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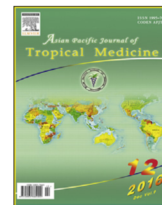

Original Research http://dx.doi.org/10.1016/j.apjtm.2016.11.004

Influence of bioactive sulfated polysaccharide-protein complexes on hepatocarcinogenesis, angiogenesis and immunomodulatory activities

Azza A. Matloub[1✉], Hadeer A. Aglan[2], Sahar Salah Mohamed El Souda[3], Mona Elsayed Aboutabl[4], Amany Sayed Maghraby[5,6], Hanaa H. Ahmed[2]

[1]*Pharmacognosy Department, National Research Centre, Dokki, Giza, Egypt*

[2]*Hormones Department, National Research Centre, Dokki, Giza, Egypt*

[3]*Chemistry of Natural Compounds Department, National Research Centre, Dokki, Giza, Egypt*

[4]*Medicinal and Pharmaceutical Chemistry Department (Pharmacology Group), National Research Centre, Dokki, Giza, Egypt*

[5]*Therapeutical Chemistry Department, National Research Centre, Dokki, Giza, Egypt*

[6]*Laboratory of Immune and Biomarkers for Infection, The Center of Excellence for Advanced Sciences, National Research Centre, Dokki, Giza, Egypt*

ARTICLE INFO

Article history:
Received 20 Jul 2016
Received in revised form 21 Aug 2016
Accepted 20 Sep 2016
Available online 9 Nov 2016

Keywords:
Jania rubens
Pterocladia capillacea
Enteromorpha intestinalis
Polysaccharide-protein complexes
Anti-tumor activity
Anti-angiogenesis

ABSTRACT

Objective: To explore the *in vivo* anticancer, anti-angiogenesis and immunomodulatory efficacies of the bioactive polysaccharide isolated from cold aqueous extract of *Jania rubens* (JCEM) and *Pterocladia capillacea* (PCEM) as well as hot aqueous extract of *Enteromorpha intestinalis* (EHEM) against hepatocellular carcinoma rat model (HCC) and to study their chemical composition.

Methods: The sugars and amino acids composition of the bioactive polysaccharides of JCEM, PCEM and EHEM were determined using gas liquid chromatography and amino acid analyzer, respectively. These polysaccharide extracts (20 mg/kg b.wt. for 5 weeks) were assessed on hepatocarcinogenesis in rats and α-fetoprotein (AFP), carcinoembryonic antigen (CEA), glypican-3 (GPC-3), hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) and Ig G levels were evaluated.

Results: The GLC analysis of JCEM, PCEM and EHEM polysaccharide revealed the presence of 10, 9 and 10 sugars, in addition the amino acid analyzer enable identification of 16, 15 and 15 amino acids, respectively. These polysaccharide extracts of JCEM, PCEM and EHEM produced significant decrease in serum AFP, CEA, GPC-3, HGF and VEGF compared with untreated HCC group. JCEM, PCEM and EHEM had an immunostimulatory responses by increasing the IgG levels as compared by naïve value (1.23, 1.53 and 1.17 folds), respectively. The bioactive polysaccharides in HCC induced rats improved the humoral immune response. The photomicrographs of liver tissue sections of the groups of HCC treated with polysaccharide extracts of *Jania rubens* and *Enteromorpha intestinalis* showed intact histological structure. Moreover, fractions HE1, HE4, HE7 obtained from polysaccharide of EHEM showed moderate cytotoxic activity against HepG2 *in vitro* with IC_{50} 73.1, 42.6, 76.2 μg/mL. However, fractions of PCEM and JCEM show no or weak cytotoxicity against HepG2 *in vitro* where the cytotoxic activity of their crude polysaccharide extract proved synergetic effect.

Conclusions: The pronounced antitumor activity of sulfated polysaccharide-protein complexes of JCEM and EHEM is due to direct cytotoxic activity, anti-hepatocarcinogenesis, and anti-angiogenesis. In addition, JCEM, PCEM and EHEM had an immunostimulatory response and improved the humoral immune response in HCC induced rats.

✉First and corresponding author: Azza A. Matloub, Pharmacognosy Department, National Research Centre, 33 El-Bohouth St. (Former El-Tahrir St.), P.O. 12622, Dokki, Giza, Egypt.
Fax: +20 33370931
E-mail: matlouba2002@hotmail.com
Peer review under responsibility of Hainan Medical University.

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common types of malignancies that carries poor prognosis worldwide. The treatment of HCC is limited because of underlying cirrhosis and a high rate of recurrence; the cumulative 5-year survival rate is 53.4% with hepatic resection, 42.0% with local ablation therapy, and 22.6% with transcatheter arterial embolization [1]. According to the rising incidence of HCC, effective therapies with improved immunity potential are urgently needed to combat the current morbidity and mortality associated with HCC without harming the host [2–4]. Therefore, development of new drugs for HCC is required.

Many non-toxic biological macromolecules including polysaccharides, sulfated polysaccharide, and polysaccharide-protein complexes, isolated from natural source such as mushrooms, fungi, yeasts, algae, lichens and plants, have attracted more attention recently in biochemical and medical areas due to their immunomodulatory and anti-cancer effects [5,6] and several involved clinical trials [4].

Referring to our previous study, polysaccharide isolated from cold aqueous extract of *Jania rubens* (*J. rubens*) (JCEM) and *Pterocladia capillacea* (*P. capillacea*) (PCEM) as well as polysaccharide isolated from hot aqueous extract of *Enteromorpha intestinalis* (*E. intestinalis*) (EHEM), were characterized as sulfated polysaccharide-protein complexes and represented as the most bioactive polysaccharides *in vitro* against hepatocarcinoma human cell line (HepG2) with an IC_{50} 10.73, 56.54 and 53.64 μg/mL [7]. This encouraged us to further explore *in vivo* their anticancer efficacy and anti-angiogenesis as well as immunomodulatory effect against chemically-induced hepatocarcinogenesis and further fractionate these bioactive polysaccharides, study their chemical composition and evaluate their cytotoxic effect against $HepG_2$ cell line.

2. Materials and methods

2.1. Materials

2.1.1. Algae material

It is described in Matloub *et al* [7].

2.1.2. Experimental animals

Sixty four adult female Wistar rats weighing (150–180) g were obtained from the Animal House Colony of the National Research Centre, Giza, Egypt and acclimatized in a specific area where temperature (25 ± 1)°C and humidity (55%). Rats were controlled constantly with 12 h light/dark cycles at National Research Centre, Animal Facility Breeding Colony. Rats were individually housed with *ad libitum* access to standard laboratory diet consisted of casein 10%, salt mixture 4%, vitamin mixture 1%, corn oil 10%, cellulose 5%, completed to 100 g with corn starch [8] and tap water. Also, they were cared for according to the guidelines for animal experiments which were approved by the Ethical Committee of Medical Research at National Research Centre, Giza, Egypt.

2.1.3. Cytotoxic activity

2.1.3.1. Culture cells for in vitro cytotoxic activity

Human hepatocarcinoma cell line ($HepG_2$) was obtained in frozen state under liquid nitrogen (−180 °C) from the American Type Culture Collection, University Boulevard, Manassas, USA. The tumor cell lines were maintained by serial subculturing in the National Cancer Institute, Cairo, Egypt.

2.1.3.2. Culture media for in vitro cytotoxic activity

HepG2 cells were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% antibiotic antimycotic mixture (10 000 U/mL K-penicillin, 10 000 μg/mL streptomycin sulfate and 25 μg/mL amphotericin B) and 1% L-glutamine (all purchased from Lonza, Belgium).

2.2. Methods

2.2.1. Extraction of water soluble polysaccharide

The cold polysaccharide extracts of *J. rubens* and *P. capillacea* as well as the hot polysaccharide extract of *E. intestinalis* were obtained as described in our previous publication [7]. The chemical composition of extracts was investigated using gas liquid chromatography (GLC) and amino acid analyzer.

