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PURIFICATION, MOLECULAR AND BIOCHEMICAL CHARACTERIZATION AND BIOLOGICAL APPLICATIONS OF HEMAGGLUTINATING LECTIN WITH ANTICANCER ACTIVITIES FROM *PLEUROTUS OSTREATUS*

Ismail M. Kamel [1], Neveen M. Khalil [1], Sherien M.M. Atalla [2] and Sara S.M. Seleem[1,*]

[1]Botany and Microbiology Department, Faculty of Science, Cairo University, Giza, 12613, Egypt

[2]Chemistry of Natural and Microbial Products Department, Pharmaceutical and Drug Industrial Research Division, National Research Centre, Dokki, Giza, 12622, Egypt

[*] Corresponding author: Sara S.M. Seleem

Email: saraseleem2@gmail.com

saraseleem@gstd.sci.cu.edu.eg

ABSTRACT

Three *Pleurotus* species were screened for their ability to synthesize mycelial, culture filtrate and fruiting bodies lectins. All tested *Pleurotus* species were able to synthesize mycelial and fruiting bodies lectins but with different degree of activity, while no extracellular lectin was detected. *P. ostreatus* Lectin (POL) from its fruiting bodies recorded the highest activity, so it was selected for further studies. Partial sequencing of lectin gene and partial amino acid sequencing were performed, resulting in 267 amino acids. POL purification involved a combination of $(NH_4)_2SO_4$ precipitation along with two anion-chromatographic steps using Q-Sepharose column and Mono Q column. POL appeared as a single band with a molecular mass of 29 kDa on SDS-PAGE. Biochemical and biological activities of pure POL were compared with crude extract from the same organism. POL activity was inhibited by xylose with a MIC of 0.1 M. It was active up to 70ºC and pH 11 then its activity completely disappeared. POL activity was inhibited by different concentrations of Hg^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Ca^{2+}, Na^{+} and K^{+} ions. POL manifested antibacterial activity, but showed no antifungal activity. It exhibited anticancer activity toward human hepatocellular and colorectal carcinoma with IC_{50} of 424 and 270 µg/ml, respectively.

***Keywords*:** Hemagglutinating lectins; oyster mushroom; anticancer and antimicrobial activities

Introduction

Lectins are non-immunogenic proteins which are capable of binding without catalysis to specific carbohydrates. They have the ability to agglutinate erythrocytes with their carbohydrate specificity, since they have at least one non-catalytic domain that reversibly binds to specific mono- or oligosaccharides. Hemagglutination assay using erythrocytes can easily detect the presence of lectins (Lam and Ng, 2011). Lectins are found in a diversity of organisms and were isolated from various sources, including plants, animals, viruses, bacteria, fungi and algae. The content and type of lectins varies in different organisms (Sharon and Lis, 2004). Lectins play a key role in various pathological and biological processes, such as cell signaling, interactions between cells, differentiation, innate immune responses, serum glycoprotein turnover, metastasis of tissues, morphogenesis, molecular recognition, defense, cell flocculation, mating, parasitism, infections and host-pathogen interactions. Plant lectins displayed anti-coronaviral activity, especially mannose-binding lectins, which affect severe acute respiratory syndrome caused by the corona virus. They interfered with viral attachment in the early stage of replication cycle and suppressed the growth by interacting at the end of the infectious virus cycle (Keyaerts *et al.* 2007).

High lectin yields from various sources may facilitate its mass production and purification (Varrot *et al.,* 2013, Muszynska *et al.,* 2018). Many lectins had been purified using traditional methods involving a series of precipitations with salts and solvents. In general, many chromatographic steps, such as affinity chromatography, ion- chromatography, hydrophobic interaction chromatography, and gel filtration chromatography have been used to purify lectins from crude extracts. The first unit operation chosen for lectin purification is precipitation with ammonium sulfate and affinity chromatography. Ion-exchange chromatography is the second most widely used unit procedures, used in 21.7% of cases and also used in all purification steps (Nascimento *et al.,* 2012).

The fungal lectins attract the attention of many scientists owing to their various carbohydrate-binding properties (Chumkhunthod *et al.,* 2006; Liu *et al.,* 2006; Thakur *et al.,* 2007), molecular masses (Liu *et al.* 2006), subunit numbers (Feng *et al.,* 2006; Jung *et al.,* 2007; Thakur *et al.,* 2007) and full or N-terminal amino acid sequences (Yang *et al.,* 2005; Chumkhunthod *et al.,* 2006) Fungi are a huge lectin reservoir, about 82 percent of the identified fungal lectins come from mushrooms (Diaz *et al.,* 2011; Varrot *et al.,* 2013).

Oyster mushrooms contain a number of biologically active compounds with therapeutic activity. Lectin is one of those compounds that are biologically active (Ng 2004). To date, approximately 60 mushroom lectins have been identified, some of which possess medicinal properties (Li *et al.* 2008, Zhang *et al.,* 2014). Molecular, biochemical and structural characteristics of hundreds of mushroom lectins along with their potential applications have been reviewed extensively by Hassan *et al.* (2015). Carbohydrate specificity of mushroom lectins has been extensively reviewed by Singh *et al.* (2019) and Hassan *et al.* (2015). Mushroom lectins have been reported to exhibit potent antiviral activity (Hassan *et al.* 2015), mitogenicity (Zhang *et al.,* 2019), modulation of immune cells and in therapeutics (Wang *et al.* 2019). There is voluminous literature pertaining to applications of lectins from mushrooms in terms of its antiproliferative activity (Singh *et al.,* 2016), immune stimulating potential, antimicrobial, antioxidant and therapeutic effects. Recent studies of the oyster mushroom's medicinal properties focused on isolated bioactive compounds. Oyster mushroom, *Pleurotus* contains 40% of the essential amino acids required for human intake and thus it considered as potential diet for people suffering from malnutrition problem (Pushpa and Purushothama 2010). There are about 40 species in the *Pleurotus* genus, including those with high economic significance as *P. ostreatus* and *P. pulmonarius.* The fruiting bodies of oyster mushrooms are of high nutritional and healthpromoting value. In addition, many species belonging to the *Pleurotus* genus have been used as sources of substances with documented medicinal properties (Golak-Siwulska *et al.,* 2018; Gregori *et al.,* 2007)

Anticarcinogenic and antitumor activities of lectins are due to diverse mechanisms mostly as direct cytotoxicity on malignant cells, inducing reductions in certain tumors, stimulation of normal growth in tumor cells, inhibition of the neoplastic effects of chemotherapy and radiation, and increasing immunogenicity of tumor cells (El-Fakharany *et al.* 2020). Additionally, many anticancer lectins usually possess low cytotoxicity to non-transformed cells. This result may be due to the distinct expression of glycans on cancer surfaces and normal cells that allow lectins to recognize cells that are distinctly malignant. In addition, lectins can interact with cell surface and/or cytosol receptors and other active cell molecules to minimize cell proliferation (Lam and Ng, 2011, Coelho *et al.,* 2017).

This study was conducted to investigate the hemagglutination activity of lectins extracted from different *Pleurotus* species in different developmental stages and to purify the most active one and determine the biochemical characteristics and the biological applications of this lectin in crude and pure state. Molecular characterization of the most active lectin and the organism from which this lectin was extracted was carried out.

Materials and Methods

Maintenance, growth and harvesting of *Pleurotus* cultures

Three mushroom species belong to genus *Pleurotus* namely *P. ostreatus*; *P. sajor-caju* and *P. columbines* were screened for their ability to produce mycelial lectin, culture filtrate lectin and fruiting bodies lectin. The most potent one was selected for further studies.

Mycelial cultures and fruiting bodies of each species were obtained from the Faculty of Agriculture Ain Shams University. Presumptive identification of each *P.* species mycelia was based on the morphology previously described by Rogers (1994) and Nwordu *et al.* (2013). Fruiting bodies were compared for morphological characters such as color, size, texture, shape and margin of fruit body, other features such as odors, stipe and stipe length, pileus length, gill attachment and spacing were considered (Appiah *et al.* 2017).

All species were maintained on 2 different growth media to compare the effect of the composition of broth medium in the production and activity of lectin. Growth media used were Potato Dextrose broth (PDB) supplemented with yeast along with Czapek Dox broth (CDB). After 10 days of growth at 25 ◦ C under static conditions. Mycelium was collected by filtration. It was washed thoroughly with phosphate buffered saline (PBS, 0.1 M, pH 7.4), and briefly pressed between the folds of filter paper. The culture supernatants of each *Pleurotus* species were separately collected. Fruiting bodies of each species were dried by sunlight (Bhari *et al.* 2016).

Extraction of lectins (extracellular and intracellular lectins)

Lectin extracted from the mycelium in 0.1 M PBS (1:1.5, w/v, pH 7.4) by ultrasound-assisted extraction with ultrasonic processor 650 W, 24 kHz, six cycles of 30s (Scientz company, NingBo, China,). The samples were kept in ice throughout the sonication cycles. The mycelium after sonication was ground in a mortar. The extract was centrifuged for 20 min at 3000 × *g* and 4◦C. The supernatant was collected as a crude extract for detection of the presence of intracellular mycelium lectin. The culture supernatant without sonication was separately collected as a crude extract for detection of the presence of extracellular lectin.

One hundred milligrams from dried fruiting bodies of each *Pleurotus* species was first homogenized in 10 ml/g 0.1 M phosphate-buffered saline (PBS), PH 7.4 and left overnight at 4ºC (Suzuki *et al.,* 2009). The homogenate was centrifuged at 4ºC for 30 minutes at 12,000 rpm and the supernatant was collected as a crude extract for detection of the presence of fruiting bodies lectin. In all cases, the extract was assayed for determination of lectin activity using 4 % (v/v) human erythrocyte suspension types A, B, AB and O (Sun *et al.,* 2014).

Assay of lectin hemagglutinating activity

Hemagglutiating activity was determined in 96-well microplate with final volume 50 µl. A serial twofold dilution of the lectin solution (25 µL) was mixed with 25µL of 4 % (v/v) suspension of human erythrocyte suspension (type A, B, AB or O) with 0.1M phosphate-buffered saline (PBS, pH 7.4) and incubated at 4 ◦C. The results were recorded after approximately an hour when full sedimentation was observed in the blank. A negative control was performed every time by mixing PBS and erythrocytes only for an easier determination of the presence of hemagglutinating activity. The reciprocal of the highest dilution showing hemagglutination, was considered as one hemagglutination unit. Specific activity defined as the number of hemagglutination units/mg protein. Human erythrocytes (A, B, AB, and O types) were donated by healthy volunteers, and

the blood type was confirmed by clinical tests. All determinations were performed in triplicate (Zhang *et al.,* 2010). *Pleurotus* species which produced the most potent lectin with the highest specific activity was molecularly identified and the produced lectin was subjected to further purification, molecular, biochemical and biological applications.

