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Research Article

Antioxidant, anti-cancer and ameliorative activities of *Spirulina platensis* and pomegranate juice against hepatic damage induced by CCl4

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Abstract

Due to the excessive impact of synthetic drugs, unravelling and employing safe, natural alternatives are now needed to resolve a number of diseases. In this research, we have evaluated hepatoprotective and antioxidant activities of *Spirulina platensis* and pomegranate juice in rats against hepatotoxicity induced by carbon tetrachloride (CCl4). Spirulina crude carotenoid extract was screened by UPLC-MS / MS. Activities of liver marker enzymes; measured aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and determined lipid peroxidation and antioxidant status as reduced glutathione (GSH) in liver homogenate. The infusion of CCl4 (2 ml/kg b.wt) greatly increases levels of liver marker enzymes and lipid peroxidation, resulting in depletion of antioxidants. Treatment of *Spirulina platensis* (Sp), pomegranate juice (Pj)or mixture (PJSP) of Spirulina water extract 10% and pomegranate juice 90% (1 ml/100 g b.wt) to CCl4-disrupted rats resulted in decreased activity of liver marker enzymes, lipid peroxidation with increased antioxidant status. Chromatographic separation showed that ß-carotene is the predominant carotenoid extract. This carotenoid extract was tested for colon carcinoma (HCT-116), liver carcinoma (HepG2) and intestinal carcinoma cell lines (CACO) LC50 for 21.8, 14 and 11.3 ug / ml, respectively. Total phenolic phytochemicals, total carotenoids and total flavonoids were also measured in Spirulina. Our study clearly demonstrates that *Spirulina platensis* and pomegranate juice had hepatoprotective effect on CCl4-caused hepatotoxicity in rats through its antioxidant activity.

Keywords: anticancer; antioxidant status; carotenoids; liver injury; pomegranate juice; *Spirulina platensis*

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Introduction

Liver is recognized to be one of the largest and most important organs in human biological system because of its pivotal role in metabolism, detoxification and excretion. It also performs an astounding role in maintenance, performance and regulation of homeostasis in biological system engaged in major biochemical growth pathways and all other biological activities (Ward and Daly, 1999). Carbohydrates, protein and lipid metabolism, detoxification, bile salt secretion, and vitamin storage are main functions of liver. Liver is constantly and variedly subjected to environmental contaminants and is impaired by unsafe medications, alcohol practices and an over-the-counter drug that can ultimately lead to numerous liver diseases, such as hepatitis, cirrhosis, liver disease (Subramonium and Pushpangadan, 1999). For now, the liver diseases are some of critical diseases in the world (Asrani *et al.*, 2019). Hence it poses significant threat to human health all over the world. However, conventional treatments have little effect on the elevation of hepatic diseases and are primarily natural preparations used for treatment of liver diseases (Karan *et al.*, 1999; Chaterrjee, 2000; Abdel-Rahim *et al.*, 2010; Abd El-Maksoud *et al.*, 2018). Hence, in the experimental animal model, several traditional herbal medicines are investigated for their possible hepatoprotective and antioxidant liver damage.

Spirulina platensis is blue-green, fresh water algae commonly used as a nutritional supplement. There are abundant proteins, carotenoids, essential fatty acids, vitamin B, vitamin E, and minerals, like copper, manganese, magnesium, iron, selenium, zinc. *Spirulina platensis* has gained significant popularity not only for its high nutritive quality; it's really signs of strong antioxidants like spirulans (sulfate polysaccharides), seleno-compounds, phenolic compounds, phycobiliproteins (C-phycocyanin and allophycocyanin) (Konícková *et al.*, 2014). Spirulina's macronutrient profile has more than 60% protein with 22 essential amino acids known to be of high biological significance, C-phycocyanin and allophycocyanin proteins were the focus of extensive Spirulina studies (Yoshikawa, 2008; de Cruz *et al.*, 2018). It has profile of high-quality fatty acids (Omega-6 and gamma-linoleic acid). Spirulina as well includes valuable antioxidant, probiotic and nutraceutical phytonutrients (Soni *et al.*, 2017). Hypolipidemic, hypocholesterolemic and antioxidant effects as well as neuroprotective and immunomodulatory roles have been correlated with Spirulina consumption (Serban *et al.*, 2015; Finamore *et al.*, 2017). In addition, its intake was correlated with reduction in age-related cerebellar tumor necrosis factors (TNF-5-007), β (TNF-β) and increment in function of the β-adrenergic receptor. Several reports indicated that metabolites generated by Spirulina found to inhibit the replication of different human viruses (Rechter *et al.,* 2006). Spirulina extracts have also been identified as antimicrobials and anticancer agents (El-Baz *et al.*, 2013; Czerwonka *et al.*, 2018). Numerous studies have tested Spirulina's antioxidant effects and capacity to scavenge hydroxyl radicals and prevent lipid peroxidation (Dartsch, 2008). Spirulina species displayed a number of biological behaviours, such as antihypertensive and antihyperilemic (Torres-Duran *et al.*, 2007). Chemopreventive agent for cancer and hepatoprotective agent for heavy metal toxicity (Ismail *et al.*, 2009; Karadeniz *et al.*, 2009). *Spirulina fusiformis* has shown hepatoprotection towards oxidative stress mediated by mercury chloride (Sharma *et al.*, 2007).

