (B), UV+AX200* (D), UV +TC100* (F), UV+AXME200* (H), and UV+AXDE200* (J) showed a significant epidermal thickness in the sections of UV-DMBA-induced group when compared to healthy and sample control groups. *, μg/kg bw.

2C,E,G,I). UV-DMBA-induced rats showed greater changes in the epidermis and dermis including irregular distribution with finger-like papilloma indicative of cancerous growth (Figure 2B). The tumors were composed of focal proliferation of squamous cells and characterized by the presence of some necrotic cells, keratinization, and epithelial pearls. AXDE-treated groups showed reduction in these lesions, although marginal epidermal thickness relative to that of the normal group was observed (Figure 2J). Only partial protection as per skin lesions was observed in AXME- and TC-treated groups, suggesting that esters can offer better protection (Figure 2F,H) than AX (Figure 2D). Tumor areas in control and treated sample sections were localized using Image J software[16] (Figure 3). Results were compared between the groups using SPSS Statistics 17.0. A one-way ANOVA test followed by a post hoc Tukey test was performed.

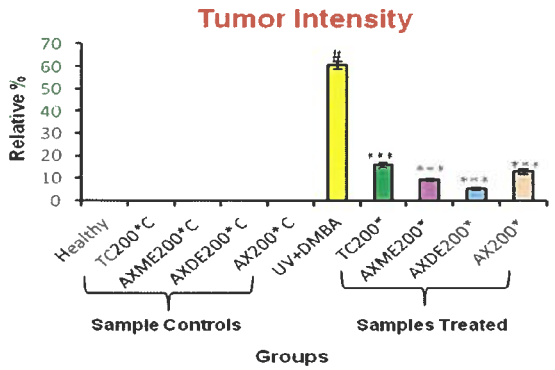

Figure 3. Analysis of tumor intensity: tumor intensity was quantitated with biometric analysis of histopathological sections of AX-, TC-, AXME-, and AXDE-treated groups. Results were compared with healthy, UV+DMBA, and sample controls. Data showed significant reduction in the tumor intensity in terms of tumor area as obtained by Image J analysis in treated groups when compared to that of only UV-DMBA-exposed group of animals. One-way ANOVA followed by post hoc Tukey test indicated the level of significance, where p values are indicated by *** $p < 0.001$. * μg/kg bw.

Hematological Changes. Hematological changes were observed in UV-DMBA-treated groups when compared to healthy control and sample control groups. Hematological parameters such as lymphocytes, mean cell hemoglobin count, platelets, mean cell volume, packed cell volume, red blood cells, mean cell hemoglobin, hemoglobin, and neutrophils were observed. Platelets, lymphocytes ,and neutrophil counts were affected significantly (Table 3).

Astaxanthin and Retinol Levels in Serum and Liver. Vitamin A is essential for a number of physiological processes, such as regulation of cell differentiation, cell proliferation, vision, and reproduction. Because carotenoids are the major sources of vitamin A/retinol, their levels were measured in the serum and liver of all groups of animals (Figure 4). The maximum levels of astaxanthin (366 ng/mL) and retinol (72 ng/mL) were found in the serum of the AXDE-treated group, followed by AXME, when compared to healthy control and other groups (Figure 4A,B). In the liver, AXDE/AX-treated groups contained 450/400 and 18/6 ng/g of liver tissue of astaxanthin and retinol, respectively, suggesting that bioconversion to retinol is much better with AXDE than with AX alone (Figure 4C,D). Furthermore, it is interesting to note that maximum depletion of AX and retinol was observed in the AXDE-treated group followed by UV-DMBA-exposed animals relative to AXDE control, indicating that the utilization of retinol is greater during the UV-DMBA-induced condition. The data further emphasize that AXDE may offer protection to animals against UV-DMBA via its conversion to retinol. Similar results were observed in the liver also.

