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Research Article

Liposome Encapsulated Astaxanthin altered Biochemical Profile in Diethylnitrosamine induced Hepato Carcinoma on Swiss Albino Mice

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ABSTRACT

Cancer is a disease in which a group of abnormal cells grows uncontrollably by disregarding the normal rules of cell division. Across several cancers, hepatocellular carcinoma (HCC) is one of the most aggressive cancers worldwide. It is held responsible for up to 1 million deaths globally per annum. HCC is inflammation-related cancer, as a chronic inflammatory state is necessary for cancer appearance. In this study, the drug astaxanthin and encapsulated astaxanthin was tested against HCC. Mice were divided into seven groups; group I: control, group II: diethylnitrosamine (DEN) induced, group III: DEN + 50 mg/kg astaxanthin, group IV: DEN + 100 mg/kg astaxanthin, group V: DEN + 50 mg/kg encapsulated astaxanthin, group VI: DEN + 100 mg/kg encapsulated astaxanthin, and group VII: DEN + 10 mg/kg sorafenib. Regular diet was given. Body weight, food intake, and water intake were noted. Other biochemical parameters, such as, alkaline phosphatase (ALP), aspartate aminotransferase (AST), albumin, proteins, and tumor necrosis factor-alpha (TNF- α), were determined. Finally, the liver was removed from each mice of different groups by sacrificing them, and histopathology was done. *In vivo* evaluation in mice models showed significant antitumor activities by both encapsulated and non-encapsulated astaxanthin at 100 mg/kg, as compared with the control, DEN induced group, and positive drug sorafenib. This research suggested that encapsulated astaxanthin can also be used as chemotherapeutic agent for the treatment of HCC.

INTRODUCTION

Various studies have been proved the links between humans and diet.^[1,2] Numerous substances naturally present in foodstuffs, particularly anti-oxidant compounds, have shown a promising effect as potential chemopreventive agents.^[3-5] Among these phytonutrients, the yellow, orange, and red carotenoid pigments have recently sparked much interest. Several naturally occurring carotenoids other than β -carotene have exhibited anti-cancer activity,^[6-9] and are being considered further as potential chemopreventive agents. Among these carotenoids, the red pigment astaxanthin is of particular interest in health management due to its unique structural and chemical properties.^[10,11] Among various carotenoids, the red pigment astaxanthin shows particular interest in the health field, is widely distributed in shrimp, salmon,

crab, and asteroidean.^[6] Astaxanthin was approved by the United States Food and Drug Administration (USFDA) in 1987 as a feed additive for use in the aquaculture production. And in 1999, it was approved for use in nutraceutical industry as a dietary supplement.^[8] When compared to other carotenoids, such as, canthaxanthin, lutein, zeaxanthin, and β -carotene, more powerful anti-oxidative property was produced by astaxanthin.^[9] The two oxygenated groups on each ring structure were responsible for its anti-oxidant features (Fig. 1).^[10] It has

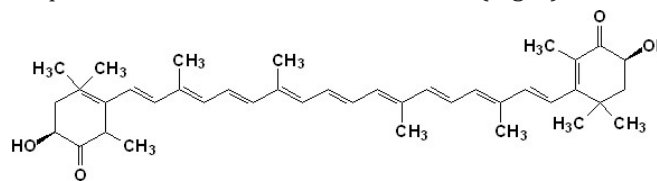


Fig. 1: Chemical structure of astaxanthin

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been proved that both *in vivo* and *in vitro* astaxanthin could protect against neurotoxin or oxidative stress-induced damage.^[11,12] Many researches have proved astaxanthin as an anti-oxidant therapeutic agent in models of brain injury^[13-15] and cardiovascular disease.^[16,17] Moreover, at least eight clinical studies have been conducted in cardiovascular disease to evaluate the dosing, bioavailability, and safety of astaxanthin.^[18] Notably, no significant side effects of astaxanthin have been reported so far. Among its potent anti-oxidative effects, evidence also suggests that astaxanthin has anti-cancer efficacy in multiple types of cancer, including oral cancer^[19] bladder carcinogenesis,^[20] colon carcinogenesis,^[21,22] leukemia,^[23] and HCC.^[24,25]

Sorafenib (Nexavar) is a small molecule that inhibits tumor-cell proliferation and tumor angiogenesis, and increases the rate of apoptosis in a wide range of tumor models. The main action of sorafenib is by inhibiting the serine-threonine kinases Raf-1 and B-Raf, and the receptor tyrosine kinase activity of 1, 2, and 3 vascular endothelial growth factor receptors (VEGFRs) and platelet-derived growth factor receptor β (PDGFR- β). Cellular signaling that is mediated by the Raf-1 and vascular endothelial growth factor (VEGF) pathways have been implicated in the molecular pathogenesis of HCC, providing a rationale for investigating sorafenib for this indication. In preclinical experiments, sorafenib had anti-proliferative activity in liver cancer cell lines, and it reduced tumor angiogenesis and tumor-cell signaling, and increased tumor-cell apoptosis in a mouse xenograft model of human HCC.^[26]

