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Original Article

Isolation and characterization of a novel lectin with antifungal and antiproliferative activities from *Sophora alopecuroides* seeds

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***Sophora alopecuroides* lectin (SAL), a novel lectin from the seeds of *Sophora alopecuroides*, was purified by ion-exchange chromatography on diethylaminoethyl (DEAE)- and carboxymethyl (CM)-Sepharose columns, followed by gel filtration on a Sephadex 75 10/300 GL column. SAL was found to be a monomer of 39916.3 Da, as determined by tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis and high-performance liquid chromatography (HPLC). The N-terminal 10-amino acid sequence of SAL, KPWALSFSFG, resembles those of other legume lectins. SAL exhibits hemagglutinating activity against rabbit erythrocytes at 11.9 μg/ml. Its hemagglutinating activity is stable in the pH range 7–11 and in the temperature range 30–90°C, and is stimulated by Mn^{2+}. The hemagglutinating activity of SAL is most potently inhibited by 50-mM D-galactose. SAL suppresses mycelial growth in *Penicillium digitatum* and *Alternaria alternata*; the IC_{50} of the antifungal activity toward *P. digitatum and A. alternata* were found to be 3.125 and 3.338 μM, respectively. SAL suppresses the proliferation of human cervical cancer cells (HeLa) at an IC_{50} of 6.25 μM ($P < 0.05$). But it has no inhibiting effect on bacteria. This is the first report of a lectin from seeds of *S. alopecuroides*.**

Keywords lectin; *Sophora alopecuroides*; antifungal activity; antiproliferative activity

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Introduction

Lectins are a class of non-covalent, carbohydrate-binding proteins of non-immune origins; possessing at least one non-catalytic domain, they can reversibly recognize and bind to monosaccharides or oligosaccharides [1]. Lectins are broadly distributed in many plants [2], animals [3], microorganisms [4], and edible fungi [5]. In the past few years, scientists have isolated >70 legume lectins, mostly derived from seeds and now called the legume lectin family [6]. Many lectins are also found in other plant tissues, including leaves [7], rinds [8], rhizomes [9], and roots [10]. These lectins have been found to be highly homologous to the seed lectins. It has been suggested that leaf lectins are similar to seed lectins in their amino acid sequences and specific carbohydrate-binding activities, but are products of distinct genes [11].

Legume lectins have been demonstrated to possess antifungal activity [12,13], and antiproliferative potency on tumor cells [10,14,15]. Legume lectins, such as pea (*Pisum sativum*) lectin and concanavalin A (ConA; from jackbean, *Canavalia ensiformis*), have been shown to confer resistance toward lepidopteran and homopteran insect species when expressed transgenically [16]. However, there is a need to search for novel lectins with significant bioactivity such as antifungal and antitumor activities.

Sophora alopecuroides is a legume used as a traditional medicinal herb in Uighur medicine in China. Tang revealed that the crude lectin of *S. alopecuroides* exhibits obvious antifungal activity [17]. At present, no other reports have been found of lectin activity in *S. alopecuroides*.

We have isolated and characterized a novel lectin (*Sophora alopecuroides* lectin, SAL) from *S. alopecuroides* seeds. SAL showed strong hemagglutination activity and antifungal and antitumor activities. Our findings suggested that SAL may have potential applications in agriculture and in medicine.