Protein determination

Protein concentration was estimated by the method of Lowry *et al*. (Lowry *et al.,* 1951) using bovine serum albumin as standard.

Molecular characterization of the selected *Pleurotus* species

For accurate study, identification of the selected *P. ostreatus* must be confirmed. So it was identified up to the species and strain levels using internal transcribed spacer (ITS) region of the rDNA (ribosomal RNA).

DNA extraction : Fruiting bodies of the selected *P. ostreatus* (20 mg) was homogenized in lysis buffer and DNA extracted according to the method of *Quick*-DNA™ Midiprep Plus Kit (Zymo Research, USA) following the manufacturer protocol (Vlasenko *et al.* 2019).

PCR amplification of the ITS region : Two universal primers, ITS1 (5'-TCC GTA GGT GAA CCT GCGG-3'), forward primer and ITS4 (5'-TCC TCC GCT TAT TGATAT GC- 3'), reverse primer were used for the amplification of the ITS regions of the selected *P.* species (Siddiquee *et al.* 2010). The PCR reaction mixture was performed in a total volume of 50 µl containing 25 µl MyTaq Red Mix, 8 µL DNA Template (change), 1 µl (20 Pico Mol) Reverse Primers, 1 µl (20 Pico Mol) Forward Primers and 15 µL Nuclease Free Water. The amplification reaction was done with a C1000 Touch thermal cycler (BioRad, USA). Method of Appiah *et al.* (2017) with slight modifications was employed for thermal cycling conditions. Initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 second, annealing at 55°C for 45 second and extension at 72 °C for 1 min and a final extension at 72 °C for 10 min**.** DNA fragments from agarose gel were purified using DNA Clean & Concentrator™-100 kits following the manufacturer's protocol.

Sequencing and analysis of ITS region : The PCR product sequenced with the same primer pair used in the PCR reactions (ITS1 and ITS4). Finally, sequencing of the PCR product was performed at the GATC Company by using ABI 3730xl DNA sequencer. The obtained sequence was submitted to the GenBank of NCBI database. The sequence data were assembled and analyzed using BioEdit version 7.2.5 and aligned with ClustalW. Nucleotide sequence comparisons were performed using Basic Local Alignment Search Tool (BLAST) network services at the National Center for Biotechnology Information (NCBI) database.

Sequencing of lectin gene and deducing of lectin amino acid sequence

The genomic DNA sample was used as template for isolation and amplification of lectin gene, Dream Taq Green PCR Master Mix (#K1082) was used in the PCR reaction. Forward and reverse primers (forward: CTTTGTGCAGAGC AGCATCG; reverse: GCTGCCCGTGAAGATTTGTT) were designed for amplification of lectin gene using PCR, they were designed with the aid of NCBI database which amplify about 1100 bp. Thermocycler (Q-Cycler, Quanta biotech) was set for the following protocol: Initial denaturation one cycle at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 second, annealing at 58 °C for 30 second and extension at 72 °C for 30 second and a final extension one cycle at 72 °C for 10 min (Adeniyi *et al.* 2018). PCR product was sequenced in forward and reverse direction on GATC Company by using ABI 3730xl DNA sequencer through combining the traditional Sanger technology with the new 454 technology, primers used in PCR were used also in process of sequencing. The resulting sequence was entered into the BLAST algorithm of National Center of Biological Information (NCBI) database to obtain closely related phylogenetic sequence and a phylogenetic tree was constructed. The obtained sequence was then submitted to the GeneBank of NCBI database

Bioinformatics analysis of lectin gene

Contig of DNA sequences was performed using the software DNA baser demo version 4.20.0. Six frame translator tool (http://www.ebi.ac.uk/Tools/st/ emboss _transeq) were utilized to deduce the sequence of amino acids. The comparison of sequences in order to find similarity was performed with the aid NCBI database (http://www.ncbi.nlm.nih.gov) by alignment of sequences using Blastn and Blastx and determining the similarities with other DNA and the deduced protein sequence. Phylogenetic tree was constructed using the neighbor-joining (NJ) method of the MEGA-X software. Also search for conserved domains in the predicted proteins was performed on NCBI. Clustal W was utilized to perform multiple sequence alignment of the sequenced gene and deduced sequence of amino acids with the similar genes and proteins that were downloaded from the NCBI.

Purification of *P. ostreatus* SS89 lectin (POL)

Lectin extracted from *P. ostreatus* SS89 fruiting bodies which showed the highest specific activity was selected for further studies.

Ammonium sulfate precipitation and dialysis : According to Dixon and Webb (Dixon and Webb 1961), purification of lectin from crude extract was carried out using 80% ammonium sulfate. The precipitate was centrifuged for 30 min in a cooling centrifuge at 14000 rpm and 4ºC. The obtained precipitate was dissolved in 5 ml of 0.1 M phosphate buffered saline (pH 7.4) and the precipitated protein was dialyzed against the same phosphate buffered saline (PBS) twice overnight in a refrigerator in order to get rid of ammonium sulfate (Sun *et al.* 2014) using dialysis bag (spectra/por® Dialysis Membrane (Nominal Flat width "50 mm", MWCO "6-8000 Da")). Then the protein was lyophilized into a powdered form. The protein content and lectin activity were determined and the specific activity was calculated.

Chromatographic techniques : The chromatographic techniques proceeded according to the method of Chan and Ng (Chan and Ng 2013) with some modifications. Fast Protein Liquid Chromatography (FPLC) system (ÄKTA Avant 150 - GE Healthcare Life Sciences) at the Central Laboratories Network, National Research Centre, Giza, Egypt was used for purification of lectin from the crude protein extract of *P. ostreatus* SS89 fruiting bodies.

The lyophilized crude protein extract was resuspended in 10 mM Tris-HCl buffer (pH 7.6) at a concentration of 5 mg/ml, then loaded onto Q-Sepharose FF (18cm x 15cm) (GE Healthcare) column pre-equilibrated with 10 mM Tris-HCl buffer (pH 7.6). Unadsorbed materials were eluted step wise with the starting buffer and discarded. Adsorbed materials were eluted with a linear gradient of 0-1 M NaCl in 10 mM Tris-HCl buffer (pH 7.6) at a flow rate of 1 ml/min and the absorbance was monitored at 280 nm. The first peak Q1 containing adsorbed fractions with hemagglutinating activity were collected, dialyzed extensively against double distilled water at 4ºC, and lyophilized into powder form.

The lyophilized powder from Q1 peak was re-suspended in 10 mM Tris-HCl buffer (pH 7.6) at a concentration of 20 mg/ml, and then subjected to FPLC-anion exchange chromatography on a Mono Q column (5/50 GL) (GE Healthcare) using an AKTA Purifier (GE Healthcare). The absorbed material was eluted with a linear gradient of 0-1 M NaCl in 10 mM Tris-HCl buffer (pH 7.6) at a flow rate of 1 ml/min and the absorbance was monitored at 280 nm. One absorbed peak M eluted from the Mono Q column constituted the purified lectin (POL), which was dialyzed extensively against distilled water at 4ºC and lyophilized into powder and stored for subsequent characterization.

Characterization of lectin from *P. ostreatus* SS89

Estimation of the molecular weight of the purified *P. ostreatus* lectin (POL) using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) : SDS-PAGE was carried out to determine the purity and molecular mass of the lectin. The procedure was based on that described by Laemmli (1970) using a 15% running gel and a 4% stacking gel. The sample buffer contained 2% (w/v) SDS and 2% (v/v) β-mercaptoethanol. A 10 µl aliquot of hemagglutinin solution was mixed with 10 µl of sample buffer and the mixture was boiled for 5 min at 100 ◦C, then the mixture was injected into the wells of the stacking gel in a Mini- protean II electrophoretic system (Bio-Rad, USA), under a constant voltage (80 V) until the dye front was near the bottom of the gel. At the end of the electrophoresis, the gel was stained with 100 ml 0.5% Coomassie Brilliant Blue in methanol solution (methanol: acetic acid: water, 40: 10:50) for 30 min and then distained with 10% acetic acid for another 30 min. The molecular mass of the hemagglutinin was calibrated with standard BLUltra prestained and broad range molecular weight markers (6.5 -270 kDa; GeneDirex cat no. PM001-0500). Linear regression of log molecular weight with the relative mobility of standard protein markers was used for the determination of the molecular weight of purified lectin.

Carbohydrate-binding specificity assay (Hapten inhibition assay) : Hapten inhibition assay was carried out against a panel of twelve carbohydrates (D (+) -glucose, L-arabinose, d-arabinose, cellulose, cellubiose, dextrose, fructose, lactose, maltose, ribose, sucrose, xylose)**.** A 25 µl of hemagglutinin solution was added to a 96-well microtitre plate and serially diluted with 25 µl of PBS. All inhibitors to be tested were dissolved in PBS at an initial concentration of 400 mM of the tested saccharides, also other concentrations were prepared and tested (200, 100, 50, 25 and 12.5 mM)**.** Saccharide solution (25 µl) was added and mixed with the hemagglutinin solutions in the wells, and then the mixture was left for 30 minutes at 4º C. A 50 µl of 4 % human erythrocyte suspension type AB was added to each well, and the plate was allowed to stand at 4º C for about an hour before the results were observed (Singh *et al.* 2008).

Button formation in the presence of carbohydrates indicated specific interaction, while mat formation indicated an absence of interaction between the lectin and the carbohydrate. The minimum inhibitory concentration (MIC) of each of the specific carbohydrates was determined by serial double dilution of the test solutions. MIC was defined as the lowest concentration of the carbohydrate that was capable of inducing complete inhibition of lectin-mediated hemagglutination.

