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ANTIMICROBIAL ACTIVITY OF LECTIN ISOLATED FROM EGYPTIAN WHEAT CULTIVAR GIZA 171

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ABSTRACT: In the current study crude lectin was isolated from Egyptian wheat seeds cultivar Giza 171 and it was characterized by several methods such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Native-PAGE, Iso-electric point evaluation, Fourier transform infrared (FTIR) spectroscopy and amino acids composition. Furthermore, antibacterial and antifungal activities were estimated against some gram positive, gram negative bacteria and pathogenic fungus. Based on Native-PAGE (unreduced) of wheat seeds lectin indicates one major band with a molecular weight of approximately 50 kDa. SDS-PAGE of wheat seeds lectin separated this high molecular weight band into 2 bands corresponding to smaller molecular weights (14,20 kDa). The iso-electric points were deduced from the protein pH-solubility curve. It is clear that the least soluble points were obtained at pH 5.5. The content of the hydrophobic amino acids residues (proline, valine, isoleucine, leucine and phenyl alanine) valued 27.8% and this represent 31.1% of the total amino acids. The minimum inhibitory concentrations (MIC) of lectin against L. monocytogenes and P. aeruginosa was 800 μg/ml, 1600 μg/ml for B. subtillis and 3200 μg/ml against E. coli. Lectin inhibited mycelial growth of F. oxysporun and F. solani at a wide concentration range (250, 500 and 1000 µg/ml). This study has elucidated that the lectin isolated from wheat seeds cultivar Giza 171 have the potent antibacterial and antifungal activities against selected pathogenic bacteria and fungai.

Key words: Wheat, lectin, antibacterial, antifungal, SDS-PAGE.

INTRODUCTION

Lectins are members of a superfamily of proteins that express the capacity to bind to specific carbohydrates reversibly without altering their covalent structure. Common dietary staples, such as cereal grains, legumes, and fruits have relatively high concentrations of a variety of lectins. Especially black beans, soybeans, kidney beans, and (whole) grains are known for significant quantities of different lectins (Matucci et al., **2004)**. In nature, lectins play a role in biological recognition phenomena involving cells and proteins and hereby protect plants against external pathogens such as fungi and other organisms (van Buul and Brouns, 2014). There are at least three reasons for the need to finding out new alternative antimicrobial substances from natural sources. The first reason is that

*Corresponding author: Tel.: +2001013612760 E-mail address: Safa.abdo903@gmail.com people nowadays concern about toxic of synthetic substances including daily contact chemicals or even drugs used in medical or healthcare purposes (Hafidh et al., 2009). Any synthetic drugs were avoided in order to keep physiological cleans as belief. Thus, natural substances were used increasingly instead as well as any drugs used for antimicrobial purposes. The second reason is that new alternative drugs are human hope for better fighting with existed diseases and pathogens. They may replace currently used drugs in points of more efficiency, more abundant, lower side-effect or safer or even lower production cost. It is a fact that most alive organisms should have some mechanisms or substances fight with all-time contacting pathogens so that they can be survived in nature. Although plenty of antibiotics were discovered after first time Fleming's declaration, they were still relatively low amounts

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compared with overall real natural antimicrobial substances. This means the natural sources still flourish with novel antimicrobial substances waiting for discovered. Additional small aspect may be raised here. The natural substances are usually good leading compound sources for the mostly synthetic drug from the long past due to their diversities are far from human imagination. New chemical structures are always found in natural resources as higher frequency than artificial deducing structures. The final reason is that the mechanism used to synthesize natural substances are available and they are usually can be imitated in small, medium, and even large scale production with present biotechnological knowledge which looks easier than newly designed plants (Karnchanatat, 2012). Natural antioxidants may have free-radical scavengers, reducing agents, complexes of prooxidant metals, quenchers of singlet oxygen, etc. Recently research has been increased considerably in finding natural occurring antioxidants for use in foods or medical products to replace synthetic antioxidants. which are being restricted due to their adverse reaction such as carcinogenicity. Where cancer is the leading cause of morbidity and mortality around the world, with approximately 14 million new cases and 8.2 million cancer related deaths worldwide in 2012 (Siegel et al., 2014). Furthermore, the number of new cases in developed countries is expected to rise up to 70% in the next decades (Siegel et al., 2014), due to population ageing and growth (Thorburn, 2008). Hence there is a need for discovery of new anticancer drugs, especially those with novel mechanisms of action that can combat resistance. The present study reports the isolation. purification, characterization, and evaluation of the antibacterial, antifungal, of a lectin isolated from Egyptian wheat cultivar Giza 171.

