

Extraction Efficiency of β -D-glucan from Waste Part of Bottom Mushroom (*Agaricus bisporus*) and its Ability to Adsorb Aflatoxin B₁

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Abstract

β -Glucans which are found in a variety of natural sources such as yeast, mushrooms, bacteria, algae, barley and oat show different biological effects. They are composed of D-glucose units linked by β -glycosidic bonds to each other. Adsorption of fungal toxins such as aflatoxin by β -glucan has been widely considered in recent years. Aflatoxins are a group of naturally-occurring carcinogens that are known to contaminate different human and animal foodstuffs. Aflatoxin B₁ is the most genotoxic hepatocarcinogenic compound among all types of the aflatoxins. The efficiency of adsorption of fungal toxins is directly related to the molecular structure, extraction method and source of β -glucan. Fungal derived β -glucan consists of β (1-3) bonds in main and β (1-6) at lateral branching point, with the specification of short shoulder length, has high ability to adsorb fungal toxins. In this study, for the first time, the efficiency of various extraction methods of β -glucan from stem cell wall of bottom mushroom (*Agaricus bisporus*) was measured and the ability to adsorb aflatoxin B₁ was evaluated. The results showed that although the yield of β -glucan from acid based extraction was higher than other methods (20.5%), the hot alkaline extracted β -glucan could adsorb and discard 90.2% of aflatoxin B₁ from contaminated samples based on HPLC analysis.

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Introduction

Today, fungal toxins have become a global problem based on the records that mention around 25% of the world cereal are contaminated with mycotoxins (Dhand, Joshi, & Jand, 1998). Among mycotoxins, aflatoxins are the most important because the diseases caused by ingesting aflatoxin

contaminated materials put forth a high risk for humans, livestock and poultry (Resnik *et al.*, 1996; Whitaker, Horwitz, Albert, & Nesheim, 1996). Aflatoxins belong to a group of compounds called furanocoumarin, which are secondary metabolites produced by certain fungi such as *Aspergillus flavus*, *Aspergillus*

parasiticus and *A. numius*. These toxins are found in various types of human foods and animal feeds including milk, cheese, peanut butter, corn, cottonseed, almonds, seasonings, figs, sorghum, and dry bread. Furthermore, eggs and meat products are sometimes contaminated with aflatoxins (Lizárraga-Paulín, Moreno-Martínez, & Miranda-Castro, 2011). Among various types of aflatoxins that have been identified so far, 4 different types of them namely B₁, B₂, G₁ and G₂ are more common, in which, B₁ has the highest toxicity in humans and animals (McLean & Dutton, 1995). Aflatoxin B₁ (AFB₁) is classified in class I as carcinogenic compounds in mammals; means exposure to this toxin leads to the development of tumors, especially in the liver (International Agency for Research on Cancer, 1992). The best and most effective way to prevent the formation of mycotoxins is to prevent the growth of fungi during planting, harvesting, storage, and transfer of plants (Doyle, Applebaum, Brackett, & Marth, 1982; Huwig, Freimund, Käppeli, & Dutler, 2001; Ramos & Hernandez, 1997). Despite the control of fungal growth in all of these stages, the incidence of infection with various fungi is unavoidable. As mycotoxin contaminations in foods are rising, it has led to an increased demand for methods to remove and eliminate toxicity (Kumar, Mahato, Kamle, Mohanta, & Kang, 2017). Among various physical and chemical methods for reduction of mycotoxins, the utilization of adsorbent compounds, which can adsorb toxins and excrete them from the gastrointestinal tract, is gaining much attention (Dixon, Kannevischer, Arvide, & Velazquez, 2008). Adsorbent compounds are mainly divided into the two groups, inorganic and organic compounds. Inorganic adsorbents include clay compounds such as aluminum silicate, sodium bentonite, potassium bentonite and zeolite which are generally called the hydrated sodium calcium alumina silicate (HSCAS) group (Dixon *et al.*, 2008). Organic adsorbents mainly include cell wall derived β -D-glucan from a wide