Materials and Methods

Materials

Seeds of *S. alopecuroides* were obtained from the suburb of Urumqi in Xinjiang. Diethylaminoethyl (DEAE)-Sepharose fast flow (2.5 × 10 cm), carboxymethyl (CM)-Sepharose fast flow (2.5 × 10 cm), and Sephadex 75 10/300 GL column were purchased from GE Healthcare (Uppsala, Sweden). Tris-buffered saline (TBS) (0.1 M Tris–HCl, 0.15 M NaCl), *Penicillium digitatum*, *Aspergillus niger*,

Alternaria alternata, *Klebsiella ozaenae*, *Acinetobacter baumannii*, *Shigella flexneri*, *Klebsiella pneumoniae*, *Pseudomonas pseudoalcaligenes*, *Proteus mirabilis*, *Shigella sonnei*, and *Escherichia coli* were provided by the China General Microbiological Culture Collection Center (Wuhan, China). The human esophageal cancer cell line (Eca109) and the human cervical cancer cell line (HeLa) were provided by the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) was from Gibco (Carlsbad, USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2h-tetrazoliumbromide (MTT) was acquired from Sigma Chemical (St Louis, USA).

Purification of lectin from seeds of *S. alopecuroides*

Seeds of *S. alopecuroides* were powdered and soaked overnight at 4°C with 0.01 M phosphate buffered saline (PBS) (pH 8.0). The mixture was then centrifuged at 4°C and 5000 *g* for 15 min. Ammonium sulfate was added to the obtained supernatant until the concentration of ammonium sulfate was 50%. The mixture was kept at 4°C for 4 h and then centrifuged at 4°C and 12,000 *g* for 10 min, and then crude protein extracts were obtained. The dialyzed crude product was loaded onto a DEAE-Sepharose column (2.5 × 10 cm) previously equilibrated with 0.01 M PBS (pH 8.0) and washed with the same buffer. The column was eluted with the same buffer containing a gradient of 0.1–0.5 M NaCl at the flow rate of 1 ml/min. Fractions exhibiting hemagglutinating activity were pooled and loaded onto a CM-Sepharose cation-exchange column (2.5 × 10 cm). The active fractions were pooled and dialyzed against 0.01 M buffer, then loaded onto a Sephadex 75 10/300 GL column pre-equilibrated with 0.01 M PBS (pH 7.2), and then eluted with 0.1–0.5 M NaCl in the same buffer, at a flow rate of l ml/min. The active fractions were collected, dialyzed, and frozen for further study, according to previous procedures [18,19].

Determination of the protein and carbohydrate content

To determine the protein concentration, the standard Bradford assay [20] was carried out, with bovine serum albumin, at a dilution of known concentration, as the standard. The elution profiles of proteins were determined by the absorbance values at 280 nm. The total neutral carbohydrate content was estimated by the method of Dubois, using glucose as the standard [21].

Assays of hemagglutinating activity

The hemagglutinating activity tests of all the collected fractions were conducted following the procedure of Petnual *et al*. [22].

Determination of the molecular mass and N-terminal amino acid sequence

In order to determine the homogeneity of the purified lectin, tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Schagger and von Jagow [23]. Samples were stained with Coomassie Blue R-250. The apparent molecular mass of the purified lectin was determined using the equilibrated TSK-G4000 PWxl HPLC column. The molecular mass of the eluted lectin was estimated from a plot of the log of the molecular weight versus the retention time of the standard markers [24]. The N-terminal sequence was determined by automated Edman degradation using a Procise Protein Sequencer 492 (Applied Biosystems, Carlsbad, USA) at Peking University (Beijing, China).

Effect of the temperature and pH on lectin hemagglutinating activity

Lectin samples were incubated at various temperatures (30–100°C at 10°C intervals) for 20 min, cooled to room temperature, and then the residual hemagglutinating activity was measured as described above.

Incubation of the lectin in buffers of different pH values was utilized to assess the pH stability. The purified lectin was mixed with buffers of different pH values (from 3 to 11; TBS buffer was used as the control) and kept for 1 h at room temperature. Samples were then adjusted back to TBS and assayed for agglutinating activity as described above.

Inhibition of haemagglutinating activity by different carbohydrates

Tests of the inhibition of hemagglutinating activity by various carbohydrates were performed as described by Wong *et al*. [25]. Carbohydrates tested included trehalose, D-xylose, maltose, D-mannose, sucrose, L-arabinose, glucose, D-galactose, methy-α-D-galactose, and ovalbumin.