MATERIALS AND METHODS

Plant Material

The wheat seeds, cultivar Giza 171 were obtained from Agricultural Research Centre, Ministry of Agricultural, Giza, Egypt, for isolation and purification of lectin.

Crue Lectin Isolation

The seeds of wheat were ground and extracted overnight at 4°C in 0.01 M phosphate

buffered saline (PBS) pH 7.2, followed by centrifugation at 3000 g at 4°C for 10 min. Solid (NH₄)₂SO₄ was added to the obtained supernatant up to 60% saturation and the proteins were precipitated at 4°C overnight. The precipitated proteins were centrifuged at 12,000g at 4°C for 15 min, and then dissolved in PBS, followed by dialysis against PBS until.

Native PAGE

Protein samples were dissolved (5 mg/ml) in a buffer (pH 6.8) containing 0.25 M Tris base and 50% glycerol and analyzed by native PAGE according to **Laemmli (1970)** in 3 and 8% acrylamide for the stacking and resolving gels, respectively. The electrode buffer (pH 8.3) was composed of 0.125 M Tris base and 0.96 M glycine.

SDS-PAGE

An amount of wheat seeds lectin (20 mg) was dispersed in 1 ml SDS 10% with 100 μ l β -mercaptoethanol for 15 min with vortexing every 5 min. The extract was centrifuged at 11,000 g for 10 min. A mixture of 20 μ l extract and 20 μ l of SDS-loading sample buffer (SDS 4%, β -mercaptoethanol 3%, glycerol 20%, Tris HCl 50mM pH 6.8 and bromophenol blue traces), was heated at 96°C for 3 min and 10 μ l aliquot (per lane) was electrophoresed by SDS-PAGE according to **Laemmli (1970)**.

Iso-electric Point (protein pH-solubility curves)

The iso-electric points were deduced from the protein pH-solubility curves as the pH at which the protein is less soluble. Protein pHsolubility curves were assayed in the pH range of 2–10 according to the procedure outlined by Chobert et al. (1991) with some modification. One hundred and twenty-five milligrams of each sample was dispersed in 25 ml of distilled water and the solution pH was adjusted to 2-10 using either 0.5 mol/l NaOH or 0.5 mol/l HCl. The slurries were mixed for 1 hr., at 30°C using magnetic bar before centrifuging at 1200 g for 20 min at 4°C. The supernatant was filtered to obtain a clear solution. Protein content in the supernatant was determined by Kjeldahl method (Horwitz and Latimer, 2000). Triplicate determinations were carried out and the solubility profile was obtained by plotting averages of protein solubility (%) against pH:

Solubility (%) = Amount of protein in the supernatant \div amount of protein in the sample \times 100