HCC is a malignant tumor of the digestive system and has a high mortality rate worldwide. Jemal and colleagues reported that the incidence of HCC has continuously increased in recent years and is the fifth most common cancer and the third leading cause of cancer death.^[27] HCC is a complex disease due to its polygenic, multifactorial, multi-stage evolution, and insidious onset, it is difficult to detect early, metastasizes easily, and is insensitive to chemotherapy.^[28,29] The protective effect of astaxanthin in human HCC cells has been rarely reported. Therefore, the application of astaxanthin in animal models requires a better understanding of its potential protective effects in human HCC and the corresponding molecular mechanisms, which may result in the development of astaxanthin for HCC patients.

Previous studies have indicated that inflammation is closely associated with HCC development. Persistent infection with either hepatitis B or hepatitis C virus, one of the major risk factors of HCC, induces chronic inflammation, and subsequent cirrhosis, thus, promoting HCC initiation and progression.^[30,31] HCC patients with high levels of inflammation, marked by increased levels of inflammatory cytokines and cells, tend to have a poor prognosis.^[32,33] Pro-inflammatory cytokines, including TNF- α , interleukin (IL)-1 β , and IL-6, are the principal mediators of tumor-

accelerating inflammation.^[34-36] Consequently, targeting tumor-accelerating pro-inflammatory cytokines may perhaps effect in HCC tumor regression.

TNF- α was initially identified by its ability to induce lysis in tumor cells.^[37] TNF- α exhibit two forms, such as, cell membrane-bound form and a soluble form. Two types of TNF- α receptors, TNF- α receptors 1 and 2, which are either cell membrane-bound or soluble, have been classified so far.^[37,38] Although TNF- α was initially identified as a tumor-killing cytokine, further studies have determined that it serves a more complex role in cancer development.^[37] Furthermore, TNF- α is a key mediator of the cancer-associated inflammatory networks that have strong tumor-promoting properties.^[39,40] Preclinical studies in various types of cancer, including breast, pancreatic, and blood cancer, have suggested that TNF- α stimulates tumor growth *in vivo* and that anti-TNF- α treatments may suppress tumor progression.^[41-43] Previous studies have demonstrated that the level of serum TNF- α and a number of other pro-inflammatory cytokines were significantly higher in patients with HCC compared with healthy individuals, thus, increased TNF- α was associated with the occurrence of HCC.^[44,45] High levels of TNF- α were also associated with increased inflammation in patients with chronic viral hepatitis C.^[46] These results suggest that TNF- α serves an important role in regulating inflammation in HCC. The present study was designed to compare the effects of encapsulated and non-encapsulated astaxanthin on HCC in mice models.

MATERIALS AND METHODS

Sample Preparation

Non-Encapsulated Astaxanthin

Astaxanthin, the red color powder, was purchased from Rudra Bio Ventures Pvt. Ltd., Bangalore.

Liposome Encapsulated Astaxanthin

The method for production of liposome-encapsulated astaxanthin was prepared using: L-phosphatidylcholine [0.04 g/mL of dimethyl sulfoxide (DMSO)] and cholesterol (0.01 g/mL of DMSO), which were liquefied in 5 mL of mixed solvent of chloroform/methanol (2:1 v/v). To this mixture, an applicable amount of astaxanthin was added. To sum up, concentration of astaxanthin was made to 1 mM. The above mixture was ultra-sonicated and concentrated under decreased pressure to totally drive-off the organic solvents until a membranous product formed on the inner wall of the concentrator. The desiccation process was carried out for another 2 hours in a vacuum drier. The product membrane was dissolved in 15% of ethanol/double distilled water. The solution was sonicated in probe sonicator for 30 minutes. The dispersal was filtered through a 0.2 μ m membrane. The filtrate



was lyophilized to obtain liposome-encapsulated astaxanthin.^[47,48]

Positive Drug

Sorafenib dose of 10 mg/kg/day was used, which leads to disclosures in the range of the respective human dose of 160 mg/day.^[49]

Preparation of DEN

A 1% DEN was prepared by using 99 mL of normal saline NaCl (0.9 percent) solution, to which was added 1 mL of concentrated DEN solution (0.01 µg/µL).

