Isolation and Antifungal Activity of Plant Lectins against some Plant Pathogenic Fungi

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ABSTRACT

Two lectins from seeds of white kidney bean (*Phaseoulus vulgaries)* and Soybean (*Glycine max)* were isolated and purified from saline crude extracts of seeds using ammonium sulfate precipitation. The two pure lectins were tested *in vitro* against two plant pathogenic fungi, *Fusarium oxysporum* and *Rhizoctonia solani*. Mycelial growth inhibition technique was performed in accordance with standard protocols to evaluate antifungal activity. Mycelial of both tested fungi were inhibited by the lectins extracts. Results revealed that, the lectin from *Phaseoulus vulgaries* (LP lectin) had a higher antifungal activity against *F. oxysporum* (EC_{50} = 5058, 4872 and 3617 mg/L, after 48, 72 and 120 h, respectively). Inaddition, the antifungal activity of LP lectin against *R. solani* was higher than lectin from *Glycine max* (LG lectin) with EC_{50} values 6786, 6646 and 5465 mg/L after 48, 72 and 120 h, respectively,

Keywords: Lectin, *Phaseolus vulgaris, Glycine max,* Antifungal activity, *Fusarium oxysporum, Rhizoctonia solani*.

INTRODUCTION

Agricultural losses are a challenging economic and food security problem. Global food security is threatened by population growth and the emergence and spread of crop pests, which are significantly increasing with climate change (Bebber *et al.*, 2013).

The search for new compounds with antifungal activity is accelerating due to increase fungal resistance to commonly prescribed fungicides. Among the molecules being investigated, plant lectins can be highlighted. Lectins are carbohydrate-binding proteins, which are highly variable in their amino acid sequences, and with different functions, structures, tissue localizations and carbohydrate-binding specificities. They bind reversibly to specific carbohydrates present on the opposing cells, which are responsible for their ability to agglutinate red blood cells, lymphocytes, fibroblasts. Lectins widely distributed in microorganisms, viruses, animals and higher plants, Plant lectins were classified into seven families according to their evolutionary and structural characteristics, one of which is the legume lectin family (Van Damme *et al.*, 1998). Lectins have been found in many plant groups, including mono- and dicotyledons, but most frequently, they have been detected in Leguminoseae and Euphorbiaceae. Lectins are distributed in various plant tissues. Many plants contain lectins, including different food crops such as wheat, rice, potato, tomato, soybean and bean. Lectin extraction is usually achieved using different methods of diffusion in aqueous solution and ammonium sulfate precipitation. Plant lectins play an important role in defense mechanisms against the attack of microorganisms (Ripoll *et al.*, 2003; S? *et al.*, 2009). These lectins have been demonstrated to inhibit the growth of several phytopathogenic fungi (Ang *et al.*, 2014).

The targets of some plant lectins are fungi that present chitin in their cell walls, resulting in inhibitory action on the growth and development of these microrganisms (Ciopraga *et al.*, 1999). A number of diverse physiological roles have been proposed for plant lectins, including, antifungal (Barrientos and Gronenborn, 2005), antiviral (Van Asbeck *et al.*, 2008) and anti-insect activities (Singh *et al.*, 2006). The *Phthirusa pyrifolia* lectin was an antifungal agent on *Fusarium lateritium* and *Rhizoctonia solani* but did not affect the growth of *Aspergillus niger*, *A. fumigatus*, *Rhizopus arrhizus*, *Paecilomyces variotti* and *F. moniliforme*. The effect of lectin on growth of *A. niger*, *F. oxysporum*, *F. moniliforme* and *Trichoderma viride* was investigated and the lectin was active only on *Fusarium* species. A stable, ion dependent and chitin-binding lectin isolated from *Opuntia ficus-indica* cladodes was able to affect the growth of *F. oxysporum* and *F. solani* (Santana *et al.*, 2009). The lectin isolated from *Ophiopogonjaponicus* rhizomes was an antifungal agent against the phytopathogens *Gibberella saubinetii* and *R. solan*i but not on *Penicillium italicum* (Tian *et al.*, 2007). The lectin of *Pisum sativum* seeds inhibited the growth of *F. oxysporum* and *Trichoderma viride* (Sitohy *et al.*, 2007).

