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Biological Activities of Soybean Galactomannan

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Abstract: Galactomannan was isolated from seed hulls of soybean (*Glycine max)* and antitumor, hemagglutinating and anticoagulating activities were investigated. The results showed that galactomannan possesses antitumor activity against EACC. Anti-tumor activity of galactomannan was proportioned with the concentration of galactomannan. The galactomannan has haemagglutinating activity for rat erythrocytes. The haemagglutinating activity is proportioned with the concentration of galactomannan. The highest activity of galactomannan (2.364) was noticed with the highest concentration (500 ppm). Also, galactomannan has anticoagulating activity in comparison with heparin. In conclusion, the soybean galactomannan possesses antitumor, hemagglutinating and anticoagulating activities.

Key words: Soybean, Galactomannan, Antitumor, Haemagglutinating, Anticoagulating, Activity

INTRODUCTION

The soybean, *Glycine max* (L.) Merrill, has been known since 3,000 BC to humans as food. Soybean seeds, are known to contain galactomannans. The galactomannans from soybeans have been purified and soybean hulls have a high content of galactomannans [1]. Galactomannans, which are one of NSP, have attracted considerable academic and industrial attention because of their unique chemical and physical properties in addition to their biological functions. Plant galactomannans are reserve polysaccharides composed of variable proportion of D-mannose and D-galactose residues. The use of these polysaccharides (also known as gums) as substances for mummification can be traced back to 3,000 BC in ancient Egypt and, hence, they are often called "Pharaoh's polysaccharides" [2]. The importance of these polysaccharides can be seen in their wide use in industry, notably in food, pharmaceuticals, cosmetics, paper products, paints, plasters, well-drilling, explosives and fire-fighting. However, there are few reports on the biological activities of isolated galactomannans [3]. Galactomannans are neutral water-soluble polysaccharides extracted from seeds of leguminous plants. Their structures are characterized by a β-(1,4) mannose (Man) backbone with α-(1,6) galactose units (Gal) as side groups. Water solubility increases when the yield in (Gal) unites increases [4-7]. Galactomannans are neutral polysaccharides composed of linear main chains of β-1>4 linked mannose units with α-1>6 linked side chains of a single galactose unit. They differ in the ratio of mannose to galactose units, M/G. The more substituted of the commercial galactomannans is guar gum (M/G ~2:1); in tara gum, the M/G is ~3:1 while in locust bean gum is ~4:1 [8,9].

However, there are few reports on the biological activities of isolated galactomannans. So that following view was generally focused on the biological activities of polysaccharides. At the end of decade of the 20th century, plant polysaccharides especially mucilages and gums has been reported to be having biological activities in human and animal. Anti-tumor, immunological, anti-complementary, anti-inflammatory, anti-coagulant, anti-viral, hypocholesterolamia and hypoglycemic activities have been observed in a wide rang of polysaccharides [10]. Tizard *et al.* [11] mentioned that the most marked biological activities of mannans (including the galactomannans and glucomananns) in mammals are activation of macrophages and stimulation of T cells. As a result, they are potent immunostimulants with significant activity against infectious diseases and tumors. Yoshiaki *et al.* [12] isolated a heteropolysaccharide from lichen, *Gyrophora esculenta* Miyoshi (Iwatake), by cold water. The heteropolysaccharide is a highly branched galactomannan-type polysaccharide, containing an

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α-(1>6)-linked D-mannan backbone. The glucan is a linear (1-6)-β-D-glucan. With regard to the anti-tumor activity, both the galactomannan and (1-6)-β-D-glucan had moderate inhibition activities on Sarcoma-180, but lower than those of branched (1>3)-β-D-glucans. Ingólfsdóttir [13] showed that the pharmacological investigations of the lichen have those polysaccharides as well as low molecular weight constituents exhibit significant biological activity. A polysaccharide with a backbone of (1-6)-linked α-D-mannopyranosyl and α-D-(1-6)-galactopyranosyl units has been isolated from an alkali extract of Iceland moss. The galactomannan exhibited pronounced enhancement of phagocytosis in both in *vitro* and in *vivo* assays.

