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Quantification of β-1, 3-D-glucan in two wild edible mushrooms collected from Andhra Pradesh, India

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ABSTRACT

Mushroom derived β-glucan polysaccharides are known for their activity as immunomodulators and anti-carcinogenic agents. In the present work, specific colorimetric method for β-1,3-D-glucan quantification was used. This method is based on interaction of a triple helix tertiary structure of β-1,3-D-glucan with Congo red dye and was detected by bathochromic shift from 488 to 512nm (>20nm) in UV-VIS spectrophotometer. Two different methods (method-1 and method-2) were used to extract the polysaccharide in various fractions of mushroom samples. The results showed that the total β-1,3-D-glucan content was found to be more in both the mushrooms using extraction method-2. *Termitomyces heimii* was found to possess high amount of total β-1,3-D-glucan (43.55mg/g dry weight) compared to *Podaxis pistillaris* (9.89mg/g dry weight).

Keywords: β-1,3-D-glucan, congo red, mushrooms, triple helix, immunomodulators

β-glucan is a cell wall structural polysaccharide, the best known mushroom-derived substance with potent antitumor and immunomodulatory properties (Ooi and Liu, 2000; Wasser, 2002; Moradali *et al.,* 2007; Zhang *et al.,* 2007). It plays a key role in some health promoting properties, such as enhancement of immune function and reduction in blood cholesterol and blood glucose levels (Sharma and Annepu, 2017). (1→3)-β-D-glucans polysaccharide from mushrooms have the ability to augment the immune system in multiple ways and are often considered pharmacologically as Biological Response Modifiers (BMRs) (Bohn and Bemiller, 1995; Ooi and Liu, 2000; Wasser, 2002; Lull *et al.,* 2005). Mushroom derived (1→3)-β-glucan polysaccharide, generally exists in a triple helical conformation which forms a complex with Congo red in dilute NaOH solution. The triple helix confirmation behavior of the isolated (1→3)-β-glucan has been evaluated by the bathochromic shift (20nm) in the visible absorption spectra of the complexes formed with Congo red whereas other conformations (α-glucan and α-galactan) would not show any spectral shift (Palacios *et al.,* 2012; Ana Villares, 2013; Semedo *et al.,* 2015).

Most β-1,3-glucan research is limited to antitumor mushrooms such as *Lentinula edodes*, *Schizophyllum commune*, *Grifola frondosa*, etc. Their β-D-glucans are already well analyzed and identified while other mushrooms have not been analyzed and little is known about their β-D-glucan content (Gupta *et al.,* 2018). Therefore, there is a need for reliable quantitative measurement of (1→3)-β-glucan with helical conformation in other wild edible mushrooms. In the present investigation, amount of (1→3)-β-glucan was measured in different

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polysaccharide fractions of two wild edible mushrooms viz., *Termitomyces heimii* and *Podaxis pistillaris* collected from Andhra Pradesh, India.

MATERIALS AND METHODS

Sample collection

The fruiting bodies of two wild edible mushrooms *viz., Podaxis pistillaris* (L. ex Pers) Fr. and *Termitomyces heimii,* Natarajan were collected from the College Campus of Sri Sathya Sai Institute of Higher Learning at Anantapur, which is the semi-arid region of Andhra Pradesh State in India. The fruiting bodies of mushrooms were collected after the rainy season. The sporophores were cleaned, sliced into thin pieces and air-dried in an oven at 40°C. Dried mushrooms were ground to a fine homogeneous powder using mortar and pestle and then stored in amber colored glass bottle at room temperature.

