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In vitro and *in vivo* Assessment of some Functional Foods against Initiation of Hepatocellular Carcinoma

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Running title:
Functional foods against hepatic carcinoma

ABSTRACT

Natural therapy is a great challenge for cancer treatment or prevention. The aim of the present work is to evaluate the protective and therapeutic effects of certain foods against initiation of hepatic carcinoma. Cytotoxic potential of cabbage, cauliflower, carrot and cinnamon against $HepG_2$ cancer cell line were evaluated. The work was extended to examine *in vivo* cytotoxic effect of their ethanol extracts (200mg/kg.b.wt) against hepatic carcinoma induced by diethylnitrosamine (DEN) (1 µl/100 g b.wt.) in rats. The protective and therapeutic values of all plants were evaluated through measuring hepatic enzymes; succinate and lactate dehydrogenases (SDH & LDH) and liver function enzymes; aspartate and alanine aminotransferases (AST & ALT), gamma glutamyl transferase (γ-GT) and alkaline phosphatase (ALP). Serum hyaluronic acid (HA) and vascular endothelial growth factor-C (VEGF-C) were estimated. Liver histopathological and electron microscopic analysis were also evaluated. Carcinogenic rats recorded drastic changes in all parameters under investigation. Plants therapy recorded more potent effect than prophylactic action. In conclusion, cinnamon followed by carrot recorded the highest *in vitro* and *in vivo* anticarciongenic effects. More detailed studies could lead to development of novel anti-tumor agents or complementary medicines for hepatic cancer treatment.

KEY WORDS: Hepatoma, biochemical markers, histopathology, tumor markers, herbal medicine, electron microscopy.

INTRODUCTION

Chemoprevention, which includes the use of synthetic or natural agents to block the development of cancer in human beings, is an extremely promising strategy for cancer prevention [1].

Cancer is the major cause of death worldwide. The epidemiological studies on cancer in Egypt have revealed the high frequency of bladder and breast as the most common cancers among Egyptian patients [2,3]. Recently, the hepatocellular carcinoma (HCC) has been increasing in Egypt with a doubling in the incidence rate in the past 10 years due to several biological (e.g. hepatitis B and C virus infection) and environmental factors (e.g. aflatoxin, AF). Other factors such as cigarette smoking, occupational exposure to chemicals such as pesticides, and endemic infections in the community, such as schistosomiasis, may have additional roles in the etiology or progression of the disease [2].

Although great advancements have been made in the treatment and control of cancer progression, significant deficiencies and room for improvement remain with a number of undesired side effects occur during chemotherapy. Natural therapies, such as the use of plant-derived products in cancer treatment, may reduce adverse side effects [4]. If these plants are already used in nutrition, it classified as a functional food. Functional food is defined as food that provides more than simple nutrition. It must consume as part of a usual diet and is demonstrated to have physiological benefits to reduce the risk of chronic diseases [5].

Brassicaceae vegetables including cabbage, cauliflower and broccoli, as famous food in Egypt, are attracting major attention as healthy foods because of their content of glucosinolates (GLs) that release the corresponding isothiocyanates (ITCs) upon myrosinase hydrolysis and showed some chemopreventive properties [6]. Brassicaceae plants recorded antidiabetic, antioxidant and antihyperlipidemic effects [7-10].

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Antibacterial and antifungal activities of Brassica family have also been reported by Guan et al [11], Lin and Ng [12] and Suido and Miyao [13].

Carrot (*Daucus carota* L.) has the highest carotenoid content among foods and is consumed in large quantities worldwide. Carotenoids (alpha and beta) have also been associated with protective effects against cancer and other chronic diseases [14]. Purup et al. [15] mentioned that the polyacetylenes (falcarinol-type) found in carrot has anti-inflammatory, antiplatelet-aggregatory, and antitumor activity as well as its activity against bacteria and mycoplasma.

Cinnamon (*Cinnamomum zeylanicum*), a widely used food spice, containing several active components such as essential oils (cinnamic aldehyde and cinnamyl aldehyde, tannin, mucus and carbohydrate) [16]. It exhibited diverse biological functions including anti-inflammatory, anti-oxidant, anti-microbial, anti-diabetic and anticancer effects [17].

In this study, the prophylactic and curative properties of *Brassica oleracea* Var, *Brassica botrytis* L, *Daucus carota* L and *Cinnamomum zeylanicum* L were studied prior or after cancer induction by diethylnitrosamine as a potent hepatocarcinogenic agent. The evaluation process takes place *in vitro* through measuring the potential cytotoxicity of each plant against human hepatic cancer cell line (HepG2) and *in vivo* through measuring liver marker enzymes; succinate and lactate dehydrogenases as well as liver function enzymes; aspartate and alanine aminotransferases, alkaline phosphatase and gamma glutamyltransferase. Serum hyaluronic acid and vascular endothelial growth factor-C (VEGF-C) were taking into consideration. Liver histopathological and electron microscopic studies were also estimated.

MATERIALS AND METHODS

Chemicals. All chemicals used were of high analytical grade, products of Sigma (US), Merk (Germany) and BDH (England).

Animals. Female Wister strain albino rats (120 – 150 g) were obtained from the animal house, National Research Centre, Cairo, Egypt and maintained on stock commercial pellet diet (El-Kahira Company for Oil and Soap) and water ad-libitum.