Experimental Animals

Swiss albino mice (20–25 grams) bought from Central Animal House Facility of Kings Institute, Chennai, India, were used to test anti-cancer activity of astaxanthin. These animals were kept in polypropylene cages, which were maintained at a temperature of $22 \pm 2^\circ\text{C}$, with a relative humidity of $60 \pm 10\%$. Standard animal diet (Biogen Foods, India) and water *ad libitum* was given. Before the starting, the experiment animals were acclimatized to laboratory conditions with 12-hour light/dark cycles. Experiments were designed and conducted in accordance with ethical norms approved by the Institutional Animal Ethical Committee.

Mice were divided into seven groups; each group consists of six animals. Mice of group I served as normal control. HCC was induced in groups II, III, IV, V, and VI, which received DEN (3.5 µL/gm) intraperitoneally, once in a week for eight consecutive weeks. After 2 weeks of DEN administration, the carcinogenic effect was promoted through subcutaneous injections of CCl_4 (3 mL/kg/week) for 8 weeks, after administration of DEN, test groups III and IV were administered orally with 50 and 100 mg/kg liposomal encapsulated astaxanthin (E.Asx), and V, VI receive with 50 and 100 mg/kg of non-encapsulated astaxanthin (NE.Asx), respectively, in the form of suspension, daily once a day, and group VII receive with 10 mg/kg of sorafenib. At the end of experiments, the body weight, water, and food intake of each mice were taken before sacrifice. The mice were killed by cervical decapitation, followed by overnight fasting. Blood was collected by heart puncture and allowed to clot before centrifugation at $1,000 \times \text{grams}$ for 10 minutes at 4°C to separate serum. The liver tissue was washed twice with ice-cold 0.1 M phosphate buffer saline (PBS, 1:9), pH 7.4, blotted, dried, and weighed. The relative liver weight was calculated as the percentage ratio of liver weight to the body weight. A small portion of the tissue was fixed in formalin for histological examination.

Serum Biochemical Analysis

Mice were anesthetized with diethyl ether, and blood samples were withdrawn from the retro-orbital venous plexus. Collected blood samples were allowed to stand for 10 minutes at room temperature, then centrifuged at

4°C using cooling centrifuge (Shizmazdu) at 3,000 rpm for 10 minutes. Sera were separated for assessment of levels of aspartate aminotransferase (AST), and alanine aminotransferase (ALT), ALP, total bilirubin, total protein, albumin, superoxide dismutase (SOD), glutathione peroxidase (GPx), and lipid peroxides (LPO), using commercially available colorimetric assay kits as previously described.^[50] Moreover, inflammatory markers TNF- α were assessed using ELISA kit (Thermo Fisher).

Statistical Analysis

The results obtained in this research were displayed as mean $\pm$ SEM. Data analysis was done using one-way ANOVA followed by Dunnett's multiple comparison test (FigurePad Prism 5 Software Inc., La Jolla, CA, USA). Values were considered significant at $p < 0.05$.

RESULTS

Animals were induced by DEN- CCl_4 to each group except the normal control, and their activities were determined using physical observations. Primary activities, like body weight, convulsion, lacrimation, salivation, defecation, locomotion, rearing, etc., were interpreted and noted in Table 1.

Body weight tends to decreases in both DEN induced group and astaxanthin treated group. Body tone was reduced in the entire mice group except normal. Urination, muscle gripness, and lacrimation were founded to be normal in all the groups. Rearing was normal in control; mild in DEN induced and slows in drug-treated groups. Locomotion was founded to be normal in control, whereas it was very slow in DEN treated and slow in drug-treated groups. Sorafenib induced group shows normal physical activities, but moderate locomotion and rearing were seen. The overall physical examination was normal in control, very poor in DEN treated, and mild in DEN induced drug-treated groups.

As shown in Fig. 2, body weight of each mice of different groups has been noted. The results analyzed by the control group were significantly similar to DEN + CCl_4 induced group. The initial weight of control and DEN induced group shows 29.13 and 28.41 grams, which tend to decreases up to 25.34 and 24.42 grams, respectively. Whereas, the