The present study reports the purification, characterization and evaluation of antifungal potential of two plant lectins isolated from two plants, white kidney bean (*Phaseoulus vulgaries)* and Soybean (*Glycine max)* against plant pathogenic fungi, root rot disease *F. oxysporum* and *R. solani*.

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MATERIALS AND METHODS

Chemicals and reagents

Bovine serum albumin (BSA) and ammonium sulphate were purchased from Sigma-Aldrich Co. (USA). Potato Dextrose Agar (PDA) medium was purchased from Oxoid Ltd. (Basingstoke, Hampshire, UK).

Microorganisms

The two fungal species used in the bioassay for lectin antifungal activity were *Fusarium oxysporum* (Family: Nectriaceae; Class: Sordariomycetes) and *Rhizoctonia solani* (Family: Ceratobasidiaceae; Class: Agaricomycetes) which provided by Microbiology Laboratory, Department of Plant Pathology, Faculty of Agriculture, Alexandria University, Alexandria, Egypt, and kept during the experiments on PDA medium at 27 ± 2°C.

Extraction of *Phaseoulus vulgaries* seeds lectin

The *P. vulgaries* (white kidney bean) seeds were obtained from Legume Research Institute, Agricultural Research Centre, Ministry of Agricultural, Giza, Egypt, for isolation and purification of lectin. White kidney beans were ground to a powder in an electric mill and filtered through 80 mesh grid. The powder was mixed with 0.15 M NaCl (1:8, w/v) for 48 h at 4°C, and filtered through 80 mesh grid. Subsequently, the filtrate was centrifuged at 9168×g for 30 min and the supernatant was used for step of purification (Hou *et al*., 2010).

Purification of *P. vulgaries* seeds lectin

The supernatant was fractionally precipitated with ammonium sulfate 60% saturation, respectively. The pellets were combined, dissolved in a minimal volume of water, and dialyzed against distilled water at 4?C.

Extraction of *Glycine max* seeds lectin

The Soybean seeds were obtained from Legume Research Institute, Agricultural Research Centre, Ministry of Agricultural, Giza, Egypt, for isolation and purification of lectins. Soybean seeds were taken and grinded in a mixer for removal of seed coats and 50 g of uncoated seed were taken for the study. The uncoated seeds were soaked in phosphate buffer saline (PBS) for overnight. Then the seeds are grinded with minimum volume of PBS and the pastes were collected in 50 ml centrifuge tubes and were centrifuged at 4 C and 7500 rpm for 20 min and the supernatant was used for step of purification as salting out process (Bhol, 2012).

Purification of *G. max* seeds lectin (LG)

The supernatant was fractionally precipitated with ammonium sulfate at 60% saturation. The pellets were dissolved in a minimal volume of water and dialyzed against distilled water at 4 C.

Determination of Protein Concentration

Lowry *et al.* (1951) method was used for protein quantification, using bovine serum albumin (BSA) as the standard. The relative protein concentration of the eluted fractions was determined by measuring the absorbance at 690 nm.

Hemagglutinating Activity Assay

Hemagglutinating activity is the most commonly used assay for the detection of lectin in a sample due to the simplicity of implementation and ease visualization of agglutination. It defined as the reciprocal of the highest dilution of sample promoting full agglutination of erythrocytes (RBC). The hemagglutinating activity occurs when the lectins binds to carbohydrate from erythrocyte surface promoting a network among them. Hemagglutination on microtiter plate was performed with the LP and LS lectins (blood groups O). The strongest hemagglutination was visible using 2% RBC. The protein is able to agglutinate RBC-O at a concentration of about 25 mg/ml. Readings were recorded after about 30 min at room temperature, when the blank had fully sedimented. Specific activity was expressed as the number of hemagglutination units per mg protein.