2.2.2. Fractionation of bioactive polysaccharide

The polysaccharides of *J. rubens* (JCEM), *Petrocladia capillacae* (PCEM) and *E. intestinalis* (EHEM) were subjected to fractionation by stepwise ethanol-precipitation from 20% to 80% [9]. The chemical composition of fractions was investigated using GLC, Fourier transform IR (FT–IR) and elemental microanalysis.

2.2.3. Chemical characterization of bioactive polysaccharide and their fraction

Carbon, hydrogen, nitrogen, and sulfur content were determined by Elemental Microanalysis (Elementary Vario EL). Monosaccharide composition was analyzed as mention in Matloub *et al* [10]. Protein content was calculated from %N using the correction factor of 6.25 and the degree of substitution (DS) was calculated from the sulfur content as mention in Matloub *et al* [7]. The amino acid composition was determined as described by Matloub *et al* [10] using an LC 3 000 amino acid analyzer (Eppendorf-Biotronik, Maintal, Germany). FT–IR was recorded with a FT/IR-6100 (JASCO, Japan) from 400 to 4 000 cm^{-1}. The samples were analyzed as KBr pellets.

2.2.4. Biological activity

2.2.4.1. Evaluation of the bioactive polysaccharide on hepatocellular carcinoma in vivo

2.2.4.1.1. Experimental design

The animals were classified into 8 groups (8 rats/group). The first group served as negative control group. The groups 2, 3 and 4 were normal rats orally administered with the polysaccharide extracts of *J. rubens*, *P. capillacea* and *E. intestinalis* (20 mg/kg b.wt), respectively for 5 weeks [11,12]. While, the groups 5–8 were orally administered with N-nitrosodiethylamine (NDEA) in a dose of 20 mg/kg b.wt, five times a week for 4 weeks and 10 mg/kg b.wt for another 1 week (total: 5 weeks) for induction of HCC [13]. Then, group 5 was left untreated; the groups from 6, 7 and 8 were treated orally with the polysaccharide extracts of *J. rubens*, *P. capillacea*, or *E. intestinalis* (20 mg/kg b.wt) respectively for 5 weeks after induction of HCC.

At the end of the experimental period, all animals were fasted for 12 h and the blood samples were collected from retro-orbital

venous plexus [14] under diethyl ether anesthesia. The blood samples were left to clot and the sera were separated by cooling centrifugation (4 °C) at 1800×*g* for 10 min and then stored immediately at −20 °C in clean plastic Eppendorf until analyzed. The whole liver of each rat was rapidly and carefully dissected then, fixed in formalin saline (10%) for histological investigation.

2.2.4.1.2. Biochemical analyses

Serum alpha fetoprotein (AFP) and carcinoembryonic antigen (CEA) were determined by enzyme linked immunosorbent assay (ELISA) technique using the kits purchased from Shinjin Medics Inc. (Korea) [13]. While, serum glypican-3 (GPC-3), hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) were determined by ELISA technique using the kit purchased from Glory Science Co., Ltd (USA) according to the manufacturer's instructions.

2.2.4.1.3. Detection of IgG levels using ELISA

The IgG sera levels in different experimental groups were detected by ELISA according to Maghraby and Bahgat [15] on plates coated with 50 μL/well sera (1:25). After coating, plates were incubated overnight at 4 °C, washed three times with PBS containing 0.05% Tween-20 (PBS-T20). Plates were blocked against non-specific binding using 100 μL/well of blocking buffer [PBS-T20 in 5% fetal calf serum (Biochrome KG; Berlin, Germany FCS)] and incubated at 37 °C for 2 h. After three washes with PBS-T20, peroxidase conjugated anti-rat IgG (KPL, Gaithersburg, MD, USA) were added 50 μL/well at dilution (1:1000) in (PBS-T20-5% FCS) to detect total IgG levels. Then the plates were incubated at 37 °C for 1 h followed by 3 washes with PBS-T20. A volume of 50 μL/well of Orthophenylenediamine (Sigma, St. Louis, Mo, USA), substrate diluted in substrate buffer was used and the plates were left for 10 min at room temperature till color development. The enzymatic reaction was stopped using 20 μL of 2 M sulfuric acid and the changes in optical density were recorded at λ max 490 nm using a multi-well plate reader (Tecan; Sunrise, Austria, GmbH).

2.2.4.1.4. Histopathological investigation of liver tissue

Samples were taken from liver of rats in the different groups and fixed in 10% formalin saline for 24 h. Washing was done in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 °C in hot air oven for 24 h. Paraffin wax tissue blocks were prepared for sectioning at 4 μ thicknesses by sledge microtome. The obtained tissue sections were collected on glass slides, deparffinized and stained by hematoxylin and eosin stains for histopathological examination through the electric light microscope [13].

2.2.4.2. Cytotoxic activity of polysaccharides fractions on hepatocarcinoma human cell line in vitro

Cytotoxic effect was accomplished on HepG2 human cell line. Cell viability was assessed by the mitochondrial dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan [7].

2.3. Statistical analysis

For *in vivo* study, all results were expressed as means ± SE of the mean. Data were analyzed by one way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) program, version 14 [16]. Difference was considered significant where $P < 0.05$.

For in *vitro* study, data were subjected to paired-samples SPSS. $P < 0.005$ was regarded as significant. Also, a probit analysis was carried for IC_{50} and IC_{90} determination using SPSS.

3. Results

The polysaccharides isolated from cold aqueous extract of *J. rubens* (JCEM) and *P. capillacea* (PCEM) as well as from hot aqueous extract of *E. intestinalis* (EHEM) were previously characterized as sulfated polysaccharide-protein complexes and their cytotoxic activity on HepG2 cell line *in vitro* were reported by Matloub *et al* [7].

Herein, the monosaccharide and amino acids composition of these bioactive polysaccharides were investigated. Furthermore, anti-tumor, anti-angiogenesis and immunomodulatory activities of these polysaccharides were evaluated *in vivo*. Also, these prepared polysaccharides were subjected to fractionation by using stepwise ethanol-precipitation method [9] and their cytotoxic activity was assessed on HepG2 cells to determine which fraction was responsible of this activity.

3.1. Chemical composition of native bioactive polysaccharide and their fractions

The fractionation of bioactive polysaccharides JCEM, PCEM and EHEM afforded 8, 8 and 5 fractions, respectively. The yields percent, elemental microanalysis, protein contents and sulfation degree of these fractions were compiled in Table 1. Fraction named JC2 (34.61%) represented the main fraction of JCEM (Table 1). The carbohydrate content of the fractions ranged from 57.09% to 74.53%. The elemental microanalysis of JCEM fractions revealed that JC1 fraction contained the highest protein content (15.18%) and lowest sulfur content (5.5%). In contrast, the other fractions (JC2–JC8) constituted higher sulfur content (11.26%–16.00%) and lower protein content (2.50%–6.87%). On the other hand, fractions PC1 (45.62%), PC5 (12.44%) & PC2 (9.66%) represented the main fractions of PCEM. The fractions constituted sugar content (62.16%–73.65%). The proportion of protein is higher in fraction PC2 (21.87%), while PC5 contain higher sulfur content (10.50%) than other fractions (Table 1). Fraction EH1 (83.85%) was represented as major fraction of EHEM. The sugar content of the fraction was ranged from 68.92% to 80.08%. Most protein content was included in EH1 & EH8 fractions and sulfur content was found in high proportion in EH7 fraction (Table 1).