Plant material. Leaves of *Brassica oleracea* Var. (Family: Brassicaceae), sprouts of *Brassica botrytis* L. (Family: Brassicaceae) and roots of *Daucus carota* L. (Family: Apiaceae) were natively collected from Egyptian country and freshly extracted. *Cinnamomum zeylanicum* L. bark (Family: Lauraceae) was purchased from local market; Hyper One Market, 6th October City, Egypt and kept in closed container until used. Voucher specimens (BOV, BBL, DCL and CZL-2010) were deposited at Therapeutic Chemistry Dept. National Research Center, Cairo, Egypt as references.

Plant Extraction- Plant materials were extracted in a Soxhlet apparatus using 95% ethanol for 72 h [18]. Solvent removal was carried out under vacuum for drying at 40C. The dried residues were stored at 4^{O}C tell used.

In vitro assay- Potential cytotoxicity of the tested plants (0- 10µg) against human hepatic cell line (HepG-2) was demonstrated by the method of Skehan et al. [19]. Cells were plated in 96-multiwell plate (10^4 cells/ well) for 24h before treatment with plants to allow attachment of cell to the wall of the plate. Different plant concentrations were added to the cell monolayer and triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the plants for 48h at 37C and in atmosphere of 5% CO_2. After 48 h cells were fixed, washed and stained with sulforhodamin B stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris EDTA buffer. Color intensity was measured in an ELISA reader. The relation between surviving fraction of hepatic cells and plant concentrations was plotted to get the survival curve of the hepatic tumor cell line.

In vivo study-

Doses and root of administration. All prophylactic and treated animals were orally received 200 mg of plant extracts/ kg body weight by stomach gavage daily for one month [7]. Diethylnitrosamine (DEN) (Sigma) was injected intraperitoneally in a dose of 1 µl (diluted 1:100 with 0.15 mol/l sterile NaCl) /100 g body weight for 7 consecutive days [20].

Experimental design- Eighty four female rats were divided into fourteen groups (six rats/group). Group 1 served as normal healthy control rats, groups 2-5 were normal healthy rats received each plant extract daily for one month, group 6 intraperitoneally injected with diluted diethylnitrosamine daily for seven days and sacrificed after thee weeks, groups 7-10 served as the prophylactic groups, where it received each plant extract for one month followed by diethylnitrosamine for one week and sacrificed after three weeks of the

last injection. Groups 11-14 served as the treated groups, where it received diethylnitrosamine for one week, left free for three weeks followed by administration of each plant extract for one month.

Preparation of tissue homogenate. Liver tissue was homogenized in normal physiological saline solution (0.9N NaCl) by a ratio 1:9 w/v. The homogenate was centrifuged for 5 minutes at 3000 xg at 4^{o}C and the supernatant was used for estimation of succinate dehydrogenase, lactate dehydrogenase, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, gamma glutamyl transferase and hepatic total protein.

Preparation of serum samples. Blood were collected by puncture of the sublingual vein, centrifuged at 3000 rpm for 5 minutes and the separated serum was used for the determination of hyaluronic acid (HA) and vascular endothelial growth factor-C (VEGF-C).

Biochemical Assays. Enzyme activities were evaluated using end point assay method. Succinate dehydrogenase: reduction of flavin adenine dinucleotide is coupled with a reduction of tetrazolium salt as 2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride (INT), the produced formazan of INT is measured colorimetrically at 490 nm [21]. Lactate dehydrogenase: the reduction of nucleoside derived amino acids (NAD) was coupled with the reduction of tetrazolium salt and phenazine methosulfate serving as an intermediate election carrier; the produced formazan of INT was measured colorimetrically at 503 nm [22]. Alkaline phosphatase (ALP) was estimated as a liberated phenol in the presence of amino-4-antipyrine and sod-arsenate as a blocking agent and potassium ferricyanide as color reagent. The developed color measured at 510 nm [23]. AST & ALT were estimated through measuring oxaloacetate and pyruvate produced respectively [24]. GGT was estimated by the method of Szasz [25], where GGT enzyme react with L-γ - glutamyl-3-carboxy-p-nitroanilide and glycyl-glycine to give L-γ – glutamyl-glycyl-glycine and 5- amino-2-nitrobenzoate. The decrease in absorbance was read at 450 nm at 1 min intervals for 3 minutes. Total protein, the Coomassie Brilliant Blue dye reacts with Bradford reagent to give a blue complex read at 595 nm [26]. Serum hyaluronic acid was done using enzyme – liked binding protein assay method by Tran et al. [27]. Vascular endothelial growth factor-C was estimated using enzyme immunoassay method by Duff et al. [28].

Histopathological studies- Slices of liver tissue of all animals were collected and fixed in 10% buffered formalin solution for histopathological studies. Paraffine embedded sections (5µm thick) were taken after fixation and slides were stained using haemoatoxylin and eosin (H&E) by the method of [29].

Electron microscopic examination- It was carried out by the method of Mercer and Birkbeck [30], where liver slices were immersed in 4% gluteraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3 for 8 h, then post fixation was carried out in 1% osmium tetroxide in the same cacodylate buffer for 2 h. Liver sections of 1 µm semi thin sections were cut, picked up on glass slides and stained with touluidine blue for light microscopic examination prior to the final examination Ultrathin sections were cut and picked up on (a formvar coated) 200 mesh copper grids. The ultrathin sections were stained with uranyl acetate for 30 min followed by lead for 15 min. The ultrathin sections were examined under an EM 10 S transmission electron microscope at 80 JV accelerating voltage.

