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Research Article

Biological Activities of Soybean Galactomannan Oligosaccharides and Their Sulfated Derivatives

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Abstract

Galactomanno-oligosaccharieds (GMO) and their sulfated derivatives (SGMO) were prepared from soybean hulls and evaluated for their biological activities as anticoagulant; antimicrobial; antitumor; fibrinolytic and prebiotics. The results indicated that the sulfating process has positive effect on the anticoagulation and fibrinolytic activities of the galactomanno-oligosaccharieds. The SGMO have prolonged clotting time more than 24h at concentration resemble that of the standard heparin. It was also found that the SGMO have fibrinolytic activity as that of the standard hemoclar and 3times higher than that of the native GMO oligosaccharides. The prepared oligosaccharides also preformed antitumor activity against human colon carcinoma cell line and the percentage of the dead cells increase from 28% to 72% by increase the concentration of the oligosaccharides from 0.005 to 0.02 mg/ml. The tested galactomanno-oligosaccharieds also act as good source for prebiotic as they have the ability to grow the beneficial bacteria 4 to 8 times higher than the pathogenic one. To our knowledge this is the first time someone report anticoagulation; fibrinolytic and direct antitumor activities for galactomanno-oligosaccharides not to mention soybean galactomanno-oligosaccharides.
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Keywords: Galactomanno-oligosaccharieds (GMO), anticoagulant; antimicrobial; antitumor; fibrinolytic and prebiotics.

Introduction

Galactomannans are naturally polysaccharides composed of β- (1→4)- mannose unit with α- (1→6) linked side chain of galactose unit in molar ratio depended on the sources (Pires, *et al*., 2001; Kashef, *et al*., 2008; Miguel, *et al*., 2009). Galactomannans have been isolated from different sources of plant seeds, particularly the Leguminosae (Miguel, *et al*., 2009); locust bean; guar; tara; tamarind; seed hulls of soy beans; sienna macranthera ; Leucaena sp. Medicago sativa (Hussein *et al*., 1998; Kashef *et al*., 2008) and microbial sources, in particular, the yeasts and fungi (shaaban *et al*., 2013). Galactomannans are important in various industrial applications such as paper, textile, petroleum-drilling, pharmaceutics, food, cosmaceutics, and explosives industries (Srivastava and Kapoor, 2005; Hassan *et al*., 2009).

Soybean, (*Glycine max*), has been known since 3,000 BC as most important source to feeding both human and animals worldwide. Soybean has played an important role in the diet of oriental civilization for many tears, as witnessed by the numerous rational soy-based foods found in the Orient. However, the galactosyl saccharides in soybean i.e., α-galactosides and β-galactomannan, are badly utilized and known as flatulence-producing (Hussein, *et al*., 1998; Hsiao, *et al*., 2006). Soybean galactomannan was also inhibiting the activity of the

digestive enzymes as α- amylase; lipase and trypsin (Kashef, *et al*., 2008).

The activity of the-amylase;digestivelipaseandtrypsin (Kashef,enzymes *et al*., as α 2008). So one possible approach to alleviating these anti-nutritional effects in soybean is the partially hydrolysis of these saccharides may be through the dietary inclusion of appropriate enzymes, i.e., α-1,6-galactosidase and β-1,4-mannanase. So partial degradation of galactomannan will be produced galactomanno-oligosaccharides.

Transferring galactomannan in soybean to galactomanno-oligosaccharides (GMO) not only improving its nutrition values but also increase its biological activity. It had been recorded by many researchers that galactomanno-oligosaccharides lower the glycemic index of food fight and/or prevent infectious diseases by preventing or reducing the adhesion of pathogenic microorganisms to human and animal epithelial cells; protect against inflammatory chronic intestinal disorders; contract the development of intestinal cancer. In general they are good source as prebiotic; modulate the immune defense system; fight and/or prevent inflammatory diseases; preventing osteoporosis ; lowering the fat body and reduced the blood pressure (Kumao and Fuji 2006; Izumi *et al*., 2008; Yin, *et al*.,2008; Wang, *et al*., 2010a). The aim of our research is to prepare soybean galactomanno-oligosaccharieds (GMO) and their sulfated derivatives (SGMO) and evaluate their biological activities as anticoagulant; antimicrobial; antitumor; fibrinolytic and prebiotics.

Materials and Methods

Materials

Heparin Sodium salt, (140IU/mg; Fluka, AG, Switzerland); Hemoclar (produced in Clin-Midy, Paris and purchase from Nile Pharmaceutical Co. Cairo, Egypt); human plasma was purchase from Egyptian Organization for Biological Products and Vaccine Production. A human colon carcinoma cell line, Caco-2, was obtained from the American Type Culture Collection (ATCC, HTB-37^{TM}), from Homo sapiens (human), organ-colon. All other chemicals are analytical grades.