The FT–IR spectrum data of JCEM, PCEM and EHEM fractions are summarized in Table 2. The spectra of all fractions scanned between wave number 4000 and 400 cm^{-1} revealed the characteristic absorption bands of polysaccharides at around (3531.99–3374.82) cm^{-1} and (2965.98–2920.66) cm^{-1} attributed to the hydroxyl group and alkyl group, respectively. Likewise, the FT–IR spectra showed peak around 1763.58–1723.09 cm^{-1} assigned for C=O stretching (esters). Meanwhile, the absorption bands at around 1685.48–1605.45 cm^{-1} and 1536.02–1525.42 cm^{-1} assigned for C=O stretching in secondary amides (amide I) and N–H deformation and C–N

Table 1

Chemical characterization of bioactive polysaccharides JCEM, PCEM and EHEM fractions (%).

Extract	Fractions	Ethanol	Fraction	Carbohydrate	Polysaccharide extract					Degree of sulfation
					C	H	N	Protein	S	
Cold extract of	JC1	0	9.56	57.09	18.46 ± 0.08	2.72 ± 0.03	2.43 ± 0.06	15.18	5.50 ± 0.05	0.67
Jania rubens	JC2	20	43.26	74.53	20.95 ± 0.05	2.99 ± 0.06	0.40 ± 0.03	2.50	15.47 ± 0.08	1.66
(JCEM)	JC3	30	9.73	72.57	22.01 ± 0.07	3.19 ± 0.04	0.54 ± 0.08	3.38	16.00 ± 0.05	1.63
	JC4	40	4.71	71.22	18.82 ± 0.03	2.49 ± 0.04	0.70 ± 0.06	4.38	11.35 ± 0.04	1.35
	JC5	50	2.50	69.76	22.64 ± 0.08	3.06 ± 0.06	1.09 ± 0.03	6.81	12.00 ± 0.08	1.19
	JC6	60	10.95	65.92	19.36 ± 0.04	2.70 ± 0.03	1.00 ± 0.05	6.25	12.59 ± 0.06	1.46
	JC7	70	8.33	67.92	19.28 ± 0.03	2.87 ± 0.05	1.00 ± 0.07	6.25	11.26 ± 0.04	1.31
	JC8	80	6.39	69.39	16.70 ± 0.06	2.32 ± 0.04	1.10 ± 0.03	6.88	12.37 ± 0.07	1.67
Cold extract of	PC1	0	50.63	67.03	25.50 ± 0.02	4.25 ± 0.06	3.20 ± 0.06	20.00	3.70 ± 0.04	0.32
Petrocladia	PC2	20	10.72	69.08	26.80 ± 0.07	4.42 ± 0.05	3.50 ± 0.06	21.87	4.80 ± 0.08	0.40
capillacae (PCEM)	PC3	30	2.89	62.16	25.00 ± 0.08	2.72 ± 0.04	1.80 ± 0.06	11.25	8.50 ± 0.04	0.77
	PC4	40	2.53	63.51	26.36 ± 0.06	3.06 ± 0.04	1.70 ± 0.05	10.63	7.50 ± 0.09	0.64
	PC5	50	13.81	71.20	20.01 ± 0.08	2.72 ± 0.05	0.60 ± 0.03	3.75	10.50 ± 0.04	1.18
	PC6	60	7.24	68.23	26.96 ± 0.05	4.42 ± 0.04	1.11 ± 0.04	6.94	8.00 ± 0.06	0.67
	PC7	70	6.35	73.65	18.47 ± 0.03	2.72 ± 0.06	0.40 ± 0.09	2.50	9.90 ± 0.05	1.21
	PC8	80	3.64	nt	nt	nt	nt	nt	nt	nt
Hot extract of	EH1	0	83.85	68.92	25.59 ± 0.05	3.65 ± 0.04	2.95 ± 0.06	18.43	1.99 ± 0.08	0.17
Enteromorpha	EH2	20	–	–	–	–	–	–	–	–
intestinalis (EHEM)	EH3	30	–	–	–	–	–	–	–	–
	EH4	40	1.19	nt	nt	nt	nt	nt	nt	nt
	EH5	50	–	–	–	–	–	–	–	–
	EH6	60	2.43	nt	nt	nt	nt	nt	nt	nt
	EH7	70	11.03	80.08	19.40 ± 0.09	2.62 ± 0.06	0.53 ± 0.07	3.31	11.07 ± 0.03	1.28
	EH8	80	1.39	63.43	23.42 ± 0.04	3.84 ± 0.05	2.95 ± 0.08	18.43	3.61 ± 0.07	0.35

– = No precipitation; nt = Not test.

Table 2

FT–IR analysis of polysaccharide fractions.

Fractions	Assignment wave number (cm^{-1})											
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
JC1	3535.84 3407.60	2929.34	–	–	–	1435.74	–	–	1118.51	887.09	–	676.89 599.75
JC2	3531.99 3403.74	2932.23	–	1684.52 1623.77	–	1436.71	–	–	1118.51	887.09	–	675.92 599.75
JC3	3396.99	2935.13	–	1665.23 1631.48	1524.45	1428.03 1413.57	–	–	1194.69 1122.37	–	754.99	653.75 602.64
JC4	3398.92	2935.13	–	1663.23 1628.59	–	1428.03	–	–	1194.69 1120.44	–	761.74	653.75 608.43
JC5	3412.42	2925.48	–	1654.62	–	1423.20	1383.67	–	1193.72 1119.47	–	754.03	655.67 607.46
JC6	3408.57	2935.13	–	1658.48 1631.48	–	1409.71	1378.85	–	1176.36 1113.69	–	748.24	617.10
JC7	3427.85	2949.59	–	1657.52 1639.20	–	1410.67	1378.85	–	1176.36 1113.69	–	796.45 758.85 721.24	693.28 661.46 617.10
JC8	3417.24	2945.73	–	1657.52 1632.45	–	1411.67	1379.82	–	1176.36 1114.65	–	796.45 735.71	673.99 618.07
PC1	3426.89	2935.13	–	1664.27 1629.55	–	1435.74	1323.89	1237.11	1099.23 1044.26	812.84	771.38 732.81	671.10 613.25
PC2	3428.81	2963.09	–	1661.37 1633.41	–	1435.74	1323.89	1244.83	1130.08 1091.51 1034.62	825.38	771.38 714.49	670.14 597.82
PC3	3427.85	2920.66	–	1657.52 1631.48	–	1414.53	1320.04	–	1133.94 1094.40	–	744.38	677.85 583.36
PC4	3405.67	2932.23	1763.58	1657.52 1633.41 1605.45	–	1413.57	1320.04	–	1197.58 1136.83 1094.40	–	757.88	656.64 602.64
PC5	3409.53	2963.09	–	1657.52 1628.59	–	1414.53	–	–	1135.87 1092.48	827.31	754.99	655.67 597.82
PC6	3404.71	2965.98	–	1661.17 1627.63	–	1430.92	1321.00	–	1127.19 1101.15	833.09	744.38	670.14 651.82 600.71
PC7	3374.82	2926.45	–	1631.48		1422.24	–	–	1199.51 1135.87 1093.44	824.42	758.85	655.67 599.75
PC8	3429.77	2923.55		1628.59	1536.02		1380.78		1091.51	818.63	750.17	699.53 609.39
EH1	3429.78	2923.56	1753.94 1723.09	1625.70	–	1438.64 1412.6	1380.78	1236.15	1133.94 1027.87	876.488	731.85	667.25 628.68 556.36
EH6	3408.57	2938.98		1652.70	–	1430.92	1320.04	1250.61	1051.98	846.59	778.13	599.75
EH7	3401.82	2939.90	–	1657.52 1625.70	1525.42	–	–	1250.61	1103.08	–	732.81	620.00 548.64

(1) The assignment for stretch vibration of O–H (hydroxyl groups). (2) The assignment for stretch vibration of C–H. (3) The assignment for C═O stretching (esters). (4) The assignment for C═O stretching in secondary amides (amide I). (5) The assignment for Amide II (N–H bending of amino acid group). (6) The assignment for symmetric stretch vibration of –COO^- (uronic acids). (7, 8) The assignment for stretching vibration of S═O (esterified sulfate). (9) The assignment for C–O–C bending mode in glycosidic linkages. (10) The assignment for bending vibration of C–O–S of the sulfate in axial position. (11) The assignment for bending vibration of C–O–S of the sulfate in equatorial position. (12) The assignment for asymmetric deformation of O–S–O groups.