Inhibitory Effect of Tyrosinase Activity. The rate-limiting enzyme tyrosinase is responsible for melanin pigment biosynthesis in human skin, which takes place within specialized organelles known as melanosomes. Skin tyrosinase has been widely used as the target enzyme for screening and characterizing potential tyrosinase inhibitors.[34] The activity was found to be increased by 7.4-fold in UV-DMBA-treated groups, and this was inhibited up to 4.5- and 3.0-fold in AXDE- and AXME-treated groups, respectively (Table 4).

Changes in Antioxidant Enzymes and Lipid Peroxidation Levels in Serum and Skin Homogenates. Effects of TC, AX, AXME, and AXDE on the antioxidant enzymes and TBARS levels were measured in all groups of experimental animals. Table 5 indicates the changes in the antioxidant enzymes and lipid peroxidation levels in the serum of UV-DMBA-induced rats. SOD levels increased by ~2-fold in the serum, and CAT and GSH levels were found to be depleted by 1-fold. Furthermore, an approximately 10-fold increase in TBARS levels in UV-DMBA-treated groups observed was normalized up to 65% upon treatment with AXEs. The antioxidant enzymes and lipid peroxidation levels were also measured in skin homogenate (Table 5). The SOD level increased in skin by 2.7-fold. CAT and GSH levels decreased by 1-fold in the UV-DMBA-treated group, which was restored to normal levels upon treatment with AX and AXEs. A 10.6-fold increase in TBARS in UV-DMBA-treated skin tissues was further recovered up to 60% upon treatment.

SGPT, SGOT, and SALP Levels in Serum and Skin Homogenates. SGPT, SGOT, and SALP levels were measured in serum and skin homogenates of UV-DMBA-induced rats (Table 6). The data revealed the normalization of these enzymes by treated samples. Toxicity profiles were also studied for AX, TC, AXME, and AXDE. SGPT, SGOT, and SALP showed enhancement of activities in serum and skin homogenates of UV-DMBA-treated groups. SGPT (1.8-fold), SGOT (1.7-fold), and SALP (1.8-fold) increased in the serum of the UV-DMBA-induced group, whereas the enzyme activities were modulated significantly in the AXDE-treated group. In the case of skin homogenates, 1.9-, 1.6-, and 2.1-fold increases in SGPT, SGOT, and SALP activities, respectively, were observed in UV-DMBA-induced groups. However, treatment with AXEs resulted in maximum recovery.

Tyrosinase Inhibitory Potentials of AX and AXEs in Vitro. It was found that AXDE and AXME had shown potent inhibitory effects on L-Dopa oxidase activity of tyrosinase and that the inhibitory activities increased with concentrations.

Table 3. Hematological Analysis of Healthy, UV-DMBA, and UV-DMBA+AX/AXE Samples[a]

group	PLT ($\times 10^3/\mu$L)	LYM (%)	LYM# ($\times 10^3/\mu$L)	neutrophils
healthy c	904 ± 6.00 b	76.6 ± 3.01 d	7.60 ± 1.23 d	19.34 ± 1.78 b
UV+DMBA	759 ± 6.31 e	86.9 ± 5.10 a	13.9 ± 1.36 a	10.45 ± 1.40 d
AX200*	993 ± 8.90 a	71.8 ± 6.72 c	6.43 ± 0.92 e	23.75 ± 1.85 a
TC200*	833 ± 6.62 d	81.3 ± 4.61 b	8.30 ± 1.04 c	14.45 ± 2.08 c
AXME 200*	868 ± 7.73 c	81.9 ± 3.40 b	9.10 ± 1.37 b	11.23 ± 1.82 d
AXDE 200*	792 ± 6.08 f	76.1 ± 3.82 d	8.2 ± 0.70 c	19.83 ± 2.31 b

[a]Each value represents the mean ± SD ($n = 6$). Values not sharing a similar letter within the same column are significantly different ($p < 0.05$) as determined by ANOVA. *, μg/kg bw. PLT, platelet count; LYM, lymphocytes; AX, astaxanthin; TC, total carotenoid; AXME, monoester of astaxanthin; AXDE, diester of astaxanthin from *H. pluvialis*.