Pomegranate (*Punica granatum*), which grows wildly in Mediterranean countries and is usually consumed as fresh fruit or beverage, has long been of interest due to its beneficial health effects. Pomegranate juice and shell have a marked antioxidant effect. Pomegranate juice consists of water (85%), sugar (10%), pectin, ascorbic acid, polyphenols (1.5%) (Aviram *et al.*, 2000). Beneficial impacts of pomegranate juice on human health are thought to have a significant antioxidant effect (Balasundram *et al.*, 2006; Rosenblat *et al.*, 2006; Laura Marín *et al.*, 2015). Pomegranate juice contains anthocyanins (delphinidine-3-glucosidase, delphinidine-3.5 glucosidase, cyanidin and pelargonidine) and ellagitanins (2 g/L as polyphenol) which scavenge free radicals and reduce their deleterious effects. Antioxidant efficiency of pomegranate belongs to Punicalagin, which is the main ellagitannine. It is rich in flavonoids (quercetin, kaemferol and luteol) glycosides and polyphenolic acids (elaic and galic) acid (Lansky, 2006; Abdel-Rahim *et al.*, 2013). Pomegranate juice's extracellular antioxidant efficacy was shown to be three times higher compared to green tea and 2-6 times greater compared to other natural beverages (Seeram *et al.*, 2005). Pomegranate juice has recently been shown

to be responsible for increasing antioxidant defense mechanisms and to have a protective impact in testicular tissue toward CCl4-caused acute toxicity in rats (Al-Olayan *et al.*, 2014). In another study, pomegranate juice intake increased glutathione levels, and reduced lipid and protein oxidation. Since antioxidant mechanisms and glutathione level play a significant role in PCT-related hepatic damage, these studies suggest that pomegranate juice may have preventive or therapeutic effects in terms of hepatic toxicity (Matthaiou *et al.*, 2014).

Carotenoids interact strongly with reactive oxygen species and work as efficient free radical quenchers, singlet oxygen scavengers, lipid antioxidants (some which are precursors of vitamin A) in plant and animal organisms (Aly *et al.*, 2012; Helmi and Mohamed, 2016; Mohamed and Akladious, 2017; Akladious and Mohamed, 2017). Apart from this physiological and medical significance, shifts in carotenoid content and structure may also serve as indicators of environmental harm (Ladislav *et al.*, 2005; Marzorati *et al.*, 2020). Additionally, chemical structure of carotenoids is a long, aliphatic, conjugated double bond system, i.e. polyene. Aspect of these are C40 hydrocarbons, generally consisting of eight isoprene units. Many natural carotenes in an all-trans position have double bonds, where R is open-chain structure or ring network. Only a handful of known carotenes have cis-trans structure. Further member of carotenoid group is a number of oxygenated carotene derivatives combinations such as hydroxyl, epoxy, alcohol, aldehyde, ketone, lactone, carboxylic, ester, phenolic. Such compounds are named xanthophyll. Several studies have shown that the intake of dietary carotenoids is connected to prevention of debilitating diseases like cancer, atherosclerosis and age-related macular degeneration in humans due to their ability to suppress oxidative lesions by scavenging free radicals and extracting singlet oxygen. Many carotenoid biological activities have also been reported, such as immune enhancement, anti-inflammation, and anti-obesity (Bernal, 2011; Nagao, 2014). In *Chlorella vulgaris*, carotenoids are β-carotene, lutein and astaxanthin whereas in *Spirulina platensis*, they are β-carotene, lutein, and zeaxanthin. Nearly 85% of primary liver tumors account for hepatocellular carcinoma (Kew, 2002). Hepatocellular carcinoma is world's fifth most prevalent malignancy, and world's third most prevalent cancer-related manner of death (Kew, 2002). Cancer is triggered by cell proliferation and apoptosis or death factor imbalances. Apoptosis may affect growth of tumors at one or more cancerous phases. Apoptosis is type of programmed cell death defined by morphological changes in cells caused by cysteine-aspartate proteases (caspases) and controlled by BCl-2 family of proteins. Implied in signal propagation (Hanson *et al.*, 2008). A strong chemopreventive agent is naturally present agent, which may cause apoptosis in cancer cells without side effects (Surh, 1999).

