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A relatively stable antifungal peptide from buckwheat seeds with antiproliferative activity toward cancer cells

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Abstract: An antifungal peptide with a molecular mass of approximately 4 kDa was isolated from buckwheat seeds by using ion-exchange chromatography on SP-Sepharose and Q-Sepharose, and gel filtration on Superdex peptide. The peptide was adsorbed on SP-Sepharose in 10 mM NH_4OAc buffer (pH 4.5) and on Q-Sepharose in 10 mM NH_4HCO_3 buffer (pH 9.4), and appeared to be highly purified after these two steps. It inhibited mycelial growth in *Fusarium oxysporum* and *Mycosphaerella arachidicola* with an IC_{50} of 35 and 40 μM, respectively. Its antifungal activity was stable between 0 and 70 °C, and between pH 1.0/2.0 and 13. It inhibited proliferation of Hep G2 (hepatoma) cells, L1210 (leukemia) cells, breast cancer (MCF-7) cells, and liver embryonic WRL 68 cells with an IC_{50} of 33, 4, 25, and 37 μM, respectively. On the other hand, the peptide was unable to evoke a mitogenic response from splenocytes or induce nitric oxide production by macrophages. It inhibited HIV-1 reverse transcriptase with an IC_{50} of 5.5 μM.

Keywords: antifungal peptide; buckwheat seeds; antiproliferative activity; cancer cells

INTRODUCTION

Plants have developed a variety of defense mechanisms including antifungal proteins to combat pathogenic microbes. A spectacular array of antifungal proteins is produced by plants. Some of them are named according to their structure, e.g. miraculin-like protein [1], thaumatin-like proteins [2,3], chitinase-like proteins [4,5], embryo-abundant protein-like proteins [6], and cyclophilin-like proteins [7], and others are named on the basis of their function, e.g. chitinases [8], peroxidases [9], glucanases [8], ribosome inactivating proteins [10–12], and protease inhibitors [13–15]. Antifungal proteins are found in seeds [16,17], fungi [18,19], rhizomes [20], bulbs [21], insects [22], tubers [23], leaves [24], and shoots [5].

Two peptides with antifungal and antibacterial activities have been isolated from buckwheat seeds. They have nearly identical amino acid sequences and molecular masses [25]. The objective of the present study was to use a simpler procedure to isolate the antifungal peptide and test it for additional biological activities including immunomodulatory and antiproliferative activities. The stability of its antifungal activity was also examined. The study disclosed that the buckwheat antifungal protein has an antiproliferative action toward the tumor-cell lines examined, and an inhibitory effect on HIV-1 reverse transcriptase. In addition, its antifungal activity remains unaffected over a wide range of temperatures and pH values.

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MATERIALS AND METHODS

Isolation of the Antifungal Protein

Buckwheat (*Fagopyrum esculentum*) seeds from Mainland China (25 g) were soaked overnight and then homogenized in distilled water (2 ml/g) using a Waring blender. NH_4OAc buffer (pH 4.5) was added to the supernatant obtained by centrifuging the homogenate, until the concentration of NH_4OAc attained 10 mM. The supernatant was then applied on a 2.5 × 16 cm column of SP-Sepharose (Amersham Biosciences), which was eluted with 10 mM NH_4OAc buffer (pH 4.5). After elution of the unbound fraction (SP1), the column was eluted with the aforementioned NH_4OAc buffer to which 1 M NaCl had been added. The fraction SP2 obtained was dialyzed and then chromatographed on a 2.5 × 10 cm column of Q-Sepharose (Amersham Biosciences). Following removal of the unbound fraction Q1 with 10 mM NH_4HCO_3 buffer (pH 9.4), the adsorbed material (Q2) was desorbed with 10 mM Tris–HCl buffer containing 1 M NaCl. Fraction Q2 was then dialyzed before gel filtration on a column of Superdex peptide (Amersham Biosciences) which had been equilibrated and was eluted with 10 mM NH_4HCO_3 buffer (pH 9.4). The single eluted peak represented the purified antifungal protein.