range of microorganisms (yeasts, molds, bacteria, fungi) as well as plants (Rahar, Swami, Nagpal, Nagpal, & Singh, 2011; Zhu, Du, Bian, & Xu, 2015). The main chain of β -D-glucan consists of β -D-glucopyranosyl units which are composed of glucose units bound by β (1-3) glycosidic bonds in the main chain and β (1-6) bonds at lateral chain points. This compound is widely distributed in the cell walls of plants (most cereals, oats and barley), yeasts (*Saccharomyces cerevisiae*, *S. fragilis*, *Candida tropicalis* and *C. utilis*), some bacteria (especially *Alcaligenes faecalis*, *Cellulomonas flavigen* and *bacillus*), algae and, in particular, fungi (Rahar *et al.*, 2011; Zhu *et al.*, 2015). The presence of β -glucan has been proven in the cell wall of some vegetables (carrots, soybeans and radishes) and fruits (banana) (Peumans *et al.*, 2000), it is also reported that β -D-glucan from different sources somehow show different biological effects (Ishibashi *et al.*, 2004) which are directly related to β -D-glucan chemical structures (types of bonds in the main backbone and branches as well as the number and length of lateral branches), solubility in water, extraction method and source of β -D-glucan. For instance Bueno, Casale, Pizzolitto, Salvano, & Oliver (2007) reported that AFB₁ was adsorbed by *S. cerevisiae* (Bueno *et al.*, 2007), but results of other studies demonstrated that the reduction efficiency of AFB₁ by various species of *S. cerevisiae* can be varied between 10 to 60% (Shetty & Jespersen, 2006). Some researchers believe that the adsorption ability of fungal toxins, such as AFB₁, is related to the β -glucan chemical structure (El-Naggar & Thabit, 2014). Accordingly, the best bioactive β -glucan is the one with specification of β (1-6) bonds in the lateral short chains, and β (1-3) bonds in the main chain (Synytsya *et al.*, 2009). Carbonero *et al.* (2012) believes that structurally, there is a similarity between β -glucan extracted from yeast and fungi, especially edible fungi whereas the chemical bounds of β -glucan extracted from these two sources in the main and lateral

branches are as β (1-3) and β (1-6) respectively. Edible fungi contain numerous compounds, including phenolic compounds, sterols, terpenoids etc. Among them, polysaccharides, and especially β -glucans, are known as a major group with medicinal properties (Ishibashi *et al.*, 2005; Kumar, Joo, Choi, Koo, & Chang, 2004). One of the most common types of edible macro-fungi is *Agaricus bisporus*, which is a white fungus commonly known as button mushroom. This species comprises one of the most economically important fungi in terms of world production, global market and consumption. This fungus is a valuable, healthy food containing polyphenols, vitamins, minerals and polysaccharides (Dubost, Ou, & Beelman, 2007; Tian *et al.*, 2012). Considering the worldwide production of this mushroom, their waste materials including, stem part, can be a valuable and inexpensive source for extraction of compounds with medicinal applications, including β -glucan. Achieving higher physicochemical properties of β -glucan is due to the chemical structure which is somehow related to the extraction methods. Therefore, an optimal extraction process is required to achieve higher efficiency and purity of β -glucan accompanying suitable functional properties (Vilkhu, Mawson, Simons, & Bates, 2008). Given the mentioned above points, this study was conducted to determine the best extraction method of β -glucan from fungal waste part which is stem cell walls of bottom mushroom (*A. bisporus*). It was also planned to find out the efficiency of each of the methods in the adsorption of aflatoxin based on high performance liquid chromatography (HPLC) analysis.

Materials and methods

The waste part of button mushroom whose stem was collected from SAYE RAS Farm (Mashhad, Iran). First they were washed and dried using a freeze dryer (Model Operon CO. FDO-8606, South Korea), then powdered with an electric mill (Model Pars Khazar, Iran). AFB₁ content was

determined by Waters 1525 HPLC (USA). All reagents and chemicals such as methanol, phosphate buffer and acetonitrile were provided from Merck (USA). Standard powder of AFB₁ and dialysis bag was obtained from Sigma (USA). Ethanol (96%) from Pars Alcohol (Iran) and, trichloroacetic acid (Carlo Erbai, Italy) was purchased.