Table 3

Monosaccharide composition of bioactive polysaccharide extracts and fractions of JCEM, PCEM and EHEM.

Samples	Relative (%)												
	Arabinose	Xylose	Ribose	Rhamnose	Mannitol	Sorbitol	Fructose	Galactose	Mannose	Glucose	Glucuronic acid	Gal/Xyl	Gal/Glu
RRt	0.68	0.69	0.71	0.76	0.88	0.89	0.91	0.98	0.99	1.00	1.32		
JCEM	5.83	17.31	0.74	4.42	7.57	–	1.43	48.78	4.01	9.65	0.20	2.80	5.05
JC1	14.26	18.86	2.82	5.09	3.05	–	0.62	16.22	4.33	33.74	0.97	0.86	0.48
JC2	6.76	11.96	4.02	8.83	3.07	–	–	29.02	13.31	18.38	4.61	2.42	1.58
JC3	4.27	14.14	7.24	7.02	4.69	–	–	33.01	12.59	15.79	1.22	2.33	2.09
JC4	7.77	13.16	3.05	8.70	4.20	–	–	34.97	9.75	16.54	1.81	2.65	2.11
JC5	6.50	15.14	1.10	9.92	3.47	0.76	1.29	31.75	9.37	20.53	0.13	2.10	1.55
JC6	15.68	6.51	1.87	8.39	6.51	0.72	1.41	19.44	22.15	12.51	4.75	2.98	1.55
JC7	5.39	17.75	0.76	7.92	8.86	0.18	1.86	12.88	32.97	11.06	0.33	0.72	1.16
JC8	6.52	14.93	–	7.98	7.08	–	–	36.82	–	23.27	3.37	2.46	1.58
PCEM	2.26	8.61	2.73	12.69	4.10	–	0.25	35.16	–	34.01	0.15	4.08	1.03
PC1	3.45	8.71	6.32	9.75	3.76	–	1.28	37.26	–	28.46	0.96	4.27	1.31
PC2	2.81	7.52	6.67	8.81	6.59	–	1.85	35.34	–	30.39	–	4.69	1.16
PC3	1.47	8.60	4.49	8.01	4.83	–	2.29	38.83	–	31.45	–	4.51	1.23
PC4	1.99	7.83	2.65	27.88	3.51	–	2.34	27.93	–	22.65	3.16	3.56	1.23
PC5	2.17	8.26	1.67	25.06	2.78	–	2.02	41.68	–	16.13	0.19	5.04	2.58
PC6	2.31	5.07	10.16	36.42	–	–	–	23.37	12.43	9.84	–	4.06	2.37
PC7	2.04	7.02	1.10	26.65	2.40	0.54	3.50	35.02	–	21.70	–	4.98	1.61
PC8	2.22	6.82	1.07	16.93	2.40	–	0.64	32.68	–	37.10	0.13	4.79	0.88
EHEM	10.13	14.24	0.45	61.03	2.62	0.15	0.08	7.66	1.30	2.30	–	1.86	3.33
EH1	7.51	16.39	0.52	61.46	1.60	–	–	10.31	–	1.99	0.19	1.59	5.18
EH6	4.38	10.48	12.27	50.56	5.26	–	–	8.83	4.62	3.33	–	1.19	2.65
EH7	5.41	16.74	1.57	44.02	9.53	4.18	0.75	4.08	3.07	10.60	–	4.10	4.10
EH8	5.81	12.44	–	54.18	–	–	–	14.51	5.23	7.76	–	0.86	1.87

stretching in –CO–NH– of protein (amide-II, respectively, this result in addition elemental microanalysis confirmed that polysaccharide fractions binding protein [17].

Moreover, bands at (1 438.64–1 409.71) cm^{-1} attributed to the symmetric stretch vibration of COO^- and the stretch vibration of C=O within COOH. Whereas, the bands at (1 199.51–1 027.87) cm^{-1} assigned to the glycosidic linkage stretch vibration of C–O–C and C–O–H [18]. While, the absorption bands at (1 383.67–1 320.04) and (1 250.61–1 236.15) cm^{-1} were attributed to S]O stretching vibration indicating the presence of esterified sulfate.

The band at 887 cm^{-1} was attributed to β-configuration of glycosidic linkage [19]. In order to identify the position of sulfate group, the bands at 846.59 cm^{-1}, (833.09–824.42) cm^{-1} and 818.63 cm^{-1} are characteristic for galactose-4-sulfate, galactose-2-sulfate and galactose-6-sulfate, respectively [7].

Absence of bands at 930 cm^{-1} and 805 cm^{-1} indicated the lack of 3,6-anhydrogalactose in the tested JCEM and PCEM fractions. In previous study, the main polysaccharide composition of *P. capillacea* was agar which composed of L-configuration for the 4-linked galactose residue [20,21]. Also, the signals (791.63–714.49) cm^{-1} might correspond to the bending vibration of C–O–S of sulfate in equatorial position, in addition they are characteristic to agar and this results indicated that the isolated polysaccharide are an agarophyte. Furthermore, (656.64–599.75) cm^{-1} attributed to the asymmetric deformation of O–S–O groups [7].

This amalgamation indicates that the isolated polysaccharides alongside are heterogeneous associate with protein and containing sulfate ester.

In terms of structural analysis, the sugar compositions of native polysaccharide extract of JCEM, PCEM and EHEM and their fractions were summarized in Table 3.

From Table 3, the GC analysis of JCEM hydrolyzate led to the identification of ten sugars which were mainly of galactose: xylose: glucose: mannose with Gal/Xyl ratio 2.8 supporting that xylogalactans are the primary macromolecules [22]. The structural features of its fractions are comparable to native polymer of JCEM and contain similar Gal/Xyl ratios, except fractions JC1 and JC7. Moreover, Gal/Glu ratio (5.05) was found much higher in native polymer than its fractions. While JC1 fraction contain lowest Gal/Glu ratio (0.48). In our previous study, JCEM had average molecular weight (2.9–28.8) kDa and composed of carbohydrate content 39.40%,

Table 4

Amino acids composition of bioactive polysaccharide extracts of JCEM, PCEM and EHEM fractions.

Algal samples	*Jania rubens* (Cold)	*Petrocladia capillacae* (Cold)	*Enteromorpha intestinalis* (Hot)
Essential amino acids			
Threonine	0.331	0.098	0.56
Valine	0.206	0.123	0.66
Methionine	0.568	–	–
Isoleucine	0.247	0.089	0.39
Leucine	0.437	0.217	1.17
Phenylalanine	0.383	0.143	0.90
Lysine	0.233	0.111	0.74
Total	2.405	0.781	4.42
Non-essential amino acids			
Aspartic acid	0.890	0.296	1.79
Glutamic acid	0.881	0.400	1.93
Serine	0.182	0.107	0.74
Glycine	0.188	0.067	0.44
Histidine	0.204	0.083	0.34
Arginine	0.187	0.152	1.23
Alanine	0.577	0.219	1.65
Proline	1.735	0.376	1.69
Tyrosine	0.406	0.145	0.66
Total	5.250	1.845	10.47
Total amino acids	7.655	7.655	14.89

Each value expressed as mg/100 mg of isolated polysaccharide.

Table 5

Effect of treatment with polysaccharide of *Jania rubens, Pterocladia capillacea*, and *Enteromorpha intestinalis* on serum AFP, CEA, GPC-3, HGF and VEGF levels in HCC model.