Amino Acid Composition

The composition of amino acids for lectin isolated from wheat seeds was evaluated according to Simpson et al. (1976) by amino acid analyzer instrument model "Eppendorf LC3000" using the following procedure: 0.2 g from lectin isolated from wheat seeds received 10 ml HCl (6N) in a sealing tube, and then put in oven at 110°C for 24 hr. Hydrolysates were transported quantitatively into a porcelain dish and HCl was evaporated to dryness at 50-60°C on a water bath. To remove the excess of hydrochloric acid, 5 ml distilled water was added to the hydrolysates and evaporated to dryness. Finally, the remains was dissolved in 10 ml distilled water and filtrate through filter membrane (0.45 µm). The filtrate was lyophilized then 10 ml of distilled water were added and the samples lyophilized a second time. One ml of sodium citrate buffer 0.2 N at pH 2.2 was added and the samples stocked frozen in a closed vial until amino acids separation by amino acid analyzer (Column: hydrolysate column Eppendorf LC $3000 (250 \times 4.6)$. Its temperature is 47°C; Sample: 30 µl; Buffer system: buffer A: Sodium acetate pH 3.3, buffer B Sodium acetate pH 3.6, buffer C: Sodium acetate pH 4.3 and buffer D: Sodium acetate pH11.0; Flow rate: 0.3 ml/min.). Ninhydrin is utilized for the revelation of amino acids at 440 nm for proline and 570 nm for the other amino acids out of an oxidative decarboxylation reaction. The peak zone and percentage of each amino acid were determinated by computer software AXXIOM CHROMATOGRAPHY-727.

Fourier Transform Infrared (FTIR) Spectroscopy

Lectin isolated from wheat seeds was prepared with potassium bromide (KBr) pellet method according the study of **Souillac** *et al.* (2002). Infrared spectra was measured with a FT-IR spectrometer (NICOLET NEXUS 470, DTGS, Thermo Scientific, Waltham, MS, USA) at 25°Cat National Research Center (NRC), Giza, Egypt. For each spectrum 256 interferograms were collected with a resolution of 4 cm-1 with 64 scans and a 2 cm interval from the 4000 to 400 cm⁻¹ region. The system

was continuously purged with dry air. Second derivation spectra were obtained with Savitsky—Golay derivative function soft as followed by **Surewicz and Mantsch (1988)**.

Antibacterial Activity

gram positive bacteria (Listeria monocytogenes and Bacillus subtilis) and two gram negative bacteria (Pseudomonas aeruginosa and Escherichia coli) were kindly gained from the Laboratory of Microbiology, Department of Microbiology, Faculty of Science, Zagazig University, Egypt. Wheat seeds lectin was tested for antibacterial activity at different concentrations (0, 200, 400, 800, 1600, 3200 and 6400 μg/ml) against two gram positive bacteria and two gram negative bacteria by conventional well-diffusion assay (Nanda and Saravanan, 2009). The clear cultures of bacterial strains were sub-cultured on nutrient broth at 37°C on a rotary shaker at 200 rpm. Every strain was dispersal uniformly onto the single plates using sterile cotton swabs. Wells of 6-mm diameter were made on Müller Hinton Agar (MHA) plates using a gel puncturing tool. Forty µl of each concentration (0, 200, 400, 800, 1600, 3200 and 6400 µg/ml) were carried into each well. After incubation at 37°C for 24 hr., the diameter of the inhibition zone was recorded by using a ruler. Minimum inhibitory concentration (MIC) of lectin was estimated as recorded earlier (Abdel-Hamid et al., 2016). The lowest concentration of the examined articles that presented visible clear zone on Mueller-Hinton agar plates was regarded as the minimal inhibitory concentration.

Antifungal Activity

The effect of the lectin isolated from wheat seeds on the mycelial growth of Fusarium oxysporum and Fusarium solani was evaluated also at different concentrations (250, 500 and 1000 μg/ml) using the poisoned food technique (Yahyazadeh et al., 2009). A 6 mm mycelial agar plug from a 7-day-old culture of Fusarium oxysporum or Fusarium solani was placed at the center of each Potato dextrose agar (PDA) plate and calculated volumes of the tested substances were added, to achieve the previously mentioned concentrations. Approximately, 0.05% (V/V) Tween-80 was then added to the media. Petri dishes were sealed with parafilm and incubated for 7 dayes at 25°C. The diameter (mm) of colony zone was measured with a caliper.

RESULTS AND DISCUSSION

Chemical Characterization

Native-PAGE and SDS-PAGE

The Native-PAGE (unreduced) of wheat seeds lectin indicates one major band with a molecular weight of approximately 50 kDa, confirming its chemical identity (Fig. 1). SDS-PAGE (reduced with β -mercapto-ethanol) of wheat seeds lectin (Fig. 1) separated this high molecular weight band into 2 bands corresponding to smaller molecular weights (14, 20 kDa).