Extraction methods of β -glucan

Hot water

In order to extract β -glucan with hot water, 2 g of dried powder were weighed and then stirred in 100 mL of distilled water for 3 h at 100 °C. After cooling, the solution was centrifuged at 5000 rpm for 20 min. Ethanol was added to the supernatant in a ratio of 4:1 (v/v), and then left to settle down for an overnight at 4 °C in order to precipitate polysaccharides including β -glucan. The precipitate was collected by centrifugation at 7000 rpm for 20 min. It was then dissolved in distilled water, dialyzed for 48 h at 4 °C in a dialysis bag (12 kDa meshes) in phosphate buffer in order to remove other small molecules such as monosaccharide. The purified precipitate was then lyophilized, weighed, and subsequently stored in moisture resistant containers (Jantaramanant, Sermwittayawong, Noipha, Hutadilok-Towatana, & Wititsuwannakul, 2014).

Acid-based

Acid-based extraction was accomplished according to the method of Szwengiel & Stachowiak (2016) with slightly modification. First, 100 mL of pure methanol was added to 2 g of mushroom powder; the mixture was stirred at 500 rpm and 60 °C for 18 h. After cooling, the solution was centrifuged at 5000 rpm for 15 min. The precipitate was dried in an oven at 46 °C to remove alcohol. The dried precipitates were then poured into a container containing 50 mL of hydrochloric acid (3.8%); the mixture was stirred at 1000 rpm and 30 °C for 5 h. The pH of sample was adjusted to 7 using

NaOH (5N); again centrifugation at 5000 rpm was done for 15 min. Next, the supernatant was mixed with ethanol in a ratio of 1:4 (v/v), and the final solution was allowed to settle down for an overnight at 4 °C. The precipitate was collected after centrifugation at 7000 rpm for 20 min. It was then dissolved in distilled water, lyophilized, weighed, and finally stored in moisture resistant containers (Szwengiel & Stachowiak, 2016).

Hot alkaline

In this method, 2 g of mushroom powder was added to the 100 mL of pure methanol; the mixture was stirred at 500 rpm and 60 °C for 2 h. The obtained solution was filtered through a whatman No. 1 filter paper. The separated material then left to dry at room temperature for 24 h. After drying, the sediment was added into 100 mL of distilled water; the mixture was then stirred at 400 rpm and 25 °C for 24 h. After separation of solid by using filter paper, it was added again into a container containing 100 mL of distilled water; this mixture was stirred at 150 rpm and 100 °C for 24 h. The filter paper separated sediments, were dissolved in 1 M NaOH by stirring at 200 rpm and 100 °C for 24 h. Centrifugation was then carried out at 7000 rpm for 15 min in order to precipitation of sediments. Next, 100 mL of the supernatant solution was dissolved in 10 g of trichloroacetic acid (10% w/v) by stirring at 100 rpm and 4 °C for 4 h. The solution was then centrifuged at 5000 rpm for 15 min, and the supernatant was mixed with the same volume of 96% ethanol. The final solution was allowed to stand overnight at 4 °C. Finally, centrifugation was carried out at 7000 rpm for 15 min, and the pellet were isolated and dried using freeze-drier afterward (Palacios, García-Lafuente, Guillamón, & Villares, 2012).

Measurement of extraction efficiency

The efficiency of extraction for each method was calculated according to the

following equation:

$$\text{Extraction efficiency} = \frac{\text{dried weight of extracted } \beta\text{-glucan (g)}}{\text{consumed powder of mushroom (g)}} \times 100 \quad (1)$$

Sample treatment

Standard solution of AFB₁ was prepared at the concentration of 1 ng/mL. It was prepared by dilution of the stock solution of AFB₁ with 80% methanol. In order to prepare aflatoxin solution with 1 ng/mL concentration, 250 μL of standard AFB₁ with 100 ng/mL concentration, was diluted to 25 mL with 80% methanol (HPLC grade). In this study to prepare contaminated samples, the protocol from Di Natale, Gallo, & Nigro (2009) was followed. To determine adsorption efficiency of extracted β-glucan, 3 samples were prepared. Each sample contained 0.05 g β-glucan from each extraction method contaminated with aflatoxin B₁ in a final volume of 5 mL solution. The concentration of AFB₁ was 1 ng/mL in each sample. The samples were then incubated at room temperature for 30 min on a shaker at 80 rpm and then centrifuged for 5 min at 3500 rpm (Di Natale *et al.*, 2009).