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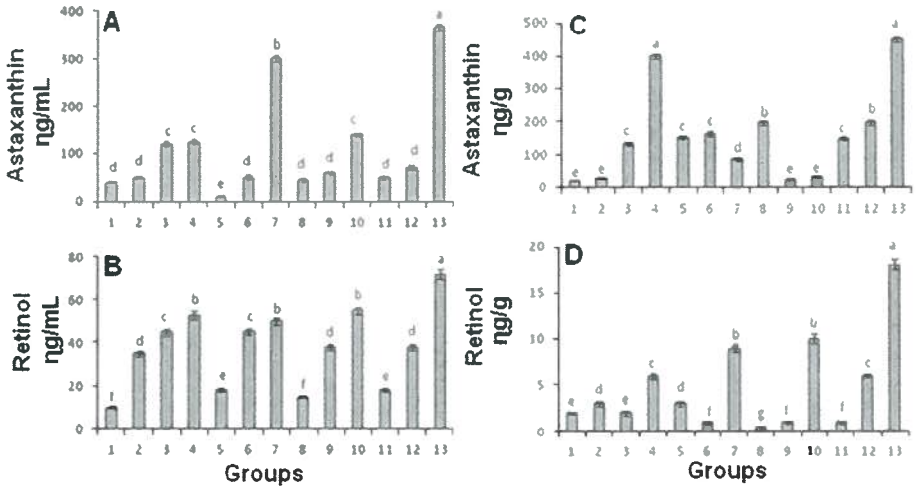


Figure 4. Astaxanthin and retinol content in the serum (A, B) and liver (C, D) of UV-DMBA-induced and sample-treated groups: UV-DMBA (1), AX100* (2), AX200* (3), AX200*control (4), TC100* (5), TC200* (6), TC200* control (7), AXME100* (8), AXME200* (9), AXME200* control (10), AXDE100* (11), AXDE200* (12), and AXDE200* control (13). Each value represents the mean ± SD ($n = 6$) of analyses. Values not sharing a similar letter between the groups are significantly different ($p < 0.05$) as determined by one-way ANOVA. *, μg/kg bw.

Table 4. Effect of AX and AXE on Tyrosinase Activity in Serum and Skin Homogenates of UV-DMBA-Induced Experimental Rats[a]

group	serum (μmol/mg protein)	skin (μmol/mg protein)
healthy c	0.19 ± 0.07 i	16.5 ± 0.40 g
UV+DMBA	21.33 ± 3.07 a	119.33 ± 9.04 a
AX100*	13.21 ± 3.14 b	72.27 ± 2.70 b
AX200*	11.03 ± 1.72 c	60.06 ± 3.40 d
AX200* c	0.32 ± 0.07 h	1.03 ± 0.14 h
TC100*	11.46 ± 2.14 c	78.57 ± 9.44 b
TC200*	9.18 ± 0.65 e	52.62 ± 7.45 c
TC200* c	0.30 ± 0.16 h	1.07 ± 0.85 h
AXME100*	10.28 ± 2.22 d	50.04 ± 8.31 c
AXME200*	8.24 ± 0.70 f	39.27 ± 6.73 e
AXME200* c	0.19 ± 0.08 i	1.25 ± 0.64 h
AXDE100*	9.94 ± 1.84 e	33.19 ± 0.50 e
AXDE200*	7.60 ± 1.26 g	26.06 ± 1.02 f
AXDE200* c	0.24 ± 0.11 h	1.169 ± 0.04 h

[a]Each value represents the mean ± SD ($n = 6$). Values not sharing a similar letter within the same column are significantly different ($p < 0.05$) as determined by ANOVA. *, μg/kg bw. C, control group; AX, astaxanthin; TC, total carotenoid; AXME, monoester of astaxanthin; AXDE, diester of astaxanthin from *H. pluvialis*.