Purpose of this investigation was to assess ability of Spirulina and pomegranate towards CCl4-induced hepatic intoxication in adult male rats in an attempt to understand their mechanism of action, antioxidant activity of the components of *Spirulina* and pomegranate and preventive role against oxidative stress and hepatic harm, as well as profile of *Spirulina* carotenoids and their anti-tumor activity, which may give lighter shade to the possibility of using it for therapeutic nutrition.

Materials and Methods

Materials and chemicals

Pomegranate (*Punica granatum* L.) fruits were purchased from local market, Cairo, Egypt. Whereas, spirulina (*Spirulina platensis*) biomass was obtained from Algae Biotechnology Unit, National Research Center, Giza, Egypt.

Commercial kits used for the determination of alanine aminotransferase (ALT); aspartate aminotransferase (AST); malondialdehyde (MDA) and reduced glutathione (GSH) were purchased from Biodiagnostic Co. Dokki, Egypt. Whereas carbon tetrachloride (CCl4) has been obtained from El-Gomhoreya Co, Cairo, Egypt. Meanwhile, 2,2-diphenyl-2-picrylhydrazile radical (DPPH) was purchased from Sigma – Aldrich Inc. (St Louis, MO, USA).

Pomegranate juice preparation

Pomegranate fruits (2.5 Kg) were washed and drained by tap water. The pomegranate fruit was sliced manually and outer leathery skin, which encloses hundreds of fleshy bags, was removed. Juice in bags was extracted using a domestic squeezer (Kenwood juicer, USA); juice obtained was stored under freezing conditions (-20 °C) until it was used and analysed (Maskan, 2006).

Spirulina aqueous extract preparation

Aqueous extract of biomass from Spirulina (*Spirulina platensis*) was prepared using described method (Abdel-Salam *et al.*, 2015). *Spirulina platensis* materials (250 g) were pulverized separately in a grinder (6% gross dry matter) for a short period. Then, pulverized content was dissolved and processed in electric blender one time for 15 min with 1000 ml of hot distilled water (50-55 °C). Suspension remained for one hour at room temperature, then filtered twice, first through the cheesecloth (50% cotton/50% polyester) and then through filter paper (Whatman No. 2). Until further usage clear aqueous extract was stored in sterile dark bottles (100 ml) at -20 °C.

Preparation the combination of pomegranate juice and Spirulina extract (PJSP)

Five separate blends of pomegranate juice and Spirulina aqueous extract were freshly prepared in varying proportions from the mixture of pomegranate juice (PJ): Spirulina aqueous extract (SP) as follows: 50:50, 60:40, 70:30, 80:20 and 90:10 %; then the sensory assessment was carried out by ten semi-trained panelists from the Ain Shams University Department of Food Science Staff; which revealed that the proportion of blends PJ: SP in ratio 90:10 was most preferred for consumption by the panelists in comparing to other blending ratios.

DPPH radical scavenging activity

Capacity of extract samples to scavenge 2,2-diphenyl-2-picrylhydrazile (DPPH) free radicals has been calculated using described method (Brand-Williams *et al.*, 1995). Percentage of scavenging effect was measured according to following equation from the reduction of absorbance at 517 nm toward the control:

Scavenging operation percentage = [(Abs control -Abs sample)/Abs control] × 100

Biological experiment design

Experiment was carried out on thirty male Albino Wistar rats from Organization of Biological Products and Vaccines (Helwan Farm, Cairo, Egypt) with average weight of 140±10 g; they were kept in special cages under controlled conditions. In compliance with the guidelines of AIN-93, animals were fed on basal diet and provided with ad-libitum water during experimental period (Reeves *et al.*, 1993). For each group, rats were randomly divided into five groups of six rats each. Class one was reserved for normal control (NC). Intraperitoneal (IP) injection with single dose of 2 ml/kg body weight was administered by mixture of (1:1 v / v CCl4 / paraffin oil) to groups of two to five rats (Małgorzata *et al.*, 2009). Group 2 was maintained as injury control (IC); each rat from group 3 to group 5 received its corresponding weight dose (depending on the weight of 15 days) through oral gavage for pomegranate juice (PJ); Spirulina aqueous extract (SP) and a combination of pomegranate juice and Spirulina aqueous extract (PJSP) at dose of 1 ml/100 g body weight per day for period of 28 days. The estimate was based on human consumption of 275 ml per day for 70 kg as recorded (Rouanet *et al.*, 2010). Weekly shifts in body weight were detected, blood samples were collected from retro-orbital eye plexus of all rats in each group a trend of experiment; the liver was removed from the body immediately after weight bleeding. Plasma was obtained by centrifugation at 1500 rpm for analysis at an ambient temperature for 15 min from blood samples. Experiment was performed in compliance with the guidelines for animal experiments of the Ain Shams University College of Agriculture. The experimental method had been approved by the Ain Shams University Ethical Committee.