Effect of:

(a) Different temperatures:

Hemagglutinin solutions were incubated in a water bath for 30 min at various temperatures (ºC): 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100. After 30 min the hemagglutinin solutions were immediately cooled on ice. The hemagglutinating activity was then tested as described above. Results were expressed by calculating the percentage of hemagglutination activity remained shown by the activity of heated aliquots compared with the activity of control sample at 20 ºC representing 100% activity (Xu *et al.* 2014)**.**

(b) Different pH

To study the effect of pH on the *P. ostreatus* lectin activity, the hemagglutinin solutions were incubated for 1 hour in the following reagents at different pH values; 0.1 M HCl for pH 1; 0.2 M glycine- HCl buffer for pH 2 & 3; 0.2 M citrate buffer for pH 4, 5& 6; 0.2 M phosphate buffer for pH 7 and 8; 0.2 M glycine-NaOH buffer, pH 9, 10 &11; and 0.1 M NaOH for pH 13, then each sample was neutralized by adding either HCl or NaCl to prevent hemolysis caused by the adverse pH which hinder hemagglutination. Hemagglutination assay was carried out as described above. The control value was the agglutination titer of the lectin in PBS, pH 7. The results were expressed by the percentage of hemagglutinating activity remained. This was shown by calculating the activity of the treated hemagglutinin solution compared with the activity of control sample at pH 7 representing 100% activity (Wang *et al.,* 2000).

(c) Different mono- and divalent cations

The hemagglutinin solutions had been dialyzed extensively against deionized water containing 10 mM EDTA for 24 hours. A 25 µl of the dialyzed hemagglutinin solution was serially diluted in 96-well microtiter plate, then mixed with equal volume of the cation solution (25 µl) in the respective concentrations (100, 50, 25, 12.5, 6.25 and 3.125 mM) and incubated at 4 ºC for two hours. The cations used were in the form of chlorides; NaCl, KCl, $CaCl_2$, $CuCl_2$, $Mncl_2$, $HgCl_2$ and $ZnCl_2$. After 2 hours, the hemagglutination activity of the lectin was determined by adding 50 µL of 4% human erythrocyte suspension type AB in PBS to each well. The plates were then allowed to stand at 4◦C for 1 hour. A control assay of the activity was done by using hemagglutinin solution without cations and the resulting activity was taken as 100% activity (Wang *et al.,* 2000).

Biological applications of lectin from *P. ostreatus* SS89

Cytotoxicity : The cytotoxic activity was carried out using Sulphorhodamine-B (SRB) assay following the method reported by Vichai and Kirtikara (Vichai and Kirtikara 2006), using the following normal baby hamster kidney cell line (BHK) and three human tumor carcinoma cell lines, namely breast cancer (MCF7); colorectal cancer (HCT) and hepatocellular carcinoma (HEPG2). They were obtained from the American Type Culture Collection (ATCC, Minisota, U.S.A.). Each type of cells were separately seeded in 96-well microtiter plates at an initial concentration of $3x10^3$ cell/well in a 150 µl fresh medium and left for 24 hours before treatment with hemagglutinin solution to allow attachment of cells to the plates and duplication of cells number to reach from 5000 to 6000 cells/well. Different concentrations (0, 62.5, 125, 250 and 500 µg/ml) of hemagglutinin solution were added. For each hemagglutinin solution concentration, 3 wells were used. The plates were incubated for 48 hours, then the cells were fixed with 50 µl cold trichloroacetic acid 10% final concentration for 1 hour at 4ºC .The plates were washed with distilled water using (automatic washer Tecan, Germany) and stained with 50 µl 0.4 % SRB dissolved in 1 % acetic acid for 30 minutes at room temperature. The plates were washed with 1 % acetic acid and air-dried. The control was the cells used without treatment with POL or crude extract that grew under the same conditions.

SRB was solubilized with 100 µl/well at 10M Tris base (pH 10.5) and optical density (O.D.) of each well was measured spectrophotometrically at 570 nm with an ELISA microplate reader (Sunrise Tecan reader, Germany). The mean background absorbances were automatically subtracted and mean values of each hemagglutinin concentration was calculated. The experiment was repeated 3 times. The inhibition of cell viability (ICV %) was calculated as follows: (1 – absorbance of treated cell / absorbance of treated cell) x 100 (Lam and Ng 2010).

Antimicrobial activity : Antibacterial and antifungal activity of POL and crude extract was determined using a modified Kirby-Bauer disc diffusion method (Bauer *et al.* 1966).

Antibacterial activity : Antibacterial activity of POL and crude extract was determined by using three gram positive species, namely *Bacillus subtilis* (ATCC: 6051; *Staphylococcus aureus* (ATCC: 12600) and *Streptococcus faecalis* (ATCC: 19433) and three gram species negative, namely *Escherichia coli* (ATCC: 11775); *Pseudomonas aeruginosa* (ATCC: 10145) and *Neisseria gonorrhoeae* (ATCC: 19424), 100 µl of the tested bacteria were grown in 10 ml of fresh broth media until they reached a count of approximately 10^8 cells/ml (Pfaller *et al.* 1988). Blank paper disks (Schleicher & Schuell, Spain) with a diameter of 8.0 mm were impregnated with 10µl of 100 µg/ml of the hemagglutinin solution, were added in the center of the plates, then plates were incubated at 35-37°C for 24-48 hours. The diameters of the inhibition zones were measured in millimeters. Standard discs of Ampicillin (Antibacterial agent) served as positive controls for antibacterial activity, but filter discs impregnated with 10 µl of solvent Dimethyl sulfoxide (DMSO) were used as a negative control.

Antifungal activity : Antifungal activity of POL and crude extract was determined by using one filamentous fungus *Aspergillus flavus* (ATCC:16872) and one yeast *Candida albicans* (ATCC: 7102), 100 µl of the tested fungi were grown in 10 ml of fresh broth media until they reached a count of approximately 10^5 cells/ml (Pfaller *et al.* 1988). The method used was the same as that used in the previous antibacterial assay. Plates were incubated at 30°C for 5 days in case of *Aspergillus flavus* and for 24-48 hours at 30°C in case of *Candida albicans*. Standard discs of Amphotericin B (Antifungal agent) served as positive controls for antifungal activity.

Statistical analysis

All the experiments were conducted in triplicates and results are expressed as mean ± standard deviation (SD). SPSS software was utilized where a significant difference in results was observed; a statistical analysis was carried out using one-way ANOVA followed by Turkey's post hoc test. Graphical representation of results was conducted using Graph Pad Prism (version 8) software.

Results and Discussion

Screening of different mushroom species belonging to genus *Pleurotus* for lectin production

The results in Table 1 showed that all the tested mycelial and fruiting bodies extracts exhibited hemaggultinating activity toward different types of human erythrocytes but with varying abilities. All of these extracts showed hemagglutinating activity toward at least two types of the used human erythrocytes except fruiting body extracts of *P. ostreatus* which exhibited specificity toward AB human blood type only. However, fruiting body extract of *P. sajor-caju* was able to agglutinate all human erythrocyte types.

Culture filtrate of the two broth media showed no hemagglutinating activity. The negative results of culture filtrate may be attributed to the complete absence of lectin in the culture filtrate or the presence of lectin in culture filtrate but not able to exhibit hemagglutinating activity. This may be due to that certain lectins contain a single carbohydrate-binding domain, like that of the monomeric mannose-binding lectin from orchids, which is not able to agglutinate red blood cells. Other lectins may not be able to agglutinate the type of erythrocytes used as in case of lectin from *Cordyceps militaris* mushroom (CML) which displayed agglutination activities towards the mouse and rat erythrocytes and not towards human erythrocytes (Pusztai 1991, Jung *et al.,* 2007). Therefore, culture filtrate was not included in further assays.

Table 1: Reaction of different lectins extracted from fruiting bodies, mycelium and culture filtrate of three *Pleurotus* species using 4 human erythrocytes types (A, B, AB, O). Two different broth medium was used (PDB and CDB) in case of mycelium and cultural filtrate lectin.

Mushroom species	Source of extraction	Hemagglutination reaction			
		A	B	AB	O
Pleurotus columbinus	1	+	-	+	+
	2	+	-	+	+
	3	-	-	-	
	4	-	-	-	-
	5	+	-	+	-
Pleurotus ostreatus	1	-	-	+	+
	2	-	-	+	+
	3	-	-	-	-
	4	-	-	-	-
	5	-	-	+	-
Pleurotus sajor-caju	1	+	-	+	-
	2	+	-	+	-
	3	-	-	-	-
	4	-	-	-	-
	5	+	+	+	+

(1): Mycelial lectin extracted from PDB supplemented with yeast.
(2): Mycelial lectin extracted from CDB supplemented with yeast.
(3): Culture filtrate lectin extracted from PDB.
(4): Culture filtrate lectin extracted from CDB.
(5): Lectin extracted from fruiting bodies.

Table 2 indicated that changing the composition of liquid culture media resulted in a change in the total protein and the activity of the produced lectin but did not affect the specificity of lectin toward the human erythrocyte type. This may indicate that changing of medium composition may affect the amount of lectin but did not affect the type of lectin. The ability to synthesize lectins is widespread among higher basidiomycetes and hemagglutinating activity is not only depending on species, but also is strain dependent (Mikiashvili *et al.,* 2006; Davitashvili *et al.,* 2008; Mikiashvili *et al.,* 2009; Davitashvili *et al.,* 2011; Davitashvili *et al.,* 2015). *P. ostreatus* fruiting bodies exhibited the highest lectin specific activity. Accordingly, this was selected for the further studies.

Many researchers studied the relationships between lectins present in fruiting bodies and mycelia. Musilek *et al.* (1990) found marked differences in the presence of lectins and in their specificity with *Flammulina velutipes*, *Kuehneromyces mutabilis*, and *Pholiota squarrosa*. Only *K. mutabilis* mycelial lectins had properties similar to those of the lectins from wild fruiting bodies. Kaneko *et al.* (1993) used human type (A) erythrocytes to study lectin activity in *P. cornucopiae* at different morphological stages (vegetatively growing mycelium, primordium, and immature and mature fruit body). No hemagglutinating activity was founded in the vegetative mycelium, but it started to appear in the primordial stage and increased during fruit body formation. In *Agrocybe aegerita*, it was found that when mycelial cultures had matured to the phase of fruiting body formation, their lectin composition was comparable to that of the wild type (Ticha *et al.,* 1985).

Table 2: Specific activity of fruiting bodies and mycelial extracts from three different *Pleurotus* species in two different broth media using human erythrocytes type AB.