Iso-electric point

The iso-electric points were deduced from the protein pH-solubility curve as the pH at which the protein is less soluble. The pH solubility curve of lectin isolated from wheat seeds is given in Fig. 2. It is clear that the least soluble points were obtained at pH 5.5. These results agree with those reported by **Mendoza-Blanco** *et al.* (2012).

Amino acids analysis

As shown in Table 1 the amino acid composition of the crude lectin is typified by high concentrations of glutamic, aspartic, serine, valine and leucine (12.9, 9.1, 9.8, 6.8 and 7.7%, respectively). The content of the hydrophobic amino acids residues (proline, valine, isoleucine, leucine and phenyl alanine) is 27.8% and this represent 31.1% of the total amino acids.

Fourier transform infrared (FTIR) spectroscopy

One of the classical methods for structure determination of small molecules is IR. This standing is due to its sensitivity to the chemical composition and architecture of molecules. The high information content in an infrared spectrum carries over also to biological systems. This makes infrared spectroscopy a valuable tool for the investigation of protein structure (Arrondo et al., 1993; Barth, 2007) of the molecular mechanism of protein reactions (McClelland et al., 2002) and of protein folding, unfolding and misfolding (Pozo Ramajo et al., 2005). In order to study proteins, the analysis of the secondary structure of protein is often required by FTIR in recent years. FTIR spectroscopy has been proven to be a powerful tool for providing conformational and structural dynamic information

of proteins. FTIR spectra of the lectin isolated from wheat seeds was shown in Fig. 3.

The infrared analysis indicated the presence of glycosylation with two typical carbohydrate absorptions at 3000–2800 cm⁻¹ and 1400–1200 cm⁻¹. The secondary structure of the protein were commonly based on the amide I band analysis (1700–1600 cm⁻¹). Amide I band peaks identified are more mature. It is the most intense absorption band of the polypeptides. ν (C= O) has a predominant role in amide I, ν (C-N) follows. There is also some in-plane NH bending contribution to amide I. The secondary structure of proteins is reflected by these bands as follows: $1610 \sim 1640$ cm⁻¹ for the β -sheet; $1640 \sim 1650$ cm⁻¹ for the random coil; $1650 \sim 1658$ cm⁻¹ for the α -helix; $1660 \sim 1700$ cm⁻¹ for the β -turn. These results agree with those reported by (Wang *et al.*, 2018).

Antibacterial Activity of Lectin Against Gram Positive and Gram Negative Bacteria

Some plant lectins have been studied for their interactions with bacteria (Santi-Gadelha et al., 2006). Some studies with lectin showed its antibacterial activity (Ayouba et al., 1991). Binding of lectins to muramic acid and Nacetylmuramic acid, carbohydrates present in the bacterial cell wall, has been reported earlier (Caldeon et al., 1997). The antibacterial activity of wheat seeds lectin was examined at different concentrations (200, 400, 800, 1600, 3200 and 6400 µg/ml) and the results are listed in Table 2 and Fig 4. The minimum inhibitory concentration (MIC) of lectin against L. monocytogenes and P. aeruginosa was 800 µg/ml, 1600 µg/ml for B. subtillis and 3200 µg/ml against E. coli. These results agree with those reported by (Saha et al., 2014). The antibacterial activity of lectin isolated from wheat seeds may be due to the hydrophobic amino acid residues. The content of the hydrophobic amino acids residues (proline, valine, isoleucine, leucine, and phenylalanine) is 27.8% and this represents 31.2% of the total amino acids. Protein hydrophobicity plays an important role in the disturbance of the bacterial cell wall and membrane (Abdel-Hamid et al., 2016).