Evaluation of liver function

Activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were spectrometry measured using process used by Reitman and Frankel (1957).

Marker of oxidative stress

Plasma lipid peroxidation was measured using thiobarbituric acid reactive substances (TBARS) method measuring malondialdehyde (MDA) formation at 534 nm (Ohkawa *et al.*, 1979). Whereas plasma reduced glutathione (GSH) has reacted with dithio – bis–2–nitrobenzoic acid (DTNB) (Beutler *et al.*, 1963).

Phytochemical evaluation of Spirulina

Total phenolics was measured spectrophotometrically (Thermo-Fischer evolution 300-USA) (Sanoner *et al.*, 1999), total carotenoids, HPLC Agilent 1200 equipped with diode array detector (Zhang and Omaye, 2001) and total flavonoids, spectrophotometrically (Thermo-Fischer evolution 300-USA) (Djeridane *et al.*, 2006) were measured in Spirulina extracts according to the described methods.

Preparation of Spirulina carotenoids extract

Crude carotenoid extract was obtained by extracting 10 g S. platensis with 250 ml n-hexane: methanol (1:1) overnight at average room temperature (Subagio *et al.*, 1996). Ten ml of 40% of KOH methanol was added to saponification mixture at room temperature overnight. After filtering with 0.45 μm Teflon membrane, extract was moved to separate funnel, washed 3 times with distilled water and evaporated in a rotary evaporator at a reduced pressure of 30 °C, then carotenoids were weighed and re-dissolved in 85% ethanol.

UPLC-MSMS identification of Spirulina carotenoids content

Extracted carotenoids from *S. platensis* was investigated by Waters, Acquity-H class system UPLC-MS / MS, configured with a Xevo-TQD triple quadrupole mass spectrometer with ESI-API source (Waters, USA). Data acquisition was carried out using Mass lynx V. 4.1 software. Separation was performed by BEH C18 column (50 mm X 2.1 mm, 1.7 μm by gradient solvent method acetonitrile-water-methylene chloride, flow rate 0.8 ml min^{-1}. Recognition of different carotenoids in *S. platensis* was performed by comparison of mass with Mass Lynx collection.

Measurement of the possible cytotoxicity of Spirulina by Sulfo-Rhodamine-B. Assay Stain (SRB)

Possible cytotoxicity of extract has been tested using (Skehan and Storeng, 1990) method. Cells were plated in 96-multi-well plate (104 cells/well) for 24 h before compound treatment to enable cell to be attached to the plate wall. With each individual dose, specific concentrations of carotenoid extract of Spirulina under test (0, 5, 12, 25 and 50 μg/ml) were applied to monolayer triplicate well cells. Monolayer cells were incubated at 37 °C, 5% CO2. Cells were fixed, washed, stained with stain from Sulfo-Rhodamine-B (SRB) after 48h. Excess stain was rinsed with acetic acid and attached stain was recovered with Tris EDTA buffer. ELISA reader determined density of colours. The relation of the remaining fraction of carotenoid algal concentrated extract. After the mentioned compound is plotted survival curve of each tumour cell line. Study was applied to following lines of tumour cells:

Colon cell carcinoma line (HCT-116)

Liver cell carcinoma line (HepG2)

Cell line of intestinal carcinoma (CACO)

Statistical analysis

Data descriptive values were presented as Mean ± SE, statistically analysed using ANOVA one-way variance analysis followed by Duncan test. In all cases the SAS program used $p<0.05$ as the statistical significance criterion (SAS, 2003).

Results and Discussion

Scavenging activity of pomegranate juice; Spirulina extract and their combination

The results of scavenging activity of the experimental materials are presented in Table (1). The results showed an increase in antioxidant activity in PJ followed by SP. While, the combination PJSP recorded the highest antioxidant activity, which revealed highly fight free radical activity 98.12%. Such results are consistent with those obtained for Spirulina (Remziye *et al.*, 2013), with high antioxidant potential due to presence of phycocyanin and phenolic compounds. It was proposed that overall peroxyl scavenging activity of Spirulina extracts shows a nearly linear association between antioxidant activity and concentration of Spirulina extract (Konícková *et al.*, 2014). The biological value of verified was in vivo antioxidant potential, which revealed a significant improvement in Spirulina-fed rats' antioxidant ability for 5 days (132 ± 22 percent, p = 0.002). Results for pomegranate juice also confirmed by (Akram *et al.*, 2016) showing that the radical scavenging behaviour of pomegranate juice is substantially greater. Pomegranate fruit juice contains significant amounts of organic acids, vitamins and polyphenols in the form of flavonoids that have antioxidant effects (Jaiswal *et al.*, 2010).