	Source of extraction	Lectin activity (U)	Total protein (mg. ml^{-1}) ±SD	Specific activity (U/mg. ml^{-1}) ±SD
P. columbinus	Mycelium (PDB + Yeast)	8	12.29 ± 0.066^{ef}	$0.001^{e} \pm 0.65$
	Mycelium (CDB)	4	12.33 ± 0.058^{de}	$0.001^{f} \pm 0.32$
	Fruiting bodies	64	20.3 ± 0.08^{b}	3.123 ± 0.006^{b}
P. ostreatus	Mycelium (PDB + Yeast)	16	12.17 ± 0.09^{f}	$\pm 012^{d}1.31$
	Mycelium (CDB)	8	11.93 ± 0.00^{g}	$\pm 0.0^{e}0.67$
	Fruiting bodies	256	$20.5 \pm .036^{a}$	12.6 ± 0.05^{a}
P. sajor-caju	Mycelium (PDB + Yeast)	4	12.46 ± 0.029^{d}	$2 \pm 0.0006^{f}0.3$
	Mycelium (CDB)	4	11.8 ± 0.00^{g}	$\pm 0.0^{f}0.34$
	Fruiting bodies	32	20.05 ± 0.0^{c}	1.6 ± 0.0^{c}

Data shown are means of three replicates; SD: Standard deviation. Means followed by the same letters are statistically non-significant.
PDB + Yeast: Potato Dextrose Broth supplemented with yeast.
CDB: Czapek Dox Broth.
Yellow highlight indicates the organism that produces the most potent lectin.

Molecular identification of the selected *Pleurotus* that produce the most potent lectin

Amplification and sequencing of fungal ITS region of rDNA resulted in 556 bp long nucleotide sequences as shown in Fig. 1A. According to molecular identification, the selected *Pleurotus* was identified as *Pleurotus ostreatus* SS89. The resulted sequence was submitted to Genbank under accession number: MN795635.

Sequencing of lectin gene and deducing of lectin amino acid sequence

Amplification and sequencing of the lectin gene from *P. ostreatus* SS89 resulted in 1094 bp long nucleotide sequences as shown in Fig. 1B. The resulted sequence of lectin gene was submitted to the NCBI database under accession number MT074428.2, *Pleurotus ostreatus* strain SS89 lectin gene, partial cds (accession no. MT074428.2) showed similarity with *Pleurotus ostreatus* lectin 1 gene, complete cds (accession no. MH106980.1) with percent identity of 100 % and 91% Query Cover. Also the amino acid sequence was deduced and given protein_id "QJQ82566.1". The open reading frame of the lectin gene consists of 801 bp encoding 267 amino acids. The deduced amino acid sequence was analyzed for similarity search by BLAST at NCBI selecting non-redundant database. The phylogenetic tree between *P. ostreatus* SS89 lectin and the other closely related lectin represented in Fig. 2.

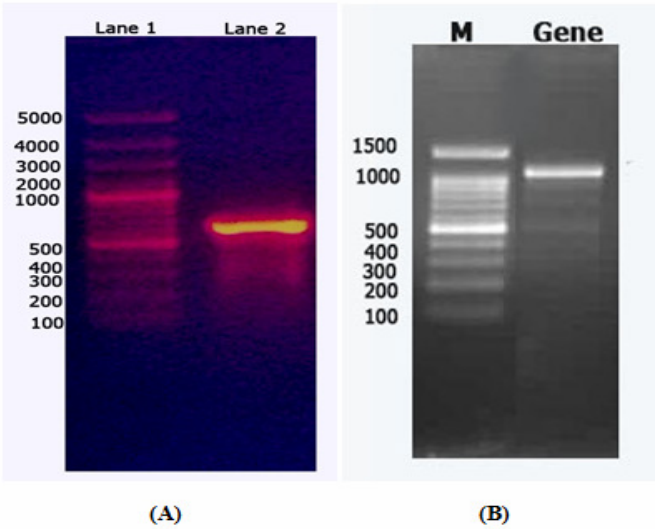


Fig. 1 : Molecular characterization of *P.ostreatus* and pure lectin. (A) Agarose gel electrophoresis of PCR product for *Pleurotus ostreatus* identification [lane 1: 1Kb plus DNA ladder; lane 2: DNA fragment of *Pleurotus Ostreatus* in 1/% agarose gel. (B) Agarose gel electrophoresis for PCR product for sequencing of lectin gene, (lane M) represent a DNA size marker (Gene Ruler 100 bp Plus DNA ladder, Frementas).

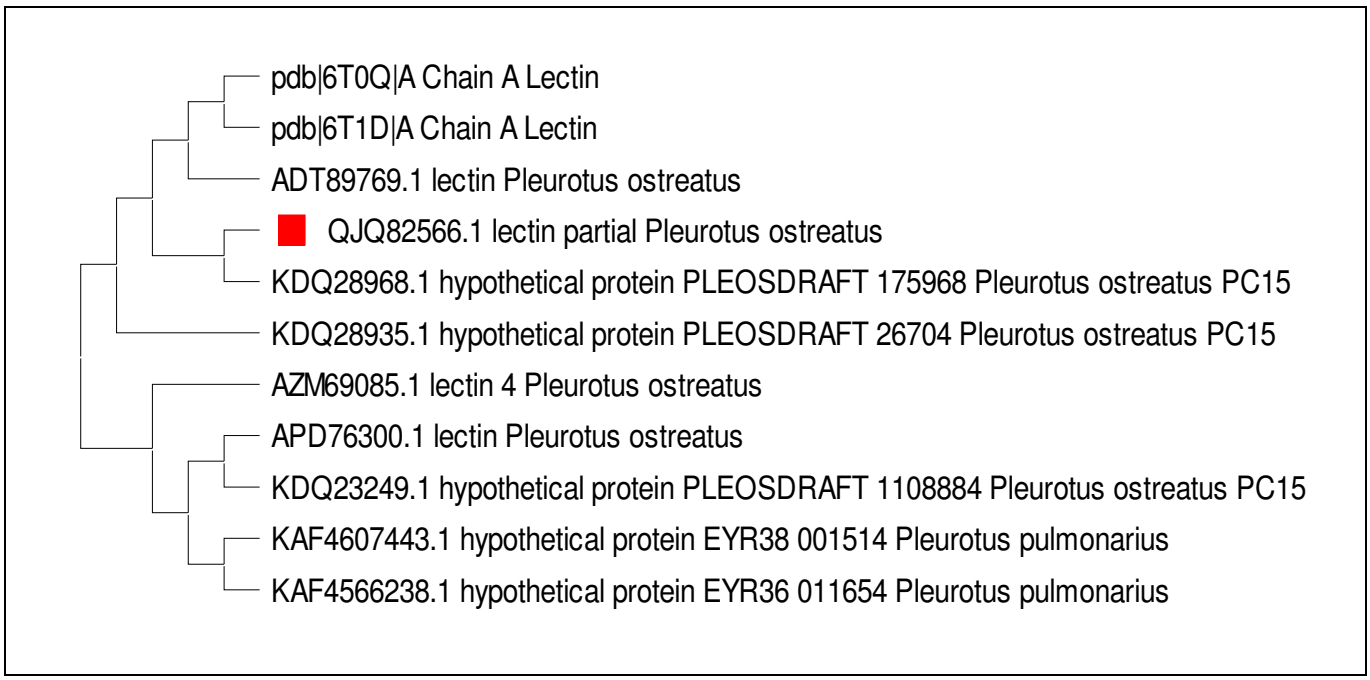


Fig. 2 : Phylogenetic tree showing a genetic relationship between the lectin from *Pleurotus ostreatus* strain SS89 (protein-id: QJQ82566.1) and other closely related lectins from other *Pleurotus* strains.

Fig. 3 showed the multiple sequence alignment between partial amino acid sequences of lectin from *P. ostreatus* SS89 and lectins from other different *Pleurotus* species that was conducted using CLC Main Workbench 20 program. BLAST analysis of the deduced amino acid sequence of lectin from *P. ostreatus* SS89 (accession no. QJQ82566.1) showed 96.12% identity with hypothetical protein PLEOSDRAFT_175968 (*P. ostreatus* PC15) (accession no. KDQ28968.1), 95.55 % with *P. ostreatus* chain A lectin (accession no. 6T0Q_A), 95.53% with Chain A, Lectin (accession no. 6T1D_A), 95.08 % with *Pleurotus ostreatus* lectin (accession no. ADT89769.1). In addition to, 75.81 % similarity was found with hypothetical protein PLEOSDRAFT_26704 (*P. ostreatus* PC15) (accession no. KDQ28935.1), 73.58% with *P. ostreatus* lectin (accession no. APD76300.1), 72.86 % with hypothetical protein PLEOSDRAFT_1108884 (*P. ostreatus* PC15) (accession no. KDQ23249.1) and 70.19% with *P. ostreatus* lectin 4 (accession no. AZM69085.1).