HPLC analysis

The supernatant was injected on HPLC to determine the remaining AFB₁ in the sample and then compared with standard. An isocratic elution containing water: methanol: acetonitrile (60:20:20% v/v) was used as the mobile phase. Fluorescence detector was also used at the wavelength of 365 and 445 nm for excitation and emission wavelength respectively. The chromatographic column was C₁₈ (250×4.6 mm, 5μm). During the analysis, the flow rate was kept at 1 mL/min, and the column temperature was maintained at 40 °C. For the derivatization of aflatoxin, a photochemical derivatization was used. The injection volume was 20 μL, and all procedures were performed in 3 replications (Iranian National

Standardization Organization [ISIRI], No. 6872, 2012).

Statistical analysis

In this study all the experiments were carried out in 3 replicates. The data were statistically analyzed based on randomized complete design (RCD) and then to determine the superior treatment, the Duncan mean comparison was used.

Results and discussion

To date, there is no scientific report that shows the optimum type of extraction of β -glucan from waste part of button mushroom and also its ability to adsorb AFB₁ in liquid conditions. The results of this study can clarify, which β -glucan extraction method has the higher efficiency among evaluated methods and also determine which method has higher ability to adsorb and discard AFB₁ from contaminated samples.

Calculation of extracted β -glucan

The efficiency of β -glucan production in each extraction method is presented in Table (1).

Table 1. Average β -glucan yield efficiency based on extraction method

Extraction method	Efficiency (%)
Hot water	3.0 ^b
Acid-based	20.5 ^a
Hot alkaline	7.0 ^b

Numbers with the same letters indicate insignificance ($P < 0.05$).

According to the obtained results shown in Table (1), the highest percentage of β -glucan yield was related to the acid-based extraction method (20.5%), which is significantly higher than hot water and hot alkaline extraction methods which are 3 and 7% respectively. Although there is no

report to compare all 3 extraction methods in a specific source, in some studies at least two methods have been compared together. Synytsya *et al.* (2009) had a comparison between acid-based and hot water for extraction of β -glucan from *A. bisporus*. They found that the yield of extraction in acid-based was 7 times higher than hot water (Synytsya *et al.*, 2009). This result confirms the findings in the present study. Despite the fact that hot water for extraction of β -glucan is stated as the most common, easier and cost-effective method (Ahmad, Anjum, Zahoor, Nawaz, & Din, 2009; Jantaramanant *et al.*, 2014), based on the results of this study, it is concluded that hot water has the lowest efficiency in β -glucan production (3%).

Calculation of the amount of aflatoxin B₁ adsorption

The amount of aflatoxin adsorption by extracted β -glucan from each method is presented in Table (2).

HPLC chromatograms of Aflatoxin B₁ for standard and contaminated sample treated with hot alkaline extracted β -glucan, was shown in Figs. (1) and (2) respectively. The obtained results in this study demonstrated that although β -glucan extracted from all methods could adsorb AFB₁, the adsorption efficiency are significantly ($P < 0.05$) different among these extracted methods, so that the lowest one, related to acid-based (5%), then after hot water (14%) and the highest attributed to the hot alkaline with 90.2% adsorption and elimination of aflatoxin B₁ in a liquid condition (Table 2). Accordingly, the optimal extraction method in terms of yield and aflatoxin adsorption can be attributed to the acid-based and hot alkaline method with the efficiency of 25 and 90.2% respectively.

Table 2. Percentage of adsorbed aflatoxin in different extraction methods

Extraction Method	Amount of AFB ₁ added to the sample (ng/mL)	Remaining amount of AFB ₁ after treatment (ng/mL)	Percentage of adsorbed AFB ₁
Hot water	1	0.860 ^b ±0.013	14
Acid-based	1	0.950 ^a ±0.010	5
Hot alkaline	1	0.098 ^c ±0.000	90.2

Numbers with the same letters indicate insignificance ($P < 0.05$).