Effect of metal ions on the lectin hemagglutinating activity

The ability of different metal ions to reduce the lectin hemagglutinating activity was tested by the method described by Suzuki *et al*. [5].

Antifungal activity assay

The antifungal activities of purified lectin toward *P. digitatum* and *A. alternata* were carried out in 90 × 12-mm Petri dishes containing 20 ml of potato dextrose agar (PDA) [26]. Suspensions (100 μl) of fungal spores were added to the PDA, sterile paper disks (6 mm in diameter) were placed on the agar, and 20 μl of the purified lectin solution was added to each disk. The dishes were incubated at 28°C

for 72 h until mycelial growth enveloped the control disks; the sizes of inhibition zones were observed and their diameters were measured.

In order to obtain a quantitative evaluation of the antifungal activity of SAL, following the method of Park *et al*. [27], equal volume proteins of different concentrations were added into a 96-well microtiter plate containing 100-μl suspensions of fungal spores (2.5×10^4 spores/ml), and incubated at 28°C for 24 h. Fungal growth was evaluated by measuring the optical density of the culture at 595 nm using a microplate reader (Bio-Rad benchmark Plus 10365, Hercules, USA). Growth inhibition was calculated by the following formula: inhibitory ratio (%) = $[1-OD_{595(lectin)}/OD_{595(control)}] \times 100\%$. IC_{50} was defined as the protein concentration at which 50% inhibition was reached.

Antibacterial activity assay

Klebsiella ozaenae, *A. baumannii*, *S. flexneri*, *K. pneumoniae*, *P. pseudoalcaligenes*, *P. mirabilis*, *S. sonnei*, and *E. coli* were cultured with agitation in Luria–Bertani broth at 37°C. The antimicrobial activities of the lectin were determined using the microdilution assays of Park *et al.* [27].

Antiproliferative activity assay

Tests were prepared by the method described by Yan *et al*. [28]. MTT colorimetry was used to detect cell survival. HeLa and Eca-109 cells in the logarithmic growth phase (5×10^4 cells/ml) were seeded independently in a 96-well plate with a final volume of 100 μl containing 5×10^4 cells per well, whereas a blank control group was cultured in DMEM. These plates were incubated at 37°C for 24 h. Cells were treated with 100 μl of DMEM with 0.625, 1.25, 3.75, 6.25, or 8.25 μM of the lectin after removal of the culture medium. At the indicated time, the medium was carefully removed and 100 μl of 0.5 mg/ml MTT solution was added to each well, and the plates were incubated at 37°C for 4 h. The medium was then replaced with 100 μl of dimethyl sulfoxide, and were quantified by measuring the absorbance at 570 nm with a microplate reader (Bio-Rad). The percentage of cell growth inhibition was calculated as follows: Inhibitory ratio (%) = $[1-OD_{570(lectin)}/OD_{570(control)}] \times 100\%$.

Statistical analysis

Data were expressed as mean ± SD from triplicate experiments. $P < 0.05$ was considered as statistically different.

Results

Purification of lectin

The extracts of *S. alopecuroides* seeds prepared as described above were initially divided into five fractions on a DEAE-Sepharose anion-exchange column (2.5 × 10 cm) [**Fig. 1(A)**]. The unabsorbed fraction (DC1) exhibiting hemagglutinating activity was collected and dialyzed with 0.01 M PBS (pH 8.0), and chromatography on a CM-Sepharose fast flow cation-exchange column (2.5 × 10 cm) was carried out [**Fig. 1(B)**]. Fraction MX2 with hemagglutinating activity was loaded onto a Sephadex 75 10/300 GL column [**Fig. 1(C)**], and one fraction, DCMx2-1, was obtained, representing purified lectin of *S. alopecuroides* (SAL).

