Journal of Medicinal Plants and By-products (2015) 2: 201-207

Original Article

Using Beta-Glucan Isolated from *Helianthus annuus* Infected by *Sclerotinia sclerotiorum* in Bakery

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Article History: Received: 11 July 2015/Accepted in revised form: 29 November 2015

Abstract

Sclerotinia sclerotiorum is a phytopathogenic fungus that attacks more than 400 plant species of them medicinal plant. In this study the extract obtained from basal stalk rot of *Helianthus annuus* L., attacked by *S. sclerotiorum*, was subjected to the analysis of FTIR to identify the presence of beta-glucan. FT-IR spectrum showed four ranges of bands in 890 cm^{-1}, two overlap band in 1047 and 1078 cm^{-1} and the last one near 1160 cm^{-1} referring to different functional groups or characteristics; beta-glycosidic linkage and pyranosyl ring. The existence of beta-glucan in the filtrate liquid culture of *S. sclerotiorum* suggests the potential of the secreted liquid drops of fungal infection in plant as a new secretory source of beta-glucan useful in food and pharmaceutical sectors. In this regard, the extracted beta-glucan was added as an additive to bread dough to elucidate its effect on the baked-bread texture as monitored by SEM micrographs. Results from the electron microscopy images of experimental bread confirmed that beta-glucan enhances the porosity of bread in the presence of normal yeast and increases fermentation period (1% dry yeast, 4.5% scleroglucan, 4 h). The solubilization of polysaccharides, primarily beta-glucan, seems to be the main strategy to improve the bread texture.

Key words: Beta-glucan, FTIR analysis, Medicinal plants, *Sclerotinia sclerotiorum*

Introduction

Most plant diseases are caused by fungi of which necrotrophic fungal *S. sclerotiorum* (Lib.) De Bary has considered an important *phytopathogenic* fungus. Several reports have been reported about the negative effect of that pathogen on medicinal plants such as *Lavandula Spica* L. [1], *Achillea millefolium* L. [2], *Trifolium repens* L. and *Trifolium pratense* [3], *Thymus vulgaris* L. [4], *Medicago Sativa* L. [5], however a full list of *Sclerotinia sclerotiorum* is accessible at some websites [6]. *Helianthus annuus* L. as a medicinal plant [7] by which its products are influenced enormously justifies more study on it in relation to *S. sclerotiorum's* potential role in damaging it.

S. sclerotiorum produces fluffy white mycelium on the infected plant parts and is favored by cool, moist conditions; however show surprisingly broad ecological distributions [8]. Although the pathogen attacks more than 400 plant species (mainly legumes, sunflowers, canola, most vegetables, tobacco, many flowering bedding plants, and stone fruits) [9] and causes huge economic loss, *H. annuus* suffers greatly as it's one of major edible oil sources and further as a medicinal plant (its oil can be used internally as a lubricant to treat constipation, and externally for wound healing and psoriasis (Medicinal plant list, College of

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pharmacy, The University of Rhode Island, USA) [7]).

S. sclerotiorum forms smaller sclerotia (0.5 to 3 mm). Sclerotia buried in the plow layer of soil can survive and remain infective for up to 5 years. Sclerotia of *S. sclerotiorum* most commonly produce a mushroom-like fruiting body termed an apothecium [7]. At soil depths of up to 2 cm, apothecia can extend from the sclerotia to reach the soil surface to distribute infection to plant parts (stalk, leaves, capitulum,…). Obviously, the infected plant affects its related products, *i.e.* sunflower oil, for its usage as medicinal oil.

Different compounds will produce upon the attack of *S. sclerotiorum* as the main/secondary metabolites of the pathogen or even as new synthesized compounds upon pathogen-plant interaction. The beta-glucan, a pathogen metabolite, is a polysaccharide formed from D-Glucose monomers with glycoside linkages. L-1,3-D-Glucan is a polyglucose and a major structural component of the cell wall of yeasts and fungi.