Agglutination is one of the most characteristic and important reactions of plant lectins, which have at least two carbohydrate binding sites. In most agglutination reactions, the main site of interaction is between the surface glycolipids of cells and the lectin [14,15]. The agglutinin present in the lectin from jack fruit react with alkali-labile asialo carbohydrate chains of various glycoproteins, which is represented by the disaccharide, 3-O-*β*-D-galactopyranosyl-N-acetyl- galactosamine linked to a protein core via serine or threonine [16].

It is well known that some natural polysaccharides and the derivatives of others possess anticoagulation activity. Good examples of such polysaccharides are the anti-coagulant heparin, a sulfated polysaccharide from animal origins. The anticoagulation activities of sulfated polysaccharides were a higher than those of the corresponding un sulfated ones [17]. Hussein *et al.* [3] reported that the crude fractionated and partially degraded galactomannans from *Leucaena* sp. (fenugreek) *Medicago sativa* (alfalfa) *G. max* (soybean) and *P. dactyliferai* (palm) exhibited considerable anti-coagulation and fibrinolytic activities. Sulfation of these polysaccharides improved the biological activities of both the native and enzymatically modified products. Pires *et al.* [18] isolated the galactomannan from the endosperm of seeds of *Senna macranthera* (from Arabic *sanβ*). The sulfation with SO_3-pyridine gave a product. It is had 45 IU/mg of anti-coagulant activity, as shown by the *in vitro* activated partial thromboplastin time (APTT), compared with 183 IU/mg for a porcine intestinal mucosa heparin. Two fractions of the sulfated derivative were obtained differing in their affinity to antithrombin III (AT III) in gel. By analogy with heparin, the anti-coagulant activity of the derivative could be expressed through binding of the polysaccharide to AT III.

Mestechkina *et al.* [19] studied the anti-coagulant activity of low-molecular-weight sulfated derivatives of galactomannan from *Cyamopsis tetragonoloba* (L.) Taub. (guar). Galactomannan was depolymerized using immobilized enzymatic preparation celloviridin. A set of fragments whose molecular weights varied from 12.6 to 245.6 kDa was obtained. Sulfated derivatives of components of all fractions were synthesized, in which the content of HSO_3^- groups was 48.05±2.31%. All preparations exhibited anticoagulant activity, which was recorded *in vitro* in two tests, anti-thrombin (aIIa) and (aXa). The anti-thrombin activity (aIIa) was high (up to 65–87 U/mg) and did not depend on the molecular weight of a sulfated derivative; in the second test (aXa), the effect of molecular weight was observed. Biospecific electrophoresis allowed detecting the ability of galactomannan sulfates to form complexes with protamine sulfate, a classic antidote to heparin.

The aim of this study was to investigate the antitumor, hemagglutinating and anticoagulating activities of soybean galactomannan.

MATERIALS AND METHODS

Materials

Plant Material: Hulls of soybean seeds of various varieties of *Glycine max* (L.) Merrill, were obtained from Soybean Factory, Food Technology Research Institute, Agriculture Research Centre, Ministry of Agriculture, Giza, Egypt.

Chemicals: RPMI 1640 medium and crystalline porcine trypsin were purchased from Sigma Chemical Co. All other chemicals were of analytical reagent grade.

Animals: Egyptian female Swiss albino mice were purchased from National Cancer Institute, Cairo, Egypt.

Extraction of Galactomannan: Galactomannan was extracted from soybean hulls using the procedure reported by Whistler and Saarino [1]. Soybean hulls were extracted with acetone for 24 hours in a Soxhlet extractor and the hulls air dried. Acetone extracted hulls were extracted twice with fresh water (ratio of water to hulls 12:1) at pH 6.5 (HCl 0.1 *M*) and at 40°C for 16 hours each. After filtration through cloth, the combined extracts were acidified to pH 4.5 and centrifuged (6,000 rpm for 60 min). The centrifuged was concentrated under reduced pressure to one-fourth the original volume and the light

yellow solution was poured into three volumes of absolute ethanol. The white precipitate was removed by centrifugation, resuspended four times in absolute ethanol and the final centrifuged precipitate freed of ethanol in a vacuum desiccator's over calcium chloride.