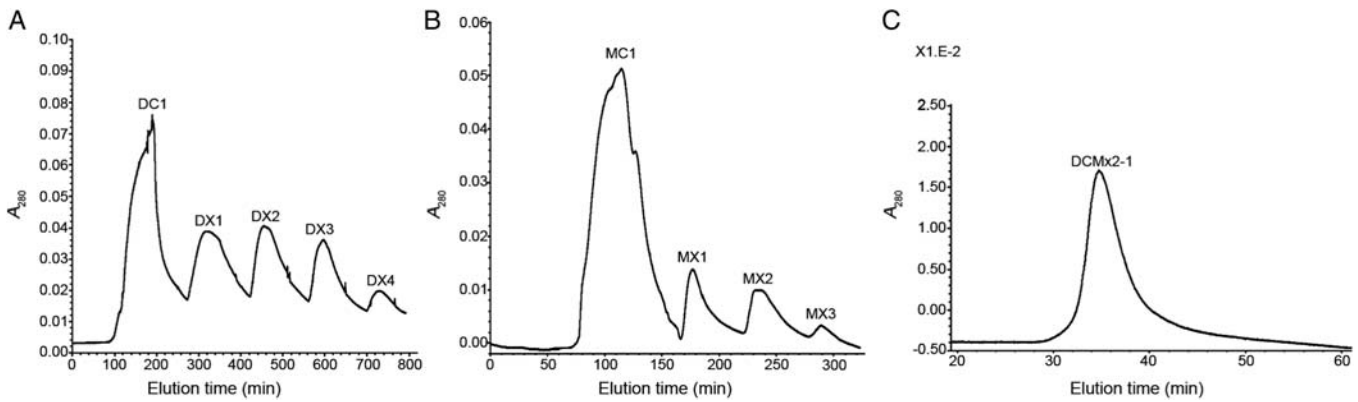

Figure 1 Purification of *Sophora alopecuroides* lectin (A) Anion-exchange chromatography of the crude product from seeds of *Sophora alopecuroides* on a DEAE-Sepharose column (2.5 × 10 cm). Sample: extracts with 50% ammonium sulfate saturation were dissolved with 0.01 M PBS (pH 8.0). A linear 0.1–0.5 M NaCl gradient was used for elution (flow rate = 1 ml/min). Five fractions were obtained and hemagglutinating activity was detected only in the unabsorbed DC1 fraction. (B) Cation-exchange chromatography of the DC1 fraction on a CM-Sepharose column (2.5 × 10 cm). Sample: the unabsorbed DC1 fraction from DEAE-Sepharose chromatography. A linear 0.1–0.5 M NaCl gradient was used for elution (flow rate = 0.5–1 ml/min). Four fractions were obtained and hemagglutinating activity was detected only in the absorbed MX2 fraction. (C) Gel filtration of the MX2 fraction on a Sephadex 75 10/300 GL. Sample: the adsorbed MX2 fraction from CM Sepharose chromatography, eluted with starting buffer containing 0.15 M NaCl (flow rate = 1 ml/min). Only one fraction, DCMx2-1, was obtained.

Yields and hemagglutinating activities at different stages of purification of SAL are summarized in **Table 1**. SAL was purified 12-fold with a recovery yield of 1.29%.

Detection of neutral carbohydrate content showed that SAL is a glycoprotein with 32.2% covalently linked carbohydrate.

Characterization of SAL

SAL showed a pure peak on the Superdex 75 and exhibited a single band with only one molecular weight on tricine-SDS-PAGE [**Fig. 2(A)**]. The molecular mass of SAL was 39916.3 Da (~40 kDa) by HPLC [**Fig. 2(B)**], suggesting that it is a monomer.

The first 10 amino acids of the N-terminal were confirmed by automated Edman degradation. SAL was found to possess an N-terminal sequence of KPWALSFSFG. The N-terminal sequence of SAL is compared with several legume lectin sequences in **Table 2**. By comparison of its N-terminal sequence with those available in the data banks, we found that it was not identical with any protein published so far. However, it exhibited 56% similarity to the N-terminal sequences of lectins from *Robinia pseudoacacia* and *Dolichos lablab*.