Depending on the source, there are clear differences in macromolecular structure between beta-glucans (Table 1). The cell wall beta-glucans of yeast and fungi consists of 1,3 beta-linked glycopyranosyl residues with small numbers of 1,6 beta-linked branches [10]. Furthermore, besides differences in type of linkage and branching, beta-glucans can vary in solubility, molecular mass, tertiary structure, degree of branching, polymer charge and solution conformation (triple or single helix or random coil). All these characteristics may influence their immune modulating effects [11].

Specifically, the scleroglucan *S. sclerotiorum* glucan (SSG) is a soluble beta-glucan obtained from the culture supernatant of *S. sclerotiorum.* It contains β-(1–6)-D-glucosyl side chains at the ratio of one residue per two main chain residues consisting of β-(1–3) bonds. SSG differs from other glucans in that it possesses antitumor effects even when administered orally [12-13].

Today, more than 70,000 ton polysaccharides uses in food sector as the thickening agent, stabilizer, gelling agent, suspension and improve the texture of the product. Although polysaccharides from the plant hydrocolloids (starch, cellulose, pectin, and guar gum), seaweed, shellfish (alginate, Carrageenan and chitosan) and microbial (xanthan) are obtained, finding new choices are still in progress.

Scleroglucan in the food industry considers as a stabilizer or gelling agent, however, xanthan has been accepted more in the market for providing similar properties and less price. It is to predict that scleroglucan will be substitute xanthan soon due to the thermal stability that bears [15-16].

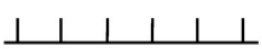
In this research, we wished to explore the presence of beta-glucan in the extract of *S. sclerotiorum*, and extract it to study its eventual usage in baking industry and analyze its benefits on the baked bread.

Material and Methods

Fungus beta-glucan isolation

S. sclerotiorum is collected from the stems of infected *H. annuus* species that had germinated and grown on potato dextrose agar at 25 °C. After 14 days, three discs (12 mm diameter) of mycelia were transferred to Erlenmeyer flasks containing 1000 mL of liquid medium (DIFCO® and BBL® CZAPEK-DOX without agar) at a constant temperature of 27 °C. Fig. 1 shows the micrograph of *S. sclerotiorum* in DIFCO®AND BBL® CZAPEK-DOX medium to confirm the growth of only this fungus.

Table 1 Differences in macromolecular structure between beta-glucans depending on the source [14].

Beta-glucan type	Structure	description
Bacterial		Linear beta 1,3 glucan (*i.e.* curdlan)
Fungal		Short beta 1,6 branched, beta 1,3 glucan (*i.e.* Schizophyllan, SSG*)
Yeast		Long beta 1,6 branched, beta-1,3 glucan (*i.e.* WGP beta-glucan, betafectin™
Cereal		Linear beta 1,3-beta 1,4-glucan (*i.e.* oat, barley, rye)

*SSG: Scleroglucan or *S. sclerotiorum* glucan

After 40 days (to obtain metabolite saturated medium), the mycelia were initially separated from the broth medium containing the extracellular metabolites by sterile gaze [17]. The solution was then filtered through a sterile 0.45 µm pore-size filter (Sarstedt Ltd., Germany). The culture filtrate was then identified through FT-IR analysis to prove the presence of beta-glucan.

Fourier Transformation Infrared Spectroscopy (FTIR)

FT-IR spectra were collected on a Bruker IFS 66 FTIR spectrometer (BRUKER, Germany) using KBr discs with an average of 128 scans and at a resolution of 4 cm^{-1}. The samples were dispersed in KBr (2 mg sample/200 mg KBr), ground, and pressed into pellets. Scans were conducted in the range 4000-450 cm^{-1} at a resolution of 2/cm [18].

Dough preparing and experimental baking

Wheat flour was obtained from Pak Ard Co. (Shahryar, Tehran, Iran). The flour was a normal one to be used for Barbari type bread.
Oval loaves were baked according to a small-scale straight dough baking test described by Faergestad et al. (2000) with adjustments [19]. Based on flour weight at 15% moisture, 1.5% NaCl, 1% vegetable oil, 2 g sugar, and 1% dry yeast (Iranmayeh yeast, Iran) and/or 4.5% SSG were added to the dough. 140 ml water was then added. Dough temperature after mixing was 27±0.5 ºC. The doughs were rested for 120 and 240 min in a fermentation cabinet at 27 ºC and 60% RH, divided into three 150 g pieces and molded in the extended manually by a wooden roller. The molded dough was baked in a rotating traditional furnace for 20 min. After cooling to room temperature for 1 h, the loaves sent for Scanning Electron Microscopy (SEM) analysis.