Determination of Antitumor Activity: The viability percentages of tumor cells were measured by the modified cytotoxic trypan blue-exclusion technique of Bennett *et al.* [20].

Animals: Female Swiss albino mice, weighing 18-22 g, 8-10 weeks old were used. Animals were kept under environmental conditions for 2 weeks then injected intraperitoneal (i.p) by Ehrlish ascites carcinoma cells (EACC). The animals were used for tumor cell preparation (cell line).

Tumor Cells (Cell Line): A line of Ehrlish ascites carcinoma resistance to endoxan [21] has been used. The parent line was first supplied through the courtesy of Dr. G. Klein, Amsterdam, Holland. The tumor line is maintained in the National Cancer Institute, Egyptian female Swiss albino mice by weekly transplantion of 2.5×10^6 cells. The cells were centrifuged at 1,000 rpm for 5 min, washed with saline then the needed number of cells was prepared by suspending the cells in the propitiate volume of saline.

Medium: The culture medium used was prepared using RPMI I640 medium, 10% fetal bovine serum and 10% L-glutamine.

Procedure: Two ml of cells (4×10^6 cells) were transferred into a set of tubes. Different volumes of examined galactomannan were added to each tube. Control was prepared with saline solution (0.9% NaCl, w/v) instead of examined galactomannan. 10 µl of each cell suspension was transferred into clean dry test tube, then 80 µl of saline solution and 10 µl of trypan blue reagent (0.4%) were added and mixed well. The viability percentages of tumor cells were measured after incubation for 2 hours. The number of living cells was calculated using a homocytometer slide.

Determination of Haemagglutinating Activity: A quantitative determination of haemagglutinating activity was carried out according to the method developed by Liener [22] by measuring the absorbance of the layer of unsedimented erythrocytes.

Reagents:

- Alsever's solution: Glucose (2.05 g), sodium citrate (0.8 g) and NaCl (0.42 g) were dissolved in 100 ml distilled water and the pH was adjusted to 6.1 with 10% citric acid.
- Anticoagulant solution: Sodium citrate (8.0 g), formaldehyde 37% (54.0 ml) and saline solution (100 ml) were mixed together.
- Stock blood suspension: Whole rat blood was added to an equal volume of Alsever's solution containing 1/30 volume of the anticoagulant solution (this suspension could be stored as long as 2 weeks at 4°C).

Preparation of Trypsinized Erythrocytes Suspension: Trypsinized erythrocytes were prepared on the day of the assay. Erythrocytes were collected from the stock blood suspension by centrifugation at room temperature (2,000 rpm, 5 min) and washed 3-4 times with saline solution (about 5.0 ml of saline solution for each ml of packed erythrocytes). The washed erythrocytes were added to phosphate buffer solution (PBS; 0.006 *M*, pH 7.4) to give a suspension with an absorbance of 2.0 at 620 nm (about 4.0 ml of cells per 100 ml of PBS). To 100 part of the suspension, one part of trypsin solution (10 mg/ml) was added and the mixture was incubated at 37°C for 1 hour. The trypsinized erythrocytes were then washed 4-5 times with saline solution as above to remove the last traces of trypsin and finally suspended in sufficient saline solution to give standard erythrocytes suspension with an absorbance of 1.0 at 620 nm.

Procedure: Serial 4-fold dilution of galactomannans was made in final volume of 1.0 ml of the saline solution in 10 x 75 mm test tube. To each tube, 1.0 ml of standard erythrocytes suspension was added. The content of each tube was placed in a rack that holds them in an exactly vertical position. After 2.5 hours at room temperature, the tubes were read at 620 nm using Jenway 6505 spectrophotometer, care being taken not to agitate the contents. Each experiment was included a set of 2-4 control tubes containing 1.0 ml of saline solution and 1.0 ml of standard blood suspension.