Antifungal Activity of Lectin

The *in vitro* antifungal activities of wheat seeds lectin (Fig. 5) against *F. oxysporun* and *F. solani* were examined at different concentrations

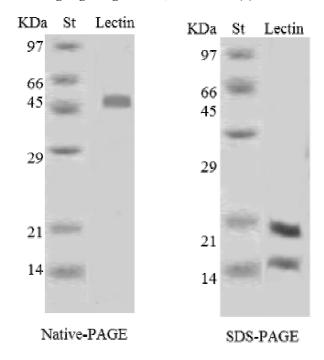


Fig. 1. Native-PAGE and SDS-PAGE of lectin isolated from wheat seeds cultivar Giza 171 as compared to standard protein (St)

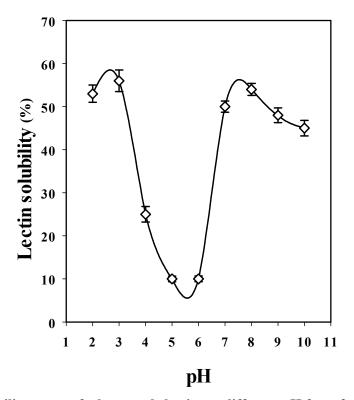


Fig 2. pH solubility curve of wheat seeds lectins at different pH from 2 to 10

Table 1. Amino acids composition of wheat seeds lec	tin
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Amino acid	Percentage (%)		
Aspartic	9.1		
Threonine	7		
Serine	9.8		
Glutamic	12.9		
Proline	3.5		
Cysteine	1.4		
Valine	6.8		
Methionine	4.9		
Isoleucine	4		
Leucine	7.7		
Tyrosine	3.3		
Phenylalanine	5.8		
Histidine	4.3		
Lysine	5.9		
Arginine	3.1		
Unknowns	10.5		

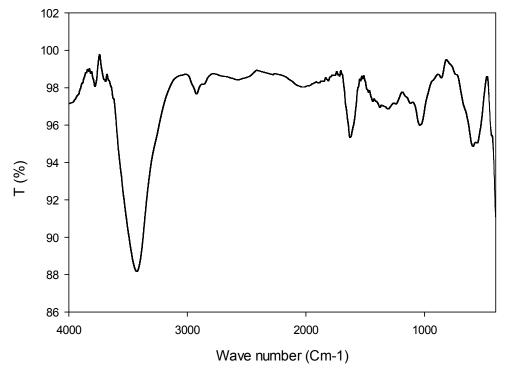


Fig. 3. FTIR spectra of lectin isolated from wheat seeds

Gram positive bacteria

Gram negative bacteria

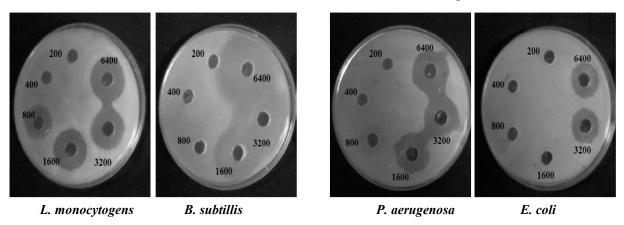
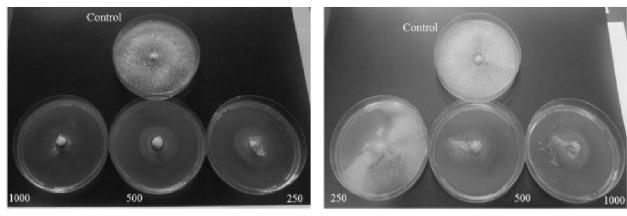


Fig. 4. The size (mm) of inhibition zones induced in Gram+ and Gram- bacteria under the influence of different concentrations (200-6400 μg ml⁻¹) of wheat seeds lectin using agar well diffusion assay

Table 2. The size (mm) of inhibition zones induced in Gram+ and Gram- bacteria under the influence of different concentrations (200-6400 μg ml⁻¹) of wheat seeds lectin using agar well diffusion assay