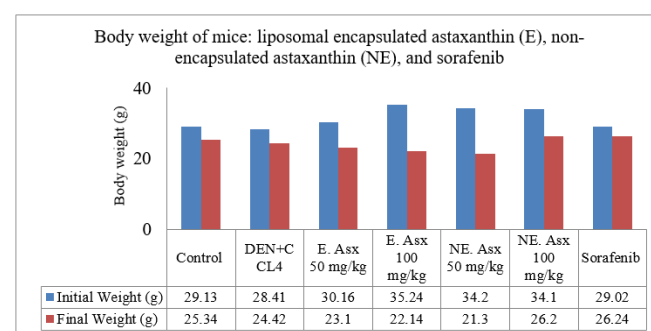
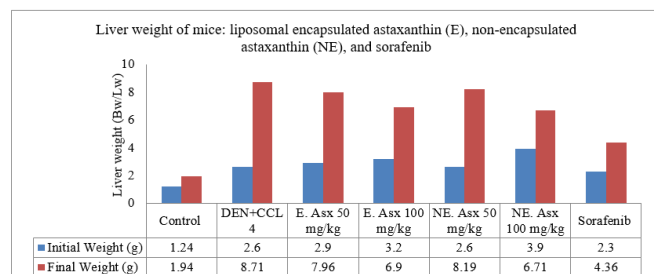


Fig. 2: Body weight evaluation of mice in different groups

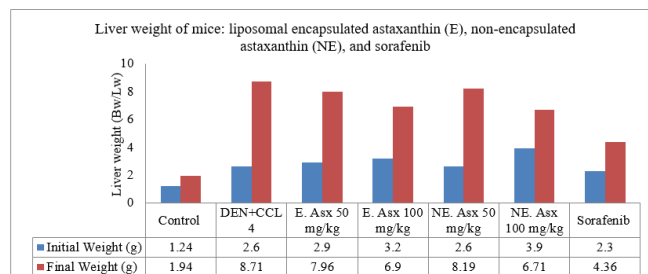
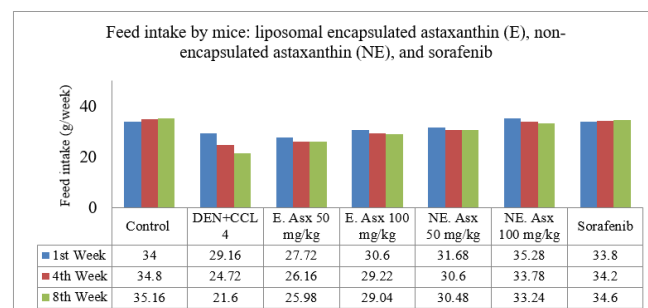
Table 1: Physical examination of normal and tested mice

Group	Control	DEN induced	E. Asx 50 mg/kg	E. Asx 100 mg/kg	NE. Asx 50 mg/kg	NE. Asx 100 mg/kg	Sorafenib 10 mg/kg
Body weight	Normal	Reduced	Reduced	Reduced	Reduced	Reduced	Normal
Assessments of posture	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Signs of convulsion	Normal	Absence of sign (-)	Absence of sign (-)	Absence of sign (-)	Absence of sign (-)	Absence of sign (-)	Normal
Limb paralysis	Normal	Reduced	Reduced	Reduced	Reduced	Reduced	Normal
Body tone	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Lacrimation	Normal	Absence	Absence	Normal	Absence	Normal	Normal
Salivation	Normal	Change in skin color	Change in skin color	Change in skin color	Change in skin color	Change in skin color	NS color change
Change in skin color	Normal	Severe erect	Moderate erect	Mild erect	Mild erect	Mild erect	Normal
Piloerection	Normal	Abnormal	Normal	Normal	Normal	Normal	Normal
Sensitivity response	Normal	Very slow	Slow	Slow	Slow	Slow	Moderate
Locomotion	Normal	Mild	Slow	Slow	Slow	Slow	Moderate
Rearing	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Urination	Normal	Normal	Normal	Normal	Normal	Normal	Normal

**Fig. 3:** Liver weight evaluation of mice in different groups

drug-treated groups (liposomal encapsulated astaxanthin 50 and 100 mg/kg; non-encapsulated astaxanthin 50 and 100 mg/kg) show lower growth in body weight. The mice which received the drug liposomal encapsulated astaxanthin of 50 and 100 mg/kg shows initial weight of 30.16 and 35.24 grams, which declines to final weight of 23.1 and 22.14 grams, respectively. Similarly, the mice induced by the drug non-encapsulated astaxanthin 50 and 100 mg/kg showed 21.3 and 26.2 grams final weight, respectively, from 34.2 and 34.1 grams, as initial weight. The positive drug-induced mice show 29.02 as initial weight and 26.24 grams as final weight, which indicates that the mice affected with HCC shows gradual decrease in the body weight.