Statistical Analysis and calculations. Analysis of data was carried out by independent student *t*-test, where the significance value was at level at $p \leq 0.05$ (Graphpad Software Computer Program).

% change = control mean - treated mean / control mean x 100.

% improvement = treated mean - injured mean / control mean x 100.

RESULTS AND DISCUSSION

Cytotoxicity of the selected plants on HEPG2 survival fractions was noticed in Fig. 1. *Brassica oleracea, Brassica botrytis, Daucus carota* and *Cinnamomum zeylanicum* recorded inhibition of carcinogenic hepatic cells by 13.63, 33.30, 81.81 and 88.67%, respectively. These results were in line with Safe et al. [31] who postulated the action of indole-3-carbinol in Brassica family in inducing overlapping and unique responses in multiple cancer cell lines and tumors through growth inhibition, apoptosis and antiangiogenic activities. Polyacetylenes (falcarinol and falcarindiol) found in carrot and cinnamaldehyde, the bioactive component of cinnamon also play vital roles in inhibiting cells proliferation with cancer origin [15,17].

Carcinogenic rats, in the present study, recoded significant decrease in liver SDH (51.52%), LDH (50.76%), AST (38.01%) and ALT (59.14%) enzyme activities (Tables 1, 2), while significant increase in ALP (57.79%), GGT (23.22%), total protein (50.10%), HA (48.39%) and VEGF (49.92%) were observed (Tables 1-3). This was in accordance with Pathak et al. [32] who noticed that feeding of carcinogens p-

diethylamino-azobenzene and phenobarbital elevated the activity of alkaline phosphatase and decreased the activity of succinate dehydrogenase. Bhatt and Bano [33] attributed the decrease in SDH to the induced toxicity of energy metabolism in exposed animals to hepatocarcinogen. Kodama et al. [34] confirmed the results on the basis of mutations in nuclear genes encoding two mitochondrial complex II subunit proteins; succinate dehydrogenase B and D. Proctor et al. [35] also recorded significant increase in serum LDH, AST, ALT, ALP and GGT in case of liver cancer. The observed decrease of AST was more manifested than that of ALT denoting that, although the latter is more specific for liver cells, yet it is less sensitive than AST in detecting liver cell damage. Moreover, the presence of considerably more AST in hepatic tissue indicated that the released ALT is too diluted in the extracellular compartment to cause significant increase in the ALT activity [36]. The same author attributed this decrease in enzyme activities to the increase of cell membrane permeability due to involved toxins which lead to leakage of the enzymes into circulation. The enhancement of protein level was also confirmed by the results of Kassie et al. [1] who motioned that tumor-associated signature protein are increased during carcinogenesis.

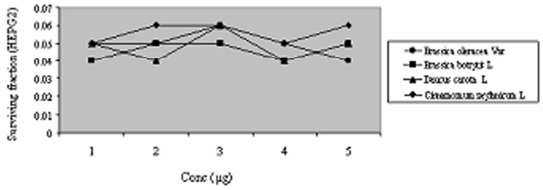

Fig. 1: *In vitro* cytotoxic potential of *Brassica oleracea* Var (a), *Brassica botrytis* L (b), *Daucus carota* L (c) and *Cinnamomum zeylanicum* L (d) on survival fraction rate of $HepG_2$.

The elevation of tumor progression index; HA and VEGF was in parallel with the results of George and Stern [20] who mentioned the role of hyaluronic acid as very early circulating indicator of acute liver injury. The same authors attributed the increase in HA to the decrease of hepatic removal and/or increased hepatic production of HA during liver inflammation. Jiang et al. [37] postulated the important role of hyaluronic acid in tissue integrity, angiogenesis, wound healing and cell motility through the interaction with receptors on cell membranes. In addition, vascular endothelial growth factor is a potent pro-angiogenic growth factor which is also known to alter tumor microenvironment by inhibiting dendritic cell differentiation and promoting accumulation of myeloid-derived suppressor cells [38]. Our results were confirmed by the same authors who recorded significant increase in VEGF in rat liver metastasis model. Jiang et al. [37] added that the anti-VEGF/ polyethyleneimine-HA complex was applied successfully as target specific antiangiogenic therapy for the treatment of diseases in the tissues with HA receptors, such as liver and kidney cancer.

Natural products seem to work in a tightly regulated manner wherein they switch their roles either towards protective or therapeutic side depending upon either the amount of the drug being used or upon the cellular phenotype [39]. In the present study, prophylactic or treatment of carcinogenic rats with the selected four plants recorded improvement levels ranged from 5.88 to 209.60% in all parameters under investigation (Table 4). It was declared that cabbage, cauliflower, cinnamon and carrots exerted either prophylactic or treatment effects in order. The same pattern of improvement was observed *in vitro* and *in vivo* studies.