Electrophoresis, Molecular Mass Determination, and *N*-terminal Sequence Analysis

The purified peptide was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for molecular mass determination in accordance with the procedure of Laemmli and Favre [26]. Gel filtration on an FPLC-Superdex peptide column, which had been calibrated with molecular mass markers (Amersham Biosciences), was conducted to determine the molecular mass of the protein. The *N*-terminal sequence of the protein was determined by using a Hewlett-Packard (HP) G1000A Edman degradation unit and an HP 1000 HPLC System [3].

Assay of Antifungal Activity

The assay of the purified peptide for antifungal activity toward *Mycosphaerella arachidicola* and *Fusarium oxysporum* was carried out in 100 × 15 mm petri plates containing 10 ml of potato dextrose agar. After the mycelial colony had developed, sterile blank paper disks (0.625 cm in diameter) were placed at a distance of 0.5 cm away from the rime of the mycelial colony. An aliquot of a solution of the purified peptide was added to a disk. The plates were incubated at 23 °C for 72 h until mycelial growth had enveloped disks containing the control and had formed crescents of inhibition around disks containing samples with antifungal activity [6]. The IC_{50} value was determined as described in [6].

Assay for Antiproliferative Activity Toward Tumor Cells

The antiproliferative activity of the purified peptide was determined as follows. The cell lines L1210 (leukemia), Hep G2 (hepatoma), MCF 7 (breast cancer), and WRL68 (liver embryonic cell) were purchased from American Type Culture Collection. The cell line were maintained in Dulbecco Modified Eagles' Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 mg/l streptomycin, and 100 IU/ml penicillin at 37 °C in a humidified atmosphere of 5% CO_2. Cells (1×10^4) in their exponential growth phase were seeded into each well of a 96-well culture plate (Nunc, Denmark) and incubated for 4 h before addition of the antifungal peptide. Incubation was carried out for another 48 h. MTT was added followed by incubation for 4 h and dissolving the MTT-formazan formed with DMSO before absorbance was read at 595 nm [27].

Assay for Mitogenic Activity Toward Splenocytes

The assay of mitogenic activity was performed as described by Ye and Ng [7]. Splenocytes were isolated from BALB/c mice. The cells were diluted with RPMI medium containing 10% FBS and then seeded (2×10^6 cells/0.2 ml/well) in 96-well microplates. The antifungal peptide was then added at various concentrations. Cells cultured in the absence of the lectin Con A served as control. The cells were incubated at 37 °C in a humidified atmosphere of 5% carbon dioxide for 24 h. The cells were viable after 24 h. During the last 6 h, the cells in one well were pulsed with 0.5 μCi of [^{3}H-methyl]-thymidine (specific activity 5 μCi/mmol, Amersham Biosciences) in 10 μl and were then harvested on to a glass fiber filter using a cell harvester. The radioactivity was determined using a Beckman scintillation counter. The proliferative (mitogenic) response was expressed as mean counts per min (cpm).

Assay of Nitric Oxide Production by Macrophages

The assay was performed as described in [28].

Assay for HIV-1 Reverse Transcriptase Inhibitory Activity

The assay for HIV reverse transcriptase inhibitory activity was carried out according to instructions supplied with the assay kit from Boehringer Mannhein (Germany). The assay takes advantage of the ability of reverse transcriptase (RT) to synthesize DNA, starting from the template/primer hybrid poly (A) oligo (dT) 15. The digoxigenin- and biotin-labeled nucleotides in an optimized ratio are incorporated into one of the same DNA molecules, which is freshly synthesized by the RT. The detection and quantification of synthesized DNA as a parameter for RT activity follows a sandwich enzyme-linked immunosorbent assay (ELISA) protocol. Biotin-labeled DNA binds to the surface of microtiter plate modules that have been precoated with strepatavidin. In the next step, an antibody to digoxigenin, conjugated to peroxidase, binds to the digoxigenin-labeled DNA. In the final step, the peroxidase substrate is added. The peroxidase enzymes catalyze the cleavage of the substrate, producing a colored reaction product. The absorbance of the samples at 405 nm can be determined using a microtiter plate (ELISA) reader and is directly correlated to the level of RT activity. A fixed amount (4–6 ng) of recombinant HIV-1 reverse transcriptase was used. The inhibitory activity of the antifungal peptide was calculated as percent inhibition as compared to a control without the protein [7].