The liver weight was evaluated for the mice of different group, since DEN was induced to cause HCC, which is represented in Fig. 3. The initial and final liver weight of control group was founded to be normal, which shows 1.24 to 1.94 Bw/Lw, but the DEN induced group shows severe weight gain in liver from 2.6 to 8.71 grams. The other test groups, such as, liposomal encapsulated astaxanthin and non-encapsulated astaxanthin of 50 and 100 mg/kg shows depleted increase in liver weight, which differs between initial and final weight of liver, but compare to DEN induced

**Fig. 4:** Water intake by mice in different groups**Fig. 5:** Feed intake by mice in different groups

the mice of drug-induced groups shows moderate liver weight gain. The liver weight of mice in sorafenib induced group also shows slight variation from 2.3 to 4.36 Bw/Lw, respectively.

Water intake by the mice of different groups has been calculated at 1st, 4th, and 8th week after inducing the drug and denoted in Fig. 4. Water intake by DEN (only) and liposomal encapsulated astaxanthin of 50 mg/kg induced mice was very poor, when compared with control, other test drug, and positive drug mice group.

Fig. 5 indicated the net amount of feed intake by mice for 1st, 4th, and 8th week after administration of DEN,



astaxanthin, encapsulated astaxanthin, and sorafenib. When compared with control, liposomal encapsulated astaxanthin of 100 mg/kg, non-encapsulated astaxanthin of 50 and 100 mg/kg, and sorafenib induced groups show similar feed intake, while other two group such as, DEN (only) and liposomal encapsulated astaxanthin 50 mg/kg shows lower feed intake, which may be due to some difficulties during HCC.

The level of ALT, AST, and ALP in control was found to be in normal range, i.e., 71.04, 124.02, and 267.28 (U/L), which tends to increase in DEN induced group [ALT: 128.21 (U/L); AST: 428.02 (U/L); ALP: 560.68 (U/L)]. But after the administration of test drug, the elevated level of ALT, AST, and ALP was suppressed in liposomal encapsulated astaxanthin 50 mg/kg [ALT: 116.41 (U/L); AST: 368.44 (U/L); ALP: 490.82 (U/L)], liposomal encapsulated astaxanthin 100 mg/kg [ALT: 97.36 (U/L); AST: 242.33 (U/L); ALP: 372.40 (U/L)], non-encapsulated astaxanthin 50 mg/kg [ALT: 106.20 (U/L); AST: 298.20 (U/L); ALP: 422.33 (U/L)], non-encapsulated astaxanthin 100 mg/kg [ALT: 90.46 (U/L); AST: 188.63 (U/L); ALP: 287.72 (U/L)], and sorafenib 10 mg/kg [ALT: 92.20 (U/L); AST: 178.36 (U/L); ALP: 279.32 (U/L)] (Fig. 6 and Table 2).

The mean values of blood factors, such as, total protein (g/dL), total bilirubin (mg/dL), albumin (U/L), SOD (U/mg protein), GPx (U/mg protein), and LPO (mmol/mg protein) were determined, and values were given in Fig. 7 and Table 3. The liposomal encapsulated astaxanthin and non-encapsulated astaxanthin suppress the elevated level of total protein, total bilirubin, and albumin, when

compared to DEN (only) induced group. Significant higher level of total protein was observed for DEN induced group than other groups. SOD and GPx activities were high in the DEN treated mice, when compared to control group,

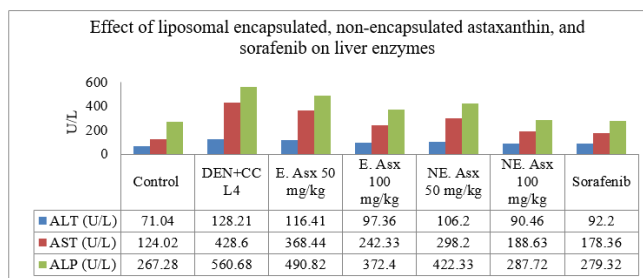


Fig. 6: Effect of treatment with liposomal encapsulated, non-encapsulated astaxanthin, and sorafenib on liver marker enzymes in hepatocellular carcinoma mice of different groups

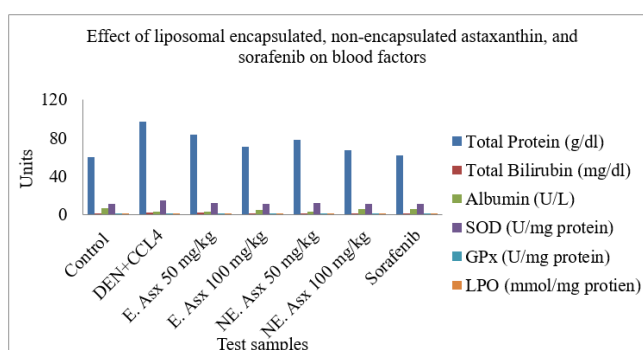


Fig. 7: Effect of treatment with liposomal encapsulated, non-encapsulated astaxanthin, and sorafenib on blood factors in hepatocellular carcinoma mice of different groups