Polysaccharide extraction

Method-1

Polysaccharides were isolated from mushrooms according to a method suggested by Ana Villares (2013) with minor modifications. Fat free dried mushroom powder of 1g was taken and methanol (80%) was added, sonicated and kept on shaker for overnight at 25°C. Mixture was centrifuged and supernatant was collected as methanolic fraction (MF). The residual pellet was re-extracted cold water in a same way as it was done with methanol, i.e., sonicated, stirred for 24 hrs, and then centrifuged and the collected supernatant was referred as cold water fraction (CWF). The residue obtained was re-extracted in hot water at 80°C, sonicated and centrifuged. The supernatant was collected and labeled as hot water fraction (HWF) while the residue obtained was subjected to alkali extraction (3% NaOH at 55°C for 3hrs) and centrifuged. Obtained supernatant was labeled as alkali soluble fraction (AKSF) and the left pellet in the centrifuge tube as alkali insoluble polysaccharide (AKI-P). Supernatant (AKSF) was neutralized and centrifuged at 20000*g* at 4°C. The pellet obtained was called alkali soluble-acid insoluble polysaccharide (AKS/I) fraction whereas supernatant collected was alkali soluble-acid soluble fraction to which methanol was added for precipitation. Mixture was centrifuged at 20000*g* at 4°C for 30min. The pellet obtained was called alkali soluble-acid soluble methanol pellet (AKS/S-MP) and was weighed and stored while the supernatant was called alkali soluble-acid soluble methanolic fraction (AKS/S-MF) and stored at 4°C. The pellet collected as AKI fraction was neutralized. The obtained pellet was washed with ddH_2O and called as alkali insoluble polysaccharide (AKIS), weighed and store at 4°C until used.

All together following seven fractions were obtained by this method and were assayed for β-1,3-D-glucan: F_1- methanolic fraction (MF), F_2- cold water fraction (CWF), F_3- hot water fraction (HWF), F_4- alkali soluble-acid soluble methanolic fraction (AKS/S-MF), F_5- alkali soluble-acid soluble methanol pellet (AKS/S-MP), F_6- alkali soluble-acid insoluble polysaccharide (AKS/I) and F_7- alkali insoluble polysaccharide (AKIS).

Method-2 (alkali extraction)

β-1,3-D-glucan from mushrooms were isolated using modified method of Magnelli *et al.* (2002). Mushroom powder (1g) was taken in a conical flask, 30ml of 3% NaOH was added and mixture was sonicated at 55°C and centrifuged at 10,000 *g* at 4°C. The supernatant was rendering the alkali soluble fraction, AKS and the pellet corresponds to the alkali insoluble residue, AKI. Both the fractions were processed further in the similar way as in method-1. All together following four fractions were obtained by this method and were assayed for β-1,3-D-glucan; alkali soluble-acid soluble methanolic fraction (AKS/S-MF); alkali soluble-acid soluble methanol pellet (AKS/S-MP); alkali soluble-acid insoluble polysaccharide (AKS/I); alkali insoluble polysaccharide (AKIS).

Determination of β-1,3-D-glucan

The colorimetric assay method to determine (1→3)-β-D-glucan was based on the specific interaction of β-D-glucan with Congo red dye to form a complex. The absorption maximum of Congo red solution (493nm) was shifted to longer wavelength upto 512nm on succeeding addition of the β-1,3-D-glucan (Wood, 1980; Ogawa *et al.,* 1972; Ogawa *et al.,* 1993). In the present study, β-1,3-D-glucan was determined by using modified method of Semedo *et al.,* (2015) and Nitschke *et al.* (2011). All absorbance measurements were carried out in triplicate which means that there were independent replicate for each concentration of the standard as well as for each mushroom sample.

The amount of β-1,3-D-glucan present in the mushroom samples was quantified by the calibration curve using 1-40 μg/ml range of β-1,3-D-glucan from barley as the standard. Calibration graph was prepared by plotting absorbance against concentration (μg/ml) of standard β-1,3-D-glucan (Fig 2). The amount of β-glucan in samples was calculated using the following equation obtained from the graph and expressed as μg/g of mushroom on a dry weight basis.

$$\text{Absorbance} = 0.005394 * \beta\text{-1,3-D-glucan} + 0.8177 \quad (R^2 = 0.97)$$

Statistical analysis

Each sample was assayed with three replicates and all the assays were performed in triplicate. The results were expressed as mean ± standard deviation (n=3). Graphs and calibration curves were prepared using trial version of GraphPad Prism8 software.