Calculation of Haemagglutinating Activity: One haemogglutination unit (HU) was defined as that amount of material which was required to cause a decrease of 50% in the absorbance of the erythrocytes suspension in 2.5 hours the conditions described above.

The reciprocal of dilution (x) corresponding to one HU was calculated from the reading of the two tubes that have optical densities nearest to half the absorbance of control (E50 ~ 0.25), one of the readings (EA) being lower and the other (EB) being higher than E50. The following equation was used:

$$\log x = \log A + \frac{E50-EA}{EB-EA} \bullet \log$$

Where: A is the reciprocal of dilution of the tube with EA

Determination of Anti-Coagulation Activity: The anticoagulant activity of galactomannan was investigated using the method of USA pharmacopoeia [23].

Preparation of Plasma: Blood was collected directly from rat into a vessel containing 8% of sodium citrate solution in the proportion of one volume to 19 volumes of blood. The mixture was immediately agitated, by gentle inversion, centrifuged and the separated canary yellow plasma was pooled.

Procedure: Hard glass, 31 x 100 mm test tubes were cleaned by immersion overnight in chromic acid. To each tubes, 0.8 ml of galactomannan solution (1%), 0.8 ml of standard heparin solution (1 USP unit/0.8 ml), or 0.8 ml of saline solution was added. To each tub, 1 ml plasma and 0.2 ml of calcium chloride solution (1%) were added. The time was immediately recorded and each tube was stopped. The contents were mixed by inverting three times in such a way that the entire inner surface of the tube was wet. The time required for clotting was determined.

Statistical Analysis: The data were analyzed by an analysis of variance (ANOVA) and the difference among means were tested for the least significant difference (LSD) at $P<0.05$. The results were processed by SAS computer program (1987).

RESULTS AND DISSCUSION

Antitumor Activity of Galactomannan: Galactomannan was tested *in vitro* study for its antitumor activity against Ehrlish ascites carcinoma cells (EACC). The viability of EACC after incubation for 120 min with galactomannan at different concentrations was evaluated. Four different concentrations of galactomannan (66.6, 133.3, 200 and 266.6 ppm) were used. The effect of galactomannan on EACC is given in Table 1. The obtained data showed that different galactomannan concentrations reduced the viability of EACC. After 2 hours of incubation, the dead cells percentage was increased from 3% to 11%, 14%, 19% and 43% for 0, 66.6, 133.3, 200 and 266.6 ppm, respectively.

Table 1: Effect of different concentrations of galactomannan on the viability of Ehrlish ascites cells carcinoma

	After 2 hours of incubation	
Treatment*	Viable cell%	Dead cell%
0 (control)**	97	3
66.6	89	11
133.3	86	14
200	81	19
266.6	57	43

* 2 ml of cells solution containing 4×10^6 cells.

** Tumor cells plus saline solution.

In general, the obtained data concerning this study could be revealed the following points:

- Galactomannan possesses anti-tumor activity against EACC.
- Antitumor activity of galactomannan, as indicated by dead cell percentages and the viability of cells, are proportioned with the concentration of galactomannan. Frankly, there is positive correlation between anti-tumor activity and galactomannan concentration.

The antitumor activity of galactomannan may be due to the following reasons:

- Interaction between galactomannan and proteins located on the tumor cells. This interaction may be induces changes in the cellular membrane, thus result in death of tumor cell.
- Interaction between galactomannan and carbohydrate moieties located on the cell surface. Consequently, this interaction may be induces change in cellular membrane and result in death of tumor cells.
- It was established the galactomannan inhibit enzyme activities, including digestive enzymes and enzymes involving glucose transport, accordingly this inhibitory activity probably affect tumor cells. The net result of this effect is death of tumor cells.

- The factors that determine whether a polysaccharide will have anti-tumor activity are unclear. However, these appear to be direct relationship between the anti-tumor activity of polysaccharides (including galactomannan) and their ability to interact with serum albumin [24-26].
- The galactomannan exhibited pronounced enhancement of phagocytosis in both *in vitro* and *in vivo* assays.
- Many researches reported that polysaccharide induced destructive effect on sarcoma cells [27,28,12]. Although, common polysaccharides, such as starch, dextran and insulin, do not have anti-tumor activity. This is abundant evidence that mannans and selected glucans are potent antitumor agents.