The observed enhancement level in case of *Brassica* vegetables was due to its content of glucosinolates, flavonoids and other phenolics [6]. The anticarcinogenic activity is related to the presence of biologically active components that modulate the activity of phase I and II detoxification enzymes and other mechanisms triggered by glucosinolates, which are formed as a result of hydrolysis and catalyzed by the enzyme myrosinase. The presence of indole-3-carbinol (I3C) and its metabolite bis (3'-indolyl) methane (DIM) induce growth inhibition and antiangiogenic activities. The mechanisms of these responses are complex and dependent on cell context. I3C and/or DIM activate or inactivate multiple nuclear receptors, induce endoplasmic reticulum stress, decrease mitochondrial membrane potential, and modulate multiple signaling pathways including kinases [31]. Kassie et al. [1] suggested that tumor chemopreventive activity of I3C might be due to the modulation of carcinogen-induced alterations in protein levels.

Table 1: Prophylactic and curative effect of *Brassica oleracea* Var, *Brassica botrytis* L, *Daucus carota* L and *Cinnamomum zeylanicum* L on hepatic enzymes and total protein content of carcinogenic rats

Groups	SDH	LDH	Total protein
Control	0.33 ±0.01	87.20 ±3.59	**33.59±1.5**
C. *Brassica oleracea* Var	0.28 ±0.04 (-15.15)	82.30 ±3.20 (-5.61)	**45.50*±3.64 (+35.45)**
C. *Brassica botrytis* L	0.30±0.03 (-9.09)	83.17± 2.71 (-4.62)	**45.00*±0.71 (+39.33)**
C. *Daucus carota* L	0.29± 0.01 (-12.12)	81.258±3.52 (- 6.82)	**49.75*±2.45 (+48.50_**
C. *Cinnamomum zeylanicum* L	0.31± 0.014 (-6.06)	82.70± 4.40 (-5.16)	**47.5*±2.50 (+41.79)**
Carcinogenic	0.16*±0.01 (-51.52)	50.76*±4.23 (-41.78)	**50.42* ±1.97 (+50.10)**
P. *Brassica oleracea* Var	0.20* ±0.01 (-39.39)	75.25*± 4.56 (-13.70)	**47.22*±1.05 (+40.57)**
P. *Brassica batrytis* L	0.21* ±0.01 (-36.36)	74.52*± 2.62 (-14.54)	**44.22*±1.05 (+31.64)**
P. *Daucus carota L*	0.22*± 0.02 (-33.33)	70.12*± 3.24 (-19.58)	**46.11*± 2.11 (+37.27)**
P. *Cinnamomum zeylanicum* L	0.23* ±0.01 (-30.30)	76.52*±3.17 (-12.24)	**42.23*±3.12 (+25.72)**
T. *Brassica oleracea* Var	0.24* ±0.01 (-27.27)	76.42* ±3.61 (-12.36)	**46.50*± 2.40 (+35.45)**
T. *Brassica botrytis* L	0.25* ±0.008 (-24.24)	74.52*± 3.81 (-14.54)	**47.52*± 3.81 +41.47**
T. *Daucus carota* L	0.27*± 0.007 (-18.18)	81.53± 2.81 (-6.50)	**45.75*±2.046 (+36.20)**
T. *Cinnamomum zeylanicum* L	**0.30± 0.05 (-9.09)**	**81.60±3.66 (-6.42)**	**44.50*±2.50 (+32.83)**

- Data are mean ± SD of six rats in each group.
- SDH and LDH are expressed as µmol /min /mg protein. Total protein is expressed as mg/g of liver tissue.
- Control (C), prophylactic (P), treated (T),
- Values between brackets are % change over control group.
- Statistical analysis is carried out by independent student *t*-test, where (*) is significant level at $p \leq 0.05$.

The recorded protective and therapeutic potential of carrot and cinnamon was in agreement with Purup et al. [15] and Young et al. [40] who postulated the role of falcarinol, falcarindiol and polyacetylenes isolated from carrot in inhibition cell proliferation of human intestinal cells, where the anticancer activity of polyacetylenes of the falcarinol-type is associated with their ability to form extremely stable carbocations and acting as alkylating agents toward biomolecules. Gichuhi et al. [14] added that carotenoids (α and β carotenes) have also been associated with protective effects against cancer and other chronic diseases. Cinnamaldehyde, the bioactive component of cinnamon, had been shown to inhibit proliferation of several human cancer cell lines including breast, leukemia, ovarian, cervical and lung tumor cells [17]. Moreover, cinnamon extract potently suppressed *in vivo* melanoma progression by mediating apoptosis and blockade of the transcriptional factors; 9NFκB and AP1 and their target genes [16]. Cinnamon polyphenols have been recently shown to play a protective role by attenuating the decline in mitochondrial membrane potential induced by ischemic injury in cancer cells [41]. In addition, it induced apoptosis through increase the intracellular calcium of mitochondrial membrane [17].

Table 2: Prophylactic and curative effect of *Brassica oleracea*, *Brassica botrytis* L, *Daucus carota* and *Cinnamomum zeylanicum* on liver function enzymes of carcinogenic rats.