Table 1. Scavenging activity % of pomegranate juice; *Spirulina* extract and their combination

Treatments	Scavenging activity %
Pomegranate juice (PJ)	91.93 ± 0.84^{b}
Spirulina extract (SP)	71.09 ± 0.26^{c}
Combination (PJSP)*	98.12 ± 0.23^{a}

Data are mean ± SE, n=3, uppercase letters in column represent statistically significant data at 5%. *Pomegranate juice (PJ) 90: Spirulina extract (SP) 10

Body weight gain and liver ratio in rats' groups

Even so, initial body weight of all rats was not substantially different after 28 days of feeding; in the hepatic injury control group (IC) administered with CCl4 body weight gain, it was marginally lower compared with normal control and other treatment groups (Table 2). In the other side, injured control rats treated with oral pomegranate juice (PJ), Spirulina extract (SP), and their combination (PJSP) significantly increased in weight gain relative to normal control and CCl4 treatment groups. With a higher increase in body weight gain compared to IC, it is presumed that treatments used with PJ, SP and their combination may boost appetite and increase weight gain. It can be seen from the same table that oral treatment of pomegranate juice (PJ), spirulina extract (SP) or their combination (PJSP) effectively lowered the liver ratio ($P<0.05$) relative to the IC group.

Table 2. Effects of pomegranate juice; *Spirulina* extract and their combination on body weight gain, liver ratio in rats

Parameters	NC*	IC**	PJ	PS	PJSP
Body weight (g)					
Initial	148.4 ± 2.4^{a}	140.3 ± 2.1^{a}	146.2 ± 2.9^{a}	141.9 ± 2.1^{a}	149.8 ± 3.1^{a}
Final	208.3 ± 3.2^{b}	178.6 ± 2.7^{c}	212.4 ± 1.9^{b}	215.1 ± 1.7^{b}	226.6 ± 2.2^{a}
Gain	59.9 ± 2.3^{b}	38.3 ± 3.2^{c}	66.2 ± 2.8^{b}	73.2 ± 1.8^{a}	76.8 ± 1.5^{a}
Liver ratio (g/100 g BW)					
	6.14 ± 0.06^{b}	8.11 ± 0.16^{a}	6.44 ± 0.02^{b}	6.75 ± 0.14^{b}	6.69 ± 0.27^{b}

Data are mean ± SE, n=6, uppercase letters in same raw represent statistically significant data at 5%. *Normal control group; **Injury control group, Pj: injured control rats treated with oral pomegranate juice, PS: injured control rats treated with Spirulina extract, PJSP: injured control rats treated with the combination between oral pomegranate juice 90% and Spirulina extract 10%.

Liver function markers

The liver plays a significant role as a main organ to detox our body from a toxicant. The current study focused on the role of pomegranate juice (PJ), spirulina extract (SP) and its combination (PJSP) against hepatic injury induced by CCl_4 and on finding possible hepatoprotection.

Rats exposed to CCl_4 produced substantial hepatocellular injury as shown by the higher plasma level of AST and ALT compared to normal control group and other treatment groups; also, oral administration of pomegranate juice (PJ), spirulina extract (SP) and its combination (PJSP) also showed a substantial reduction compared to the liver injury management group of rats (Table 3). As these enzymes are stored in cytoplasmic region of cell and released into circulation in event of cell damage increased plasma of hepatic markers has been associated with liver injury (Manal, 2011). Some studies have shown that spirulina (García-Martínez *et al.*, 2007; Dartsch, 2008) significantly improves the liver markers after receiving gradient doses of Spirulina. Pomegranate juice (Faria *et al.*, 2007; Nirwane and Patil, 2012; Sadia *et al.*, 2016) also showed a substantial decrease in AST and ALT in rats suffering from CCl4 induced liver damage when fed with pomegranate fruit juice.

Table 3. Liver functions of different experimental groups

Groups	AST (U/L)	ALT (U/L)
NC*	49.82 ± 3.2^{c}	45.19 ± 2.1^{b}
IC**	108.22 ± 2.8^{a}	126.03 ± 2.5^{a}
PJ	58.71 ± 1.27^{b}	48.04 ± 1.7^{b}
SP	57.16 ± 1.29^{b}	47.22 ± 5.8^{b}
PJSP	56.13 ± 0.8^{b}	44.88 ± 3.2^{b}

Data are mean ± SE, n=6, uppercase letters in same column represent statistically significant data at 5%. *Normal control group; **Injury control group. Pj: injured control rats treated with oral pomegranate juice, PS: injured control rats treated with Spirulina extract, PJSP: injured control rats treated with the combination between oral pomegranate juice 90% and Spirulina extract 10%.