RESULTS AND DISCUSSION

In the present study two extraction methods were optimized in order to obtain β-1,3-D-glucan PS from the dried fruiting bodies of *Podaxis pistillaris* and *Termitomyces heimii*. From each of the mushroom samples, seven polysaccharide fractions were collected using method-1 and four fractions using method-2. Amount of fractions in liquid and pellet forms was measured and used for quantification of β-1,3-D-glucan. Yield of each polysaccharide fraction is measured and given in table 1 using method-1and in table 4 using method-2.

Polysaccharide content

Polysaccharide was measured using all the solid fractions. The results showed that polysaccharide obtained in various fractions using method-2 was relatively higher than the method-1 (Table 3). Total polysaccharide yield was found more in *Podaxis pistillaris* (49.10%; 64.44%) as compared to *Termitomyces heimii* (34.68%; 45.28%). Significant amount of PS was obtained from AKIS (alkali insoluble polysaccharide) fraction from both the mushrooms using both the extraction methods (Table 3). Dong and Yao, (2008) reported high yields of polysaccharides from both the natural and cultivated mycelia of *Cordyceps sinensis* (30.46% and 39.11%, respectively) whereas Thetsrimuang *et al.* (2011)

Table 1. Yield of polysaccharides in different fractions of mushrooms using method-I

Mushrooms	Extraction yield[a] (ml or g biomass) in various fractions[b]							
	F_1 (ml)	F_2 (ml)	F_3 (ml)	F_4 (ml)	F_5 (g)	F_6 (g)	F_7 (g)	Total (g)
Podaxis pistillaris	26	29.5	25.5	>50	0.10	0.19	2.69	2.98
Termitomyces heimii	26	29.5	25.5	>50	0.06	0.21	3.34	3.61

[a] Each value is expressed as mean (*n* = 3).
[b]Fractions: F_1 - methanol fraction (MF); F_2 - cold water fraction (CWF); F_3 - hot water fraction (HWF) F_4 - alkali soluble-acid soluble methanolic fraction (AKS/S-MF); F_5 - alkali soluble-acid soluble methanol pellet (AKS/S-MP); F_6 - alkali soluble-acid insoluble polysaccharide (AKS/I) and F_7 - alkali insoluble polysaccharide (AKIS)

Table 2. Determination of β-(1→3)-D-glucan in fractions of mushrooms using congo red method

Mushrooms	β-(1→3)-D-glucan[a] (mg/g dry weight) in seven fractions[b]							
	F_1	F_2	F_3	F_4	F_5	F_6	F_7	Total
Podaxis pistillaris	0	0.260±0.49	0	0	1.25±1.95	0	0	01.51
Termitomyces heimii	0	0	0	0	10.01±0.94	0	0	10.01

[a] Each value is expressed as mean ± standard deviation ($n = 3$).
[b]Fractions: F_1 - methanol fraction (MF); F_2 - cold water fraction (CWF); F_3 - hot water fraction (HWF) F_4 - alkali soluble-acid soluble methanolic fraction (AKS/S-MF); F_5 - alkali soluble-acid soluble methanol pellet (AKS/S-MP); F_6 - alkali soluble-acid insoluble polysaccharide (AKS/I) and F_7 - alkali insoluble polysaccharide (AKIS)

Table 3. Total polysaccharides in different fractions of mushrooms using method-1 and method-2

	Polysaccharide yield[a] (%) in various fractions[b]							
	Method-1				Method-2			
Mushrooms	AKS/S-MP	AKS/I	AKIS	Total	AKS/S-MP	AKS/I	AKIS	Total
Podaxis pistillaris	1.65	3.13	44.32	49.10	4.74	4.53	55.17	64.44
Termitomyces heimii	0.58	2.02	32.08	34.68	5.18	3.25	36.85	45.28