AXDE had the highest tyrosinase inhibitory activity with an IC_{50} of 2.12 μg/mL followed by AXME (IC_{50} = 3.5 μg/mL) and was found to be 2.4−2.8-fold better than AX and TC, respectively (Figure 5).

Toxicity Studies. Body weight and relative organ weight at the termination of the experiment have been observed. There is no significant difference in the body weight gain profile, and the corresponding values of low and high doses were comparable with those of the control. The oral administration of TC, AX, AXME, and AXDE did not cause any apparent changes in clinical signs such as survivability or any gross visible changes attributable to toxicity in the organ weights of rat. There was no significant difference either in the biochemical profile or in the serum or skin homogenate of various groups of animals or in behavioral aspects. Similar results were observed when astaxanthin-rich *H. pluvialis* biomass was fed to rats and no adverse effects on animals were found.[10]

Melanoma is a relatively common and one of the most malignant tumors in humans. The social impact of skin cancer leading to melanoma is significant because it has a very poor prognosis;[35] more importantly, many melanoma cases occur in young individuals, and there is little effective treatment available once it becomes metastatic.[36] During the transformation of normal melanocytes into malignant ones, several steps of reactions, including imbalance in the regulation of proliferation, apoptosis, galectin-3 expression, and the enhanced activation of tyrosinase enzyme, are key factors, and hence they can be used as promising targets for management of the disease.[37] In the current study, we examined the role of carotenoids from *H. pluvialis*, a microalga which produces varieties of carotenoids, particularly the presence of ~80% of mono- and diesterified forms of astaxanthin of total carotenoids as opposed to either free astaxanthin in other plant sources. It is of current interest to find out the bioactive potency of esterified astaxanthin in comparison with astaxanthin alone. Differences in bioavailability and bioconversion to vitamin A in comparison with the standard astaxanthin are also of interest because astaxanthin primarily functions as a precursor of vitamin A and vitamin A exhibits anticancer property. UV-DMBA-induced skin cancer model has been employed during the study. Histopathological, biochemical, and hematological parameters have been analyzed to determine either the diagnostic or prognostic value of AX and AXEs.

The results of the study indicated for the first time that AXE exhibited a 3-fold higher potential in inhibiting skin cancer incidences, and the efficacy could be attributed to increased bioavailability as revealed by higher levels of vitamin A as retinol in the serum of AXE-treated animals than in those animals ingesting AX or TC alone. Results were substantiated by histopathological studies, where 2−3-fold increased reduction of papillomas was observed in groups of animals treated with AXEs when compared to AX-treated animals. Furthermore, a normal histological pattern in the skin of control as well as AX- and AXE-receiving rats indicated no adverse effect on the skin. In UV-DMBA-treated rats, all of the

Table 5. Effect of AX and AXEs on Antioxidant/Antioxidant Enzymes and TBARS Levels in Serum and Skin of UV-DMBA-Induced Experimental Rats[a]