Groups parameters	AFP(ng/mL)	CEA (ng/mL)	GPC-3 (pg/mL)	HGF (ng/mL)	VEGF (ng/mL)
Negative control	24.1 ± 1.6	0.140 ± 0.009	10.0 ± 0.9	4.20 ± 0.30	90.3 ± 1.0
Jania rubenes	25.5 ± 1.1	0.150 ± 0.005	11.2 ± 0.3	4.50 ± 0.30	90.9 ± 5.1
Pterocladia capillacea	26.6 ± 1.2	0.165 ± 0.007	12.5 ± 0.6	4.67 ± 0.40	92.0 ± 5.1
Enteromorpha intestinalis	25.9 ± 0.7	0.150 ± 0.008	11.6 ± 0.6	4.55 ± 0.40	91.0 ± 4.6
Positive control (HCC untreated group)	45.4 ± 0.8[a]	0.660 ± 0.010[a]	27.2 ± 2.6[a]	25.80 ± 2.10[a]	126.4 ± 0.8[a]
HCC + *Jania rubenes*	30.0 ± 1.5[bc]	0.380 ± 0.010[bc]	18.8 ± 1.3[bc]	13.80 ± 0.80[bc]	98.1 ± 3.6[b]
HCC + *Ptercladia capilleace*	35.3 ± 0.8[b]	0.450 ± 0.020[b]	23.1 ± 2.0[b]	18.50 ± 0.90[b]	107.2 ± 5.1[b]
HCC + *Enteromorpha intestinalis*	30.9 ± 2.7[bc]	0.390 ± 0.004[bc]	19.2 ± 1.3[bc]	15.00 ± 0.60[bc]	100.1 ± 2.9[b]

Data are represented as mean ± SE of eight rats/group. [a]$P < 0.05$ compared with negative control group. [b]$P < 0.05$ compared with HCC untreated group. [c]$P < 0.05$ compared with HCC + *Pterocladia capillacea* group.

protein 12.06% and sulfur content 8.88% [10]. In parallel of to our results, Navarro and Stortz [22], extracted xylogalactans (15 kDa) from *J. rubens* by boiling water which constituted of carbohydrate (71%), protein (15%), sulfate (8%) contents which was characterized as agarans that have β-D-xylosyl groups attached at O-6-position of D-galactose units (agar-like xylogalactans).

The GC analysis of PCEM hydrolyzate yielded nine sugars and mainly formed from galactose, glucose, rhamnose and xylose (Table 3). PCEM had average molecular weight (2.56–

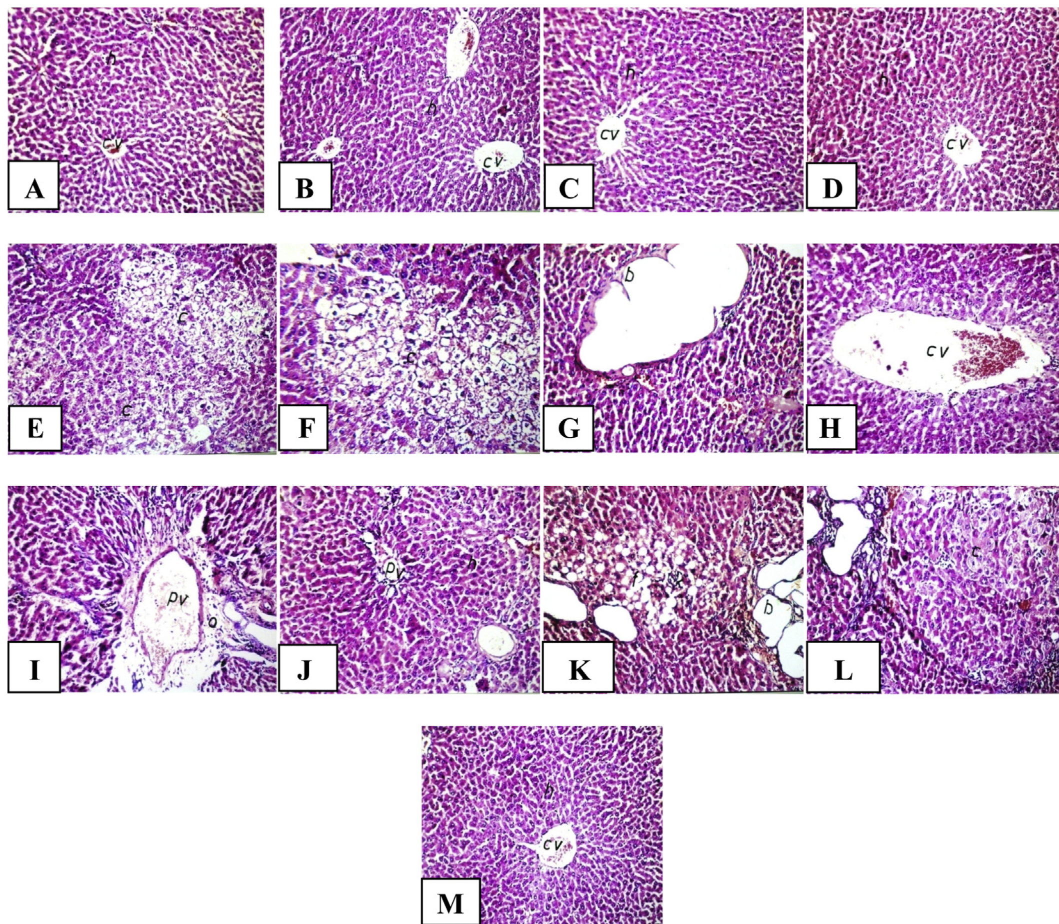

Figure 1. Photomicrographs of liver section H&E × 40.
A. Negative control group shows normal histological structure of the central vein (cv) and surrounding hepatocytes in hepatic parenchyma (h). **B**. The group administered with *Jania rubens* shows normal intact histological structure. **C**. The group administered with *Pterocladia capillacea* shows normal intact histological structure. **D**. The group administered with *Enteromorpha intestinalis* shows normal intact histological structure. **E**. The untreated HCC group shows focal area of hyperplasia with vacuolated hepatocytes. **F**. The untreated HCC group shows the magnification of Figure (E). H&E × 64. **G**. The untreated HCC group shows hyperplastic cystic bile duct (b). **H**. The untreated HCC group shows sever dilatation and congestion in central vein (cv). **I**. The untreated HCC group shows dilatation in portal vein (pv) with edema and few inflammatory cells infiltration (o) in portal area. **J**. The HCC group treated with *Jania rubens* shows normal intact histological structure. **K**. The HCC group treated with *Pterocladia capillacea* shows focal fatty change in the hepatocytes of the parenchyma (f) with multiple cystic hyperplastic bile ducts (b). **L**. Photomicrograph of liver section of the HCC group treated with *Pterocladia capillacea* shows fine fibroblastic proliferation surrounding the degenerated hyperplastic hepatocytes with nodular formation (c). **M**. The HCC group treated with *Enteromorpha intestinalis* shows normal intact histological structure.

21.00) kDa and comprised carbohydrate (44.85%), protein (13.13%) and sulfur (7.50%) [7].

The GC analysis of EHEM hydrolyzate led to identify ten monosaccharides which were mainly of rhamnose: xylose: arabinose: galactose with molar ratio 6.1: 1.4: 1: 0.76. The structural features of the main fractions EH1 and EH7 were similar to native polymer. From our previous study, EHEM had average molecular weight 51.9 kDa and constituted of carbohydrate (55.2%), protein (15%) and sulfur (3.30%) [7].

The amino acids composition of JCEM, PCEM and EHEM were summarized in Table 4 and revealed the presence of 16, 15, 15 amino acids, respectively. The non-essential amino acids; proline, aspartic and glutamic were the major amino acids in the binding protein. Furthermore, leucine, phenylalanine and valine were predominant essential amino acids in PCEM and EHEM proteins while, methionine and leucine were predominant essential amino acids in JCEM protein.