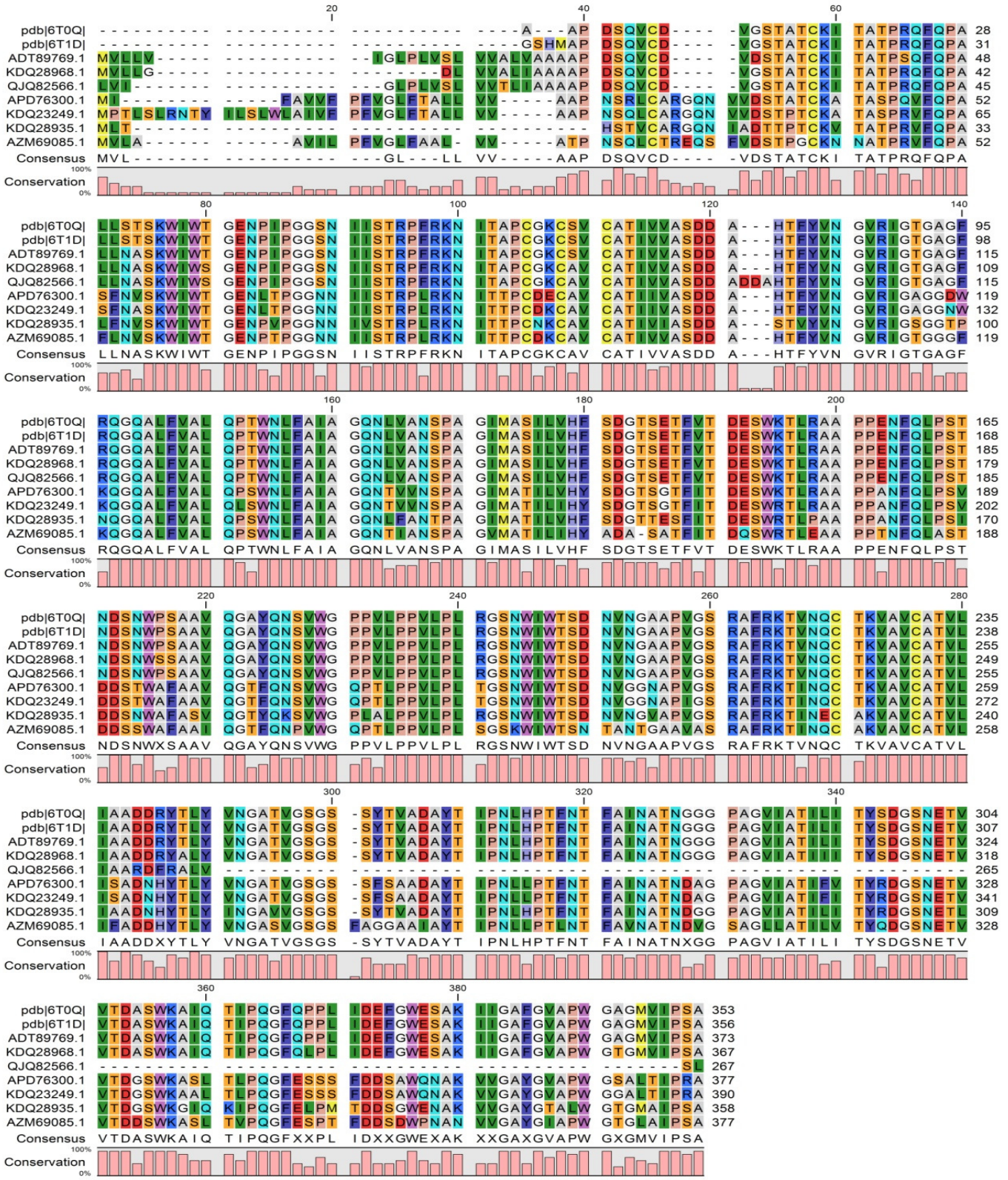


Fig. 3 : Multiple alignments of the POL sequence with sequences from other *Pleurotus ostreatus* lectins using the CLC Main Workbench 20 program. The sequence data were obtained from the NCBI sequence database. Accession numbers are as follows: KDQ28968.1, 6T0Q_A, 6T1D_A, ADT89769.1, KDQ28935.1, APD76300.1, KDQ23249.1 and AZM69085.1.

The deduced partial amino acid sequence of POL was scanned for conserved residues by the Conserved Domain Database (CDD). The CDD results revealed the presence of a conserved domain of Alpha-L-rhamnosidase N-terminal domain, Spanning residues between amino acid number 146 and 195 (CDD accession: cl07232) as shown in Fig. 4. This family consists of bacterial rhamnosidase A and B enzymes. This domain is probably involved in substrate recognition.

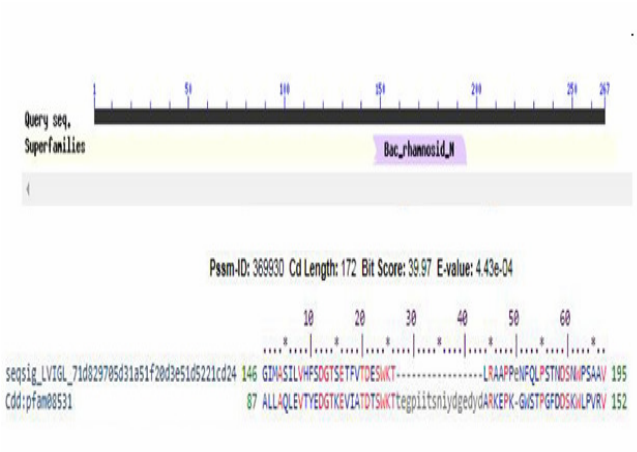
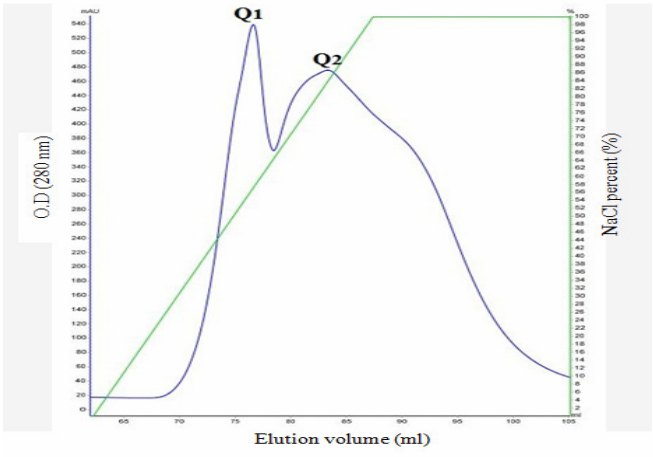


Fig. 4: Graphical view showing the conserved domain of *Pleurotus ostreatus* SS89 lectin. The deduced partial amino acid sequence of *P. ostreatus* SS89 lectin (protein_id QJQ82566.1) contains a conserved domain of Bac_rhamnosid_N superfamily (CDD accession: cl07232).

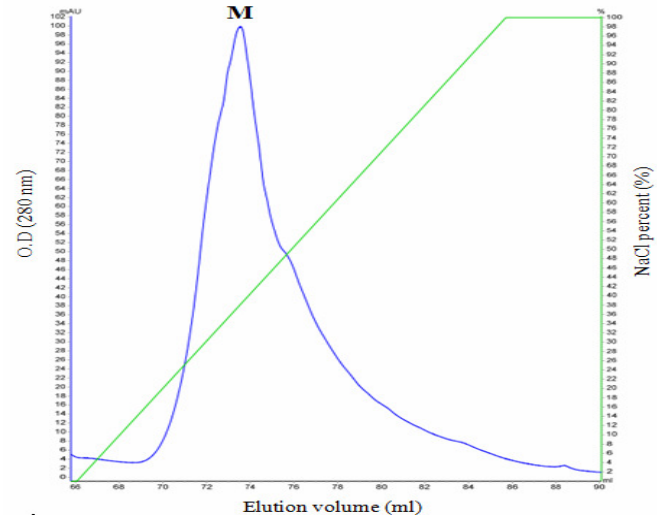
Amino acid sequences of several fungal and mushroom lectins have been determined and analyzed for similarity and homology with other lectins and proteins. The amino acid sequence of *Xerocomus chrysenteron* lectin showed 69 and 64% homology with *Agaricus bisporus* and *Arthrobotrys oligospora*, respectively (Birck *et al.* 2004). The amino acid sequence of *Pleurocybella porrigens* lectin showed similarity with ricin-B-chain (33%), lectin from *Polyporus squamosus* (36%) and hemagglutinin from *Clostridium botulinum*, HA-1 (40%) (Suzuki *et al.,* 2009). Analysis of lectin from *P. ostreatus* by Perduca *et al.* (2020) revealed that the complete sequence of lectin consists of 353 amino acid chain. In a study by Suzuki *et al.* (2009) on lectin from mushroom *Hygrophorus russula* the open reading frame of the cDNA consisted of 528 bp encoding 176 amino acid and the sequence showed homology to the lectin from the mushroom *Grifola frondosa* (GFL; 38%) and the lectin from Japanese sago palm *Cycas revoluta* (CRLL; 27%).

Purification of *P. ostreatus* lectin (POL)

POL was purified following a purification protocol that entailed two consecutive steps of ion exchange chromatography. The first step was cation exchange chromatography on Q-Sepharose FF which yielded two major adsorbed peaks (Q1 and Q2) as shown in Fig. 5A. Hemagglutination activity was residing in the first peak Q1, only 4 fractions showed the highest hemagglutinating activity and therefore they were pooled and lyophilized. The specific activity of these collected fractions was 204.8 U/mg representing 16.4 fold increases over the crude extract and the recovery percentage was 24 % as recorded in Table 3. These fractions were subjected to the second step which was FPLC-cation exchange chromatography on Mono Q this step yielded one major adsorbed peak (M peak) as shown in Fig. 5B. Only five fractions showed the highest hemagglutinating activity so they were pooled and lyophilized. The specific activity was 512 U/mg, which represents 40.99-folds purification of the crude extract. The hemagglutinin recovery percentage was 20% as recorded in Table 3. Several methods had been used for purification of mushroom lectin. Similarly, most lectins were purified using two ion- exchange chromatography following the precipitation with ammonium sulfate. *P. ostreatus* fruiting body lectin was purified by Wang *et al.* (2000) using ammonium sulfate and two ion-exchange chromatographic of DEAE- cellulose and CM-cellulose and finally subjected to fast protein liquid chromatography on a Superpose 12 with 48.7-fold purification and 28.1 % hemagglutination recovery. Also *Paxillus involutus* lectin was purified using two ionic chromatography, DEAE-cellulose and Q-Sepharose followed by Superdex 75 HR 10/30 column, resulting in pure lectin with purification fold of 38.8 and 42.8% recovery of activity (Wang *et al.* 2013). Many other studies used more than two ionic-exchange chromatography as in the purification of lectin from the fruiting body of mushroom Boletus speciosus by Masri *et al.* (2017) which adsorbed successively on DEAE-cellulose, CM-cellulose, and Q-Sepharose columns, then the purified fraction was obtained through gel filtration on a Superdex G-75 HR 10/30 column. Other lectins were purified using single step techniques such as the purification of *Ganoderma lucidum* lectin using single step of hydrophobic interaction chromatography (Thakur *et al.,* 2007).



(A)



(B)

Fig. 5: Typical elution profile of *Pleurotus ostreatus* hemagglutinin on (A) Q-Sepharose FF column (18cm x 5 cm) (GE Healthcare) and (B) Mono Q column (GE Healthcare) (5/50GL) using an AKTA Purifier (GE Healthcare) previously equilibrated with 10 mM Tris-HCl buffer, pH 7.6. Elution was performed using a linear gradient of 0-1 M NaCl in 10 mM Tris-HCl buffer (pH 7.6) at a flow rate of 1 ml/min and the absorbance was monitored at 280 nm.

Table 3: Results of the purification steps of hemagglutinine (lectin) from fruiting bodies of *Pleurotus ostreatus*

Purification Step	Total activity * (U)	Total protein (mg) ± SD	Specific activity ** (U/mg) ± SD	Purification Fold ***	Recovery **** (%)
Crude extract	25600	2050 ± 10.82[a]	12.51 ± 0.047[e]	1	100
Ammonium sulfate precipitation	8704	180 ± 11[b]	48.48 ± 2.97[d]	3.87	34
Dialysis	7680	66 ± 4.36[c]	116.71 ± 8[c]	9.32	30
Column 1 (Q-Sepharose)	6144	30 ± 1.2[d]	202.97 ± 11.36[b]	16.4	24
Column 2 (Mono Q)	5120	10 ± 0.45[e]	512.69 ± 23.098[a]	40.99	20

Data shown are means of three replicates. SD: Standard deviation, Means followed by the same letters are statistically non-significant.
*Total activity: hemagglutinating activity of lectin in total protein.
** Specific activity: hemagglutinating activity/ mg protein.
*** Fold purification: calculated as the ratio between specific activities of the fractions with the crude extract.
**** Recovery (%): percentage of the ratio between hemagglutinating activities of the fractions with the crude extract.