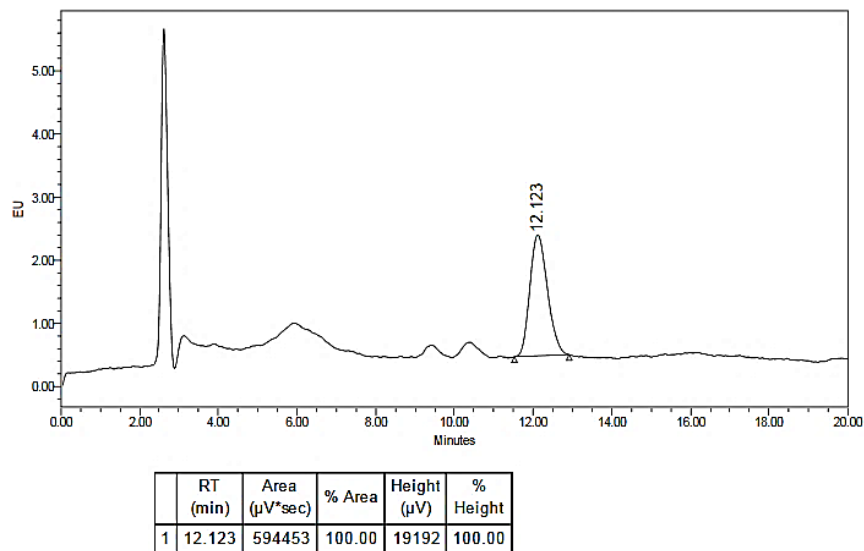


Fig. 1. HPLC chromatogram of standard solution of AFB₁ with a concentration of 1 ng/mL

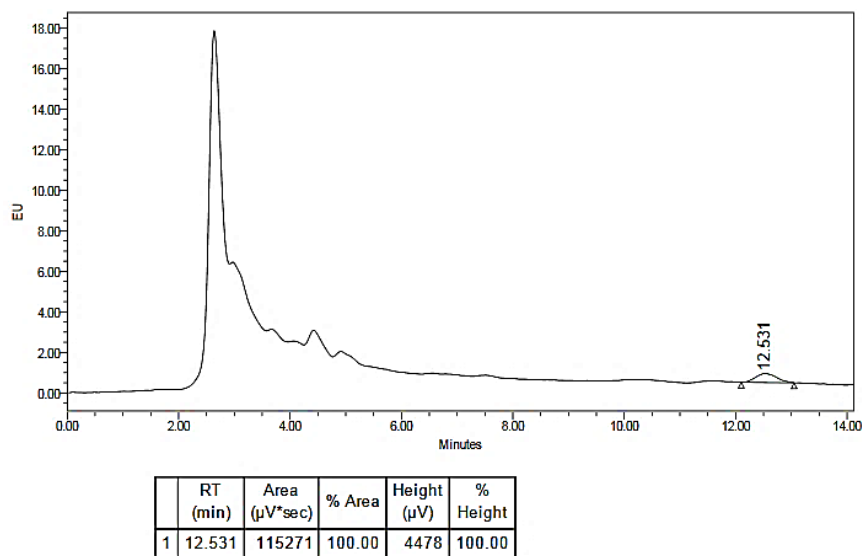


Fig. 2. HPLC chromatogram of aflatoxin B₁ in contaminated sample after treatment with hot alkaline extracted β -glucan

The results of this study also revealed that the highest ability to adsorb AFB₁ was achieved by β -glucan extracted through hot alkaline method (90.2%), which followed the protocol described by Palacios *et al.* (2012). As above mentioned the source and method of β -glucan extraction can affect chemical structure and, consequently, biological activity of β -glucan such as adsorption of aflatoxin B₁. For example, Palacios *et al.* (2012) used hot alkaline and hot water extraction methods to extract β -glucan from edible fungi. In that study, the GC-MS and H-NMR results showed that the sample extracted with hot water,

contained linear glucose units linked β (1-4) bonds with no branches, while the sample extracted through hot alkaline, contained glucose units linked by β (1-3) bonds in the main chain and β (1-6) bonds at lateral branching point. In case of acid-based extraction method, Szwengiel & Stachowiak (2016) demonstrated that the obtained β -glucan from edible fungi has higher yield efficiency as compared to the hot water, but contains protein impurities which can have effects on β -glucan functionalities. According to the explanations given earlier, it is clear that the ability of AFB₁ adsorption is related to

β -glucan that contains glucose units with the β (1-3) bonds in the main chain and β (1-6) bonds at the branching point. In this study the priority of hot alkaline derived β -glucan to adsorb AFB₁, can be attributed to the specific chemical structure of β -glucan, in which the length of lateral branches is shorter and the number is higher as compared to the other extracted β -glucans (Elaine R Carbonero *et al.*, 2012).