Hemagglutinating activity of SAL

The minimum agglutinative concentration of SAL toward rabbit erythrocytes was 11.9 μg/ml. Its hemagglutinating activity was inhibited by D-galactose at a concentration of 0.05 M. The hemagglutinating activity of SAL was stable in the temperature range 30–90°C (**Fig. 3**); even at 100°C, it had some hemagglutinating activity. The hemagglutinating activity of SAL was stable in the pH range 7–11 (**Fig. 4**); at pH 12, 70% agglutination activity was observed. The effects of different metal ions on the hemagglutinating activity of SAL were tested. In Mn^{2+} solution, SAL exhibited maximal agglutinating activity; the minimum concentration of SAL for agglutination was 0.1 μg/ml. This indicated that SAL may be a Mn^{2+}-dependent protein.

Table 1 Yields and hemagglutinating activities of SAL at different stages of purification from 100 g of *Sophora alopecuroides* seeds

Purified periods	Chromatographic fraction	Yield (mg)	Purification fold	Specific activity (HU/mg)[a]	Recovery of activity (%)[b]
	Crude extract	66.4	1.00	0.48	100
DEAE-Sepharose FF	DC1	8.25	6.38	3.06	12.42
CM-Sepharose FF	MX2	1.71	14.60	7.01	2.58
Sephadex G-75	DCMx2–1	0.86	21.38	10.26	1.29

[a]HU, hemagglutinating unit, i.e. the reciprocal of the highest complete hemagglutination. Specific activity (HU/mg) = 25 μl × concentration of lectin (μg/μl)/2^n/quality of total lectin (mg).

[b]Recovery of activity(%) = quality of lectin (mg)/quality of total lectin (mg).

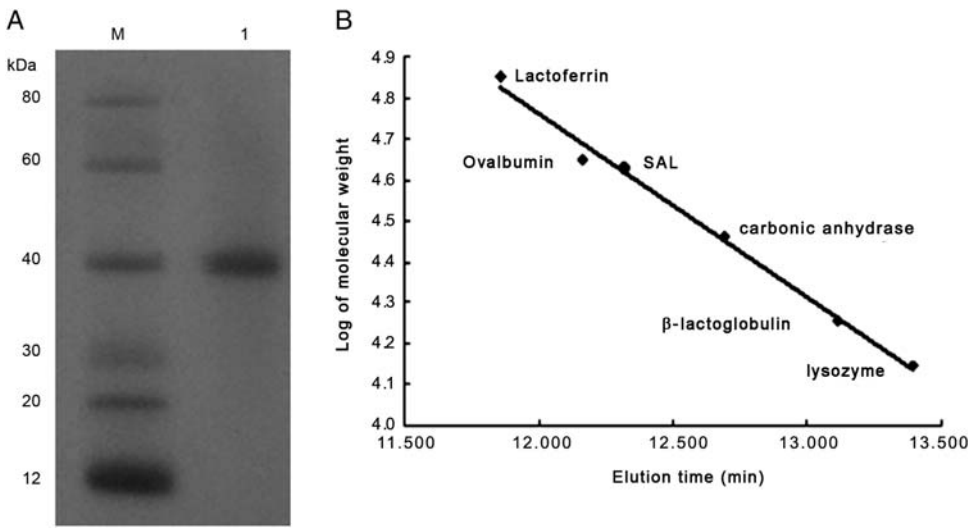

Figure 2 Determination of molecular mass of SAL (A) Tricine-SDS-PAGE pattern of SAL. M, six purified recombinant proteins ranging from 12 to 80 kDa (12, 20, 30, 40, 60, and 80 kDa). 1, the DCMx2-1 fraction from Sephedex G-75. (B) Native molecular mass estimation of SAL on HPLC. Standard markers: Lactoferrin (71 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18 kDa), and lysozyme (14 kDa).