Characterization of lectin from *P. ostreatus* SS89

Estimation of the molecular weight of the purified *P. ostreatus* lectin (POL) by SDS-PAGE : The purified lectin produced from FPLC-cation exchange chromatography (Mono Q) appeared as a single band with a molecular mass of 29 KDa in SDS-PAGE as shown Fig. 6. This suggested that POL is a monomeric protein composed of one subunit. In a close study carried out by Zhang *et al.* (2019) *Agaricus bitorquis* lectin was found to be a monomeric protein with a molecular mass of 27.6 kDa. Mushroom lectins isolated from different mushroom species vary in molecular masses, subunit number and carbohydrate specificity (Yang *et al.* 2009). The molecular weights of most lectins isolated from edible mushrooms range from 12-kDa to 190-kDa and consist of one or more subunits which might be identical (homo) or non-identical (hetero) (Singh *et al.,* 2020). Most of lectins isolated from edible mushroom are dimeric and subunits held together by non-covalent interaction as in lectin purified by Wang *et al*. (2000) from fruiting bodies of edible mushroom *P. ostreatus* that was heterodimeric lectin composed of 40-kDa and 41-kDa subunits. Lectins from *Polyporus squamosus* and *Xylaria hypoxylon* are homodimeric lectins with a molecular weight of 28 and 28.8 kDa, respectively (Thakur *et al.,* 2007). A few lectins are multimeric such as the novel hexameric lectin isolated from *Ganoderma lucidum* with a molecular weight of 114-kDa. To date, a lectin from *Pholiota squarrosa* possesses the lowest molecular mass of 4.5 kDa (Zhang *et al.,* 2019).

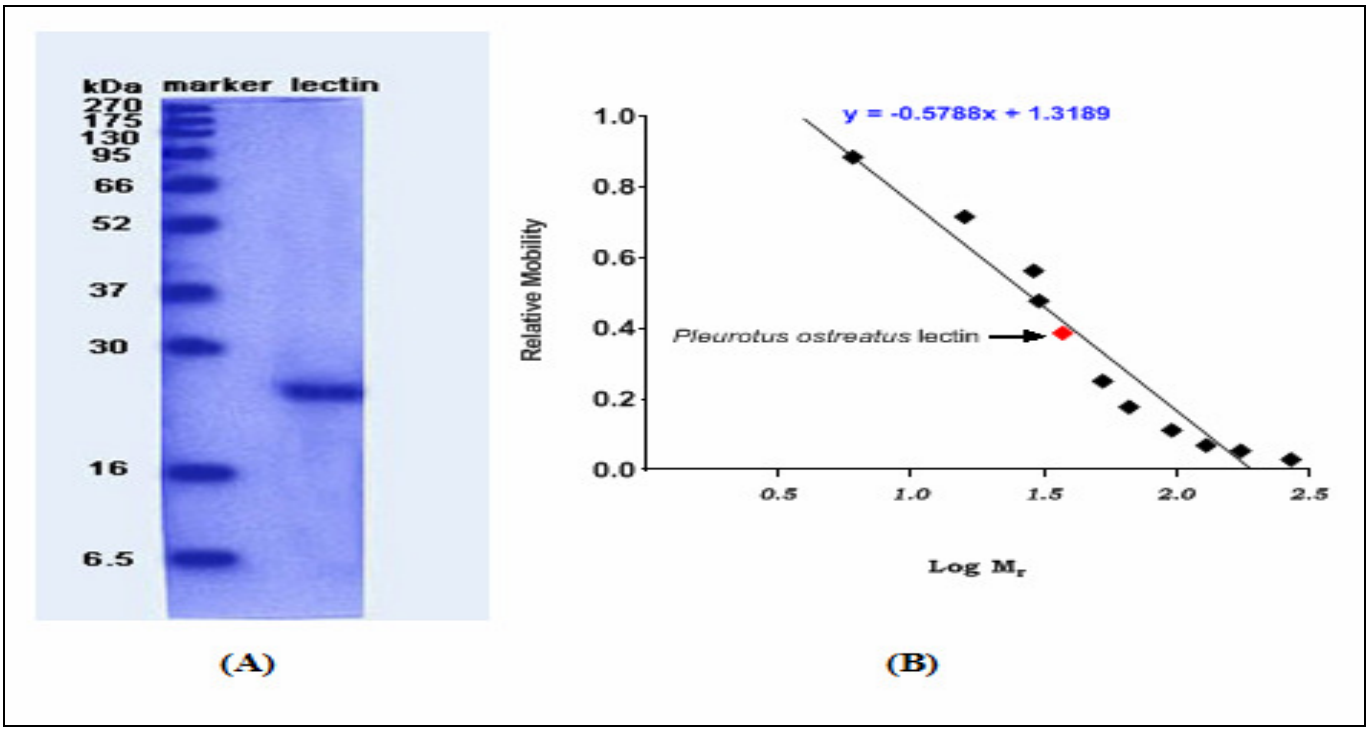


Fig. 6: (A) SDS-PAGE of *Pleurotus ostreatus* lectin. (Lane1: The molecular weights of the marker proteins; Lane 2: The molecular weight of purified lectin). (B) SDS-PAGE regression curve depicting the position of *Pleurotus ostreatus* lectin as a red dot. Relative mobility of standard reference proteins was plotted against the log molecular weight. Molecular weight standard used were: (6.5, 16, 30, 37, 52, 66, 95, 130, 175 and 270).

Carbohydrate-binding specificity assay (Hapten inhibition assay) : The results in Fig. 7 showed that POL and crude extract from *P. ostreatus* SS89 had the ability to interact with xylose which was indicated by reduction in hemagglutination activity of both POL and crude extract. The minimum inhibitory concentrations of xylose in case of POL and crude extract were 100 mM and 600 mM respectively, as shown in Table 4. Other sugars tested had no effect on the hemagglutination activity of both POL and crude extract. In agreement with the current study the hemagglutinating activity of *Xylaria hypoxylon* lectin was inhibited by xylose (Singh *et al.* 2015). Lectin from mushroom *Stropharia rugosoannulata* like POL did not exhibit any change at various concentrations from 0.87 mM to 200 mM of different sugars except inulin with minimum concentration of 50 mM (Zhang *et al.,* 2014). *Agaricus bisporus* lectin inhibited by N-acetyl-D-galactosamine, while lectins from other *Agaricus* species, including *A. blazei*, *A. campestris*, and *A. edulis*, were not affected by common monosaccharides (Singh *et al.* 2015). Another *P. ostreatus* lectin purified by Wang *et al.* (2000) was affected by most of tested sugar including, inulin, melibiose, lactose, D- galactose Raffinose, N-acetylneuraminic acid and α-Methyl-D-galactopyranoside.

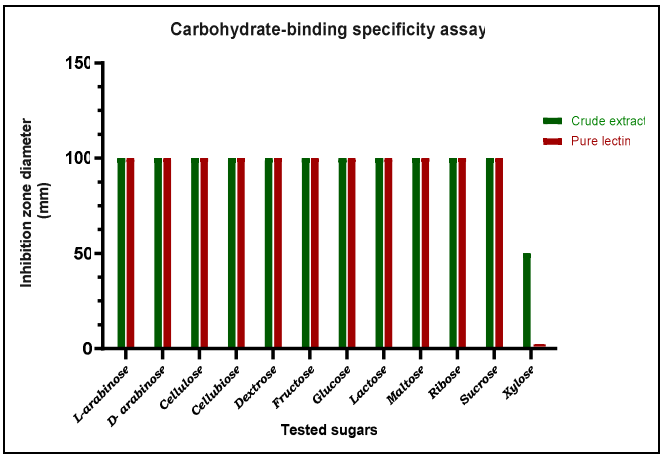


Fig. 7 : Comparison between hemagglutination inhibition assay of crude extract and pure lectin from *Pleurotus ostreatus* SS89 using 12 different types of sugars (concentration of sugar = 200 m M)

Table 4: Effect of different concentrations of xylose on hemagglutinating activity of pure lectin and crude extract from *Pleurotus ostreatus.*

Concentration of xylose (mM)	Hemagglutinating activity remained (%)	
	Pure lectin	Crude extract
12.5	50	100
25	25	100
50	12.5	100
100	0 (MIC)	100
200	0	50
400	0	25
600	0	0 (MIC)
800	0	0

Data shown are means of three replicates; MIC: Minimum Inhibitory concentration of xylose that completely inhibit hemagglutinating activity; MIC of xylose in case of: (pure lectin = 100 mM) and (crude extract = 600 mM).