Table 2: Activity of test drug on liver marker enzymes

Sample/ Test name	ALT (U/L)	AST (U/L)	ALP (U/L)
Control	71.04 ± 0.213	124.02 ± 0.036	267.28 ± 0.032
DEN + CCL4	128.21 ± 0.223	428.6 ± 0.089	560.68 ± 0.063
E. Asx 50 mg/kg	116.41 ± 0.132	368.44 ± 0.023	490.82 ± 0.162
E. Asx 100 mg/kg	97.36 ± 0.162	242.33 ± 0.017	372.4 ± 0.102
NE. Asx 50 mg/kg	106.2 ± 0.006	298.2 ± 0.045	422.33 ± 0.213
NE. Asx 100 mg/kg	90.46 ± 0.035	188.63 ± 0.061	287.72 ± 0.310
Sorafenib	92.2 ± 0.106	178.36 ± 0.092	279.32 ± 0.156

NS-Not significant; **($p > 0.01$); *($p > 0.05$); $n = 10$; values are mean $\pm$ SD (one-way ANOVA followed by Dunnett's test)

Table 3: Activity of test drug on blood factors

Sample/ Test name	Total protein (g/dL)	Total bilirubin (mg/dL)	Albumin (U/L)	SOD (U/mg protein)	GPx (U/mg protein)	LPO (mmol/mg protien)
Control	59.98 ± 0.03	0.72 ± 0.008	6.8 ± 0.067	11.02 ± 0.122	0.0125 ± 0.230	0.0035 ± 0.235
DEN + CCL4	96.82 ± 0.256	1.72 ± 0.003	2.4 ± 0.032	14.32 ± 0.231	0.1325 ± 0.031	0.0689 ± 0.003
E. Asx 50 mg/kg	83.36 ± 0.120	1.46 ± 0.13	3.1 ± 0.128	11.55 ± 0.032	0.0312 ± 0.138	0.0426 ± 0.038
E. Asx 100 mg/kg	71.09 ± 0.003	1.06 ± 0.361	4.8 ± 0.004	10.59 ± 0.561	0.0281 ± 0.209	0.0389 ± 0.162
NE. Asx 50 mg/kg	78.22 ± 0.23	1.24 ± 0.031	2.9 ± 0.01	12.06 ± 0.106	0.0356 ± 0.213	0.0416 ± 0.189
NE. Asx 100 mg/kg	66.78 ± 0.162	0.96 ± 0.009	5.4 ± 0.318	11.33 ± 0.267	0.0302 ± 0.003	0.0341 ± 0.056
Sorafenib	62.03 ± 0.145	0.89 ± 0.021	5.7 ± 0.019	10.65 ± 0.268	0.0198 ± 0.009	0.0127 ± 0.046

NS-Not significant; **($p > 0.01$); *($p > 0.05$); $n = 10$; values are mean $\pm$ SD (one-way ANOVA followed by Dunnett's test)

drug-treated group, and positive drug sorafenib. Due to chronic cell changes, anti-oxidant response had been activated. Such alterations could be due to DEN induced inflammation or increased apoptosis. The findings indicate that encapsulated and non-encapsulated astaxanthin exerted an anti-inflammatory action against DEN-induced.

Treatment with liposomal encapsulated, non-encapsulated astaxanthin and sorafenib showed a significant decrease in the inflammatory marker (TNF- α), thereby indicating

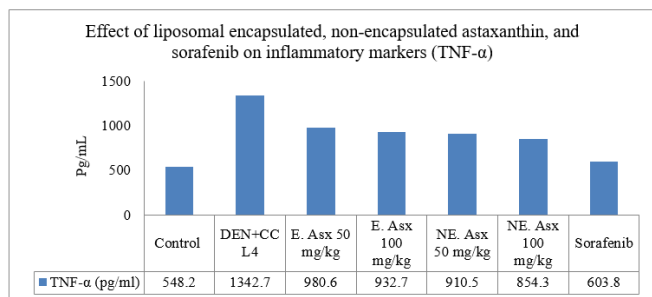


Fig. 8: Effect of treatment with liposomal encapsulated, non-encapsulated astaxanthin, and sorafenib on inflammatory markers (TNF- α) in hepatocellular carcinoma mice of different groups