Results

Beta-glucan isolation

Beta-glucans are present as major structural components of the cell walls of yeast, fungi, some bacteria and even in the endosperm cell wall in cereals. However its total amount and biological properties are differ in different resources.
It should be noted that the particular fractionation procedure scheme in each case depends on the polysaccharide composition of the original material, involving their molecular weight, branching degree and pattern of branches [10]. In Fig. 1 the microscopic observation of fungus isolated from *H. annuus* is provided and the cell organization confirms the growth of only *S. sclerotiorum*.

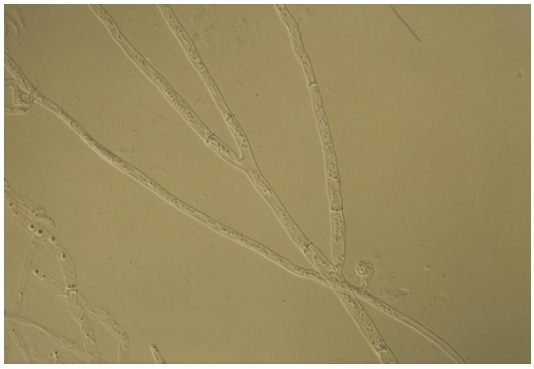


Fig. 1 Micrograph of *S. sclerotiorum* in DIFCO®AND BBL® CZAPEK-DOX medium to confirm the growth of only this fungus (light microscopy 400 X)

FT-IR analysis

Fig. 2 demonstrates the absorbance of the *S. sclerotiorum* extract at frequency region of 4000 – 450 cm^{-1} to study the spectra for existence of polysaccharides in which three zones of absorption bands were identified. First of them is in the range of 3400 cm^{-1} that showed existence of (O-H), the second in the range of 2800 and 2900 cm^{-1} showed the existence of (C-H) and the last one in the range of 1080 cm^{-1} confirming the presence of C=O or C-O [18].

Bread texture improvement qualification:

The impact of using either SSG or yeast and investigation of their synergistic effect on dough and bread was carried out through scanning electron microscopy (SEM). The breads in the presence of improving agent (SSG) and passing different fermentation periods (2 or 4 hours) were studied (Fig. 3).
The porosity and the balanced distribution of micro/macro holes in the loaves were increased upon the usage of SSG accompanied with normal yeast (*S. cerevisiae*) and longer fermentation time. The orientation of porous, their number and harmonization in their size (over the chemical properties) are considered crucial factors through which bread keep its final form and its shape for longer time, more adorable by the consumer.

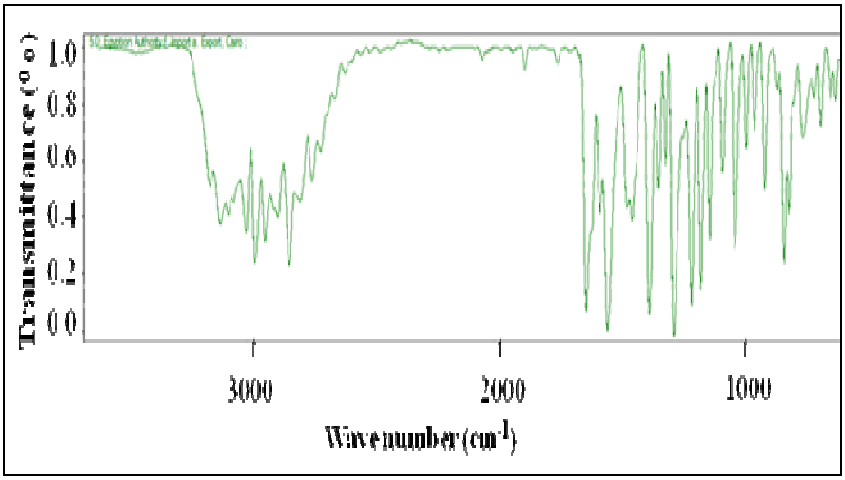


Fig. 2 FT-IR spectrum of *scleroglucan*, the extracellular beta-glucan from *S. sclerotiorum*