Antifungal activity of purified lectin

The antifungal activity was tested using mycelia radial growth technique (El-Ghaouth *et al.*, 1992). The compounds were dissolved in water and serial concentrations ranged from 50 to 3000 mg/L were tested. The aliquots of the stock solutions were added to the PDA medium and then transferred to. After solidification, Petri dishes were inoculated with a 5 mm in diameter mycelium fungi and incubated in the dark at 27 ± 2 C. Fungal growth was measured when the control had grown to the edge of the plate. The inhibition of fungal growth was calculated as the percentage of inhibition of radial growth compared to the control after different time intervals (48, 72 and 120 h). The effective concentration that inhibits 50% of mycelial growth (EC_{50}) for each compound was estimated by probit analysis (Finney 1971) using SPSS 21.0 software.

Statistical analysis

Statistical analysis was performed using SPSS 21.0 software (Statistical Package for Social Sciences, USA). All experiments were repeated 3 times.. The log dose-response curves allowed determination of the EC_{50} values for the fungal bioassay according to the probit analysis (Finney 1971). The 95% confidence limits of EC_{50} values were determined by the least-square regression analysis of the relative growth rate (%

control) against the logarithm of the compound concentration.

RESULTS AND DISCUSSION

Extraction and Purification of lectin from *P. vulgaries* seeds

The lectin contents in 250 g of *P. vulgaries* seeds were ranged from 300-500 mg. The total protein concentrations in the pure lectin were in the range 118 – 120 mg/mL. Lectins are found in abundance in legume seeds. *Phaseolus vulgaris* is an herbaceous annual plant grown worldwide for its edible beans, popular in both dry and green bean forms. Lectins or hemagglutinins have been purified from different varieties of *P. vulgaris*. The lectin contents are low in some varieties and high in other varieties. The lectin contents in some parts of plants are higher, e.g., 390 and 75 mg of the purified lectin was recovered from 100 g *Remusatia vivipara* tubers (Bhat *et al.*, 2010) and *Astragalus mongholicus* roots (Yan *et al.*, 2005), respectively. The lectin content in non-legume plants is low, e.g., 3.3 mg lectin from 100 g Hibiscus *mutabilis* seeds (Lam and Ng, 2009). In this study, lectin extracted from seeds of *P. vulgaries* with 0.15 M NaCl for 48 hrs as shown in Figure 1. Isolation of lectins achieved by an ammonium sulfate 60% to precipitate lectins as schematized in Figure 1. Lectins can be extracted from plant tissue with water, 0.15 M NaCl or buffer solutions when it is necessary to control pH for the maintenance of hemagglutinating activity. The temperature and extraction time depends on stability and solubility of the lectin and may vary from 4 to 27? C, from minutes to hours. Isolation of lectins can be achieved by a combination of different purification techniques. Acids (e.g., acetic acid used by Naeem *et al.*, (2007), organic solvent (e.g., acetone used by Medeiros *et al.*, (2010) or salt (e.g., ammonium sulfate) can be used to precipitate lectins.

Extraction and Purification of lectin from *G. max* seeds

The present study represents the investigation on the purification of a lectin from *G. max* seeds. 60 % saturation ammonium sulphate precipitation and dialysis as shown in Figure 2. The lectin contents in 250 g of *G. max* seeds were ranged from 500-700 mg. The total protein concentrations in the pure lectin were in the range 160 –163 mg/mL. The soybean (*G. max*) is a legume species native to East Asia, which is highly cultivated for its edible seed. Soybean is one of the most important bean among all in the world, which provides vegetable protein for millions of human and ingredients for thousands of chemical products. Soybean lectin isolated from *G. max* is a carbohydrate binding protein highly specific to terminal non-reducing *N*-acetyl-D-galactosamine but less to D-galactose.