Oxidative stress markers

Lipid peroxidation (MDA) is a process of autocatalysis and is natural consequence of cell death. It can induce inflammatory injury to peroxidative tissue (Bandyopadhyay *et al.*, 1999).

In the current study, as shown in Table (4), plasma lipid peroxide concentrations were decreased in the treatment group relative to the liver injury group; the percentage of reduction was slightly lower in the PJSP group at 40.55% compared to the other treatment groups with 28.62% and 31.74%% respectively in the PJ and SP groups. This finding is consistent with that of (Ahmed *et al.*, 2011). At the other hand, glutathione improved in plasma in groups administered orally pomegranate juice (PJ) or spirulina extract (SP) and their combination (PJSP) by 19.9, 14.6 and 35.8 percent, respectively, relative to the injury group (IC). Ultimately, it could be noticed that the PJSP-consuming group had the largest substantial difference relative to the injury group. Attack GSH (reduced glutathione) may be the primary agent involved in redox protein thiol control (Sies, 1999). In studies performed by Vadiraja *et al.* (1998) Intraperitoneal administration of single dose of Spirulina phycocyanin to rats pretreated with CCl_4 significantly decreased hepatotoxicity. Losses of microsomal cytochrome P450, aminopyrine-N-demethylase, and glucose-6-phosphatase have been significantly reduced, thereby providing protection to liver enzymes by phycocyanin.

In a study conducted by Manal (2011), pomegranate juice significantly increased hepatic GST activity and preserved glutathione GSH levels even after treatment with CCl_4. The mechanism of hepatoprotection by an extract of pomegranate against CCl_4 toxicity may be due to restoration of GSH level.

Table 4. Oxidative stress markers of different experimental groups

Groups	MDA (nmol/ml)	GSH (mg/dl)
NC*	3.66±1.27[b]	26.15±1.24[b]
IC**	5.45±1.35[a]	21.87±1.77[c]
PJ	3.89±0.21[b]	26.23±1.13[b]
SP	3.72 ±0.66[b]	25.07±2.21[b]
PJSP	3.24 ±0.44[b]	29.71±3.11[a]

Data are mean ± SE, n=6, uppercase letters in same column represent. Statistically significant data at 5%. *Normal control group; **Injury control group Pj: injured control rats treated with oral pomegranate juice, PS: injured control rats treated with Spirulina extract, PJSP: injured control rats treated with the combination between oral pomegranate juice 90% and Spirulina extract 10%.

Table 5. Chemical composition of *Spirulina platensis*

Component	mg/g dry weight
Total phenolics	16.0 ± 0.7
Total carotenoids	161 ± 7.0
Total flavonoids	79.6 ± 3.3

Data in Table 5 showed that the content of phytochemicals in Spirulina whereas the total carotenoids was higher followed by total flavonoids and total phenolics. Katsuya *et al.* (2007) showed that the total phenolics in *Spirulina* are five times higher than other species in blue- green algae. Also, *Spirulina* contains free carotenoids and carotenoid esters.

Table 6. UPLC-MSMS analysis of major components in methanolic extract of *Spirulina platensis*

Main Components	Area %	Rt	M+
β – Cryptoxanthin	3.0	1.02	551
Zea-xanthine	2.8	1.24	569
*trans*β – Carotene	57.3	2.94	536
*cis*β – Carotene	31.0	4.40	536

Rt: retention time, M+: positive molecular ion

The chromatogram showed in Figure 1 Identify the major beaks at Rt of 2.92 and 4.4 min and comparison of the mass chart with National Institute of Standards and Technology (NIST) samples reveals that the major compounds in the extract are trans and cis-β-carotene. In current study, carotenoid extract analysis using UPLC-MS/MS in *Spirulina platensis* presented high levels of trans-and cis-β-carotene accompanied by β-cryptoxanthin and zeathanthin (Table 6). Carotenoids are one of the key pigment dependents who have assessed their nutritional and biological function, as per comprehensive research in field of algal-natural products. *Spirulina platensis* extract analysis revealed that it occurred same sequence β-carotene, zeaxanthin, myxoxanthophyll, chlorophyll. Other pigments have been known, such as pheophytin-like compounds, siphonin and astaxanthin along with other minor carotenoids (Mendiola *et al.*, 2005). Similarly, *Spirulina platensis* displayed high β-carotene content 30 times higher than that contained in carrots (Yin *et al.*, 2017). Carotenoids recorded antioxidant properties as active compounds but their efficacy varies between source to source (Sallam *et al.*, 2017; Cipolatti *et al.*, 2019; El-Beltagi *et al.*, 2018, 2019a-b). Past studies have been conducted on impact of naturally present and food-approved carotenoids (e.g. ß-carotene, canthaxanthin) on single oxidation of vegetable oils (Panek *et al.*, 2001). Single-oxygen quenching levels of food-approved carotenoids increased with numbers of conjugated double carotenoid bonds (Jung and Min, 1991; Hamed *et al.*, 2019).