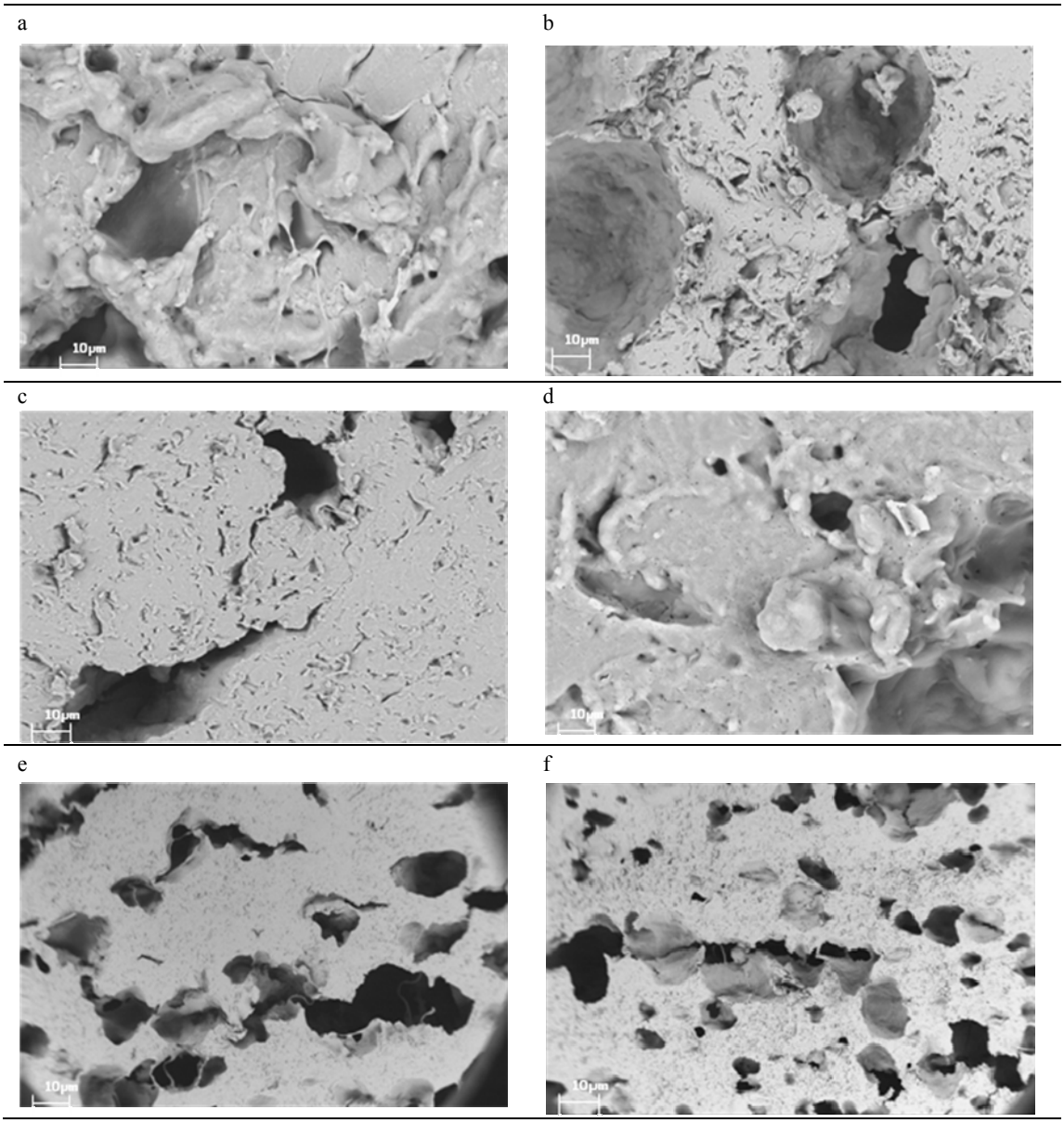


Fig. 3 Scanning electron micrographs of baked breads: (a) control baked 1 (with normal *Saccharomyces cerevisiae* yeast, no SSG, 2 h fermentation); (b) control baked 2 (with normal *S. cerevisiae* yeast, no SSG, 4 h fermentation); (c) sample baked 1 (no yeast, *Pleurotus florida's* beta-glucan, no fermentation rest); (d) sample baked 2 (no yeast, with SSG, 2 h fermentation); (e) sample baked 3 (with normal *S. cerevisiae* yeast, with SSG, 2 h fermentation); (f) sample baked 4 (with normal *S. cerevisiae* yeast, with SSG, 4 h fermentation).

Discussion

Upon phyto-pathogens attack, especially when they are necrotrophic, whole plant losses its usage and goes out from consumption cycle. This situation will have greater impact when the candidate plant is a medicinal plant. In such case, alternative procedures to make weaker the economical losses through using byproducts or isolates some metabolites to use in other sectors seems promising. In the present research, we extracted an extracellular polysaccharide from an invader fungal *phytopathogen*, *S. sclerotiorum* that attacked sunflower, and we confirmed the presence of beta-glucan in the extraction by FT-IR analysis. *S. sclerotinum* is a pathogen fungus from *ascomycete* family which affects 400 plants in once [8]. Upon the attack of pathogen, the secreted liquid drops because of the presence of fungal infection in plant in which 56 proteins existing in drops which are critical in amino acids metabolism, carbohydrates metabolism and formation of secondary metabolites such as beta-glucan [20]. The beta-glucan presented in this fungi is in the form of beta (1, 3) glucan with peripheral branches of (1, 6) linkages. Furthermore beta-glucan is produced from cell walls of different spices. However, it has been confirmed that other fungal plant pathogen, *Botryosphaeria rhodina*, includes three different typed of beta-glucan in its cell wall structure [21]. The beta-glucan isolated from *S. sclerotiorum* culture