Groups	AST	ALT	ALP	GGT
Control	1.36±0.08	2.57±0.25	4.81±0.22	**80.60±0.4**
C. *Brassica oleracea* Var.	1.20±0.03 (-11.76)	1.83*±0.03 (-28.79)	4.84±0.33 (+0.62)	**82.50±0.50 (+2.35)**
C. *Brassica botrytis* L	1.32±.03 (-2.94)	2.33*±0.16 (-9.34)	5.66*±0.79 (+17.67)	**78.66±0.47 (-2.41)**
C. *Daucus carota* L	1.00*±0.05 (-26.47)	2.12*±.17 -17.32	5.76*±0.78 (+19.75)	**81.23±0.816 (+0. 78)**
C. *Cinnamomum zeylanicum* L	1.10*±0.06 (-19.11)	1.69*±0.17 (-34.12)	5.24±0.22 (+8.93)	**80.5±0.5 (-0.124)**
Carcinogenic	0.84*±.050 (-38.01)	1.05*±0.48 (-59.14)	7.59*±0. 98 (+57.79)	**271.80*±3.05 (+23.22)**
P. *Brassica oleracea* Var	0.97*± 0.08 (-28.67)	2.30±0.25 (-10.50)	6.50*± 0.46 (+35.13)	**185*± 3.56 (+129.52)**
P. *Brassica batrytis* L	0.96*±0.04 -29.41	2.49*±0.20 (-3.11)	5.59*±0.40 +16.22	**175.00*±2.61 (+117.12)**
P. *Daucus carota L*	0.90*±0.06 (-33.82)	2.25±0.21 (-12.45)	6.20*±0.44 +28.8	**180*± 2.98 (+123.32)**
P. *Cinnamomum zeylanicum* L	0.93*±0.08 (-31.61)	2.20*±0.43 (-14.39)	5.74*±0.36 (+19.33)	**178.65*±1.55 (+121.65)**
T. *Brassica oleracea* Var	0.95* ±027 (-30.14)	2.38±.317 (-7.39)	5.74*±0.36 (+19.33)	**116.71*± 1.10 (+44.80)**
T. *Brassica botrytis* L	0.98*±0310 (-27.94)	2.18*±.23 (-15.17)	5.90*±0.16 (+22.03)	**117.62*±4.2 (+45.89)**
T. *Daucus carota* L	0.92*±0.07 (-32.35)	2.02*±.14 (-21.32)	5.49*±0.26 (+14.13)	**132.67*±14.76 (+64.60)**
T. *Cinnamomum zeylanicum* L	**1.14±0.14 (-16.17)**	**2.48 ±.34 (-3.63)**	**5.39± 0.31 (+12*.05)**	**102.86*±2.08 (+27.61)**

- Data are mean ± SD of six rats in each group.
- Values are expressed as µmol /min /mg protein.
- Control (C), prophylactic (P), treated (T),
- Values between brackets are % change over control group.
- Statistical analysis is carried out by independent student t-test, where (*) is significant level at $p \leq 0.05$.

Light and electron microscopic analysis of liver section of DEN treated rats after prophylactic or treatment with the selected four plants confirmed the improvement observed in all the biochemical parameters. Histological examination of normal control liver tissue under light microscope revealed the presence of hexagonadal or pentagonadal lobules with central veins and peripheral hepatic triads or tetrads embedded in connective tissue (Fig. 2a). Acute inflammatory cells with vacuolated cytoplasmic changes in DEN group were seen in Fig. 2b. Other liver sections c, d and e showed the effect of DEN on the structural integrity of the cells. Cell necrosis was observed in Fig. 2c, while hepatic cells with oval or elongated nuclei and lightly eosinophilic cytoplasm were observed in Fig. 2 d and e. Theses marked changes in the hepatic architecture could be explained on the basis that DEN treatment manifested its toxic effects through the generation of ROS. The resulting effect was the production of elevated amounts of malondialdehyde and conjugated dienes, which caused deleterious effects on the membranous components of hepatocytes [42]. Liver section of normal rats treated with plant extracts showed more or less normal hepatic cells architectures, revealing extracts safety on hepatic cells (Fig. 2 a, d, i, j). Liver sections of DEN rats prophylactic with *Brassica Oleracea*, *Brassica botrytis*, *Daucus carota*, or *Cinnamomum zeylanicum* showed enlarged hepatocytes with vacuoles. In most hepatocytes, the structure of nuclei was normal as shown in Fig.3 b, e, j, m. Liver sections of rats treated with the same plants showed more or less normal lobular pattern, contain a large spherical nucleus as compared to the normal control group (Fig.3c, f, k, n). The recovery of necrosis due to the treatment of plant extracts may be due to diminution of oxidative stress and free radicals elevation, which are indicative of hepatic injury [43].

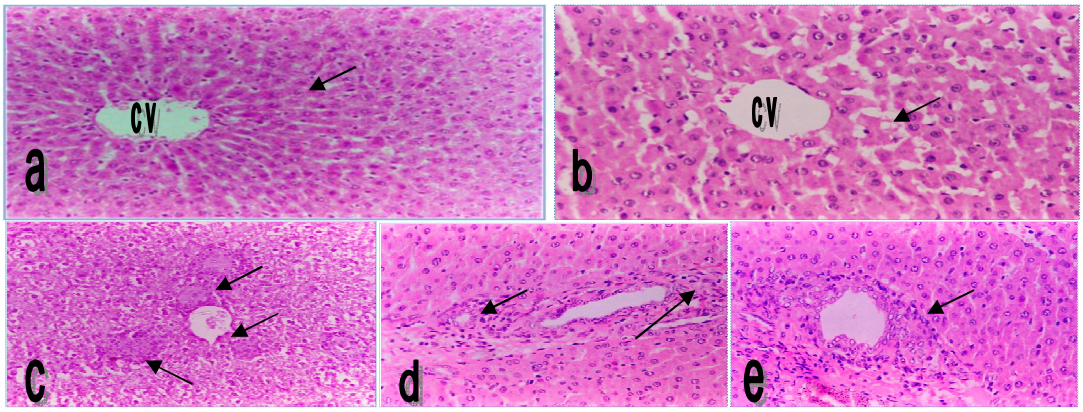

Fig. 2: Light photographs of H&E stained liver sections (200X) of control (a) and DEN treated group (b, c, d and e) groups.