Effect of:

(a) Different temperature:

Both POL and crude extract exhibited moderate thermostability as shown in Fig. 8A. In case of POL, hemagglutinating activity was 100% at 0 ^{o}C up to 50 ^{o}C, after that the hemagglutinating activity decreased as the temperature increased to reach 25% at 60 ^{o}C and 6.25% at 70 ^{o}C. The activity of POL disappeared at 80 ^{o}C. The crude extract was slightly less thermostable than POL. Crude extract activity was 100% at 0 ºC up to 40 ^{o}C, then decreased to 50% at 50 ^{o}C and to 25% at 60 ^{o}C. The activity of the crude extract completely disappeared at 70 ^{o}C. The activity of both POL and crude extract was 100% at 37 ^{o}C which is the physiological temperature of the plasma, so it can be used as a drug for injection (Wong *et al.,* 2010). In a closed study, Sun *et al*. (Sun *et al.* 2014) found that hemagglutinating activity of *Boletus specious* lectin (BSL) was stable up to 60 ^{o}C then declined to 12.5% of the original value at 70 ^{o}C and completely inhibited at and above 80 ^{o}C. Based on the properities of POL, it was more thermostable than *Xylaria hypoxylon* lectin (Liu *et al.* 2006), that was stable up to 35 ^{o}C, also the hemagglutinating activity of *P. ostreatus* lectin that purified by Wang *et al*. (2000) was reduced at and above 40 ^{o}C and *Paxillus involutus* lectin was stable up to 32 ◦C while no activity was detected at 42 ^{o}C or higher (Wang *et al.* 2013). *P. ferulae* lectin (PFL) purified by Xu *et al*. (2014) lost 50% of the original activity at 70 ^{o}C; it also reduced further to be 6.25 % at 80 ^{o}C then completely destroyed at and above 90 ^{o}C

(b) Different pH:

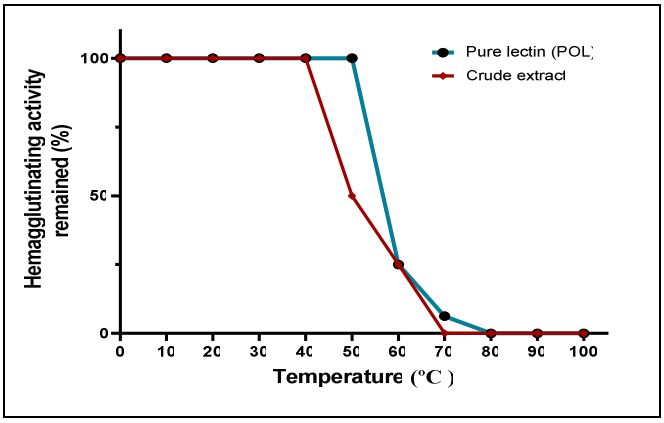
Both POL and crude extract exhibited moderate pH stability as shown in Fig. 8B. In case of POL, no hemagglutinating activity was recorded at pH 1 and 2. At pH 3 POL activity was 50% and increased by increasing the pH value to reach 100% over the pH range (7-9). After pH 9, POL activity decreased again to reach 12.5% at pH 10, after that the activity completely disappeared. In case of crude extract, also no activity recorded at pH 1 and 2. The activity of crude extract was 50% over the pH range (3-5), and then increased to reach 100% over the pH range (6-8). After pH 8 the activity decreased to 25% at pH 9 and 12.5% at pH (10 and 11), then no activity could be detected. Absence of hemagglutinating activity of both POL and crude extract at pH 1 and 2, and low activity at pH 3 preclude its development as an orally administered drug, due to the pH range of gastric juice from 1 to 3 (Wong *et al.,* 2010).

Stability of mushroom lectin in different pH values differ from one another. In a Similar study to the present work, Xu *et al*. (2014) revealed that no activity of *P. ferulae* lectin was found at pH 1-2, while activity was stable at pH range 6-9. Below pH 9 no activity was shown and at pH 5 only 50% of the lectin activity remained. The hemagglutinating activity of lectin from *Boletus* species (BSL) was not detected at very low pH 0.7-1.3, while activity was stable at pH range 2.2 to 12.1 and diminished to reach 50%of the original activity at pH 12.4 and 12.7. Reduction continued to reach 25 % at pH 13 and 1.6 (Sun *et al.* 2014).

(c) Different mono- and divalent cations:

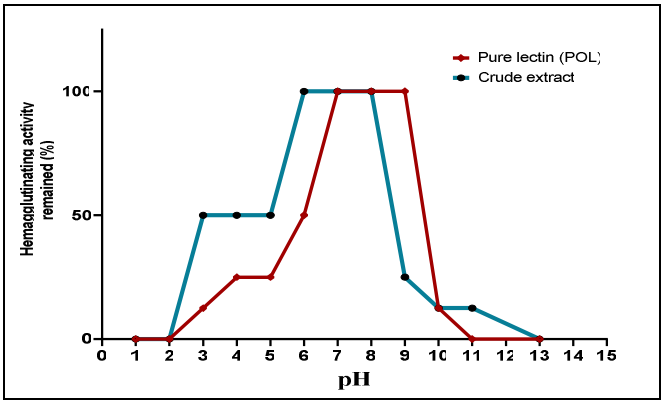
The hemagglutinating activity of POL and crude extract from *P. ostreatus SS89* was affected by different concentrations of different cations from 3.125 mM to 100

mM as shown in Fig. 8C. In case of POL, Cu^{2+}, Hg^{2+} and Zn^{2+} were the strongest inhibitor that completely inhibited POL activity at all concentrations followed by Mn^{+2} which inhibit POL with MIC value of 6.25 mM, then K^{+} (MIC= 12.5 mM) and finally Na^{+} and Ca^{2+} (MIC= 25 mM) as recorded in Table 5. In case of crude extract, the activity completely inhibited by Hg^{2+} and Zn^{2+} at all concentrations, followed by Cu^{2+} and Mn^{2+} with MIC value of 6.25 mM, then Na^{+} and Ca^{2+} (MIC= 25 mM) and finally K^{+} (MIC=50 mM) as shown in Table 5. The way by which cations affect the hemagglutinating activity of mushroom lectins differ from one another. The fact that no increase in POL hemagglutinating activity was detected in the presence of the metal ions suggests that the active region of POL may not contain any cations. The inhibitory effect of Zn^{2+}, Hg^{2+} and Cu^{2+} ions indicates that POL hemagglutinating activity may be reduced by heavy metals due to protein denaturation. Another lectin from *P. ostreatus* purified by Wang *et al.* (2000) was inhibited by 1 mM and above of Ca^{2+}, Mg^{2+} and Mn^{2} but was not affected by Zn^{+2}. Trivalent cations like Fe^{3+} completely inhibited activity of *P. ostreatus* lectin at all concentrations. Unlike the current study, Sun *et al.* (2014) showed that the hemagglutination activity of *Boletus specious* lectin (BSL) was not affected in the presence of most tested metal ions K^{+}, Cd^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Al^{3+}, and Fe^{3+} (1.25–10mM). It was reduced by Fe^{2+} (5–10mM), Ca^{2+} (5–10 mM), and Pb^{2+} (2.5–10mM) and potently reduced by Hg^{2+} (1.25–10mM) ions. *Polyporus adusta* lectin showed no effect when incubated with different metal ions $ZnCl_2$, $MgCl_2$, $MnCl_2$ and $CaCl_2$, while $FeCl_3$ activated lectin activity at 10 mM (Wang *et al.*, 2003).

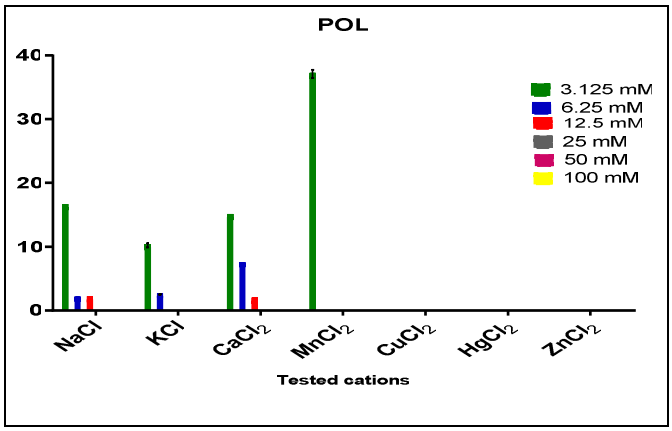


(A)

Figure 8

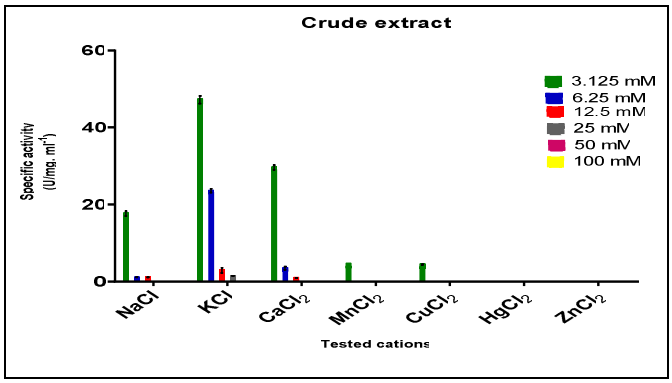


(B)



(C)

Figure 8



(D)

Figure 8: Effect of different (A) temperature and (B) pH on *Pleurotus ostreatus* lectin-induced hemagglutination in case of POL and crude extract. Effect of different concentrations of mono- and divalent cations in the form of chlorides on hemagglutinating activity of (C) POL and (D) crude extract from *P.ostreatus*. Bars show means of three replicates. Error bars show ± SD.

Table 5: Minimum Inhibitory Concentration (MIC) of different cations that inhibit hemagglutinating activity of *Pleurotus ostreatus* pure lectin and crude extract.

Cations tested	MIC (mM)	
	Crude extract	Pure lectin
NaCl	25	25
$CaCl_2$	25	25
KCl	50	12.5
$MnCl_2$	6.25	6.25
$CuCl_2$	6.25	N. D.
$ZnCl_2$	N. D.	N. D.
$HgCl_2$	N. D.	N. D.

N.D. : Not detectable

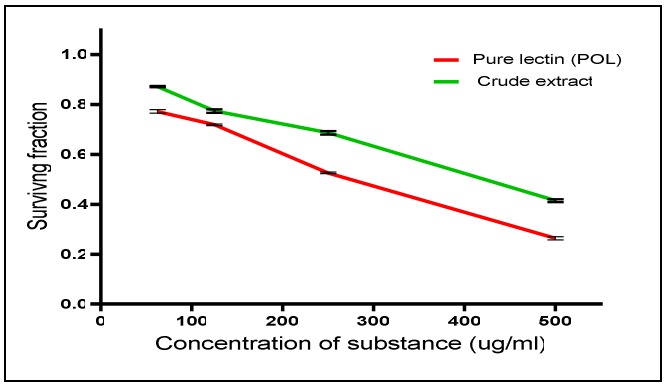
Data shown are means of three replicates

Biological applications of *P. ostreatus* SS89 lectin (POL)

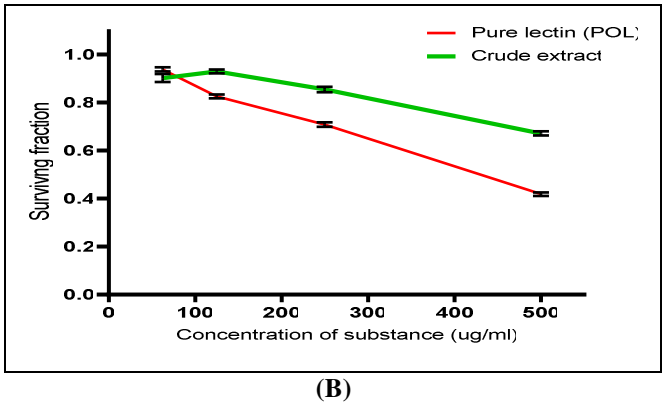
Cytotoxicity : POL showed a promising anticancer activity toward two human tumor cell lines HepG2 & HCT cell as shown in Fig. 9A & 9B. POL was able to inhibit the proliferation of HepG2 & HCT cells at different concentrations from 62.5 to 500 µg/ml with IC_{50} value of 424 µg/ml and 270 µg/ml respectively. At 500 µg/ml of POL the percent of inhibition of both HepG2 & HCT cells was very high (58.2 % and 73.7 % respectively). POL exhibit weak anticancer activity toward MCF7 cells with IC_{50} value > 500

µg/ml as shown in Fig. 9C. Crude extract showed anticancer activity toward HCT cells with IC_{50} value of 417µg/ml while, slightly inhibited the proliferation of HepG2 cells. Crude extract did not exert any inhibitory effect on MCF7 cells. The inhibitory effect of POL and crude extract on cancer cell proliferation was found to be dose dependent. Both POL and crude extract showed no obvious harmful effect on normal baby hamster kidney cells (BHK cells) as shown in Fig. 9D. These results suggested that POL is non- toxic compound which can be safely used as an anticancer drug in cancer therapy and other biomedical applications including drug delivery. The mechanism of the anticancer effect of the hemagglutinin involves the binding of hemagglutinin to a cell surface receptor such as Fas or tumor necrosis factor receptor (TNFR) that induces subsequent signal transduction and finally leads to apoptosis of tumor cells (Wong *et al.,* 2010).