has identified correctly through FT-IR (Fig. 2). As regard to the spectrum of the extract of S. sclerotiorum consisted of 3386.67 cm^{-1} band which is sign of OH presence, 2921.08 cm^{-1} that addresses CH existence and 1072 cm^{-1} which means the presence of (C-O) band. Following the confirmation of polysaccharide presence as a metabolite of *S. Sclerotiorum* through wave number in the range 800-1200 cm^{-1} and taking into consideration that carbohydrates are able to be absorbed in the 800-1200 cm^{-1} range, a more liable fingerprint determination of beta-glucans comes to be real. Beta-glucan's FT-IR spectrum was showed four ranges of bands in 890 cm^{-1}, two overlap band in 1047 and 1078 cm^{-1} and the last one near 1160 cm^{-1}. Absorption near 890 cm^{-1} showed the presence of ß–glycosidis linkage [10]. Two different overlap bands of 1048 $cm^{-1}$and 1078 cm^{-1} in fungal samples showed pyranosyl presence [10]. A band near 1160 cm^{-1}in *S. sclerotiorum* sample was also detected. Other sources of beta-glucan such as *Saccharomyces cerevisiae* needs more elaboration to obtain pure beta-glucan. Liu et al., reported the necessity of performing preliminary purification using sieves and autolysis induction procedure through sodium chloride and heating process and finally treating with organic solvents to obtain only cell walls including beta-glucan [22]. However, it quiet certain that isolation of beta-glucan from *S. sclerotiorum* culture does not need further purification steps over the steps appeared in the materials and methods since this source provides secretory beta-glucan.

In the next step, we used the beta-glucan as a texture enhancement in bread making and visualized bread porosity under scanning electron microscopy. Since physico-chemical properties of bakery products changes after the cooking process (in both crust and main texture) which are associated with irreversible changes in amylopectin structure of bread and affect organoleptic properties, flavor and aroma, as well.