[a] Each value is expressed as mean ($n = 3$).
[b]Fractions: AKS/S-MP - alkali soluble-acid soluble methanol pellet; AKS/I - alkali soluble-acid insoluble polysaccharide and AKIS - alkali insoluble polysaccharide

Table 4. Yield of polysaccharides in different fractions of mushrooms using method-2

Mushrooms	Extraction yield[a] (ml or g) in various fractions[b]				
	AKS/S-MF(ml)	AKS/S-MP(g)	AKS/I(g)	AKIS(g)	Total
Podaxis pistillaris	>50	0.288	0.275	3.349	3.912
Termitomyces heimii	>50	0.539	0.338	3.836	4.713

[a] Each value is expressed as mean ($n = 3$).
[b]Fractions: AKS/S-MF - alkali soluble-acid soluble methanolic fraction; AKS/S-MP - alkali soluble-acid soluble methanol pellet; AKS/I - alkali soluble-acid insoluble polysaccharide and AKIS - alkali insoluble polysaccharide

Table 5. Determination of β-(1→3)-D-glucan in fractions of mushrooms using congo red method

Mushrooms	β-(1→3)-D-glucan[a] (mg/g dry weight) in various fractions[b]				
	AKS/S-MF(ml)	AKS/S-MP(g)	AKS/I(g)	AKIS(g)	Total
AKS/S-MF	**AKS/S-MP**	**AKS/I**	**AKIS**	**Total**	
Podaxis pistillaris	0	09.89±0.95	0	0	09.89
Termitomyces heimii	0	43.55±0.91	0	0	43.55

[a] Each value is expressed as mean ± standard deviation ($n = 3$).
[b] Fractions: AKS/S-MF - alkali soluble-acid soluble methanolic fraction; AKS/S-MP - alkali soluble-acid soluble methanol pellet; AKS/I - alkali soluble-acid insoluble polysaccharide and AKIS - alkali insoluble polysaccharide

extracted 5.9% (mycelia), 5.7% (dried fruiting bodies) and 5.2% (fresh fruiting bodies) crude PS from *Lentinus polychrous.* Similarly, total PS content was measured in hot water fractions of *Agaricus bisporus* (74.4%), *A. brasiliensis* (45.9%), *Ganoderma lucidum* (27.6%) and *Phellinus linteus* (62.6%) by Kozarski *et al.* (2011). Palacios *et al.* (2012) had also reported 2% (cold fraction), 1.5% (hot fraction) and 4.5% (alkali fraction) polysaccharide yield from fruiting bodies of *Pleurotus ostreatus*. This difference in PS content in mushrooms fruiting body or in natural or cultivated mycelium depends on the species and extraction methods used.

Polysaccharides (PS) are the major component found more than 80% of the fungal cell wall. Various PS fractions have been isolated from fruiting bodies and mycelia of many mushroom species of higher Basidiomycetes which have been extensively studied for its uses as therapeutic adjuvant or dietary supplements for cancer treatment (Borchers *et al.,* 2004; Zekovic *et al.,* 2005; Sullivan *et al.,* 2006). In the recent past, pure β-glucans and β-glucans protein complex PS are very popular nutraceuticals in Japan, China and other Oriental regions (Mizuno *et al.,* 1995; Ooi and Liu, 1999; 2000) and have been approved for the clinical treatment of cancer patients.