RESULTS

Ion-exchange chromatography of the crude buckwheat seed extract on SP-Sepharose yielded a broad unadsorbed fraction (SP1) without antifungal activity and a sharp adsorbed fraction (SP2) with antifungal activity (Figure 1). Chromatography of fraction SP2 on Q-Sepharose resolved it into a broad and shallow unadsorbed fraction (Q1) and a sharp adsorbed fraction (Q2). Antifungal activity resided in fraction Q2 (Figure 2). Fraction Q2 gave rise to a single absorbance peak upon gel filtration on Superdex peptide (Figure 3). Its mass spectrum disclosed a molecular mass of about 4 kDa (Figure 4). Its *N*-terminal sequence is presented in Table 1. It suppressed mycelial growth in *Fusarium oxysporum* and *Mycosphaerella archidicola* with an IC_{50}

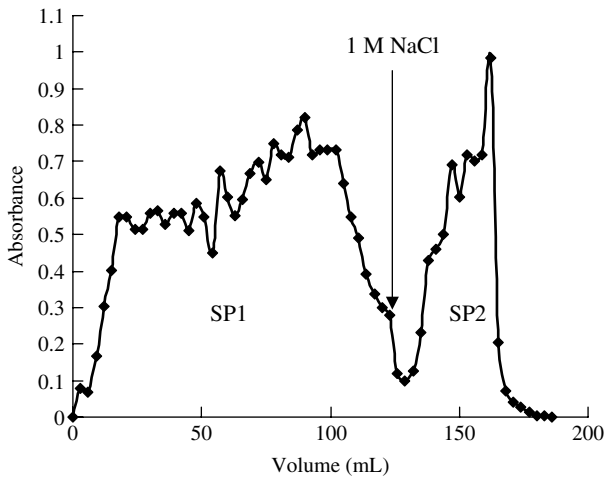

Figure 1 Results of cation-exchange chromatography of buckwheat on a SP-Sepharose column (2.5 × 16 cm) in 10 mM NH_4OAc buffer (pH 4.5). Antifungal activity resided in the second fraction (SP2) eluted with 1 M NaCl.

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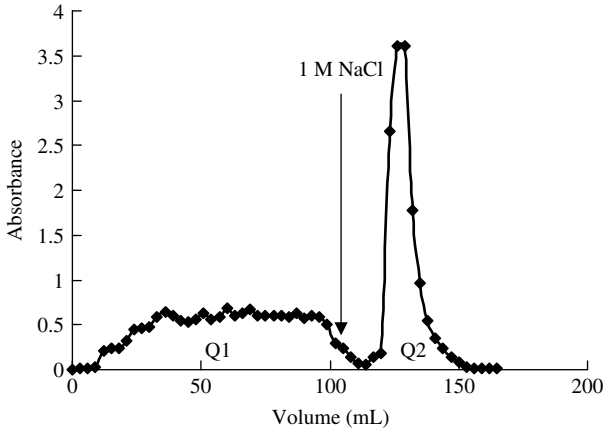

Figure 2 Results of anion-exchange chromatography of fraction SP2 on a Q-Sepharose column (25 × 16 cm) in 10 mM NH_4HCO_3 buffer (pH 9.4). The fraction (Q2) eluted with 1 M NaCl was the only antifungal fraction.