group	SOD (U/mg protein)	catalase (nmol H_2O_2/mg protein)	GSH (μg GSH/mg protein)	TBARS (μmol/MDA/mg protein)
		Serum		
healthy c	11.33 ± 2.65 m	0.48 ± 0.00 a	2.82 ± 0.40 a	0.45 ± 0.11 g
UV+DMBA	33.61 ± 3.53 a	0.21 ± 0.03 e	1.71 ± 0.24 d	4.56 ± 0.81 a
AX100*	23.18 ± 1.94 c	0.26 ± 0.02 e	2.30 ± 0.16 bc	3.11 ± 0.13 c
AX200*	19.04 ± 2.01 f	0.31 ± 0.03 d	2.28 ± 0.44 c	3.22 ± 0.12 c
AX200* c	13.36 ± 3.46 j	0.35 ± 0.07 c	2.34 ± 0.17 bc	0.46 ± 0.13 g
TC100*	24.01 ± 6.60 b	0.23 ± 0.05 e	2.11 ± 0.20 bc	3.60 ± 0.10 b
TC200*	19.89 ± 1.13 e	0.34 ± 0.03 c	2.17 ± 0.41 bc	3.08 ± 016 c
TC200* c	17.18 ± 3.01 g	0.36 ± 0.03 c	2.19 ± 0.12 bc	0.47 ± 0.06 g
AXME100*	24.01 ± 2.07 b	0.31 ± 0.07 c	2.33 ± 0.50 bc	2.34 ± 0.23 d
AXME200*	13.81 ± 1.73 i	0.41 ± 0.08 ab	2.41 ± 0.36 a	2.15 ± 0.21 d
AXME200* c	12.77 ± 2.76 k	0.42 ± 0.10 ab	2.94 ± 1.84 a	0.46 ± 0.05 g
AXDE100*	20.73 ± 0.58 d	0.29 ± 0.01 cd	2.52 ± 0.38 b	1.82 ± 0.10 e
AXDE200*	15.84 ± 1.81 h	0.36 ± 0.02 b	2.68 ± 0.14 bc	1.59 ± 0.23 f
AXDE200* c	11.58 ± 2.20 l	0.45 ± 0.10 a	2.73 ± 0.14 a	0.35 ± 0.10 g
		Skin		
healthy c	164.89 ± 17.35 l	0.16 ± 0.02 a	18.95 ± 2.54 a	0.33 ± 0.08 h
UV+DMBA	452.50 ± 24.70 a	0.07 ± 0.01 d	9.14 ± 1.74 k	3.51 ± 0.15 a
AX100*	280.45 ± 7.50 d	0.11 ± 0.01 c	15.12 ± 5.00 h	3.14 ± 0.06 b
AX200*	243.65 ± 75.90 f	0.12 ± 0.03 c	15.57 ± 7.68 f	2.93 ± 0.14 c
AX200* c	182.40 ± 20.10 j	0.14 ± 0.02 b	16.92 ± 3.21 c	0.32 ± 0.03 h
TC100*	309.40 ± 14.62 b	0.08 ± 0.01 d	13.14 ± 0.37 j	3.16 ± 0.10 d
TC200*	279.63 ± 21.91 b	0.12 ± 0.09 c	14.89 ± 1.53 i	2.89 ± 0.15 c
TC200* c	168.73 ± 16.73 m	0.14 ± 0.03 b	16.94 ± 3.04 c	0.30 ± 0.08 h
AXME100*	292.02 ± 19.50 c	0.11 ± 0.04 c	14.50 ± 0.60 i	2.39 ± 0.03 d
AXME200*	248.97 ± 21.58 f	0.13 ± 0.05 c	16.07 ± 1.03 e	2.31 ± 0.17 d
AXME200* c	208.08 ± 30.27 i	0.15 ± 0.03 a	16.97 ± 4.81 c	0.68 ± 0.14 g
AXDE100*	275.09 ± 26.21 e	0.12 ± 0.06 c	15.38 ± 3.77 g	2.16 ± 0.06 e
AXDE200*	223.12 ± 10.36 h	0.13 ± 0.02 c	16.66 ± 4.31 d	1.90 ± 0.10 f
AXDE200* c	173.04 ± 13.11 k	0.16 ± 0.02 a	17.03 ± 0.68 b	0.31 ± 0.10 h

[a]Values are expressed as the mean ± SD. Values are not sharing a similar letter within the same column are significantly different ($p < 0.05$) as determined by ANOVA. *, μg/kg bw. C,control; AX, astaxanthin; TC, total carotenoid; AXME, monoester of astaxanthin; AXDE, diester of astaxanthin from *H. pluvialis*.

tumors were confirmed to be papillomas, whereas the extent of hyperchromatia in the tumors of the rats that received AXEs was observed to be approximately 2−3-fold less than that of the TC/AX treatment. AXDE showed better potency than AXME and AX. Results thus indicate the chemopreventive role of AXEs in the potential management of skin cancer.