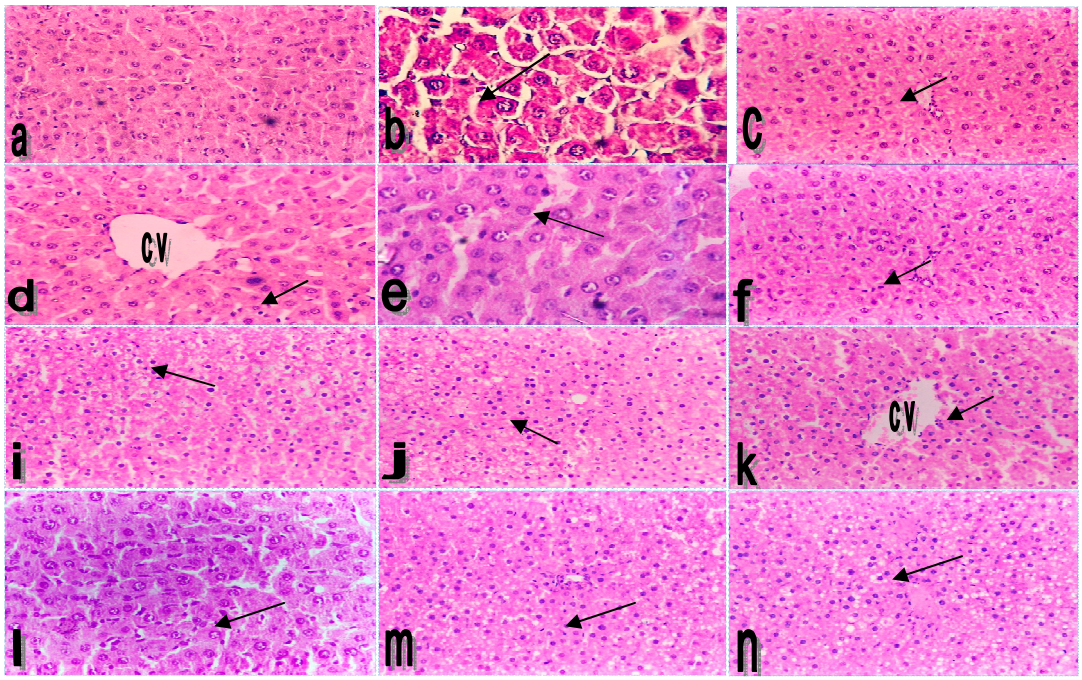

Fig. 3: Light photographs of H&E stained liver sections (200X) of control rats treated with *Brassica Oleracea* (a), rats treated with *Brassica Oleracea* then given DEN (400X) (b), rats given DEN then treated with *Brassica Oleracea* (c), control rats treated with *Brassica Botrytis* (200X) (d), rats treated with *Brassica botrytis* then given DEN (e), rats given DEN then treated with *Brassica botrytis* (f), control rats treated with *Daucus carota* (200X) (i), rats treated with *Daucus carota* then given DEN (prophylactic) (j), rats given DEN then treated with *Daucus Carota* (k), control rats treated with *Cinnamomum zeylanicum* (200X) (l), rats treated with *Cinnamomum zeylani*cum then given NDEA (m) and rats given NDEA then treated with *Cinnamomum zeylanicum* (n). Central vein (CV).

Table 3: Prophylactic and curative effect of *Brassica oleracea*, *Brassica botrytis* L, *Daucus carota* and *Cinnamomum zeylanicum* on hyaluronic acid and vascular endothelial growth factor in carcinogenic rats.

Groups	HA	VEGF
Control	90.11±2.10	**6.73±1.06**
C. *Brassica oleracea* Var.	89.23±2.73 (-0.98)	**6.22±1.12 (-7.57)**
C. *Brassica botrytis* L	88.50±3.11 (-1.78)	**6.15±1.20 (-8.62)**
C. *Daucus carota* L	86.50±4.31 (-4.01)	**6.10± 1.14 (-9.36)**
C. *Cinnamomum zeylanicum* L	85.56±7.22 (-5.04)	**6.22±1.32 (-7.57)**
Carcinogenic	133.72*± 4.13 (+48.39)	**10.09*±3.04 (+49.92)**
P. *Brassica oleracea* Var	123.22* ± 5.25 (+36.76)	**8.20±2.13 (+21.84)**
P. *Brassica botrytis* L	98.41 ± 9.70 (+9.21)	**8.63*±2.85 (+28.23)**
P. *Daucus carota L*	120.50* ± 9.10 (+33.72)	**8.34±2. 11 (+23.92)**
P. *Cinnamomum zeylanicum* L	97.20±3.24 (+7.86)	**8.11*±2.83 (+20.50)**
T. *Brassica oleracea* Var	114.37* ±6.32 (+26.92)	**9.02*± 2.64 (+34.02)**
T. *Brassica botrytis* L	96.67 ± 6.47 (+7.27)	**9.31* ±2.67 (+38.33)**
T. *Daucus carota* L	110.34*± 5.45 (+22.45)	**9.11±1.22 (+35.36)**
T. *Cinnamomum zeylanicum* L	**95.93 ± 4.60 (+6.45)**	**9.00 ±1.21 (-33.72)**

- Data are mean ± SD of six rats in each group.
- Hyaluronic acid expressed is expressed as ng/ml. VEGF is expressed as Pg/ml
- Control (C), prophylactic (P), treated (T),
- Values between brackets are % change over control group.
- Statistical analysis is carried out by independent student t-test, where * is significant level at $p \leq 0.05$.