Microorganisms

a-Bacterial strains

*** Beneficial bacteria**

Seven bacterial strains, attained from the culture collection of National Research Centre, Cairo, Egypt; known to be as important probiotics, were used for the study and investigation of the prebiotic activity of isolated oligosaccharides . These included: *Bifidobacterium bifidum; Lactobacillus casei; Lactobacillus reuteri; Lactobacillus bulgaricus; Lactobacillus helveticus; Lactobacillus rhamnosus* and *Lactobacillus acidophilus*. Beneficial bacteria were maintained on MRS agar slants ((De Man- Rogosa-Sharp- medium); at pH 6.5 and incubation aerobically at 37°C for 24h before stored at 4°C and transferred monthly (De Man, *et al*., 1960).

*** Pathogenic bacteria**

A pathogenic isolated bacterium, namely *Escherichia coli* was used for comparison with probiotics bacteria for their capability of utilizing prebiotic isolated oligosaccharides. Other three pathogenic isolated bacterial strains: *Staphylococcus aureus, Streptococcus pyogens and Pseudomonas aeruginosa* (in addition to *E. coli)* were used for attempted assay of antibacterial activity of the isolated oligosaccharides. The four pathogenic bacterial strains were obtained from the clinical laboratory of Nozha International Hospital (Cairo, Egypt).All pathogenic strains were maintained on nutrient agar slants (at pH 7) and stored at 4°C after incubated at 37°C for 24h and transferred monthly.

b-Yeast strain

Pathogenic yeast (*Candida albicans*) was also used in the antibacterial activity test and obtained from the clinical laboratory of Nozha International Hospital, Cairo, Egypt. The culture was maintained on potato dextrose agar slant after incubation at 30°C for 7 days and stored at 4°C.

Culture media

Probiotic bacteria

M.R.S. broth medium at pH6.5 was used as the culture and/or inoculums medium for fermentation and prebiotic tests of beneficial bacteria.

Pathogenic microorganisms

Nutrient broth at pH7 was used as the culture and/or inoculums medium for fermentation; in antibacterial and prebiotic tests for pathogenic microorganisms.

Media for prebiotic tests

The carbon source of MRS medium was replaced by the isolated soy bean galactomanno-oligosaccharides, GMO, (MRS_1) or their sulfated derivatives, SGMO, (MRS_3) at

concentration 1.5 % and the final pH value adjusted at 6.2 –6.6 and used as medium for probiotics growth. The second medium for prebiotic test was prepared by replacing the carbon and the nitrogen sources of MRS broth medium by GMO, (MRS_2) or SGMO (MRS_4) at the same concentration and pH value as indicated above.

Cell Culture

A human colon carcinoma cell line, Caco-2, was obtained from the American Type Culture Collection (ATCC, HTB-37^{TM}), from Homo sapiens (human), organ-colon and cultivated in Eagle's minimum essential medium (ATCC) supplemented with 20% fatal bovine serum (FBS, ATCC) and 1% antibiotic antimycotic solution (containing 10,000 units/ml penicillin G, 10 mg/ml streptomycin sulphate and 25 µg/ml amphotericin B, Sigma-Aldrich, St. Louis, MO). Confluent mono layers were sub cultured by incubating with 0.05% trypsin and 0.2% EDTA in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS, Sigma-Aldrich). Cultures were incubated at 37°C in a humidified atmosphere of 95% air, 5% CO_2. For all experiments, cells were seeded at high density (10^6 cells/ml, 0.2 ml/well) onto test surfaces contained within 96-well plates and proliferated for 2 days.

Plant Sources

Soybean (SB): this was a commercial sample purchased from local Egyptian market (ISIS Company).

Determination of some analytical characters of soy bean

a-Ash was obtained upon direct flaming of one gram of air dried material of known moisture content for several hours until a constant weight was attained (A.O.A.C, 1970). b-Organic nitrogen of soy bean was determined by Kjeldal's method. c-Total Lipids were isolated by soxhlet extraction with petroleum ether (B.P. 60-90) and determined according to Lin and Chen (2004). d-Total carbohydrate content was determined adapting to the phenol-sulfuric acid method (Dubois, *et al*., 1956) after complete acid hydrolysis of soy bean sample.