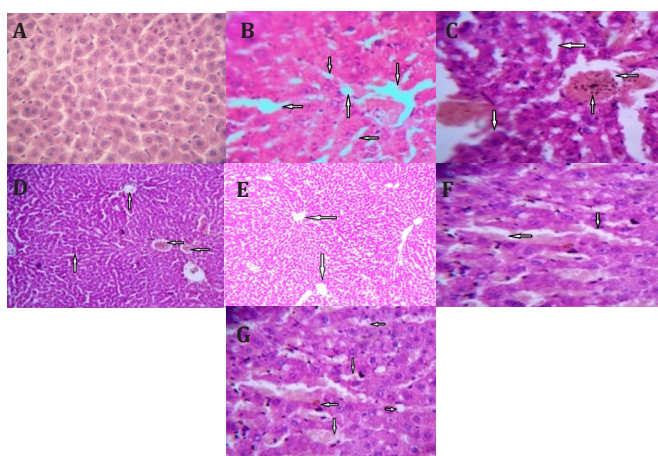


Fig. 9: Histopathology image of liver: control group and DEN treated group

Note: **A**-Control; **B**-DEN induced; **C**-Non-encapsulated astaxanthin (50 mg/kg); **D**-Non-encapsulated astaxanthin (100 mg/kg); **E**-Encapsulated astaxanthin (50 mg/kg); **F**-Encapsulated astaxanthin (100 mg/kg); **G**-Sorafenib (10 mg/kg)

Table 4: Activity of test samples on inflammatory marker

Sample/Test name	TNF- α (pg/mL)
Control	548.2 $\pm$ 0.137
DEN + CCL4	1,342.7 $\pm$ 0.203
E. Asx 50 mg/kg	980.6 $\pm$ 0.032
E. Asx 100 mg/kg	932.7 $\pm$ 0.103
NE. Asx 50 mg/kg	910.5 $\pm$ 0.006
NE. Asx 100 mg/kg	854.3 $\pm$ 0.034
Sorafenib 10 mg/kg	603.8 $\pm$ 0.612

NS-Not significant; ** ($p > 0.01$); * ($p > 0.05$); $n = 10$; values are mean $\pm$ SD (one-way ANOVA followed by Dunnett's test)

the anti-inflammatory activity of astaxanthin, both in encapsulated and non-encapsulated form. These results indicate similar effect on the HCC by astaxanthin, when compared with control and positive drug sorafenib. Control group shows 548.2 pg/mL of TNF- α in mice blood sample. Elevated level of 1,342.7 pg/mL was found in DEN induced group, this tend to decrease to the level of 980.6, 932.7, 910.5, 854.3, and 603.8 pg/mL in E.Asx 50 mg/kg, E.Asx 100 mg/kg, NE.Asx 50 mg/kg, NE.Asx 100 mg/kg, and sorafenib 10 mg/kg, respectively (Fig. 8 and Table 4).

Liver section of each group mice was tested for histopathological analysis. From the result, the control group mice showed normal liver structural design and normal appearance of the hepatocytes. The liver sinusoids were seen in between the adjacent plates. Kupffer cells were also seen associated with the sinusoidal lining cells (Fig. 9A). However, DEN + CCL₄ is highly mutagenic in mice showed loss of lobular architecture, hence, showed stronger fibrosis and tumor necrosis of hepatic tissues, which was represented in Fig. 9B.

The histopathological changes in the livers of the liposomal encapsulated astaxanthin of 50 mg/kg bw treated mice give the impression of neoplastic changes in liver section and reduced the severity of lesions in liver, when compared with untreated control and DEN treated group (Fig. 9C). Group treated with higher dose of liposomal encapsulated astaxanthin of 100 mg/kg bw after the induction of HCC showed reduced degeneration and distortion of hepatocytes and marked transformation of hepatic manner with restoration of bile duct structure. Similarly, less fatty changes and minimal pleomorphic was also noted compared to normal control and HCC control (Fig. 9D).

In parallel, the histopathological image of liver treated with non-encapsulated astaxanthin of 100 mg/kg bw after the formation of liver tumor showed nucleated cells with slight improvement in liver cells than 50 mg/kg bw of non-encapsulated astaxanthin which showed steatosis, micro and macro vesicles, cytoplasmic degeneration, Kupffer cells activation, hemorrhage, and infiltration of inflammatory cells (Figs 9E and F).

The group treated with positive drug sorafenib after the administration of DEN + CCL₄ were also examined. It discovered the fewer pathological changes compared to HCC control group, i.e., DEN + CCL₄ treated group. The liver sections of sorafenib treated group revealed less fatty changes, less disarrangement, minimal pleomorphic, vacuolation, and degeneration of hepatocytes, and the result of this research reveals that there is no much difference between the test drug and sorafenib-administered group (Fig. 9G).

DISCUSSION

Natural products have been considered as important sources that could create prospective chemotherapeutic



agents. Apparently, natural products are an extremely important source of medicinal agents. While, there are some new approaches to drug discovery, such as, combinatorial chemistry and computer-based molecular modeling design, none of them can replace the reputation of natural products in drug discovery and development.

