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

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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.

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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

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