Isolation and purification of galactomanno-oligosaccharides

Galactomanno-oligosaccharides (GMO) from soybean hulls were extracted according to Hussein, *et al*., (1998) and Whister and Saarino (1957) after soaking the seeds at 4°C for 12 hours with constant shaking. The GMO were purified from the precipitate sugars by using preparative paper chromatography techniques (Jayme and Knolle, 1956; Partridge, 1949).

Characterization of the isolated oligosaccharides

a- The percentage (%) of the isolated oligosaccharides was determined calorimetrically by phenol–sulfuric acid method (Dubois, *et al*., 1956) with glucose as standard. b- Determination of the mono sugars compositions of the isolated oligosaccharides. This was carried out in two steps:

i- Complete acid hydrolysis according to the method of Perida and Bishop (1961).

ii- Quantitative paper chromatography for the hydrolysis products according to the method of Wilson (1959) using galactose, glucose and mannose as authentic samples.

Preparation of sulfated derivative of the isolated oligosaccharides

Sulfating of the isolated oligosaccharides was carried out with chloro-sulfonic acid according to Hussein (1994) and the products were isolated with 3 volumes of methanol. Purification of the sulfated oligosaccharides was performed by repeated dissolution in water and re-precipitation with methanol. The prepared sulfated derivative was replaced the carbon source or carbon and nitrogen sources in MRS medium at different concentration ($MRS_{2;}$ MRS_4 respectively) to examined the response of both beneficial and pathogenic bacterial strains toward the sulfated derivative of the isolated oligosaccharides.

Determination of biological activities of the isolated oligosaccharides

The determinations of anticoagulant, antimicrobial, antitumor, fibrinolytic and prebiotic activities of the isolated oligosaccharides and their sulfated derivatives were carried as indicated below.Unless otherwise stated all the experiments were carried out in triplicate.

Anticoagulant activity

The anticoagulation activity of the isolated oligosaccharides and their sulfated analogs at different concentrations (500µg/ml–1000µg/ml) were investigated following USA Pharmacopoeia, (1960) with sodium Heparin as standard.

Antimicrobial activity

The agar diffusion technique described by Mitcher, *et al*., 1972**,** was used in this experiment. Positive control experiments were preceded in the same way using the penicillin (200,000 IU/ml) as antibacterial agent and fluconazole (33.3 mg/ml) as antifungal agent. The antimicrobial activity was recorded by measuring the diameter of the clear inhibition zone around the pores at the end of incubation periods.

Antitumor activity

The isolated oligosaccharide and its sulfated derivative were tested in vitro study for their antitumor activity.

Cytotoxicity Assay of Tumor Cells

The cytotoxicity of galactomanno-oligosaccharide and its sulfated derivative were measured by using a methylene blue assay method (Felice *et al*., 2009). Human colon carcinoma cell line, Caco-2 cells were plated at the density of 10000 cells/ well, 100µl well, in 96-well plates and left for 24 h to attach., then the medium was replaced with 100 µl aliquots suspension of each purified soybean oligosaccharide (native and sulfated, at concentration 0.005; 0.01 and 0.02 mg/ml dissolved in Mem Earl's medium) then inoculated in each 8 well and incubated for 24 h in CO_2 incubator at 37ºC. After incubation, the medium was aspirated and each well was gently rinsed with phosphate-buffered saline (PBS) twice. Cells were stained and fixed by adding 50µl methylene blue solution (HBSS + 1.25% glutaraldehyde + 0.6% methylene blue) to each well. After 1 h incubation, plates were rinsed by gently submerging in distilled water six times. Plates were drained and air-dried before addition of 100 µl elution solution (50% ethanol + 49% PBS + 1% acetic acid) to each well and homogenized by agitating plates at room temperature for 1 h, to fully dissolve the stained materials. The absorbance was measured at 570 nm by a microplate reader (Thermo Labsystems, Helsinki, Finland)

Fibrinolytic activity

This was performed by exposing a plasma clot (prepared according to USA Pharmacopoeia, 1960) to the effect of the investigated oligosaccharides and their sulfated derivatives at concentration resample that of the standard hemoclar (2mg/tube). The lyses percentage of the plasma clots at 37°C were recorded with each sample and compared with that of the standard hemoclar.