However, the use of beta-glucan does not play a direct role in the delay staling, but to promote more balanced porosity of bread and the creation of a more spongy structure in bread, delaying glamorization of pectin and amylopectin, use of beta-glucans seem justifiable. This result is in agreement with the report for beta-glucan originated from barely cell wall in the baked bread [23]. Cleary et al., evaluated the effect of added beta-glucan on the dough rheology, quality of bread, digestion of bread (*in vitro*) and micro structure of baked breads. Although, many authors have reported some deficiencies in bread after adding beta-glucan, such as loss of height, volume and increased firmness [24-27], adding beta-glucan directly to flour and before bread making. This means that b-glucan could tightly bind appreciable amounts of water, making it less available for the development of the gluten network and reduced loaf volume.

Results from the electron microscopy images of experimental bread suggest that beta-glucan enhance the porosity of bread in the presence of normal yeast and longer fermentation period (as it shown in Fig. 3f the better distinction of structure in the bread has obtained). However in samples 1 and 2 which dough includes no yeast, beta-glucan from *Pleurotus florida's* and SSG, respectively the bread structure were uneven with fewer starch granules exposed. These results are in accordance with findings of Cleary et al. who illustrate that loss of dough and bread quality are related to beta-glucan molecular weight, the higher the molecular

weight of barley b-glucan, the greatest changes to dough and bread characteristics [23] .

Indeed the synergistic effect of SSG and yeast in providing more flexible texture of the baked bread has been revealed. The solubilization of polysaccharides, primarily beta-glucan, has greater impact to improve the bread texture. Beta-glucan was solubilized to a greater extent after baking, and the presence of natural gluten in wheat flour increased the solubility of beta-glucan further. Furthermore, the addition of beta-glucan to the bread dough caused a notable increase in the viscosity of dough. This result indicates that utilization of a beta-glucan concentrate that is less soluble during bread preparation may be effective in fortifying bakery products to achieve beta-glucan's health benefits resulting from its unique properties.

Acknowledgment

This work was supported by the grant from the National Institute of Genetic Engineering and Biotechnology, Tehran, Iran (Grant No. 433).