Table 2 Comparison of the N-terminal amino acid sequences (1–10 amino acids) of SAL and other legume lectins

Lectin	N-terminal sequence	Percent identity
Sophora alopecuroides lectin (SAL)	KPWALSFSFG	100
Robinia pseudoacacia lectin	TGSLSFSFPK	56
Dolichos lablab lectin (FRIL)	AQSLSFSFTK	56
Sophora flavescens lectin (SFL)	SLSFTFSDFD	44
Phaseolus vulgaris cv. lectin (EAPL)	ANEIYFSFQR	33

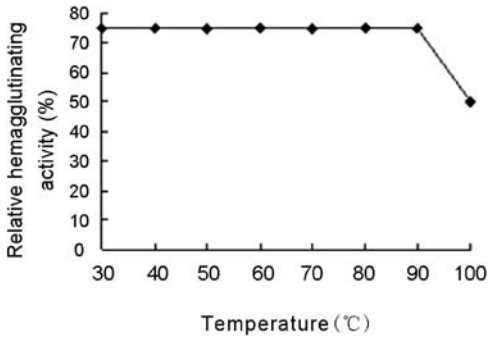

Figure 3 Effect of temperature on the hemagglutinating activity of SAL toward rabbit erythrocyte suspension in TBS Full activity (100%) corresponds to 0.048 mg/ml of SAL.

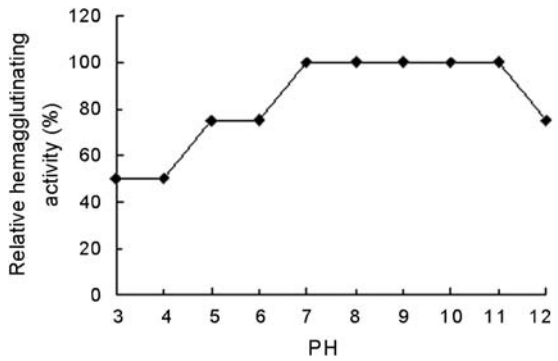

Figure 4 Effect of pH on the hemagglutinating activity of SAL toward rabbit erythrocyte suspension in TBS Full activity (100%) corresponds to 0.024 mg/ml of SAL.

Antimicrobial activity of SAL

SAL inhibited mycelia growth of *P. digitatum* and *A. alternata* after treatment for 24 h (**Fig. 5**), at an IC_{50} of 3.125 and 3.338 μM, respectively ($P < 0.01$, vs. control) (**Fig. 6**). However, SAL was devoid of antibacterial activity against *K. ozaenae*, *A. baumannii*, *S. flexneri*, *K. pneumoniae*, *P. pseudoalcaligenes*, *P. mirabilis*, *S. sonnei*, and *E. coli*.

Antiproliferative activity of SAL

The antiproliferative effects of SAL against HeLa and Eca109 cells were determined using MTT assay (**Fig. 7**). Similarly to some other lectins, SAL showed significant inhibitory effects on the proliferation of HeLa at an IC_{50} of 6.25 μM for 24 h ($P < 0.05$). When Eca-109 cells were incubated with 8.75 μM of SAL for 24 h, the inhibitory ratio reached 46.21%. The inhibition ratio for SAL showed a concentration-dependent pattern for the two cell lines. The maximal inhibition of cell proliferation was observed toward HeLa cells ($P < 0.05$).

Discussion

The *S. alopecuroides* seed lectin (SAL) has been purified for the first time using a DEAE-Sepharose column, CM-Sepharose column, and Sephadex 75 10/300 GL column (**Fig. 1**). Multi-step ion-exchange chromatography and gel filtration have been commonly employed to obtain other legume lectins [25,29].