Prebiotic activity

The prebiotic activity of the isolated oligosaccharides was investigated by comparing the ability of beneficial bacteria and pathogenic *E. coli* to utilize these oligosaccharides as carbon and /or nitrogen sources. The growth of beneficial bacteria (*Bifidobacterium bifidum; Lactobacillus casei; lactobacillus reuteri*; *Lactobacillus bulgaricus*; *Lactobacillus helveticus; Lactobacillus rhamnosus* and *Lactobacillus acidophilus*) on MRS broth and pathogenic bacteria, *E. coli* on nutrient broth for 24h at 37 °C were used to inoculate the medium of prebiotic tests (MRS1; MRS2; MRS3; MRS4) at level of 0.15 ml inoculums per 10 ml of prebiotic medium. After incubation at 37°C for 24 h, the resulted bacterial growth were measured spectrophotometrically at 660 nm (Bausch and Lomb; spectronic, USA). The "prebiotic index" (I) was used as the indication on the prebiotic effect of the tested oligosaccharides (Hussein, *et al*., 2010), and calculated as follow:

Prebiotic Index (I) = O.D. of bifidogenic bacterial culture / O.D. of *E. coli* culture.

Results and Discussions

Analytical characterization of soy beans

The whole soybean seed was found to contain approximately 36.5% protein; 30.2% total carbohydrates; 13.5% lipids and 2.4% ash. Our results closed to that recorded by USDA (2009). Characterizations of the isolated oligosaccharides indicated that; the isolated oligosaccharides represent 7.6% of the total carbohydrates. The preparative paper chromatography of the isolated oligosaccharide and their hydrolysis products clarify that the mono sugar compositions was galactose and mannose in a ratio of 1:2 with traces of glucose units.

Biological activities of GMO and their sulfated derivative

Anticoagulation activity

The results in table (1) showed that the anticoagulation activities of the native GMO at different concentrations (500-1000µg/ml) were lower than that of standard heparin. While the SGMO were possessed very high anticoagulation activities at concentration ranged from 800µg to 1000µg (clotting time more than 24h). At conc. 700µg SGMO had anticoagulation activities almost the same as that of the standard heparin. The results

indicated the influence of the chemical structure of the tested oligosaccharides on their biological activities and also indicated that the sulfate group has great influence in anticoagulation activity. This was in agreement with that reported by other authors (Hussein *et al.,* 2011; Wang, *et al.,* 2010b). It had been reported that soy bean galactomannan and/or its sulfated derivative have low anticoagulation activities comparing to that of the standard Heparin (Hussein *et al.,* 1998; Mestechkina *et al.,* 2008; Hassan *et al.,* 2009), comparing this with our results, we can concluded that the depolymerization of galactomannan and transferred to oligosaccharides and/or sulfated derivatives improved the anticoagulation activities to very high extent. Mestechkina *et al.,* (2008) recorded anticoagulant activity of low-molecular-weight sulfated derivatives of galactomannan from Cyamopsis tetragonoloba (L.) seeds.

Antimicrobial activity

The results revealed that all the samples gave no inhibition zone (negative effect) for both all bacterial strains and yeast *(Candida albicans*), which mean that none of the tested oligosaccharides exhibited any antimicrobial activity.

Antitumor activity

The *in vitro* effect of soy bean, GMO and SGMO at different concentration (0.005mg/ml - 0.02mg/ml) in human colon carcinoma cell, Caco-2 was shown in table (2). The data indicated that the % of the dead cells was increased from 28.4% to 72% by increasing the oligosaccharides concentration from 0.005mg/ml to 0.02mg/ml respectively. The data clarify that the antitumor activity of native and sulfated soybean galactomannan oligosaccharide was almost the same and was proportioned to oligosaccharides concentration. The effect of galactomannan and galactomannan oligosaccharide to act as antitumor, antioxidant and to enhance immune system was reported by many researchers (Hsiao, *et al*., 2006; Krizkova, *et al*., 2006; Hassan, *et al*., 2009; Wismar, *et al*., 2010).

Fibrinolytic activity

Data in table (3) indicated that native GMO had fibrinolytic activities almost 1/3 that of the standard hemoclar while the sulfated derivatives (SGMO) had fibrinolytic activities resembled that of the standard, which indicates the influence of the chemical structures of the oligosaccharides on the fibrinolytic activity, as in the case of anticoagulation activity, (Hussein, *et al.,* 2011; Hashem, *et al.,* 2013). Hussein, *et al.,* (1998) reported a very weak fibrinolytic activity for soy bean galactomannan and/or its sulfated derivative. He also recorded that sulfated galactomannan of lower degree of polymerization exhibited higher fibrinolytic activity than that of the higher degree of polymerization so our results were logically in agreement with these data.