References

1. Garibaldi A, Bertetti D, Pensa P, Franco Ortega S, Gullino ML. Disease note First report of *Sclerotinia sclerotiorum* on butterfly lavender in Italy. J Plant Pathol. 2015;97:391-403.
2. Winslow SR. Plant materials technical note: western yarrow *Achillea millefolium* var. *occidentalis,* A native forb for conservation use in Montana and Wyoming. 2014; NRCS-Montana-Technical Note-Plant Materials-MT-103
3. Vleugels T, Baert J, Van Bockstaele E. Evaluation of a diverse red clover collection for clover rot resistance (Sclerotinia trifoliorum). Commun Agric Appl Biol Sci. 2013;78:519-22.
4. Moghtader M. Antifungal effects of the essential oil from *Thymus vulgaris* L. and comparison with synthetic thymol on *Aspergillus niger*. J Yeast Fungal Res. 2012;3:83-88.
5. Frate CA, Long RF. *Sclerotinia* in alfalfa: biology and control in the central valley. Proceedings, California Alfalfa and Forage Symposium, 12-14 December, 2005, Visalia, CA, UC Cooperative Extension, Agronomy Research and Extension Center, Plant Sciences Department, University of California, Davis
6. http://www.chaseagriculturalconsultingllc.com/resources/pdfs/articlesPdf/28HerbDiseases.pdf
7. http://web.uri.edu/ pharmacy/facilities/medicinal-garden/medical-plant-list/
8. Persley D, Stirling G. Common diseases of vegetable crops. 2010; pp. 15-35. In: Persley D, Cooke T, House S. (eds.), Diseases of Vegetable Crops in Australia. CSIRO PUBLISHING, 150 Oxford Street (PO Box 1139), Collingwood VIC 3066, Australia.
9. Dilantha Fernando WG, Nakkeeran S, Zhang Y. Ecofriendly methods in combating *Sclerotinia sclerotiorum* (Lib.) de Bary. Recent Res. Devel. Environ. Biol. 2004;1:329-347.
10. Stone BA. Chemistry of β–Glucans. 2009; pp.14-55. In: Bacic A, Fincher GB, Stone BA (eds.), Chemistry biochemistry and biology of 1-3 beta glucans and related polysaccharides, First edition. Academic Press is an imprint of Elsevier, NY, USA.
11. Li W, Cui SW, Kakuda Y. Extraction, fractionation, structural and physical characterization of wheat β-D-glucans. Carbohydr. Polym. 2006;63:408-416.
12. Ohno N, Adachi Y, Suzuki I, Sato K, Oikawa S, Yadomae T. Characterization of the antitumor glucan obtained from liquid-cultured Grifola frondosa. Chem Pharmaceu Bulletin. 1986;34:1709-1715.
13. Suzuki I, Hashimoto K, Ohno N, Tanaka H, Yadomae T. Immunomodulation by orally administered beta-glucan in mice. Inter Immunopharmaco. 1989;11:761-769.
14. Lee, HH, Lee JS, Cho JY, Kim YE, Hong EK. Structural Characteristics of Immunostimulating Polysaccharides from Lentinus edodes. 2009;19:455-461.
15. San Ei. Quality improvement of bakery products with sclerogum, Japanese Kokai Tokkyo Koho. Chem Industries Ltd. 1982;57:163-432.
16. Cun Z, Mizuno T, Ito H, Shimura K, Sumiya T, Kawade M. Antitumor activity and immunological property of polysaccharides from the mycelium of liquid-cultured Grifola frondosa. J Japanese Society Food Sci Techno. 1994; 41:724-733.
17. Tahmasebi Enferadi S, Vannozzi GP, Gomez Sanchez D, Baldini M. Results of screening sunflower for resistance to toxic metabolites produced by *Sclerotinia sclerotiorum* (LIB). De Bary and prospects for the use of biochemical markers in breeding. Agr. Med. 1998;128:47-58.
18. Seyed Hassan Tehrani S, Hashemi Sheikh Shabani S, Tahmasebi Enferadi S, Rabiei z. Growth Inhibitory Impact of *Peganum harmala* L. on Two Breast Cancer Cell Lines. Iran J Biotech. 2014;12:8-14.
19. Færgestad EM, Molteberg EL, Magnus M. Interrelationships of protein composition, protein level, baking process and the characteristics of hearth bread and pan bread. J. Cereal Sci., 2000;31:309-320.
20. Liang Y, Strelkov SE, Kav NN. The proteome of liquid Sclerotial exudates exudates from Sclerotinia J. Proteome Res. 2010;9:3290-3298.
21. Corradi da Silva MD, Fukuda EK, Vasconcelos AFD, Dekker RFH, Matias AC, Monteiro NK, Cardoso MS, Barbosa AM, Silveira JLM, Sassaki GL, Carbonero ER. Structural characterization of the cell wall D-glucans isolated from the mycelium of Botryosphaeria rhodina MAMB-05. Carbohydrate Res. 2008;343:793-798.

22. Liu XY, Wang Q, Cuic SW, Liu HZ. A new isolation method of b-D-glucans from spent yeast *Saccharomyces cerevisiae*. Food Hydrocolloids. 2008;22:239-247.
23. Cleary LJ, Andersson R, Brennan CS. The behaviour and susceptibility to degradation of high and low molecular weight barley b-glucan in wheat bread during baking and in vitro digestion. Food Chem. 2007: 102:889-897.
24. Cavallero A, Empilli S, Brighenti F, & Stanca AM. High (1-3, 1-4)-b-glucan fractions in bread making and their effect on human glycemic response. J Cereal Sci. 2002;36:59-66.
25. Gill S, Vasanthan T, Ooraikul B, Rossnagel B. Wheat bread quality as influenced by the substitution of waxy and regular barley flours in their native and extruded forms. J Cereal Sci. 2002;36:219-237.
26. Knuckles, B. E., Yokoyama, W. H., & Chiu, M. M. Molecular characterization of barley b-glucans by size exclusion chromatography with multiple-angle laser light scattering and other detectors. Cereal Chem. 1997; 74:599-604.
27. Symons LJ, rennan CS. The influence of a (1-3) (1-4)-b-D-glucan rich fraction on the physico-chemical properties and in vitro reducing sugar release of white wheat breads. J Food Sci. 2004;69:463-467.