3.2. Anti-tumor, anti-angiogenesis, and immunomodulatory activities of bioactive polysaccharides in HCC rats model

To the best of our knowledge, there are no reports studying the direct relationship between these three extracts and their anti-hepatocarcinogenesis, anti-angiogenesis and the immune response using NDEA induced hepatocarcinogenesis (HCC) in rats.

3.2.1. Anti-tumor and anti-angiogenic activities of bioactive polysaccharide extracts

In view of the data of the current study, the groups of healthy rats administered (20 mg/kg b.wt for 5 weeks) with polysaccharides of JCEM, PCEM or EHEM showed insignificant change ($P < 0.05$) in the serum levels of AFP, CEA, GPC-3, HGF and VEGF as compared with the negative control group (Table 5). Furthermore, the photomicrographs of liver tissue sections of healthy rats administered with JCEM, PCEM and EHEM showed that there were no histopathological alterations as recorded respectively in Figure 1B–D.

Our data revealed that oral administration of NDEA produced significant increase ($P > 0.05$) in serum AFP, CEA, GPC-3, HGF and VEGF levels (Table 5) by 88.4%, 371.4%, 172%, 514.3% and 40% respectively, in comparison with the negative control group.

The photomicrographs of liver tissue sections of the untreated HCC group revealed that the focal area of the hepatic parenchyma showed hyperplasia with vacuolated hepatocytes (Figure 1E and F), associated with cystic hyperplastic bile ducts (Figure 1G). Also, there was congestion in the central and portal veins in association with edema and few inflammatory cells infiltration in the portal area (Figure 1H and I). Treatment of HCC groups with polysaccharide of JCEM, PCEM or EHEM caused significant decrease ($P > 0.05$) in serum levels of AFP (by 33.9%, 22.2% and 31.9%, respectively), CEA (by 42.4%,

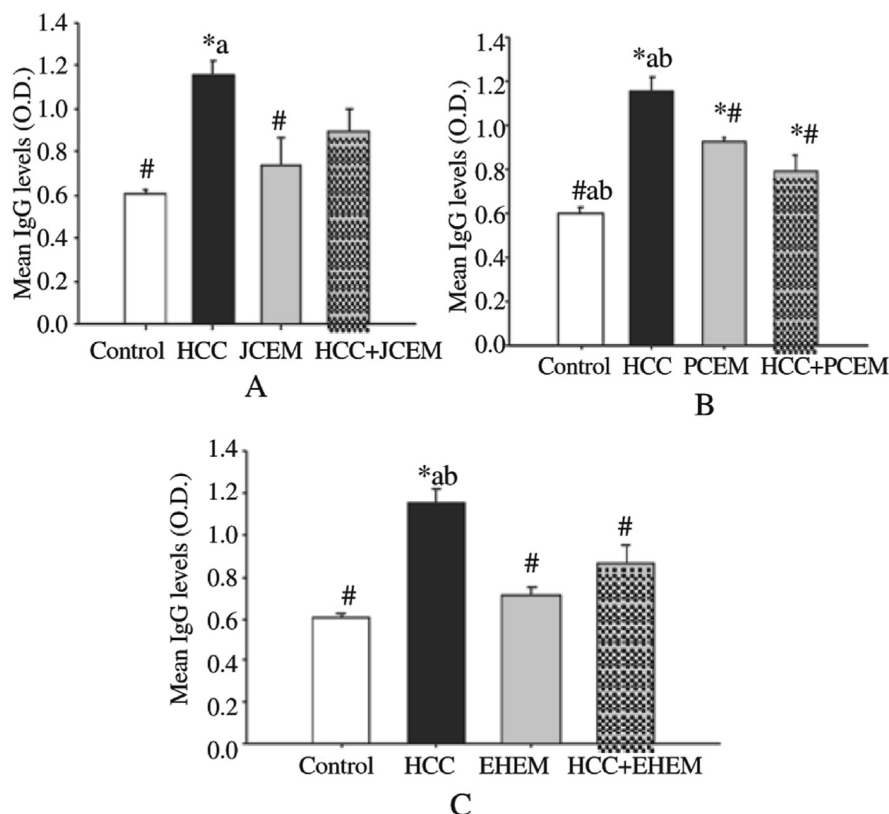

Figure 2. Determination of the IgG level in sera from different groups.
A: Control untreated rats (control), rats induced with hepatic carcinoma (HCC), rats injected with JCEM (20 mg/kg), rats both induced with hepatic carcinoma (20 mg/kg) and received JCEM (20 mg/kg); **B**: Rats injected with PCEM (20 mg/kg), rats both induced with hepatic carcinoma (20 mg/kg)and received PCEM (20 mg/kg); **C**: Rats injected with EHEM (20 mg/kg), rats both induced with hepatic carcinoma (20 mg/kg) and received EHEM (20 mg/kg). Data presented as mean ± SEM ($n = 8$). $^{*}P < 0.05$ compared with untreated rats; $^{\#}P < 0.05$ compared with hepatic carcinoma-induced rats; $^{a}P < 0.05$ compared with the IgG levels of rats receiving extract only; $^{b}P < 0.05$ compared with rats that were induced with hepatic carcinoma and were injected with the extract.

31.8% and 40.9%, respectively) and GPC-3 (by 30.9%, 15.1% and 29.4%, respectively) in comparison with the untreated HCC group. Furthermore, they produced significant decrease ($P > 0.05$) in serum levels of HGF (by 46.5%, 28.3% and 41.9% respectively) and VEGF (by 22.4%, 15.2% and 20.8% respectively) in comparison with the untreated HCC group (Table 5). Noteworthy, the groups of HCC rats treated with either polysaccharide JCEM or EHEM showed significant decrease ($P > 0.05$) in serum AFP, CEA, GPC-3, HGF and VEGF levels in comparison with that treated with PCEM.

Furthermore, the photomicrographs of liver tissue sections of the groups of HCC rats treated with JCEM or EHEM showed intact histological structure as recorded in Figure 1J and M, respectively. While the photomicrographs of liver tissue sections of the group of HCC rats treated with PCEM showed that there was focal area of fatty change in the hepatocytes associated with cystic hyperplasia in the bile ducts (Figure 1K). In addition, to fine fibroblastic cells proliferation surrounded focal area of degenerated hyperplastic hepatocytes with nodular formation has been observed (Figure 1L).

3.2.2. Immunomodulatory effect of bioactive polysaccharide extracts

In the current study, we investigated the immunomodulatory effect of polysaccharides of *J. rubens* (JCEM), *Pterocladia capilleace* (PCEM) and *E. intestinalis* (EHEM) in normal rats and those induced with hepatic carcinoma. Interestingly, JCEM, PCEM and EHEM had an immunostimulatory response by increasing the IgG levels in sera of naïve rat. The serum IgG levels of rats administrated orally (20 mg/kg b. wt) daily with PCEM for 5 weeks exhibited the highest IgG level and were significantly ($P < 0.05$) higher (1.53-fold) than the naive group (Figure 2B). In a similar manner both JCEM and EHEM possessed increased production of IgG levels by 1.23