Absorption spectroscopy of (1→3)-β-D glucan-congo red complex

β-D-glucan polysaccharide displays single helix, triple helix and random coil conformations. It was reported that triple-helix conformer (1→3)-β-D-glucans are usually more stable than the single coil conformer and are potent immunomodulators and anticarcinogenic agents (Falch *et al.,* 2000). In the present work, the specific interaction of triple helical β-D-glucan (from barley) with Congo red was detected by bathochromic shift from 488 to 512nm (>20nm) in the visible absorption maximum of Congo red. Absorption spectra (300-650nm) were carried out of 50 μM Congo red complex with β-1,3-D-glucan solution in the range of 1-40 μg/ml. The spectra were also obtained for all the alkali soluble-acid soluble methanol pellet (AKS/S-MP) fractions of both the mushroom which all exhibited a bathochromic shift (Fig.1). Similar absorption spectra were obtained by Nitschke *et al.* (2011) reporting the bathochromic shift of 0.08% Congo red when interacted with schizophyllan solutions in a concentration of 50-150 μg/ml. Semedo *et al.* (2015) reported that 224 μM Congo red when interacted with β-1,3-D-glucan solutions in a range of 25-70 μg/ml also resulted in bathochromic shift from 488 to 516 nm. Similar reports

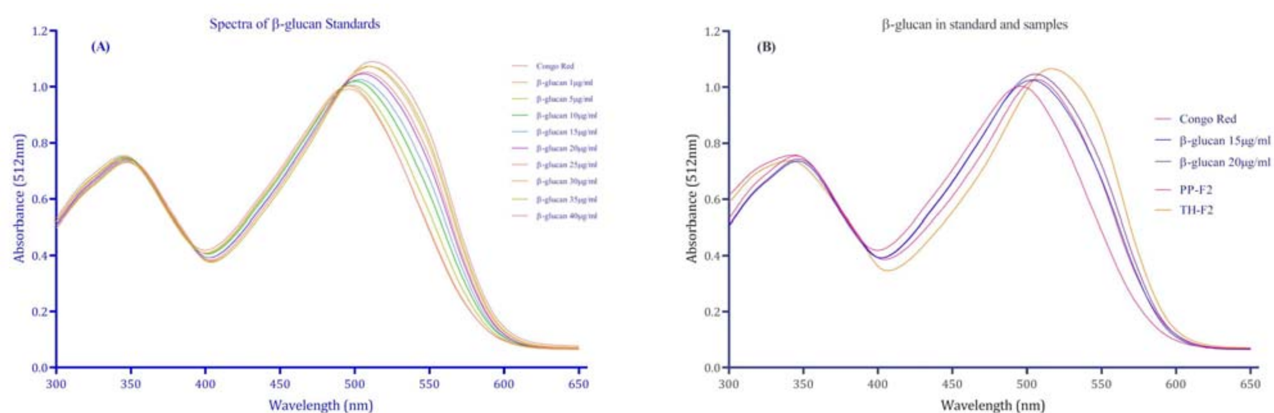

Fig. 1. Absorption spectra of (A) Congo red alone and with β-(1→3)-D-glucan in a range of 1-40 μg/ml (B) Congo red alone and with AKS/S-MP fraction of *Podaxis pistillaris* and *Termitomyces heimii* in the range of 300-650nm.

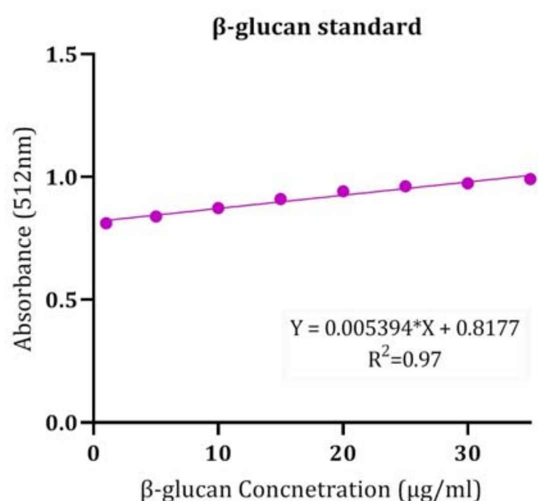

Fig. 2. Calibration curve of standard β-(1→3)-D-glucan from barley in the range of 1-40 μg/ml at 512nm

of bathochromic shift due to Congo red and triple helical β-1,3-D-glucan complex were given by Palacios *et al.* (2012); Ogawa *et al.* (1972) and Wood, (1980).