Microorganism -	Lectin concentration (µg ml ⁻¹)						
	200	400	800	1600	3200	6400	
-	Inhibition zone diameter (mm)						
		I	Gram ⁺				
L. monocytogens	-	-	15	20	22	26	
B. subtilis	-	-	-	33	35	48	
			Gram ⁻				
P. aerugenosa	-	-	10	18	21	24	
E. coli	-	-	-	-	15	17	



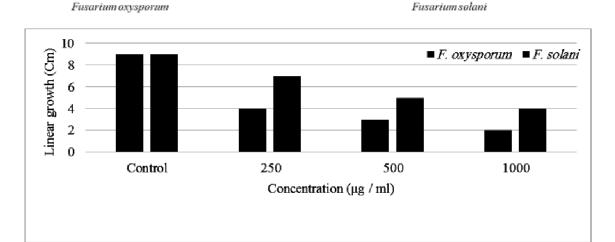


Fig. 5. Linear growth (cm) of *Fusarium oxysporum* and *Fusarium solani* after 7 days at 25°C in the presence of wheat seed lectin at different concentrations (250, 500 and 1000 µg/ml)

(250, 500 and 1000 μg/ml). Lectin inhibited mycelial growth at a wide concentration range (250, 500 and 1000 μg/ml). On the other hand, lectin showed a concentration-dependent inhibitory action on the fungal growth. Some studies with lectin isolated from different sources showed its antifungal activity against pathogenic fungal species (Maria das Graças et al., 2002; Yan et al., 2005; Tian et al., 2008; Chen et al., 2009; Kheeree et al., 2010).

REFERENCES

Abdel-Hamid, M., H.A. Goda, C. De Gobba, H. Jenssen and A. Osman (2016). Antibacterial activity of papain hydrolysed camel whey and its fractions. Int. Dairy J., 61:91-98.

Arrondo, J.L.R., A. Muga, J. Castresana and F.M. Goñi (1993). Quantitative studies of the

structure of proteins in solution by Fourier-transform infrared spectroscopy. Progress in Biophysics and Molecular Biol., 59:23-56.

Ayouba, A., C. Chatelain and P. Rougé (1991). Legume lectins interact with muramic acid and N-acetylmuramic acid. FEBS letters, 289: 102-104.

Barth, A. (2007). Infrared spectroscopy of proteins. Biochimica et Biophysica Acta (BBA)-Bioenergetics, 1767:1073-1101.

Caldeon, A., G. Buck and R. Doyle (1997). Lectin-microorganism complexes. Electronic Lectin J.: Lectins, Biol. Biochem., Clinical Biochem., 12:87-984583.

Chen, J., B. Liu and N. Ji (2009). A novel sialic acid-specific lectin from Phaseolus coccineus seeds with potent antineoplastic and antifungal activities. Phytomed., 16:352-360.

- Chobert, J-M., A. Touati, C. Bertrand-Harb, M. Dalgalarrondo, M-G. Nicolas and T. Haertle (1991). *In vitro* proteolysis and functional properties of reductively alkylated β-casein derivatives. J. Dairy Res., 58:285-298.
- Hafidh, R.R., F. Abas, A.S. Abdulamir, F. Jahanshiri, F.A. Bakar and Z. Sekawi (2009). A review: Cancer research of natural products in Asia. Int. J. Cancer Res., 5:69-82.
- Horwitz, W. and G. Latimer (2000). Official Methods of Analysis of AOAC International, Gaithersburg MA, USA. Assoc. Official Anal. Chem.
- Karnchanatat, A. (2012). Antimicrobial activity of lectins from plants. Antimicrobial agents. In. Tech., 145-178.
- Kheeree, N., P. Sangvanich, S. Puthong and A. Karnchanatat (2010). Antifungal and antiproliferative activities of lectin from the rhizomes of *Curcuma amarissima* Roscoe. Appl. Biochem. and Biotechnol., 162: 912-925.
- Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nat., 227:680-685.
- Maria das Graças, M.F., V.M. Gomes and R.E. Corsini (2002). Isolation and partial characterization of a novel lectin from *Talisia esculenta* seeds that interferes with fungal growth. Plant Physiol. and Biochem., 40: 61-68.
- Matucci, A., G. Veneri and C.D. Pellegrina (2004). Temperature-dependent decay of wheat germ agglutinin activity and its implications for food processing and analysis. Food Control, 15: 391-395.
- McClelland, J., R. Jones, S. Bajic, J. Chalmers and P. Griffiths (2002) Handbook of Vibrational Spectroscopy. John Wiley and Sons, Ltd.
- Mendoza-Blanco, W., L. Ponce-Soto and S. Marangoni (2012). Purification and primary structure of a lectin from Buddleja coriacea seeds. Sciéndo (Trujillo), 15: 81-88.
- Nanda, A. and M. Saravanan (2009). Biosynthesis of silver nanoparticles from *Staphylococcus aureus* and its antimicrobial activity against