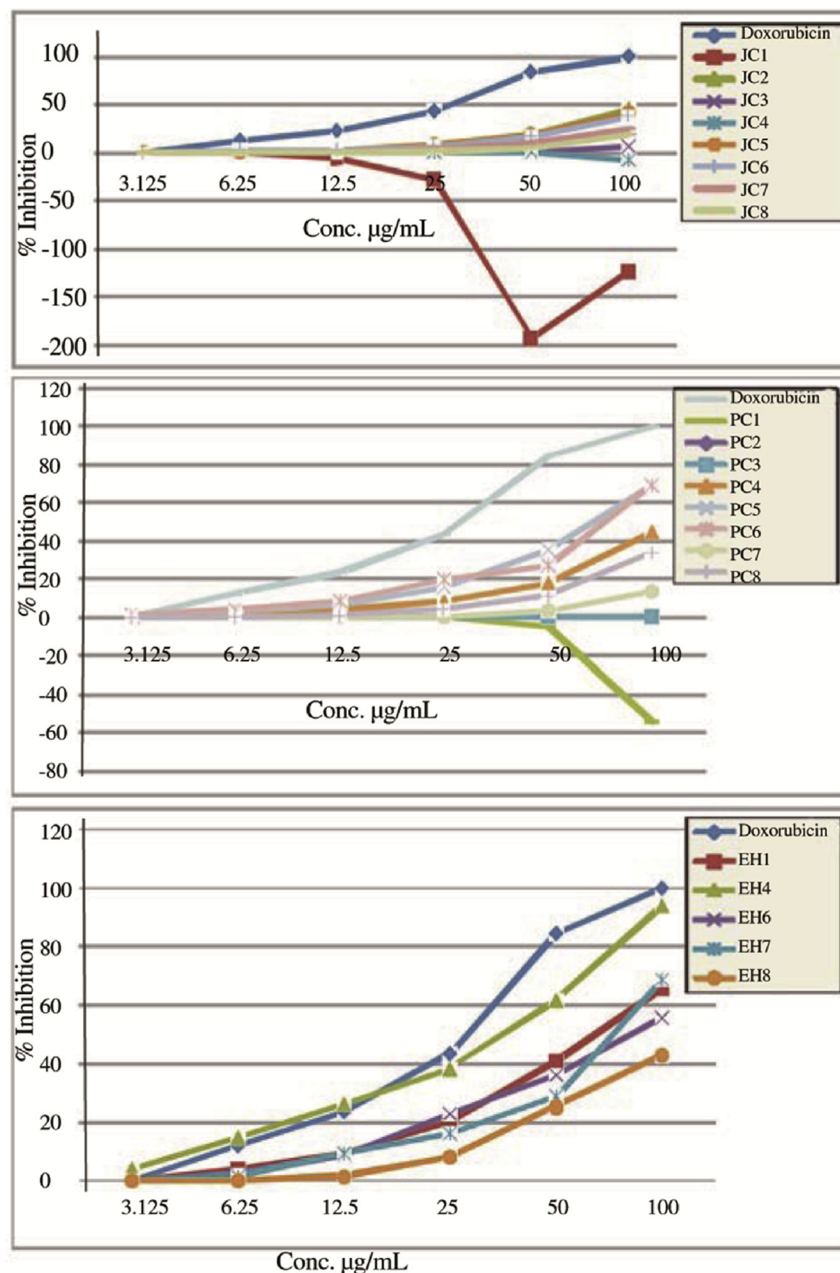

Figure 3. Cytotoxic activity of bioactive polysaccharide fractions on HepG2 human cell line *in vitro*.

and 1.17-fold compared to the control level suggesting the different immunostimulatory potential of three extracts (Figure 2A and C). Furthermore, the sera from HCC rat model demonstrated a significant increase ($P < 0.05$) in the IgG levels when compared to the control uninduced rats by 1.91-fold (Figure 2).

The IgG sera levels of HCC rats model receiving JCEM demonstrated a decrease of 0.78-fold compared to the hepatic carcinoma-induced rats (positive control) (Figure 2A). Further, the rats induced with hepatic carcinoma and were administrated with PCEM demonstrated a significant ($P < 0.05$) decrease in IgG sera levels by 0.69-fold compared to positive control (Figure 2B). In addition, similarly, HCC rats treated with EHEM demonstrated a significant ($P < 0.05$) decrease of 0.75-fold in the IgG sera levels compared to the rats induced with hepatic carcinoma (Figure 2C).

3.3. Cytotoxic activity of polysaccharides fractions in vitro

Assessment of cytotoxic activity of the JCEM, PCEM and EHEM polysaccharide fractions on HepG2 cells using the MTT assay was illustrated in Figure 3a–c, respectively. The IC_{50} value of fractions JC2 and PC4-PC6 were 99.5, 100.8, 74.0 and 72.30 μg/mL, respectively. While, the IC_{50} values of other fractions could not be determined even at the maximum concentration (100 μg/mL). On the other hand, the percentage inhibition at the maximum concentration (100 μg/mL) of tested fractions was 41.9% for JC5 > 38.4% for JC6 > 25% for JC7 > 19.8% for JC8 while JC1 and JC4 displayed proliferative effect 123% and 12%, respectively. In the same study, the percentage inhibition at the maximum concentration tested (100 μg/mL) of 33.7% for PC8 > 13.9% for PC7 while PC1 showed proliferative effect 54.2% whereas PC2 and PC3 did not show any effects on HepG2 cells.

Further, the cytotoxic activity of fractions of polysaccharide isolated from EHEM revealed that EH1, EH4, EH6 and EH7 fractions exhibited a moderate cytotoxic activity against HepG2 cell lines in a dose-dependent manner and their IC_{50} value was 73.1, 42.5, 79.3 and 76.2 μg/mL, respectively.

4. Discussion

Referring to our previous study, polysaccharide isolated from cold aqueous extract of *J. rubens* (JCEM) and *P. capillacea* (PCEM) as well as polysaccharide isolated from hot aqueous extract of *E. intestinalis* (EHEM), were characterized as sulfated polysaccharide-protein complexes [7]. The polysaccharide-protein complexes were isolated previously from mushrooms and algae [5,10]. The structural feature of native polymer (JCEM and PCEM) and their fractions were seemed identical which comprised nearly similar Gal/Xyl ratios indicated they belong to xylogalactans family of polysaccharides, which confirm the previously reported, from *J. rubens* and *P. capillacea* by Navarro and Stortz [22] and Errea and Matulewicz [23], respectively.

The GC analysis of EHEM refers to polysaccharides of Ulvaceae named Ulvans [24]. Ray isolated Ulvans from *Enteromorpha* which composed of α-(1 → 4)-, α-(1 → 3)-, α-(1 → 3,4)-, and α-(1 → 2,3,4)-linked Rha units in addition β-(1 → 4)- and β-(1 → 2,4)-linked Xyl units [25]. Rhamnose was reported previously as main content in polysaccharides isolated from different *Enteromorpha* species [25,26].

These polysaccharides represented as the most bioactive polysaccharides *in vitro* against hepatocarcinoma human cell line (HepG2) with an IC_{50} 10.73, 56.54 & 53.64 μg/mL [7] and subjected for *in vivo* anticancer and anti-angiogenesis as well as immunomodulatory study against chemically-induced hepatocarcinogenesis.

NDEA as a well known potent hepatocarcinogenic agent, is an environmental and dietary hepatocarcinogen [27]. Exposure to NDEA leads to impairment of the nuclear enzymes involved in DNA repair/replication. It has been widely used as a carcinogen to induce HCC in animal models [28] and to augment oxidative stress in liver. This oxidative stress may be attributed to the metabolization of NDEA in the liver by cytochrome p450 enzymes. The formation of NDEA reactive metabolites is primarily responsible for its hepatotoxic effects. Our data revealed that oral administration of NDEA produced significant increase ($P > 0.05$) in serum AFP, CEA and GPC-3 levels in comparison with the negative control group, which is in agreement with Xu *et al*, Ramakrishnan *et al* and Nakatsura *et al*, respectively [29–31].

Further, angiogenesis, which is the growth new blood vessel from preexisting vessels, is imperative in malignant tumor growth. It is regulated by a balance of pro-angiogenic and anti-angiogenic factors which, upon the switch of tumor cells to an angiogenic phenotype, leads to tumor growth and progression [32]. Several endogenous proangiogenic factors are expressed in HCC [33] and evidenced to play a role in HCC pathogenesis. Hepatocyte growth factor is one of the pro-angiogenic factors. While, vascular endothelial growth factor was identified as a positive regulator of angiogenesis [34]. In the light of the current data, NDEA administration produced significant ($P > 0.05$) increase in serum HGF and VEGF levels by 514.3% and 40%, respectively, in comparison with the negative control group which is greatly supported by Burr *et al* and El Mesallamy *et al*, respectively [35,36]. The observed increase in serum HGF might be attributed to the decreased clearance of HGF by the liver [37]. While, the increase in serum VEGF level could be allied to the high angiogenic activity in NDEA-induced hepatocarcinoma rats, in addition to the increase of nitric oxide activity, thus enhancing the angiogenesis by stimulating the synthesis of VEGF [38].