Haemagglutinating Activity of Galactomannan: The galactomannan under investigation was subjected to examine its haemagglutinating activity. In this study, one type of erythrocytes was used, rat's erythrocytes. Four concentrations of galactomannan were employed. Regarding the haemagglutinating activity of galactomannan for rat's erythrocytes, the obtained data were recorded in Table 2. Briefly, the obtained data showed that galactomannan has haemagglutinating activity for rat's erythrocytes. The haemagglutinating activity is proportioned with the concentration of galactomannan, i.e. positive correlation between them (concentration and haemagglutinating activity). The highest activity of galactomannan (2.364) was noticed with the highest concentration (500 ppm). The lowest activity (1.328) was found with the lowest concentration (200 ppm) concentration. On the other hand, no significant differences in case of (200, 300 ppm) and (400, 500 ppm). There is no direct reason for the haemagglutinating activity of galactomannan, but we can say that this activity may be due to the interaction between polysaccharides and polysaccharides. Galactomannan as polysaccharide may be interact with carbohydrate moieties, which carried on cell membrane of erythrocytes. This interaction, of course, led to haemagglutination. Therefore, galactomannan possesses haemagglutinating activity. This explanation is in agreement with that proposed by Belogortseva *et al.* [29].

Anticoagulating Activity of Galactomannan: It was known that many polysaccharides isolated from plants have anti-coagulation activity [3]. In this part of our study, there was an important assay to evaluate the anticoagulating activity of galactomannan. Data concerning the anti-coagulating activity of galactomannan is shown in Table 3. Heparin was used as standard anti-coagulant. Briefly, the obtained results could be revealed the following:

Table 2: Haemagglutinating activity of galactomannan for rat erythrocytes

Concentration (ppm)	Activity*
200	1.328^{a}±0.15
300	1.658^{a}±0.19
400	2.159^{a}±0.24
500	2.364^{a}±0.253
L.S.D	0.679

-Value are means of three replicates ± SE, Numbers in the same raw followed by the same letter are significant at $p > 0.05$

* Haemagglutinating activity was presented as haemagglutination unit

Table 3: Anticoagulating activity of galactomannan

Polysaccharide	Anticoagulating activity
Blank	-
Heparin	++++
Galactomannan	++

++++ Plasma clotting after 22 min.

++ Plasma clotting after 11 min.

- Plasma clotting after 8 min.

- Heparin showed higher anticoagulating activity than galactomannan, where plasma clotting was occurred after 22 min.
- Galactomannan caused clotting for plasma after 11 min.

In the present study, galactomannan of soybean hull was examined for its anticoagulating activity. Rakhimov *et al.* [30] found that the polysaccharide isolated from the epigeal part of *L. usunachmaticus* possessing pronounced anti-coagulant activity. Hussein *et al.* [3] reported that the crude galactomannan from *P. dactylifera* and *G. max* exhibited anticoagulating activities comparable to that of standard heparin sodium. On the other hand, Srivastava and Kulshreshtha [17] reported that neutral polysaccharides have no effect as anticoagulating activity. Sulfation of polysaccharides improved the biological activities of the modified products [3]. Many heparinoids, prepared by sulfation of polysaccharides from plant or obtained directly from animal tissue, have been studied. However, such products showed weak anticoagulant action [31-33]. It appears

that the sulfate group plays an important role in anticoagulating activity. The effect of galactomannan, which possesses anticoagulating activity, may be due to their contents of uronic acid. The polysaccharides, which contain the uronic acids, carrying negative charge, have ability to binding the calcium ions, therefore prevent the formation of clot [3].

CONCLUSION

Finally, it could be concluded that galactomannan under investigation possesses antitumor, hemagglutinating and anticoagulating activities. It can be used in medical and pharmaceutical fields as an adjuvant in cancer therapies and anticoagulant agent.

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