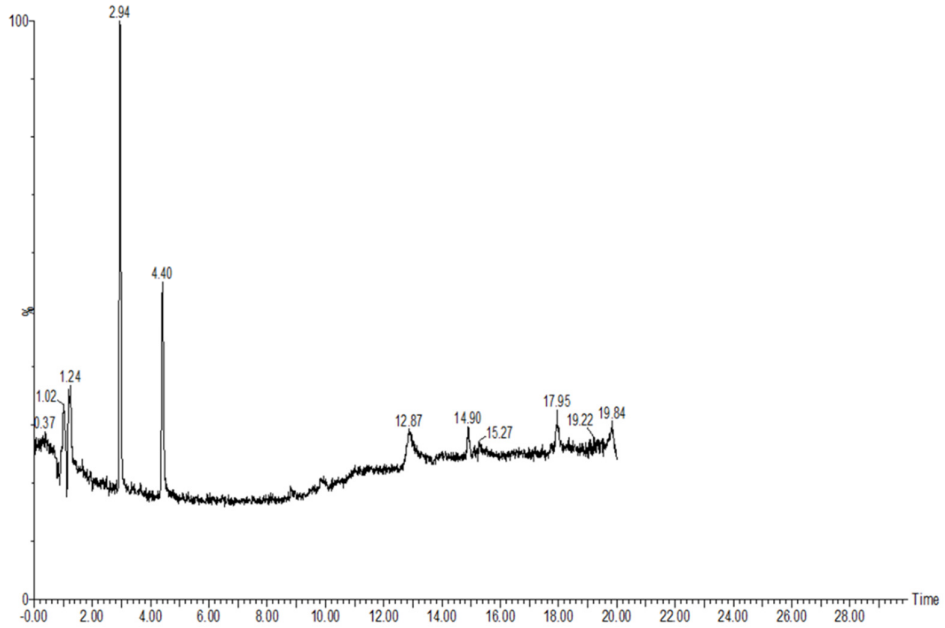

Figure 1. Chromatogram of carotenoids analysis by UPLC MS/MS

Effect of carotenoids extract from Spirulina platensis on tumor cell lines

In this part of study, crude extract of *Spirulina* carotenoids assayed to human carcinoma cell lines to evaluate its activity as anticancer which add value to the dietary Spirulina. Data provided in Table 7 and illustrated in Figure 2 stated that a dose dependent of carotenoids extract from *Spirulina platensis* and reduction the surviving fraction of intestinal followed by liver and finally colon tumor cell lines. Phycocyanobilin (PCB) and chlorophylls are among other potentially bioactive substances of particular interest due to their structural similarity to bilirubin, potent antioxidant, atheroprotective, anti-proliferative agent (Konícková *et al.*, 2014).

The cytotoxicity exhibited by Spirulina extract to cancer cell lines might be due to the presence of phytopigments (carotenoids, chlorophyll, phycocyanin) as well as polysaccharides that were reported previously as constituents of the extract. So crude extracts of Spirulina can be used as a source to develop anticancer drugs (Hernandez *et al.*, 2017).

Table 7. Lc_{50} of spirulina carotenoids extract in tumor cell lines of different types of cancer

Tumor cell line	LC50 ug/ml
Colon (HCT116)	21.8
Liver (HEPG2)	14
Intestinal (CACO)	11.3