Conclusions

In this research, for the first time, β -glucan was extracted from waste materials produced from button mushroom (*Agaricus bisporus*). *A. bisporus* is a fungal source with the worldwide production and consequently huge amount of stem parts as waste materials. The results of this study demonstrated that although the highest extraction efficiency was related to acid extraction (25%), the highest rate of toxin adsorption belongs to

the sample extracted through the hot alkaline method (90.2%). The present study reconfirmed that method of extraction has a major effect on the molecular structure and biological properties of β -glucan. On another note, β -glucan from edible fungi should be evaluated to be used as an AFB₁ adsorption agent, instead of yeast β -glucan. Fungal β -glucan is suitable for use in the food industry as it has additional benefits including antiviral, anti-bacterial and anti-allergic properties, besides having the ability to stimulate and modulate the immune system. As above mentioned, the highest percentage of toxin adsorption by *S. cerevisiae* yeast had previously been reported to be 60%, while a rate of 90.2% was obtained in the present study. On the other hand, the use of live yeast in food products as an adsorption agent may lead to undesirable reactions.

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بازدهی استخراج بتا-دی-گلوکان از ضایعات قارچ دکمه‌ای (آگاریکوس بیسپوروس) و توانایی آن در جذب آفلاتوکسین B₁

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چکیده

بتاگلوکان‌ها در گستره وسیعی از موجودات مختلف از جمله مخمر، قارچ، باکتری، جلبک، جو و جو دوسر یافت می‌شود و اثرات بیولوژیکی متنوعی را از خود نشان می‌دهند. بتاگلوکان‌ها از پیوند بتاگلوکوزیدی واحدهای D-گلوکز به یکدیگر تشکیل شده‌اند. توانایی جذب سموم قارچی از جمله آفلاتوکسین‌ها توسط بتاگلوکان در سال‌های اخیر بسیار مورد توجه قرار گرفته است. آفلاتوکسین‌ها گروهی از ترکیبات سمی با قدرت سرطان‌زایی بالا هستند که به‌عنوان یک عامل آلوده‌کننده مواد غذایی برای انسان و حیوان، شناخته می‌شوند. آفلاتوکسین B₁ سمی‌ترین ترکیب در ایجاد سرطان در بین انواع آفلاتوکسین‌هاست. توانایی جذب سموم قارچی توسط بتاگلوکان به‌طور مستقیم به عواملی مانند ساختمان مولکولی، روش استخراج و منبع حاوی بتاگلوکان بستگی دارد. بتاگلوکان مستخرج از منبع قارچی دارای اتصالات بتا (۱-۳) در رشته اصلی و بتا (۱-۶) در رشته جانبی و در محل انشعاب‌هاست. وجود انشعاب‌های زیاد با طول کوتاه با ساختاری شانه‌مانند، سبب افزایش جذب سموم قارچی توسط این ترکیب می‌شود. در تحقیق حاضر، برای اولین بار میزان بازدهی چندین روش استخراج بتاگلوکان از دیواره سلولی ساقه‌های دورریز قارچ دکمه‌ای (آگاریکوس بیسپوروس) اندازه‌گیری و توانایی جذب آفلاتوکسین B₁ نیز سنجیده شد. نتایج نشان داد علی‌رغم اینکه میزان بازدهی استخراج بتاگلوکان به روش اسیدی بیشتر از سایر روش‌هاست (۲۰/۵ درصد)، اما براساس سنجش به روش HPLC، بتاگلوکان استخراج‌شده با استفاده از قلیای داغ، توانایی جذب و خارج کردن ۹۰/۲ درصد آفلاتوکسین B₁ را از نمونه آلوده‌شده را دارد.

واژه‌های کلیدی: آفلاتوکسین B₁، آگاریکوس بیسپوروس، بتا-دی-گلوکان، روش استخراج، ساقه‌های دورریز