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Short Communication

Inhibition of *Astragalus membranaceus* polysaccharides against liver cancer cell HepG2

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We have studied the inhibition of polysaccharides of *Astragalus membranaceus* against liver cancer cell HepG2. The polysaccharides at higher doses (25 mg/ml) have stronger antitumour effects, decreasing more than 40.5% (24 h) and 67.3% (48 h) of liver cancer HepG2 cell viability. A high percentage of apoptotic HepG2 cells was found at 25 mg/ml of *A. membranaceus* polysaccharides. 23.9 and 38.2% of cells experienced apoptosis when HepG2 cells were treated for 24 and 48 h with 25 mg/ml of *A. membranaceus* polysaccharides. Consequently, the results of the *in vitro* assays suggest that the *A. membranaceus* polysaccharides possesses strong antitumour activities, which is benefical to treatment of liver cancer.

Key words: Astragalus membranaceus polysaccharides, Antitumour, HepG2, MTT.

INTRODUCTION

Astragalus membranaceus (Fisch) Bunge (AM), Maxim of the Leguminosae family, is a traditional Chinese medicinal herb originated in Northern China. The dried root of AM, Huangqi, contains 2′4′-dihydroxy-5,6-dimethoxyisoflavone, kumatakenin, choline, betaine, polysaccharides, saponins, glucuronic acid, sucrose, amino acids, traces of folic acid and astraisoflavanin (Bensky and Gamble, 1993; Ma et al., 2002; Wu and Chen, 2004). Huangqi is the Chinese name for the root of AM. AM (root), also known as Huangqi in Chinese and Radix Astragali (RA) in Latin, is the dry root of *A. membranaceus* (Fisch) Bge. or *A. membranaceus* var. Mongholicus (Bge.) Hsiao of the Fabaceae family. AM grows mainly in Northern China, Mongolia and Siberia. Modern analytic techniques have identified more than 100 compounds that are contained in AM (root), such as flavonoids, polysaccharides (astragalan, APS), saponins (astragalosides), sucrose, amino acids and phenolic acids (Wu and Chen, 2004). The major bioactive constituents of AM (root) are flavonids, APS and saponins, and each has its own therapeutic properties.

Primary liver cancer is the fifth most common malignancy in the world, with a global annual incidence of about one million new patients. In 2004, the American Cancer Society estimated 18,920 new cases of Hepatocellular Carcinoma (HCC) and the estimated deaths were 14,720 (Shah and Bhowmick, 2006). The disease prevails in parts of Asia and Africa; yet appears rife in many European countries in recent years. Although, substantial advances have been made in chemotherapy regimen for HCC, the efficacy of drugs is often hampered by a range of adverse side-effects imposed on patients (Zein and Zein, 2002). Accordingly, it is urged to explore a new approach for development of an effective therapy against this disease.

In the present study, the polysaccharides of *A. membranaceus* were tested for its anti-tumour activity using liver cancer cell lines HepG2.

MATERIAL AND METHOD

Material

A. membranaceus was purchased from a local herb shop (Taizhou, China). The *A. membranaceus* was identified and authenticated by Institute of Botany, zhejiang Province, China. A voucher specimen (Number 20090876) was deposited in the herbarium of the Institute. Human liver cancer cell lines HepG2 was kindly provided from institute of pathology, our university.

Sample preparation

About 100 g of *A. membranaceus* sample was ground into powder

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Table 1. Inhibition rate (%) of *A. membranaceus* polysaccharides against HepG2 cell growth.

	Concentration					
	0 (Control)	**3 mg/ml**	**15 mg/ml**	**25 mg/ml**	**35 mg/ml**	**45 mg/ml**
Inhibition rate (%)	-	3.2±0.1	18.4±1.4	40.5±2.8	49.4±3.3	50.8±2.1
	-	4.2±0.2	29.1±1.5	67.3±4.9	76.2±6.1	78.1±5.3

Table 2. *A. membranaceus* polysaccharides-induced HepG2 cell death rate (%).

	Concentration					
	0 (Control)	**3 mg/ml**	**15 mg/ml**	**25 mg/ml**	**35 mg/ml**	**45 mg/ml**
Cell death rate (%)	1.3±0.1	2.3±0.1	10.6±1.4	23.9±1.5	40.9±3.3	47.8±2.3
	1.6±0.1	3.4±0.2	18.4±1.2	38.2±1.9	52.5±4.6	54.2±4.5

in a mortar, and was then extracted with 0.3 mol/L, pH 8.0, phosphate buffer containing a appropriate ratio of trypsinase at 40℃ for a given time. Then, protein in the extract was removed by Sevag method. The result material then was extracted with distilled water at 100℃. After filtration, the suspension was centrifuged for 10 min. The residues were extracted twice for 2 h with distilled water (100℃) and concentrated to dryness. The crude extract was then dialyzed against tap water, deionized with mixed ion exchange resins and dried under reduced pressure to give desire product. HPLC analysis showed that *A. membranaceus* polysaccharides contained glucose (34%), mannose (2.7%) and xylose (7.9%).