In addition, as it is known that AX exerts a beneficial effect against several disorders by antioxidative properties, the current results of anticancer potentials of skin cancer were correlated to antioxidant capacities of AX and AXEs, which was established from our laboratory previously. Results, however, reveal that the protective effect is not directly proportional to the antioxidant capacities because AXEs showed lowered antioxidant activity relative to AX and TC.[15] The data thus may open up the possibility that metabolites released into the serum from AX- and AXE-treated group may be more antioxidative in nature or may be going through a nonantioxidative route such as tyrosinase inhibition. This interpretation may be supported by one of our previous studies that had revealed that photooxidized lutein has more melanoma cell killing effect than the lutein per se.[38] Also, observed results of the efficacy of AXDE < AXME < AX are also supported by increased inhibition of lipid peroxidation and enhancement of antioxidant and antioxidant enzyme levels in AXDE-treated groups followed by AXME- and AX-treated groups. Lipid peroxidation has been reported to play an important role in the control of cell proliferation and to induce cytotoxicity and cell death[39,40] in the case of normal cellular environment. Contradictory to this, tumor cells were found to be more resistant to antioxidant (GSH) and antioxidant enzyme levels (superoxide dismutase, peroxidase, etc.), which were found to be depleted in UV-DMBA-induced tumorigenic animals. Similarly, results were obtained when astaxanthin was exposed to UV-A light; it was protected against UV-A light induced oxidative stress in in vivo models when compared with other carotenoids.[41−44] AX is a very efficient antioxidant due to the unique structure of the terminal ring moiety.[45,46] It is therefore feasible that AX has an affinity for the superoxide free radical and thus may act as a satisfactory antioxidant, ultimately preventing an increase in basal SOD activity.

Increased retinol conversion (increased bioavailability) rate by AXDE relative to AX (Figure 2) may offer enhanced anticancer potential. Increased levels of AXDE and retinol in the AXDE control groups of animals suggest that there is an increased uptake of AXDE in vivo followed by AXME and other carotenoids. Furthermore, within the bioavailable AX and AXEs, maximum depletion of 1.4-fold of retinol was observed in only AXDE followed by AXME, suggesting that AXDE may

Table 6. Effect of AX and AXEs on SGPT, SGOT, and SALP in the Serum and Skin of UV-DMBA-Induced Experimental Rats[a]

group	SGPT (U/mg protein)	SGOT (U/mg protein)	SALP (U/mg protein)
		Serum	
healthy c	108.89 ± 21.61 m	105.72 ± 3.28 l	227.56 ± 22.60 l
UV+DMBA	195.33 ± 16.45 a	184.66 ± 3.21 a	409.33 ± 19.10 a
AX100*	173.10 ± 11.77 b	153.79 ± 18.58 d	352.34 ± 26.67 d
AX200*	166.26 ± 21.62 c	145.72 ± 10.42 f	322.70 ± 14.94 f
AX200* c	113.08 ± 16.32 j	111.03 ± 14.51 j	249.96 ± 11.91 j
TC100*	168.28 ± 9.87 c	171.54 ± 12.19 b	370.94 ± 17.39 b
TC200*	156.94 ± 31.64 e	152.69 ± 23.26 e	329.99 ± 18.72 e
TC200* c	110.35 ± 12.56 l	106.16 ± 15.56 k	247.80 ± 15.42 k
AXME100*	161.98 ± 13.55 d	159.03 ± 19.17 c	359.33 ± 12.90 c
AXME200*	144.90 ± 5.79 g	138.40 ± 29.01 g	307.89 ± 23.63 g
AXME200* c	111.91 ± 12.55 jk	112.39 ± 73.18 i	257.51 ± 19.40 i
AXDE100*	148.35 ± 16.02 f	153.61 ± 13.05 d	323.69 ± 9.13 f
AXDE200*	138.63 ± 14.21 h	121.77 ± 8.17 h	287.16 ± 21.07 h
AXDE200* c	114.61 ± 16.05 i	107.48 ± 13.50 k	226.26 ± 11.18 l
		Skin	
healthy c	72.68 ± 15.06 fg	319.25 ± 25.44 i	329.31 ± 25.61 j
UV+DMBA	126.17 ± 7.41 a	668.84 ± 32.80 a	591.93 ± 23.09 a
AX100*	101.99 ± 23.97 c	549.87 ± 35.32 b	516.62 ± 34.21 c
AX200*	95.89 ± 15.60 d	421.40 ± 28.15 g	489.60 ± 21.90 e
AX200* c	77.95 ± 24.35 fg	295.92 ± 15.61 j	390.18 ± 15.23 h
TC100*	115.05 ± 8.13 b	547.59 ± 18.82 b	528.50 ± 25.12 b
TC200*	95.23 ± 6.14 b	495.16 ± 21.93 c	498.76 ± 31.03 d
TC200* c	75.05 ± 27.48 g	316.12 ± 36.88 i	365.06 ± 34.49 j
AXME100*	93.09 ± 12.51 d	557.73 ± 14.91 b	499.00 ± 14.03 d
AXME200*	82.85 ± 12.97 e	484.05 ± 11.51 c	442.59 ± 12.10 f
AXME200* c	78.53 ± 16.11 fg	311.27 ± 16.29 i	378.39 ± 25.78 i
AXDE100*	100.49 ± 29.97 c	536.06 ± 18.92 b	484.47 ± 14.27 e
AXDE200*	79.75 ± 3.09 f	447.20 ± 3.85 f	418.47 ± 18.25 g
AXDE200* c	71.58 ± 14.42 h	331.53 ± 17.30 h	378.64 ± 20.95 i