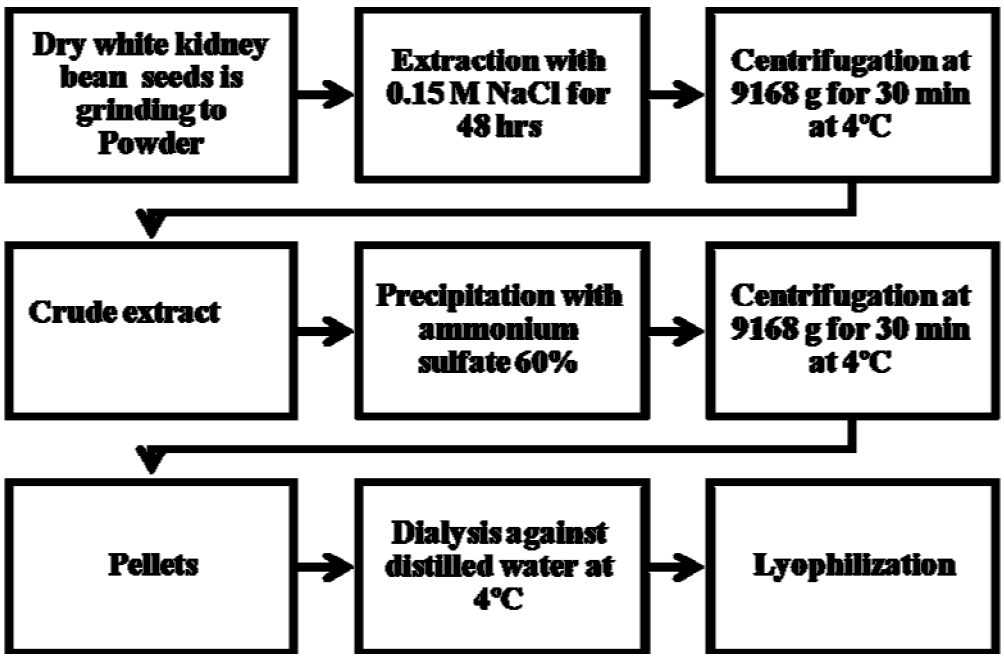

Figure 1. Scheme for isolation and purification of lectin from white kidney bean

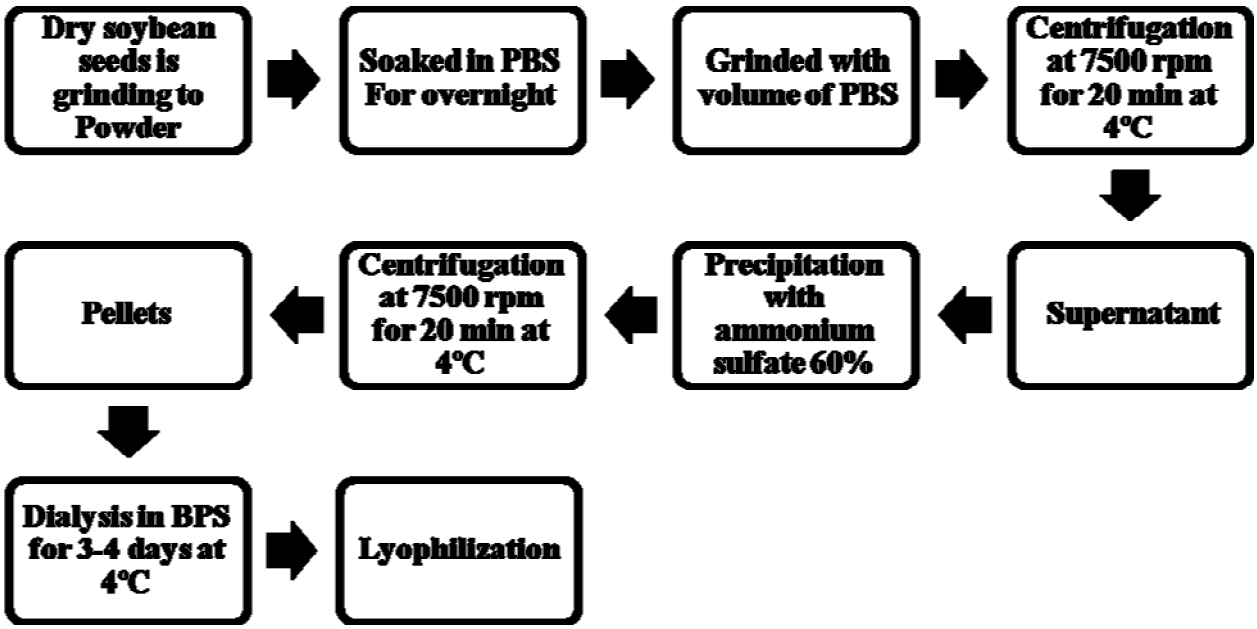

Figure 2. Scheme for isolation and purification of lectin from soybean

The *in vitro* antifungal activity of pure lectins

The *in vitro* antifungal activity of pure lectins from *G. max* (LG) and *P. vulgaries* (LP) against *F. oxysporum*, and *R. solani* fungi was estimated by determination the concentration causing 50% mycelial growth inhibition (EC_{50}) as shown in Tables 1 and 2. The mycelial growth inhibition was recorded after different time intervals, 48, 72 and 120 hrs. All lectins had higher inhibition against the tested fungi according to the type of lectin and microorganism. The antifungal activity of pure lectins against the soil borne fungus *F. oxysporum* (Table 1) indicated that the LP was the most potent among the tested compounds with EC_{50} of 5058, 4872 and 3617 mg/L, after 48, 72 and 120 hrs, respectively. In contrast, the LG was the lowest active compounds with EC_{50} of 13982, 9449 and 5635 mg/L after 48, 72 and 120 hrs against the same fungus, respectively. It can be concluded that the mycelial growth inhibition is time dependent.

The antifungal activity against the soil borne fungus *R. solani* (Table 2) showed that the LP (EC_{50} of 6786, 6646 and 5465 mg/L after 48, 72 and 120 hrs, respectively) was more active than the LG (EC_{50} of 13982, 9449 and 5635 mg/L after 48, 72 and 120 hrs, respectively). In general, these results revealed that myselial growth inhibition was time dependent and the soil-borne pathogenic fungus *F. oxysporum* was more susceptible to the tested lectins than the soil borne fungus *R. solani*.

The expression of *Gastrodia elata* lectins in the vascular cells of roots and stems was strongly induced by the fungus *Trichoderma viride,* indicating that lectin is an important defense protein in plants (S? *et al.*, 2009). Following insertion of the precursor gene of stinging nettle isolectin I into tobacco, the germination of spores of *Botrytis cinerea, Colletotrichum lindemuthianum*, and *T. viride* was significantly reduced (Ferreira *et al.