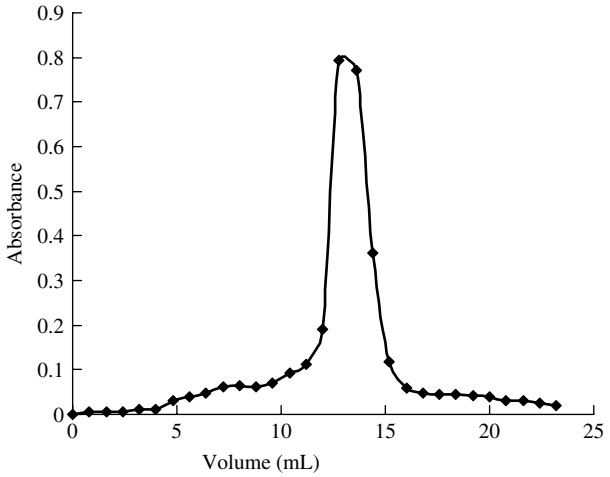

Figure 3 FPLC-gel filtration of fraction Q2 adsorbed on Q-Sepharose column on Superdex peptide HR 10/30 column in 10 mM NH_4HCO_3 buffer (pH 9.6).

of 35 and 40 μM, respectively (Figure 5). The inhibitory effects of the peptide on proliferation of L1210 leukemia cells, MCF-7 breast cancer cells, Hep G2 liver cancer cells, and WRL 68 embryonic liver cells are shown in Figures 6–9. The IC_{50} values were, respectively, 33, 4, 25, and 37 μM. The peptide had no effect on the mitogenic response of splenocytes and nitric oxide production by macrophages when tested at 33 and 50 μM, respectively (data not shown). The antifungal activity of the peptide toward *Mycosphaerella arachidicola* was stable over the pH range 1.0–13.0 and its activity toward *Fusarium oxysporum* was stable over the pH range 2.0–13.0. There was no activity when the pH was raised to 14 or lowered to 1.0. The antifungal activity was stable between 0 and 70 °C for both types of fungi (data not shown). The antifungal peptide inhibited HIV-1 reverse transcriptase with an IC_{50} of 5.5 μM (Figure 10).

Table 1 *N*-terminal sequence of buckwheat antifungal protein

		Reference
Buckwheat antifungal protein	AQCGAQGGGATCPGG	This study
Fα - AMP1	AQCGAQGGGATCPGG	24
Fα - AMP2	AQCGAQGGGATCPGG	24

DISCUSSION

Fujimura *et al.* [25] reported the isolation of two antibacterial peptides with extremely similar sequences from buckwheat seeds. Their isolation procedure comprised extraction with aqueous buffer, $(NH_4)_2SO_4$ precipitation, gel filtration on Sephadex G75, second $(NH_4)_2SO_4$ precipitation, ion-exchange HPLC on SP-Cosmogel, and reversed-phase HPLC on Mightysil RP-4. The antifungal peptide isolated in the present study is probably the same as or very similar to the peptide reported by Fujimura *et al.* [25], as judged by the similarity in amino acid sequence and molecular mass. In the present investigation, the purification scheme did not involve $(NH_4)_2SO_4$ precipitation. It was a much simpler procedure and yielded a highly purified preparation of antifungal peptide after only two ion-exchange chromatographic steps, the first one on SP-Sepharose and the second one on Q-Sepharose. The third chromatographic step on Superdex peptide revealed that the active fraction adsorbed on Q-Sepharose appeared as a single absorbance peak and SDS-PAGE disclosed that it was a single band.

The buckwheat antifungal peptide isolated by Fujimura *et al.*, [25] was inhibitory to *Fusarium oxysporum* and *Geoticehum candidum* and a number of Gram-positive and Gram-negative bacteria. The buckwheat antifungal peptide obtained in the present study was inhibitory to *Fusarium oxysporum* and *Mycosphaerella arachidicola*. By comparison, some antifungal proteins, such as that from shallot bulbs, were active against only one out of several fungal species tested [29].

The thermostability and pH stability of the bulk of antifungal proteins isolated to date have not been reported. One of the exceptions is the demonstration of the optimum pH and optimum temperature for the chitinase activity of the mung bean chitinase which has antifungal activity [30]. However, in that study the effects of pH and temperature on the antifungal activity of the chitinase were not examined. The antifungal activity of buckwheat seed antifungal peptide is preserved up to 70 °C and over a wide pH range of 1 or 2 to 13. This indicates that it has fairly high stability.