The HPLC and tricine-SDS-PAGE results have indicated that the molecular mass of SAL is ~40 kDa; the lectin is thus a monomer (**Fig. 2**). To our knowledge, most known legume lectins are homodimers or homotetramers [30]. *Phaseolus coccineus* lectin, for example, is a dimer of 56 kDa [31]; a lectin from Chinese black soybeans is a dimer protein with molecular mass of 50 kDa [14], and ConA, a well-known lectin, is a homotetramer [32]. Unlike most legume lectins, SAL is a monomer of 40 kDa; and although a monomer, it showed hemagglutination activity. This finding expands the known structural diversity of the legume lectin family and provides valuable data for research into monomer lectins.

It is worth noting that SAL was less susceptible to alterations in temperature and pH than the lectins of *Astragalus mongholicus* and *Curcuma longa* [22,29]. The hemagglutinating activity of SAL required Mn^{2+}; this is consistent with a lectin from *C. longa* [22]. Of all the sugars tested, 50 mM D-galactose most potently inhibited the hemagglutinating activity of SAL; this is the same as for AMML (a lectin from *A. mongholicus*) [2].

The N-terminal sequence of SAL, KPWALSFSFGR, resembles those of other legume lectins (**Table 2**). Although some amino acids have been found to be highly conserved, their biochemical characteristics are distinctive [10,33,34]. In contrast, despite the fact that SAL and AMML (with an N-terminal sequence of ESGINLQGDA) belong to the same legume lectin family with identical binding specificity for D-galactose-type residues, their N-terminal amino acid sequences are completely disparate [2]. As mentioned above, our results indicate that SAL may well be a novel legume lectin. However, N-terminal homology searching by the BLAST method from NCBI

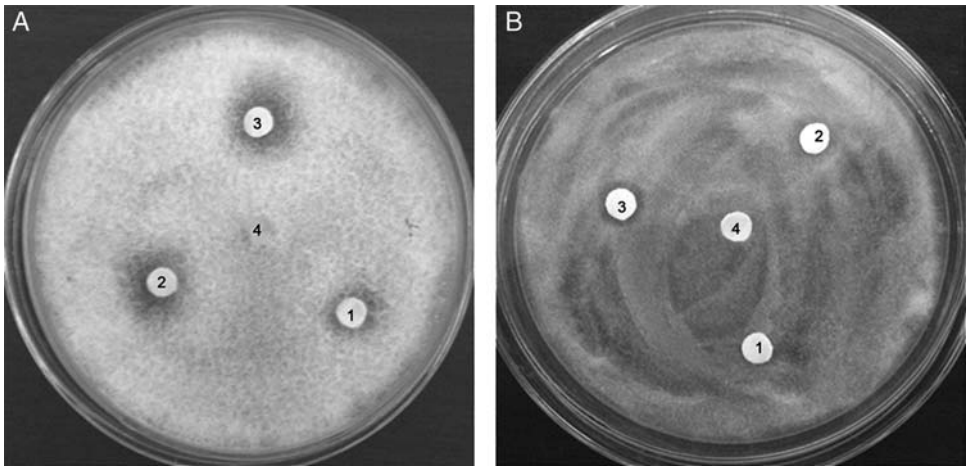

Figure 5 Growth inhibition of SAL against the two fungi *Penicillium digitatum* (A) and *Alternaria alternata* (B) 1, 20 μl of SAL (10 μM); 2, 20 μl of SAL (20 μM); 3, 20 μl of SAL (30 μM); 4, 20 μl of PBS (pH 7.2, 20 mM).