Total β-1,3-D-glucan

In the present investigation triple-helical β-1,3-D-glucan in the mushroom sample was measured using specific colorimeter method using Congo red. The β-1,3-D-glucan in each fraction, extracted by both the methods was determined using linear regression equation obtained from the calibration curve of standard β-1,3-D-glucan using Congo red method (Fig 2). The amount of total β-1,3-D-glucan is given in table 2 and table 5. Out of seven fractions collected using method-1, β-1,3-D-glucan was found only in two fractions *i.e.,* F-2- cold water fraction (CWF) and F5 - alkali soluble-acid soluble methanol pellet (AKS/S-MP) of *Podaxis pistillaris* where as only one fraction of *Termitomyces heimii*, F5 - alkali soluble-acid soluble methanol pellet (AKS/S-MP) has shown β-1,3-D-glucan. Total β-1,3-D-glucan obtained from *Termitomyces heimii* was 10.01mg/g DW which was found to be higher than *Podaxis pistillaris* 1.51mg/g DW (Table 2). Similarly, using alkali extraction (Method-2), β-1,3-D-glucan was obtained only in one of the fractions in both the mushrooms. This fraction was alkali soluble-acid soluble methanol pellet (AKS/S-MP) which represents the total content of β-1,3-glucan in the samples (Table 5). In the present investigation, *Termitomyces heimii* showed significantly higher amount of total β-1,3-D-glucan (43.55 mg/g DW) as compared to *Podaxis pistillaris* (9.89 mg/g DW).

When both the methods were compared for total content of β-1,3-D-glucan in mushroom samples, method-2 (alkali extraction) was found be better than extraction method-1. The results revealed that total β-1,3-D-glucan in *Termitomyces heimii* using method-2 is 43.55 mg/g which is significantly higher than 10.01 mg/g obtained using method-1. The amount of β-1,3-D-glucan reported by Semedo *et al.* (2015) using the similar assay in different fractions had shown relatively less amount of β-1,3-D-glucan in the range of 1.17 x 10^{-2} to 8.95 x 10^{-1} mg/g in young fruiting bodies, stipe and mycelia of their 10 mushroom samples compared to presently investigated mushrooms from Andhra Pradesh. Nitschke *et al.* (2011) also reported total β-1,3-1,6-glucan content (0.41-12.91 g/100g DW) using Congo red method in the mycelia and fruiting bodies of mushrooms.

The antitumor and immuno pharmacological activities of β-1,3-D-glucan depends on its helical confirmation. Lentinan, shizophyllan and glucan moieties of PSK all have triple-helix structure. According to Falch *et al.* (2000), Pachyman, (1→3)-β-glucan from *Poria cocos* is a single-helix conformer and is biologically inactive against tumor growth but the cytokine stimulation activity of (1→3)-β-glucan was found to be related to the triple helix conformation.

CONCLUSION

The results of the current study are the first information on quantification of triple helical β-1,3-D-glucan content using Congo red dye colorimetric assay in wild edible mushrooms from Andhra Pradesh of India. The colorimetric assay revealed that alkali

soluble-acid soluble methanol pellet fraction (AKS/S-MP) extracted from both the methods have good amount of β-1,3-D-glucan. These wild edible mushrooms are good source of β-1,3-D-glucan. *Termitomyces heimii* evaluated with high amount of total β-1,3-D-glucan (43.55 mg/g dry weight) compared to *Podaxis pistillaris* (9.89 mg/g dry weight) using alkali extraction method. Since the triple-helical β-1,3-D-glucan from many mushrooms with or without bound protein are known for antitumor and immunomodulatory activities, isolated β-1,3-D-glucan from these mushrooms could be the best candidate for antitumor drugs.

The authors declare that there are no conflicts of interest.

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