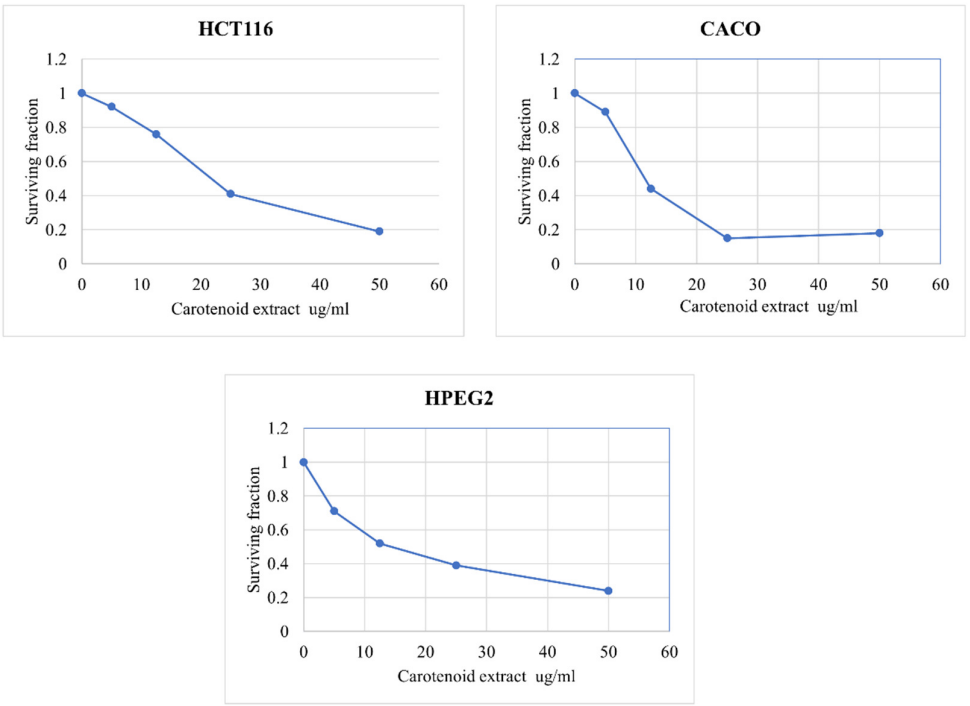

Figure 2. The relationship between surviving fraction and concentrations of carotenoid extracts of *Spirulina platensis* for treating (HCT116), (HEPG2) and (CACO) cell lines

Spirulina bilirubin's anticancer activity has been attributed to its effects on mitochondria and intracellular signaling (Keshavan *et al.,* 2004; Ollinger *et al.*, 2007). Bilirubin has also been shown to be a commonly used protein phosphorylation inhibitor. Previous study showed that the therapeutics tested exerted strong antioxidant effects with substantial reductions in ROS levels of mitochondrial reactive oxygen species, which strengthened the overall cell redox status as shown by observed improvement in parameters of redox glutathione (Konícková *et al.*, 2014). These results tend to be related to bilirubin's inhibitory effects on superoxide formation and the mitochondrial metabolism in general (Nakamura *et al.*, 1987). It has also been proposed that pyrrole groups of bilirubin present in our compounds interact with NADH at active mitochondrial dehydrogenase sites suggesting possible mechanisms for these effects. Spirulina aqueous extract had greater effect on HepG2 and HSC than chlorella. Spirulina's LC50 value for HepG2 was 60 at complete phenolic concentration (Li-Chen *et al.*, 2005). It also had significant cytotoxic effect of on human acute leukemia Kasumi-1, chronic myelogenous leukemia K-562 and e human lung cancer A549 cell lines (Hernandez *et al.*, 2017; Czerwonka *et al.*, 2018). In addition, *Spirulina platensis* water extracts showed antiproliferative properties against breast cancer adenocarcinoma cell line (MCF-7) and mice intestine carcinoma cell line (L20B) suggesting that new promising anticancer natural products from blue-green algae are possible (Fayyad *et al.*, 2019). The *Spirulina platensis* derived phycocyanin pigments have more active functional groups with potential anticancer, anti-diabetic and anti-inflammatory action and it could be considered as an alternate functional food for food and drug industry (Prabakaran *et al.*, 2020).

Conclusions

Based on results obtained, it was assumed that conventional and common dietary Spirulina and pomegranate juice had antioxidant activity and significantly reduced hepatic damage caused by CCl4, and that the combination of both Spirulina and pomegranate juice did not significantly affect the hepatoprotective properties of Spirulina and pomegranate juice, meaning that it could be used as a therapeutic natural and benefitted beverage. In addition, Spirulina carotenoids showed anti-cancer activity in the liver, colon and intestine tumor cell lines.

Authors' Contributions

Conceptualization, I.S.A. and K.MA.R.; Methodology, K.MA.R.; Formal analysis, F.D., H.S.E., I.S.A. and K.MA.R.; Funding acquisition, F.D., H.S.E. and K.MA.R.; Investigation, F.D., H.S.E. and K.MA.R.; Resources, I.S.A. and K.MA.R.; Data curation, H.S.E., I.S.A. and K.MA.R.; Project administration, F.D., H.S.E., A.M.H. and E.AH.S.E., Writing-original draft preparation, I.S.A. and K.MA.R.; Software, H.S.E. and K.MA.R.; Supervision, H.S.E. and K.MA.R.; Validation, I.S.A. and K.MA.R.; Visualization, H.S.E. and K.MA.R.; Writing-review and editing, F.D., H.S.E., I.S.A. and K.MA.R.; All authors have read and agreed to the published version of the manuscript.

Ethical approval (for researches involving animals or humans)

Ethical approval, for all the practiced experimental protocols, was obtained from Medical Research Ethics Committee, National Research Center (NRC-MREC; number, 20/150) according to Egyptian Network of Research Ethics Committee (ENREC) regulations.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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