*, 2007). Thus, lectins may be introduced into plants to protect them from fungal attack. Plant lectin may have indirect effects produced by the binding of lectins to carbohydrates on the fungal cell wall surface.

Chitinase-free chitin-binding stinging nettle (*Urtica dioicalectin*) impeded fungal growth. Cell wall synthesis was interrupted because of attenuated chitin synthesis and/or deposition (Munro *et al.*, 2003). The effects of nettle lectin on fungal cell wall and hyphal morphology suggest that the nettle lectin regulates endomycorrhizal colonization of the rhizomes. Severa1 other plant lectins inhibit fungal growth. The first group includes small chitin-binding merolectins with one chitin-binding domain, e.g., hevein from rubber tree latex and chitin-binding polypeptide from *Amaranthus caudatus* seeds (Broekaert *et al.*, 1992). A part from seeds, lectins were also found in both reproductive and vegetative tissues, such as fruits, leaves, flowers, roots etc. Lectins from various part of the plant are different in its efficiency against particular pathogen. The

inhibition of fungi growth can occur through lectin binding to hyphas resulting in poor absorption of nutrients as well as by interference on spore germination process (Zvereva and Vysotskaya, 2005). Lectins from *Alliaceae* (Roy *et al.*, 2002) and *Zingeberaceae* (Chen *et al*., 2005) have been reported for their insecticidal and anti-fungal properties that can be used for various applications. The activity of *Archidendron jiringa* lectin against the *in vitro* growth of *Exserohilum turcicum*, *F. oxysporum* and *Colletotrichum cassiicola*, showing that 5.7 µg/mL of purified protein resulted in complete inhibition (Charungchitrak *et al.*, 2011). The polysaccharide chitin is constituent of fungi cell wall and chitin-binding lectins showed antifungal activity; impairment of synthesis and/or deposition of chitin in cell wall maybe the reasons of antifungal action (Selitrennikoff, 2001).