Electron microscopic examination of normal control liver revealed normal hepatic architecture and normal appearance of hepatic cell organelles (Fig. 4 a, b). Diethylnitrosamine treated liver revealed extensive changes in hepatic architecture and severe damages in different cell organelles (Fig 4 c, d, e, f). The recorded observation were in parallel with the illustration of Suga [44] who noticed initial hepatic responses to carcinogenic compounds represented by proliferation of endoplasmic reticulum and peroxisomes. In addition, extensive appearance of hypertrophied nuclei with irregular nuclear membrane, nucleolar fragmentations, atrophied mitochondria, hypertrophied of Golgi apparatus, alteration in rough endoplasmic reticulum and dilatation in smooth endoplasmic reticulum. Hayashi et al. [45] added that cell proliferation, an important factor in carcinogenesis, is regulated by various tumor suppressor genes and growth factors such as hepatocyte growth factor, epidermal growth factor and transforming growth factor α and β. If the balance of these factors is altered by diethylnitrosamine, this could be favorable for the promotion of preneoplastic cells, resulting in tumor development. In addition, hepatic H_2O_2 levels were increased slightly by this chemical agents and H_2O_2-degarading enzymes; catalase and glutathione peroxidase were decreased and cannot be able to counteract the increment in H_2O_2 level, resulting sever damage and tumor initiation. Prophylactic treatment of hepatoma cells showed marked pathological alterations represented by liver sinusoidal capillarization, mesenchymal inflammatory reactions in the lobules, portal and periportal areas. In addition, Kupffer and sinusoidal endothelial cells were increased in the hepatic lobules (Fig. 5 a, b, c, d). Treatment of hepatic carcinomas showed recovery and restoration of

the nuclear shape dense glycogen granules, microbodies and active Kupffer cell (Fig.5 e, f, i, j). This was in parallel with the observation of Li et al. [46]. In addition, administration of vitamin A and β-carotene, a food colorant, may attenuate lipid accumulation in sinusoidal cells of the liver [47].

Table 4: Improvement percentages of certain biochemical parameters after prophylactic and treatment of carcinogenic rats with different plants.

Parameters	P. *Brassica oleracea* Var	P. *Brassica botrytis* L	P. *Daucus carota* L	P. *Cinnamomum zeylanicum* L	T. *Brassica oleracea* Var	T. *Brassica botrytis* L	T. *Daucus carota* L	T. *Cinnamomum zeylanicum* L
SD	12.12	15.15	18.18	21.21	24.24	15.15	33.33	**42.42**
LDH	28.08	27.24	22.20	29.54	29.42	27.24	35.28	**35.36**
T. Protein	9.52	18.45	12.83	24.38	11.67	8.63	13.90	**17.62**
AST	6.62	8.82	4.41	9.55	8.08	10.27	5.88	**22.05**
ALT	48.63	56.03	46.69	44.74	51.75	43.96	37.74	**55.64**
ALP	22.66	41.58	28.89	38.46	38.46	35.13	43.65	**45.73**
GGT	107.69	120.09	113.89	115.57	192.41	191.29	172.61	**209.60**
HA	11.65	41.93	14.67	40.52	21.47	41.11	25.94	**41.93**
VEGF	**28.08**	**21.69**	**26.00**	**29.42**	**15.89**	**11.58**	**14.56**	**16.19**

- Improvement percentages = (treated mean – carcinogenic mean) / Control mean x 100