Prebiotic activity

Prebiotic activity of the isolated GMOS and their sulfated derivatives SGMOS were recorded in table (4). The result showed that all the probiotic microorganisms had the ability to utilize both native GMOS and their sulfated derivatives SGMOS in equal manner in all the tested media. The prebiotic index was in the range of 3.6 (for *Lactobacillus casei*) –8.1 (for *Lactobacillus bulgaricus*) when the carbon source of MRS medium replaced by GMO and SGMO (MRS_1 and MRS_3). When the carbon and nitrogen sources in MRS medium replaced by GMO and SGMO (MRS_2 and MRS_4) the prebiotic index was decreased and the lower value, was 2.8 for *Bifidobacterium bifidum* and *Lacfobacillus casei* while the highest value, 4.6 was for *Lactobacillus bulgaricus.* The value of the prebiotic index indicated that the isolated galactomanno- oligosaccharides inhabited the growth of pathogenic microorganisms as *E. coli* and the most important they are growth stimulator for probiotic microorganisms which known to inhibit the growth of other pathogenic microorganisms such as *Clostridia difficle* ; *Clostridia perfringens* and *Enterobacteriaceae*, through the production of short chain fatty acid, lowering of colonic pH, production of antimicrobial compounds and competition for growth substrates and adhesion sites. So they have beneficial effect on the large bowel function by improving the intestinal micro biota and hence the immune defense against cancer and inflammatory diseases and these recorded by other authors (Kumao and Fuji 2006; Izumi, *et al*., 2008; Yin, *et al*., 2008; Wang, *et al*.,2010a).

Conclusions

The evaluation of the biological activities of the isolated soy bean galactomanno-oligosaccharides and their sulfated derivatives proved that they have anticoagulant; antitumor; fibrinolytic and prebiotics activities resemble or even higher than that of the standard compounds. So we can say that we successfully found new cheap save

Table (1): Anticoagulation activity of native and sulfated soybean galactomanno-oligoisaccharieds.

Samples concentration	Anticoagulation activity			
	Native oligosaccharides		Sulfated oligosaccharides	
	min.	sec.	min.	sec.
500 µg/ml	-----		5	4
600 µg/ml	-----		40	7
700 µg/ml	-----		84	5
800 µg/ml	-----		*Prolonged	
900 µg/ml	-----		*Prolonged	
1000 µg/ml	3	10	*Prolonged	
Standard heparin (1000 µg/ml)	90			

*Prolonged: clotting time more than 24h.

Table (2): Antitumor activity of native and sulfated soybean galactomanno-oligoisaccharieds.

Samples	No. of viable cells	No. of dead cells	Cytotoxicity (%)
Control	10000	0	0
0.005mg/ml			
GMO	7116	2884	28.80
SGMO	7162	2838	28.40
0.01mg/ml			
GMO	6312	3688	36.88
SGMO	6091	3909	39.19
0.02 mg/ml			
GMO	2819	7181	71.80

SGMO	2796	7204	72.04

GMO: native galactomanno-oligosaccharides. SGMO: sulfated galactomanno-oligosaccharides.

Table (3): Fibrinolytic activities of native and sulfated soybean galactomanno- oligoisaccharieds.

Samples	Lyses of plasma clot (%)
Hemoclar	75
GMO	25
SGMO	75

Samples concentration was 2mg/tube.

Table (4): Prebiotic Effect of soybean oligosaccharide (native and sulfated) on the growth of tested bacteria.

Bacteria	Native oligosaccharides		Sulfated oligosaccharides	
	MRS_1	MRS_2	MRS_3	MRS_4
	I	I	I	I
Bifidobacterium bifidum	6.0	2.8	6.0	2.8
Lactobacillus casei	3.6	2.9	3.5	2.8
Lactobacillus acidophilus	6.5	3.9	6.4	3.8
lactobacillus reuteri	5.9	3.8	5.9	3.8
lactobacillus bulgaricus	8.1	4.6	8.0	4.6
Lactobacillus helveticus	5.8	4.0	5.8	3.9
Lactobacillus rhamnosus	4.9	3.5	4.9	3.5
Escherichia coli	----	-----	----	----

MRS_1: MRS media with soybean GMO as carbon source.
MRS_2: MRS media with soybean GMO as carbon and nitrogen source.
MRS_3: MRS media with sulfated soybean SGMO as carbon source.
MRS_4: MRS media with sulfated soybean SGMO as carbon and nitrogen source.
Probiotic Index (I) = O.D.of probiotic growth / O.D. of *E. coli* growth.

source for compounds have beneficial effect on the life of large group of people from the health and medical point of view.

To our knowledge this is the first time someone report anticoagulation; fibrinolytic and direct antitumor activities for galactomanno-oligosaccharides not to mention soybean galactomanno-oligosaccharides.

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