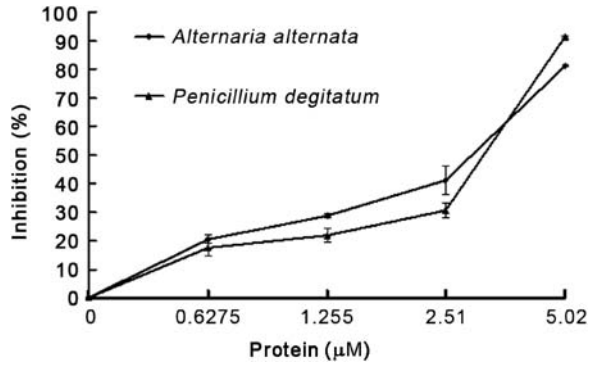

Figure 6 Antifungal activity of SAL Percentage of growth inhibition was measured at different concentrations of SAL with the following test organisms: *Alternaria alternata* (◆), *Penicillium digitatum* (▲).

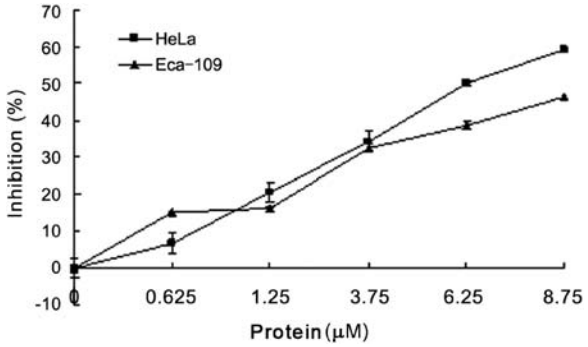

Figure 7 Effects of SAL on the proliferation of HeLa (■) and Eca109 (▲) cells

cannot prove conclusively that SAL is novel. A further study will aim at the molecular cloning and sequence analysis of SAL to gain more information.

Many lectins do not exert antifungal activity; these include lectins from *Phaseolus vulgaris* cv. *Bauhinia variegata* and Chinese black soybeans [14,35,36]. Only a few lectins have been reported to possess remarkable antifungal activity [12]; these include lectins from *P. vulgaris*, *P. coccineus*, and *A. mongholicus* [2,31,37]. Similar to these antifungal lectins, SAL showed inhibition activity on *P. digitatum* and *A. alternata*, at an IC_{50} of 3.125 and 3.338 μM. This indicates that SAL may have a promising fungal disease resistance potential for utilization in agriculture. The antibacterial activity assay showed that SAL had no inhibitory effect on the tested bacteria (data not shown).

Some lectins are able to bind to chitin, and ultimately lead to inhibition of fungal growth [26,38]. Some lectins can combine with specific sugar residues exposed on the surface of fungal cells, and can thus interfere with fungal cell wall generation and influence formal cell metastasis [12]. However, further studies of the detailed mechanisms of the antifungal activities of SAL would validate the feasibility of SAL as a biocontrol agent of microbial plant pathogens.

Many plant lectins possess antiproliferative activity toward tumor cell lines; these include the lectins from *P. vulgaris* cv. and *P. vulgaris* [34,37]. In contrast, lectins from *A. mongholicus* and *C. longa* showed no inhibiting effect on tumor cells [22,29]. The antiproliferative activity of SAL on HeLa cells with an IC_{50} of 6.25 μM has been found *in vitro*. In comparison, SAL showed strong efficacy in inhibiting the proliferation of tumor cells.

This study indicated that SFL (a lectin from *S. flavescens*) can induce the apoptosis of HeLa cells in a caspase-dependent pathway [10]. Yan *et al.* [28] showed that AMML displayed the outstanding characteristics of apoptosis on HeLa cells. Fang *et al.* [34] revealed that a lectin from the seeds of extra-long autumn purple beans could inhibit the proliferation of human tumor cells by inducing the production of apoptotic bodies. Further studies are required to uncover the detailed mechanisms of the antiproliferative activity of SAL.

In summary, we have effectively isolated and characterized a novel lectin from *S. alopecuroides* seeds for the first time. This lectin possesses antifungal activity and antiproliferative activity against human cancer cells. In future, it may contribute to the development of a strategy for the biological control of fungal pathogens, and could be exploited pharmaceutically in the treatment of cancer diseases.

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