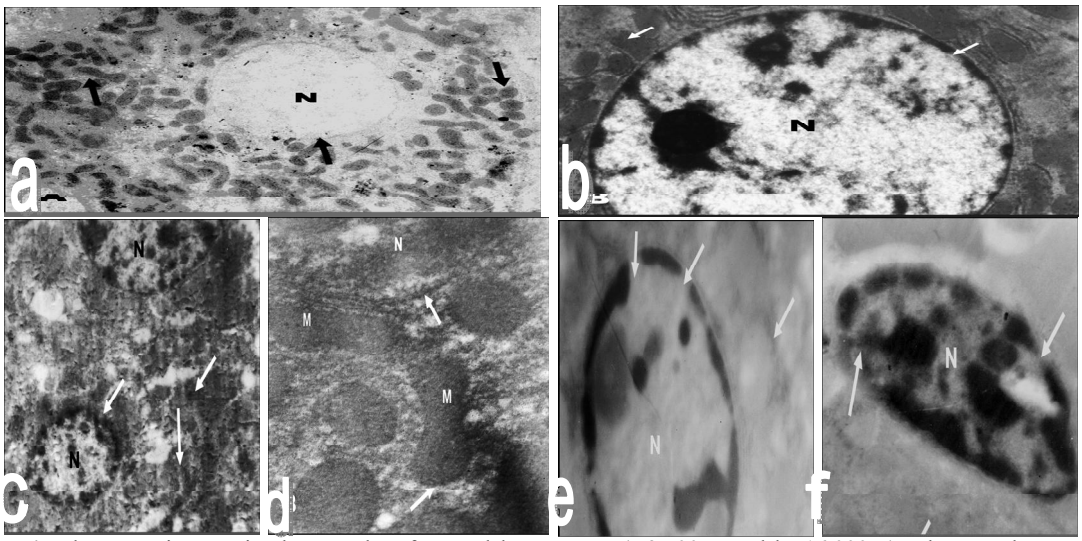

Fig. 4: Electron microscopic photographs of normal hepatocytes (a-3.500 x and b- 16.000 x). The cytoplasm was crowded with organelles, particularly rough endoplasmic reticulum, smooth endoplasmic reticulum, Golgi apparatus, ribosomes, mitochondria and glycogen particles. Nucleus and cytoplasmic organelles were clearly identified. Treatment with diethylnitrosamine; the hepatocytes revealed severe and extensive appearance of hypertrophied nuclei with irregular nuclear membrane (c-5000 x), and nucleolar fragmentations; atrophied mitochondria. The mitochondria changed their size and shape, i.e. they became polymorphous (d-5000 x). Nuclear pyknosis with compact and peripheral distribution of the chromatin and organelle compaction were observed. Hepatocytes showing lyses of their cytoplasmic membrane and organelles, as well as rupture of their nuclear membrane and degeneration of hepatocytes (e-10.000 x) and (f- 8000 x).

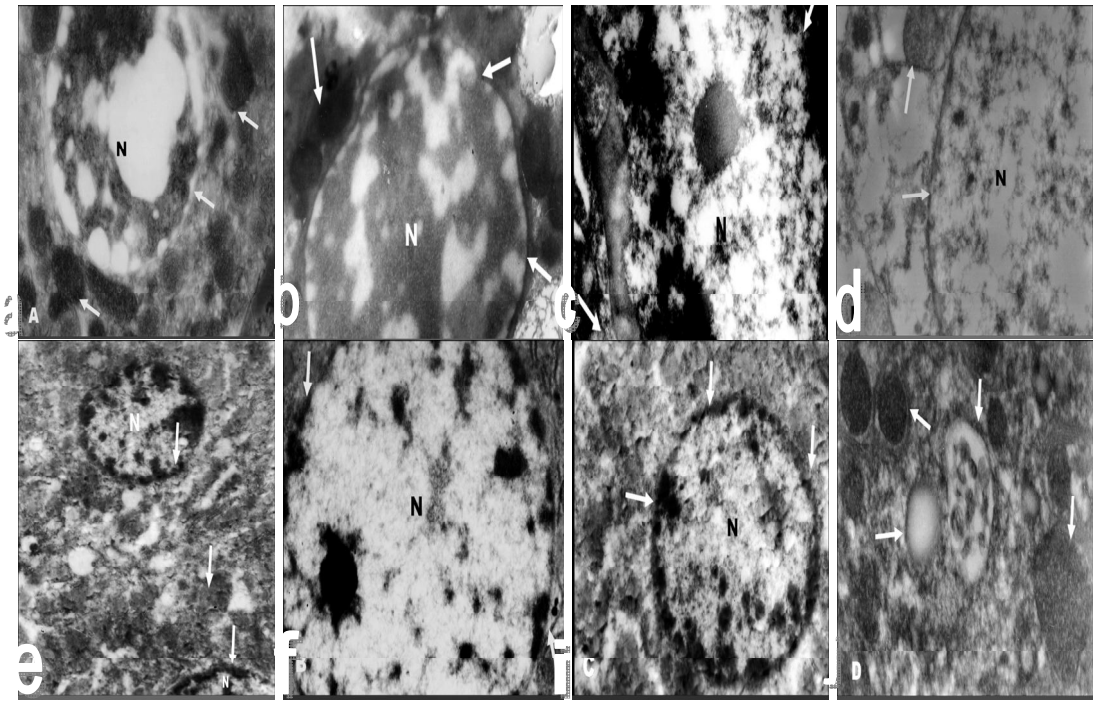

Fig. 5: Electron microscopic photographs of prophylactic rats with different plants showed some hepatocytes with hydropic degeneration characterized by more or less dilated endoplasmic reticulum, irregular cell surface without confirming the nuclei and cell organelles, some organelle-containing portions of cytoplasm around the centrally located nucleolus. The cytoplasm, microtubules and mitochondria turned normal. More clearly and little fatty vesicles were found. Healthy normal epithelial cells were detected. Cinnamon- 12.5000 x (a), cauli-flower 16.000 x (b), carrot- 8000 x (c), cabbage-10.000 x (d). Treatment of rats with different plants showed hepatocytes recovery and restoration of the nuclear shape and different cell organelles. Dense glycogen granules were observed. Cabbage-5000 x (e), cauliflower-15. 000 x (f), carrot-12.500 x (i), cinnamon 3.150 x (j).

Conclusions

Cinnamomum zeylanicum exerted *in vitro* and *in vivo* anticancer effect followed by *Daucus carota*. Treatment with these plants is a great challenge than prophylactic use. Cinnamon and carrot extracts could lead to development of potent anti-tumor agents or complementary and alternative medicines for hepatic cancer treatment.

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Declaration of interest

The authors report no declarations of interest.

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