[a]Values are expressed as the mean ± SD. Values not sharing a similar letter within the same column are significantly different ($p < 0.05$) as determined by ANOVA. *, μg/kg bw. C, control; AX, astaxanthin; TC, total carotenoid; AXME, monoester of astaxanthin; AXDE, diester of astaxanthin from *H. pluvialis*.

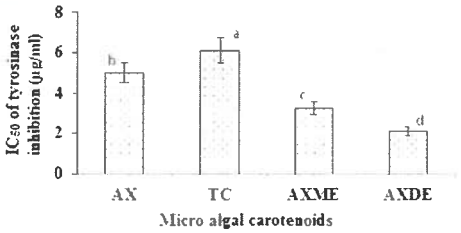


Figure 5. Inhibition of skin tyrosinase activity in vitro by AX and AXEs. AX, astaxanthin; TC, total carotenoid; AXME, monoester of astaxanthin; AXDE, diester of astaxanthin from *H. pluvialis*. Each value represents the mean ± SD ($n = 3$). Values not sharing a similar letter between the groups are significantly different ($p < 0.05$) as determined by one-way ANOVA. *, μg/kg bw.

be more bioavailable, yielding more retinol, which may be utilized in animals during their exposure to UV-DMBA. Thus, AXDE may confer better anticarcinogenic potency than other compounds tested. AXEs may offer protection against skin cancer at least partly by an antioxidative route, probably via mediation of their metabolites rather than exhibiting antioxidants by them, per se, and partly by tyrosinase inhibitory potentials due to increased bioavailability of AXDE.

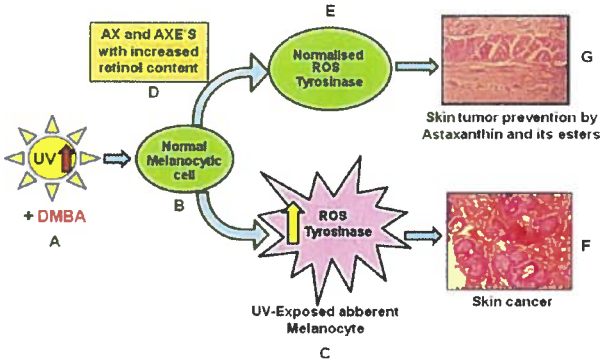
Our observed data are strongly encouraged by the observations made previously by Camera et al.[12] and Savouré et al.;[13] these two independent studies revealed that AX has better anticancer potency than other carotenoids, canthaxanthin and β-carotene. Furthermore, it was noted that UV-induced skin cancer is via modifications of polyamine metabolism, particularly by epidermal ornithine decarboxylase (ODC). ODC induction was amplified by several-fold in the skin of vitamin A-deficient animals relative to vitamin-normalized animals and was not protected effectively by carotenoids. However, AX had a stronger inhibitory effect than other carotenoids on polyamine accumulation, suggesting an alternate mechanism of protection against skin cancer besides retinol activity by AX. Now with the observed results that AXEs are more protective against cancer induction by UV-DMBA than AX, it is important to address the role of AXEs on polyamine accumulation in comparison with that of AX, which can further strengthen the use of AXE-rich *H. pluvialis* against skin cancer.