Moreover the histopathologic investigation of liver supported the biochemical results. Scherer and Emmelot reported that in NDEA administered rats, carcinomas are preceded by the development of so-called altered foci and hyperplastic nodules [39]. Also, Burr *et al* found that there was necroinflammation with perivenular inflammatory cells and macrophages in liver tissue after NDEA exposure [35]. In addition, NDEA administration produced vacuolization, loss of normal hepatocellular architecture and presence of pycnotic nuclei [40]. These findings might be attributed to that NDEA is primarily metabolized in the liver and the reactive metabolites generated thereby are known to damage hepatocytes.

Treatment of HCC groups with polysaccharide of JCEM, PCEM or EHEM caused significant decrease ($P > 0.05$) in serum levels of AFP, CEA, GPC-3, HGF and VEGF in comparison with the untreated HCC group. These findings might be attributed to the anti-metastatic, anti-angiogenic [41], antioxidant [42] and anti-inflammatory [43] activities of the sulfated polysaccharides. Also, it has been reported that sulfated polysaccharides have the ability to induce apoptosis in several cancer cell lines and stimulate immune system cells to induce tumor cell death [44]. Moreover, sulfated polysaccharides could inhibit angiogenesis by

interfering with the binding of VEGF [44] and basic fibroblast growth factor to their respective receptors [44]. Additionally, they have antimetastatic activity blocking the interactions between cancer cells and the basement membrane [45].

The study of Liu *et al* proved that the diseased liver increases immunogenesis, subsequently led to high Ig production through activating plasma cells proliferation [46] which is in agreement with our results. In addition, it has been reported that IgG are not only produced by B lymphocytes and plasma cells, but also produced by non-lymphoid lineage cells such as cancer cells (breast, colorectal, prostate, *etc*.) as well as soft tissue tumors, human umbilical endothelial cells, epididymal epithelial cells, in addition to neurons and eyes of human and mouse. Interestingly, IgG secreted by human cancers were reported to promote growth and survival of tumor cells. Moreover, the detection of IgG levels in different neoplasms has been associated with proliferation markers and tumor grades [47,48]. Wang *et al* reported that siRNA silencing resulting in knock down expression of IgG resulted in suppression of cancer cell through inhibition of growth and proliferation of different cancer cell types both *in vivo* and *in vitro* [48].

Determination of cytotoxicity revealed that the native polysaccharide JCEM (IC_{50} value 10.73 μg/mL) exhibited a potent cytotoxicity effect as well as the native polymer of PCEM (IC_{50} value 56.54 μg/ml) displayed a moderate cytotoxic activity according to Matloub *et al* and Sajjadi *et al* [7,49] on the contrary to their fractions. Although the structure features of fraction similar to native polysaccharide, the behavior of cytotoxic activity of fractions may due to their synergistic effect on $HepG_2$ cell line. The native polysaccharide EHEM showed moderate cytotoxic activity with an IC_{50} value 53.64 μg/mL [7]. Wang *et al* referred the growth inhibition growth *in vitro* of HepG2 to the polysaccharide isolated from *E. intestinalis* induced apoptosis in HepG2 cells including a loss of mitochondrial membrane potential and then release of cytochrome *c* to the cytosol [50].

Structural activity relationship, the behavior of sulfated polysaccharides-protein complexes as anti-tumor and immunomodulatory agents is still in progress in many laboratories. Many study supported that monosaccharide composition, backbone type and/or molecular conformation, molecular weight, sulfate sites heterogeneity, position of the sulfate moiety of polysaccharides were played critical role in the anti-tumor and immunomodulatory activities [51]. The polysaccharide-protein complexes were isolated previously from mushrooms, which have anticancer and immunomodulation effects [5]. The antitumor activity of these polysaccharides against various tumors due to activating different immune responses which influenced by sugar composition, protein contents and their solubility in water [51]. Furthermore, Huang *et al* found that arabinose, mannose, xylose and galactose rather than glucose as well as amino acids; aspartic, glutamic, serine, glycine and threonine played a key role in the immunomodulatory and antitumor activities of polysaccharides-protein complexes [52].

The therapeutic effect exerted by *J. rubens* might be explained by that the sulfated galactans obtained from red algae have shown very organized chemical structures built-up of regular oligomeric repetitive units within well-defined sulfated patterns [53]. Additionally, the reason for the observed variable effects exerted by the studied extracts might be related to the sulfation levels which are important to their success as anti-angiogenic agents.

From our observation, it is notable that the anti-tumor and anti-angiogenic activities of native polysaccharide 'xylogalactan' isolated from JCEM showed higher activity than PCEM, that may be JCEM constituted highest Gal/Glu and Gal/Xyl ratios than PCEM, in spite of they had nearly the same structural feature, molecular weight, protein and sulfur contents. However, the fraction composed from highest protein, lowest sulfur contents as well as highest Gal/Glu and Gal/Xyl ratios play a key role in their cytotoxic activity.

Nevertheless, the degree of sulfation in sulfated polysaccharides was not a prerequisite for the manifestation of cytotoxic effect [54]. While, the sulfation of polysaccharides achieved the importance in eliciting immune response [55].

Further, Kaeffer *et al* found a relationship between the molecular weight of polysaccharide and anticancer activity of ulvans, reporting that ulvans with molecular weight lower than 5×10^3 g/mol exhibited strong growth inhibition of cancer cells [56].

On the other hand, most studies support that sulfated polysaccharides can enhance the innate immune response by promoting the tumoricidal activities of macrophages and natural killer cells [57].

Numerous studies have suggested that polysaccharides can inhibit tumor growth and exert immunomodulating effect through the following mechanisms: by prevention of oncogenesis and tumorigenesis, direct anti-tumor activity, immunopotentiation activity and preventive effect on tumor metastasis [58].

The algal polysaccharides are still tremendous opportunities to find new types of anti-cancer, anti-angiogenic and immunomodulatory agents. Sulphated polysaccharide are involved with the surface of animal cells in different biological activities, such as cell recognition, cell adhesion, and regulation of receptor functions, which are of great interest in medicine.

The sulfated polysaccharide-protein complexes isolated from cold aqueous extract of red algae *J. rubens* and *P. capillacea* which referred as sulfated galactans and polysaccharide isolated from hot extract of green alga *E. intestinalis* which referred as ulvan, were bounded with protein.

Noteworthy, the most pronounced therapeutic effect against hepatocellular carcinoma was exerted by cold polysaccharide extract of *J. rubens* and hot polysaccharide extract of *E. intestinalis*. This efficacy may be due to direct cytotoxic activity, anti-hepatocarcinogenesis and anti-angiogenesis.

Moreover, the polysaccharides isolated from JCEM, PCEM, and EHEM all possessed an immunostimulatory effect compared to naive level, with PCEM having the highest effect and EHEM with the lowest effect through increasing the humoral response. In addition, individual oral administration of the extracts for 5 weeks in hepatocarcinoma induced rats resulted in suppression of the IgG sera levels with variable degrees compared to HCC rats, suggesting the immunomodulatory role of PCEM followed by EHEM and JCEM that they may play during hepatocarcinoma.

Our investigation emphasized that sulfated polysaccharides–protein complexes can have impact on disease progression and outcome including tumor progression and metastasis.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgments

The authors acknowledged the National Research Centre for the financial support. Grant (No: 9080104).

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