In this study, we demonstrated the antitumor effects of astaxanthin and encapsulated astaxanthin against HCC. According to our interpretation, both encapsulated and non-encapsulated astaxanthin at higher dose of 100 mg/kg shows moderate anti-cancer activity in mice while a lower dose of 50 mg/kg of encapsulated and non-encapsulated astaxanthin showed mild activity. This was compared with the control, DEN induced, and positive drug sorafenib. The positive drug sorafenib shows good anti-cancer activity when compared with other groups.

Signaling pathways that are connected with HCC carcinogenesis promote angiogenesis, enhance growth, and inhibit apoptosis, which can result in uncontrolled tumor cell growth.^[51] HCC signal transduction may occur through different pathways. (1) angiogenesis-related pathways (e.g., vascular endothelial growth factor, VEGF; platelet-derived growth factor, PDGF; and fibroblast growth factor, FGF),^[52] (2) growth-related pathways (e.g., hepatocyte growth factor receptor, HGFR; epidermal growth factor receptor, EGFR; insulin-like growth factor 1 receptor, IGF-1R; PI3K/AKT/mTOR, and Wnt- β catenin),^[53,54] and (3) HGF/ c-Met pathway.^[55]

Previous studies have reported that sorafenib is a multi-kinase and tyrosine kinase inhibitor for the treatment of advanced HCC.^[52] A 2008 phase III trial has shown that sorafenib significantly extends the survival of patients with advanced HCC.^[56] Sorafenib is a multi-targeted small molecule that inhibits the activity of VEGF, PDGF, and EGF, as well as, Raf to block tumor proliferation and angiogenesis.^[57] The median overall survival in the sorafenib-treated group was 10.7 months, compared with 7.9 months in the placebo group.^[52,56] However, the survival benefit was only a few months. In addition, numerous patients required a dose reduction or cessation of treatment because of the adverse effects of the drug, and some patients with renal cancer experienced tumor rebound after discontinuing the drug.^[57,58]

HCC is complex pathogenesis involving aberrant signaling in several molecular pathways. Sorafenib is a targeted therapy, currently used as a standard treatment for patients with advanced HCC. Sorafenib is a multi-kinase inhibitor that demonstrates activity against several tyrosine (VEGFR and PDGFR) kinases and serine/ threonine (Raf) kinases.^[52] The administration of sorafenib has generated promising results for the treatment of HCC patients, and the mechanisms behind the antitumor activities of sorafenib have been well-explored.^[59] In a previous study, the administration of more than 400 mg/day of sorafenib for one-month

prolonged progression-free survival in Japanese patients with advanced RCC.^[60] Therefore, the management of sorafenib dose to prevent the occurrence of side-effects is a very important topic.

The anti-cancer properties as anti-oxidants have been inviting attention, with research concentrated on energy metabolism and oxidative stress in cancer research. Research by previous studies has proven that ASX at higher doses is non-toxic to mice and human endothelial cells.^[61-64] Related clinical studies have also been conducted into cardiovascular disease to assess the dosing, bioavailability, and safety of ASX.^[19] To date, no significant side effects related to ASX have been reported.^[65] Therefore, this powerful anti-oxidant may be a novel and potential drug for inhibiting the proliferation of carcinoma cells.^[66,67] ASX may play an efficient role against cancer by enhancing the immune response in mice, as described by Jyonouchi and colleagues in 2000.^[68] Kowshik and other researchers found that ASX induced intrinsic apoptosis not only in oral cancer cells, but also in skin cancer, breast cancer, and neuroblastoma SH-SY5Y cells.^[11,68-71] In 2010, Tripathi DN explored the effects of ASX on early hepatocarcinogenesis in rats.^[72] In addition, Song and colleagues established that astaxanthin induced mitochondria-mediated apoptosis in rat HCC CBRH-7919 cells by an IC₅₀ of 39 μ M through inhibition of the JAK/STAT3 signaling pathway.^[24,25] Also, another study showed that oral astaxanthin intake during the early developmental stage suppressed MNU-induced rat mammary carcinogenesis.^[73]

CONCLUSION

Our studies were correlated with the previous reports, which include the role of encapsulated astaxanthin along with non-encapsulated astaxanthin. So far, pure form of astaxanthin was alone confirmed against HCC; the encapsulated astaxanthin was not performed. Hence, our findings demonstrate that both non-encapsulated and encapsulated astaxanthin has a promising potential therapeutic agent for human HCC, and has anti-oxidant and anti-inflammatory activity.

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