Our observed data in the current paper indeed show the most potent anticancer form of carotenoids probably better than AX, although the latter by itself is more potent than other reported carotenoids such as cantaxanthin and β-carotene. Studies thus throw light on the possible application of AXEs and AX-rich *H. pluvialis* if one compares the mechanism of action of AX and AXEs with that of known anticancer drugs and prognostic factors in various types of malignancies. Furthermore, the neutrophil to lymphocyte ratio (NLR) has

been documented as a simple index of systematic inflammatory response in critically ill malignancy patients. Similarly, the preoperative platelet to lymphocyte ratio (PLR) has been also suggested as an independent significant prognostic indicator in pancreatic cancer. In this perspective, AXDE was effective in restoring normal levels of NLR and PLR ratios, suggesting the role of AXDE as immunomodulator during inhibition of skin cancer in the studied model.

With the observed results, therefore, Scheme 1 has been proposed to explain the mechanism of protection of UV-

Scheme 1. Exposure of Experimental Animals to UV-DMBA (A) Induces Skin Cancer (F) via Up-regulation of Tyrosinase Enzyme in the Melanocyte[a]



[a]Increased exposure of normal melanocyte (B) to UV radiation and DMBA (A) results in the aberrant melanocyte (C) due to increased ROS and tyrosinase. This results in skin cancer (F), astaxanthin (AX) and its esters (AXEs) (D) have been shown to down-regulate tyrosinase and ROS (E) with increased retinol content resulting in protection to skin against cancer (G). AXEs > AX from *H. pluvialis* thus are known to protect skin against UV-DMBA-induced skin cancer.

DMBA-induced cancer in animals by AX and AXEs of *H. pluvialis*. Multistep action such as inhibition of accumulation of ROS and inhibition of tyrosinase enzyme activity may result in inhibition of UV-DMBA-induced skin cancer. Subsequently, the mentioned properties may prevent uncontrolled proliferation of melanocytes and prevention of accumulation of melanocytes and melanin pigments in addition to the inhibition of polyamine accumulation. Immunomodulatory action may potentiate the anticancer property of *H. pluvialis*.

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Notes

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ABBREVIATIONS USED

AX, astaxanthin; AXEs, astaxanthin esters; AXME, astaxanthin monoesters; AXDE, astaxanthin diesters; TC, total carotenoid; UV, ultraviolet; DMBA, 7,12-dimethylbenz(*a*)anthracene; TLC, thin layer chromatography; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography–mass spectrometry; APCI, atmospheric pressure chemical ionization; ROS, reactive oxygen species; CAT, catalase; SOD, superoxide dismutase; GSH, glutathione; MDA, malondialdehyde; TBA, thiobarbituric acid; SGPT, serum glutamate pyruvate transaminase; SGOT, serum glutamate oxaloacetate transaminase; SALP, serum alkaline phosphatase; LYM, lymphocytes; PLT, platelet count; HBCs, human buccal cells; NLR, neutrophil to lymphocyte ratio; PLR, platelet to lymphocyte ratio; EDTA, ethylenediaminetetraacetic acid; MDA, malondialdehyde; TBARS, thiobarbituric acid reactive substances

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