- MRSA and MRSE. Nanomedicine: Nanotechnol., Biol. and Med., 5: 452-456.
- Pozo Ramajo, A., S.A. Petty, A. Starzyk, S.M. Decatur and M. Volk (2005). The α -helix folds more rapidly at the C-terminus than at the N-terminus. J. Ame. Chem. Soc., 127: 13784-13785.
- Saha, R.K., S.H.M. Tuhin, N. Jahan, A. Roy and P. Roy (2014). Antibacterial and antioxidant activities of a food lectin isolated from the seeds of *Lablab purpureus*. Ame. J. Ethnomed., 1: 8-17.
- Santi-Gadelha, T., C.A. de Almeida Gadelha and K.S. Aragao (2006) Purification and biological effects of *Araucaria angustifolia* (Araucariaceae) seed lectin. Biochem. and Biophysical Res. Communications, 350: 1050-1055.
- Siegel, R., J. Ma, Z. Zou and A. Jemal (2014). Cancer statistics, CA: A Cancer J. Clin., 64:9-29.
- Simpson, R.J., M.R. Neuberger and T. Liu (1976) Complete amino acid analysis of proteins from a single hydrolysate. J. Biol. Chem., 251: 1936-1940.
- Souillac, P.O., C.R. Middaugh and J.H. Rytting (2002). Investigation of protein/carbohydrate interactions in the dried state. 2. Diffuse reflectance FTIR studies. Int. J. Pharm., 235: 207-218.
- Surewicz, W.K. and H.H. Mantsch (1988). New insight into protein secondary structure from resolution-enhanced infrared spectra. Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymol., 952: 115-130.
- Thorburn, A. (2008). Apoptosis and autophagy: regulatory connections between two supposedly different processes. Apoptosis, 13: 1-9.
- Tian, Q., W. Wang and C. Miao (2008). Purification, characterization and molecular cloning of a novel mannose-binding lectin from rhizomes of *Ophiopogon japonicus* with antiviral and antifungal activities. Plant Sci., 175:877-884.

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van Buul, V.J. and F.J. Brouns (2014). Health effects of wheat lectins: A review. J. Cereal Sci., 59: 112-117.

Wang, Z., A. Tu, D. Tang and Y. Shan (2018). Effectively preparing soluble ovomucin with high antiviral activity from egg white. Int. J. Biol. Macromolecules.

Yahyazadeh, M., R. Zare, R. Omidbaigi, M. Faghih-Nasiri and M. Abbasi (2009). Control of Penicillium decay on citrus fruit using

essential oil vapours of thyme or clove inside polyethylene and nano-clay polyethylene films. J. Hort. Sci. and Biotechnol., 84: 403-409.

Yan, Q., Z. Jiang, S. Yang, W. Deng and L. Han (2005) A novel homodimeric lectin from *Astragalus mongholicus* with antifungal activity. Archives of Biochem. and Biophysics, 442:72-81.

النشـــاط المضـــاد للميكـروبـات للكتيـن القمح المصري صنف جيـــزة ١٧١ صفا عبدالوهاب مصطفى ـ هيثم على بدر ـ على عثمان ـ خالد محمد وهدان قسم الكيمياء الحيوية الزراعية ـ كلية الزراعة ـ جامعة الزقازيق ـ مصر