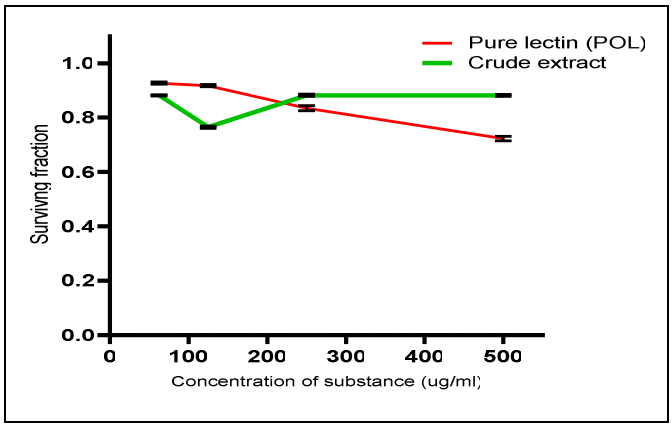
In the case of colon cancer, terminal Gal residue position C-3 exhibits reduced sulfation and thus an increased expression of sialyl-Lewis a (SLea) glycan occurs. These glycans can act as potential binding sites for different lectins that exert anti-proliferative activity afterwards. In the case of breast cancer alteration of L-fucose in cancer cells occurs. Whereas in the case of hepatoma, GDP-L-fucose synthase, GDP-L-fucose and 1,6 fucosyltransferase are involved in alterations (Singh *et al.,* 2020). Inulin-specific lectin from *P. adiposa* demonstrated antiproliferative activity towards both Hep G2 and MCF7 cells with an IC_{50} value of 2.1 and 3.2 µM, respectively (Zhao *et al.,* 2009). Mushroom *Boletus speciosus* BSH showed antiproliferative activity towards human hepatoma Hep G2 cells with IC_{50} of 4.7 µM (Sun *et al.* 2014). The same effect was shown by Zhang *et al.* (2014). *Paxillus involutus* lectin did not inhibit lung cancer (A-549) cells, colon cancer (HCT-8) cells (Wang *et al.,* 2013). *A. bisporus* lectin (50 mg/ml) can inhibit the proliferation in human colon cancer HT29 cells without cytotoxicity (Singh *et al.,* 2010).



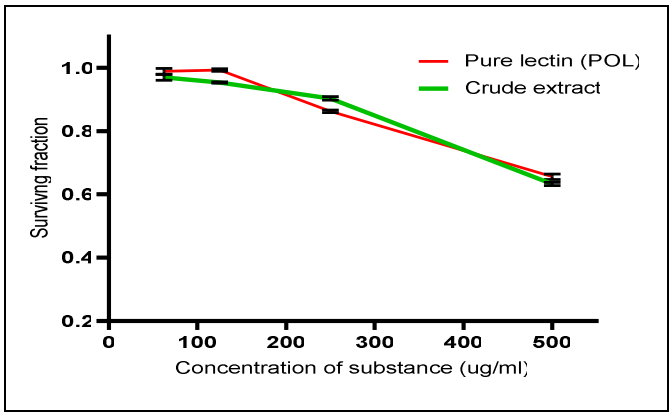
(A)



(B)



(C)



(D)

Figure 9: *In vitro* inhibitory effect of *Pleurotus ostreatus* lectin (POL) and crude extract on the proliferation of cancer cell line. (A) Inhibitory effect of POL and crude extract on hepatoma (HepG2) cell line; (B) Inhibitory effect of POL and crude extract on colorectal cancer (HCT) cell line; (C) Inhibitory effect of POL and crude extract on breast cancer (MCF7) cell line and (D) Inhibitory effect of POL and crude extract on Normal Baby hamster kidney cell line (BHK) cell line.

Antimicrobial activity

Antibacterial activity : Results in Fig. 10 showed that POL recorded antibacterial activity toward all the tested G^+ and G^- bacterial species except *Neisseria gonorrhoeae*. It showed the highest antibacterial activity toward *Escherichia Coli* with inhibition zone diameter of (11 ± 0.87 mm) followed by *Staphylococcus aureus* which recorded inhibition zone diameter of (10.03 ± 0.0.25 mm), *Streptococcus faecalis* (10 ± 0.5 mm), *Bacillus subtilis* (9.33 ± 0.306 mm) and *Pseudomonas aeruginosa* (9 ± 0.5 mm) as shown in Fig. 10. Crude extract did not show any growth retarding effect on all the tested bacterial species of both types (G^+ and G^-). Some lectins may exert their antibacterial action by binding cell surface glycans over bacterial cell walls. Gram positive bacterial cell walls consist of teichoic acids, murein (N-acetyl-glucosamine and N-acetylmuramic acid residues), and other surface proteins. Antibacterial activity against these types of bacteria may be due to interaction with these bacterial cell wall residues. Gram negative bacteria possess lipid A (an antigenic portion) in its lipopolysaccharide layer, antibacterial activity of these types may be due to the interaction of lectin with this lipopolysaccharide, resulting in the transition of lectin orientation from a random coil to –helix (Singh *et al.,* 2020).

Antifungal activity

AS shown in Fig. 10. POL and crude extract were devoid of any growth retarding effect on *Aspergillus flavus and Candida albicans*, indicating the inability of POL to bind to any of fungal cell wall component. Antifungal activity of lectins might be due to its carbohydrate binding specificity towards any of fungal cell wall components as L-fucose, N-acetylglucosamine, Mannan and laminarin, causing poor nutrient absorption, or disrupting of cell wall synthesis (Singh *et al.,* 2020). Waithaka *et al*. (Waithaka *et al.* 2017) studied the antimicrobial properties of mushrooms *Agaricus Bisporus* lectin extract and found that mushroom extract inhibit the growth of Erwinia spp. with zone of inhibition ranged from (18 ± 0.1 mm to 12 ± 9.2 mm), *Ralstonia spp*. (14 ± 0.3-11 ± 0.1), and *Enterococcus faecalis* (15 ± 0.3-09 ± 0.2).Unlike POL, *Agaricus bisporus* lectin inhibits growth of tested fungi. Like POL, *Paxillus involutus* lectin did not exert any antifungal activity (Wang *et al.,* 2013) also *Pholiota adiposa* lectin lacked antifungal activity (Zhang *et al.,* 2009).

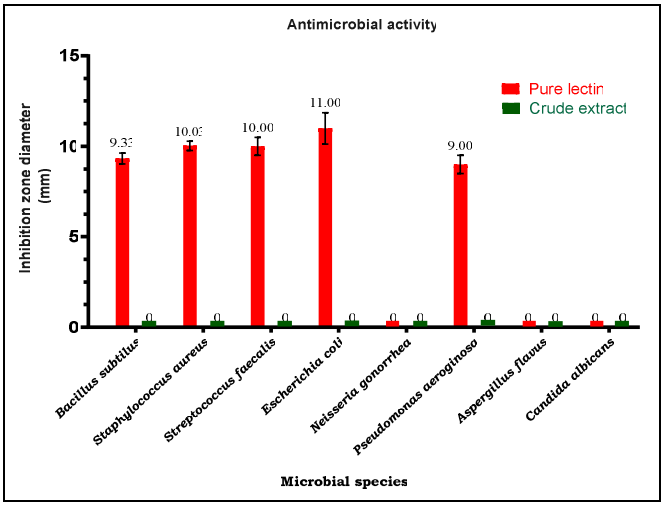


Fig. 10: Antimicrobial activity of *Pleurotus ostreatus* lectin and crude extract against three Gram positive bacteria, three Gram negative bacteria, one mold and one yeast. Bars show means of three replicates. Error bars show ± SD.

Conclusion

In summary, a lectin with a distinctive amino acid sequence was purified from *Pleurotus ostreatus* SS89. POL was a xylose-specific lectin with a molecular mass of 29 kDa and prefered human type AB erythrocytes. Additionally, it remained active over a wide range of temperature and pH. It was affected by all tested cations and was completely inhibited by Zn^{2+}, Hg^{2+} and Cu^{2+}. Comparing with the crude extract from the same organism, pure extract was slightly more stable. It manifested significant antiproliferative activity towards colorectal cancer cells (HCT) and hepatic cancer cells (HepG2), and antibacterial activity towards *Staphylococcus aureus, Escherichia Coli, Streptococcus faecalis, Bacillus subtilis* and *Pseudomonas aeruginosa.* However, in the future, the functional understanding of POL will be of high interest. Meanwhile, the safety of using the present purified lectin (POL) should be more confirmed by an *in vivo* lectin cytotoxicity test by using an animal model. Furthermore, studying the antiviral activity of POL will be taken into consideration.

Author contributions

ISK contributed in conceiving the ideas, designing and supervising the research and contributed in data analysis, discussion, writing and editing of the manuscript. NMK contributed in conceiving the ideas, designing and supervising the research and contributed in data analysis, discussion, writing and editing of the manuscript. SMMA contributed in conceiving the ideas, designing and supervising the research and contributed in data analysis, discussion, writing and editing of the manuscript. SSMS performed the experiments and contributed in data analysis, discussion, writing and editing of the manuscript.

Conflicts of interest

The authors have declared no conflict of interest.

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