Probably the carbohydrate-binding property of lectin is involved in the antifungal mechanisms and lectins of different specificities can promote distinct effects. The role of lectins in the defense mechanism of plants may have evolved from the ability to lectins to agglutinate and immobilize microorganisms. The supporting evidence for this proposed role in defense against pathogens falls into two main observed categories, namely (a) the presence of lectins at potential sites of invasion by infectious agents, and (b) the binding of lectins to various fungi and their ability to inhibit fungal growth and germination.

Table 1. The *in vitro* antifungal activity of pure lectins against *F. oxisporum* by using mycelial radial growth technique

Pure lectins	EC_{50}[a] (mg/L)	95% confidence limits		Slope [b] ± SE	Intercept [c] ± SE	(χ^2)[d]
		Lower	Upper			
48 hrs						
LG	13982	9272	31106	1.1±0.2	-4.56±0.73	2.59
LP	5058	3891	7330	1.0±0.18	-3.72±0.64	1.77
72 hr						
LG	9449	6769	17221	1.07±0.19	-4.23±0.68	1.01
LP	4872	3554	7832	0.82±0.18	-3.02±0.62	1.90
120 hr						
LG	5635	4293	8516	1.0±0.18	-3.73±0.64	0.05
LP	3617	2653	5088	0.87±0.18	-3.08±0.62	0.86

[a] The concentration causing 50% mycelial growth inhibition.
[b] Slope of the concentration-inhibition regression line ± standard error.
[c] Intercept of the regression line ± standard error.
[d] Chi square value.
LG, lectin from *G. max;* LP, lectin from *P. vulgaries*

Table 2. The *in vitro* antifungal activity of pure lectins against *R. solani* by using mycelia radial growth technique

Pure lectins	EC_{50}[a] (mg/L)	95% confidence limits		Slope[b] ± SE	Intercept[c] ± SE	(χ^2)[d]
		Lower	Upper			
48 hrs						
LG	15563	10765	30475	1.45±0.24	-6.06±0.87	1.36
LP	6786	5543	9022	1.54±0.20	-5.88±0.73	2.16
72 hrs						
LG	11611	8412	20311	1.32±0.21	-5.35±0.76	0.81
LP	6646	5455	8738	1.56±0.20	-5.97±0.73	1.12
120 hrs						
LG	7781	5789	12829	1.08±0.19	-4.19±0.67	0.21
LP	5465	4386	7381	1.25±0.19	-4.66±0.67	0.78

[a]The concentration causing 50% mycelial growth inhibition.
[b]Slope of the concentration-inhibition regression line ± standard error.
[c]Intercept of the regression line ± standard error.
[d]Chi square value.
LG, lectin from *G. max;* LP, lectin from *P. vulgaries*

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الملخص العربي

عزل اللكتينات النباتية و فاعليتها ضد بعض الفطريات الممرضة للنبات

سعاد فوزي عيد محسن، مصطفى عبد اللطيف عباسي، انتصار ابراهيم ربيع، حمدي قطب ابو طالب

تم عزل اللكتين من بذور الفاصوليا البيضاء وبذور فول الصويا والتنقية بواسطة الترسيب بكبريتات الامونيوم وتم دراسة التاثير الابادي الفطري لهما على الفيوزاريوم اوكسيسبورم و الرايزوكتونيا سولاني واتضح من الدراسة ان لهما تاثير مثبط على نمو كلا الفطريين والتاثير الاقوى كان للكتين المستخلص من الفاصوليا على الفيوزاريوم حيث كانت قيم التركيز المسبب لتاثير تثبيطي ٥٠% هي ٥٠٥٨ ، ٤٨٧٢ و ٣٦١٧ مجم/لتر بعد ٤٨، ٧٢ ،١٢٠ ساعة على التوالي .كما ان دراسة التاثير الابادي ضد فطر الرايزوكتونيا سولاني اوضحت ان لكتين الفاصوليا البيضاء كان اكثر تاثيرا في تثبيط النمو الهيفي من لكتين الصويا بقيم تركيزات مسببة لتاثير تثبيطي ٥٠% لنمو الهيفات ٦٧٨٦ ٦٦٤٦، و ٥٤٦٥ مجم/ لتر بعد ٢٤ ،٧٢ و ١٢٠ ساعة على التوالي.