Gas chromatography–mass spectrometry (GC–MS)

Each of the *A. membranaceus* polysaccharides was dissolved and hydrolyzed in 200 µl 4.0 M trifluoroacteic acid (TFA) for 30 min in a sealed vial at 120℃. After hydrolysis reaction, excess TFA was removed by purging the sample with a stream of nitrogen at 2.0 ml/min. The hydrolyzed samples were derivatized using trimethylsilylation (TMS). The hydrolyzed samples were dissolved in 500 µl pyridine with vigorous shaking and/or sonication. 200 and 100 µl of trimethylchlorosilane (TMCS) was added. The mixtures were shaken vigorously for 1.0 min and were allowed to stand overnight to ensure complete derivatization of the sample. The final TMS-derivatized solutions were centrifuged for 1 min. 10.0 µl of the supernatant liquid was extracted and diluted with 990.0 µl pyridine. 1.0 µl of the sample solution was injected into the GC–MS system.

Treatment with antitumour agents and MTT colorimetric assay

Human liver cancer cell lines HepG2 (5×10^3 cells/0.33 cm^2) was plated in 96-well plates "Nunclon TM MicrowellTM" (Nunc) and was incubated at 37℃. After 24 h, cells were treated with *A. membranaceus* polysaccharides (final concentration 3-25 mg/ml). Untreated cells were used as controls. Microplates were incubated at 37℃ in humidified atmosphere of 5% CO_2, 95% air and then cytotoxicity was measured with colorimetric assay based on the use of tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). The results were read on a multiwell scanning spectrophotometer (Multiscan reader), using a wavelength of 570 nm. Each value was the average of 8 wells (standard deviations were less than 20%). Results are expressed as percent of cell proliferation inhibition calculated according to the following:% inhibition rate of tumour cells (%) = (Absorbance of control - Absorbance of test) / Absorbance of control × 100%

Flow cytometry

Apoptosis induction of human liver cancer cell lines HepG2 by *A. membranaceus* polysaccharides was verified by using a BD FACSCalibur flow cytometer (Becton–Dickinson) and staining with Annexin V-PE and 7-AAD. Cell samples (5×10^6 cells/ml) were induced with *A. membranaceus* polysaccharides (3-25 mg/ml). The cells were incubated for 24 and 48 h in medium. After incubation, an aliquot of cells was taken, washed, and resuspended in PBS prior to analysis. Cells were identified by forward and side scatters as well as the fluorescence Annexin V-PE and 7-AAD to discriminate apoptotic cells from live and dead cells.

RESULTS

Cell treatment with *A. membranaceus* polysaccharides at the concentration range of 3-45 mg/ml markedly affected HEPG2 cell growth. Cell exposure to aqueous polysaccharides of *A. membranaceus* induced an inhibition of cell proliferation and the effect increased in a dose-dependent manner. Compared with untreated control, the concentration of 45 mg/ml of *A. membranaceus* polysaccharides resulted in 50.8% growth inhibition HEPG2 after 24 h of exposure and in 78.1% growth inhibition after 48 h of exposure (Table 1).
Flow cytometric analyses (Table 2) indicated that 47.8% of cells experienced apoptosis when HEPG2 cells were treated for 24 h with 45 mg/ml of *A. membranaceus* polysaccharides. In the control cells, only a minor fraction of the cell population (1.3%) experienced apoptosis. When HEPG2 cells were treated for 48 h with 45 mg/ml of *A. membranaceus* polysaccharides, 54.2% of apoptotic cells were detected as compared to the untreated control with 1.6% apoptotic cells.

DISCUSSION

Despite the decrease in incidence, liver cancer remains the second leading cause of cancer related death worldwide. Prevention is likely to be the most effective means of not only reducing the incidence but also mortality from this disease. In recent years, attention has been focused on the anticancer properties of plant-derived dietary constituents of food, an important role in the prevention of disease (Block et al., 1992; Lambert and Yang, 2003; Wang et al., 2009; Wei et al., 2009; Xu et al., 2009; Yoon et al., 2009).

In this study, we identified the antitumor activity of *A. membranaceus* polysaccharides by the MTT method, and determined the high level (40.5% for 24 h and 67.3% for 48 h) of inhibition of liver cancer HEPG2 cell survival (at a dose range of 25 mg/ml).

Apoptosis is characterized by a number of well-defined features (Hengartner, 2000). In contrast to necrosis, apoptotic cell death is thought to be physiologically advantageous (Shacter et al., 2000; Anderson et al., 2002; Lauber et al., 2004). In this study, *A. membranaceus* polysaccharides induced apoptotic cell death in liver cancer HEPG2 cells in a dose-dependent manner. Our results showed that *A. membranaceus* polysaccharides, at doses higher than 25 mg/ml, reduced more than 23.9% (24 h) and 38.2% (48 h) of the HEPG2 cells viability, respectively.

Our results showed that *A. membranaceus* polysaccharides could inhibit HEPG2 cells proliferation, at least partly through apoptosis. The inhibition rate and the percentage of apoptotic cells were all dose-dependent. *A. membranaceus* polysaccharides at 25 mg/ml dosages had a prominent inhibition rate than the lower dosages.

Consequently, the results of the *in vitro* assays suggest that the *A. membranaceus* polysaccharides possesses strong antitumour activities, which is benefical to treatment of liver cancer.

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