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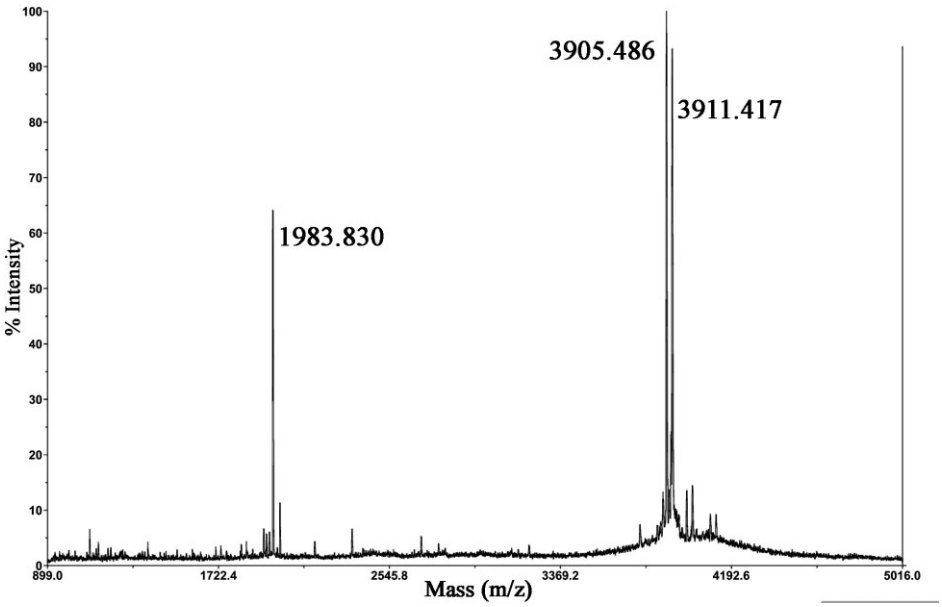

Figure 4 Mass spectrometry result showing that the molecular mass of buckwheat antifungal peptide was 3.9 kDa.

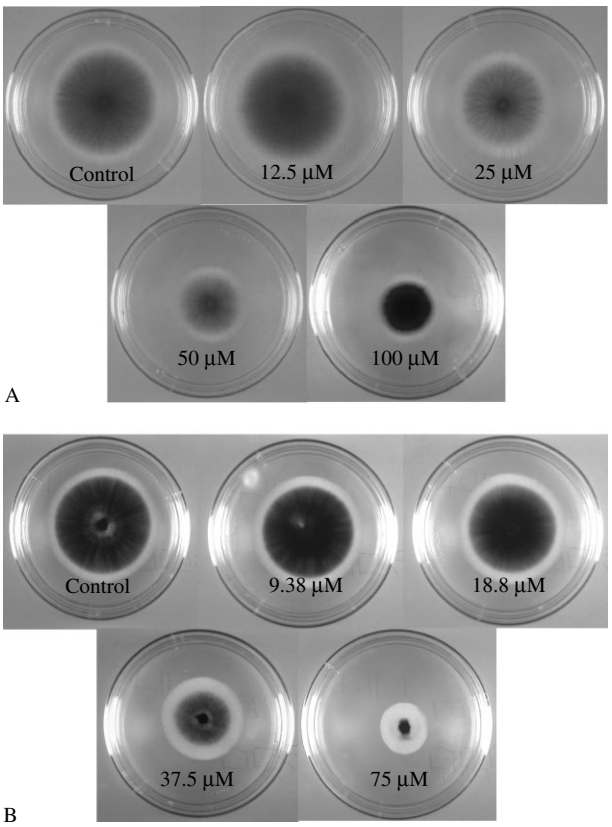

Figure 5 Inhibitory effect of buckwheat antifungal peptide on (A) *Fusarium oxysporum* and (B) *Mycosphaerella arachidicola*. The IC_{50} toward *Fusarium oxysporum* was 35 μM and the value toward *Mycosphaerella arachidicola* was 40 μM.

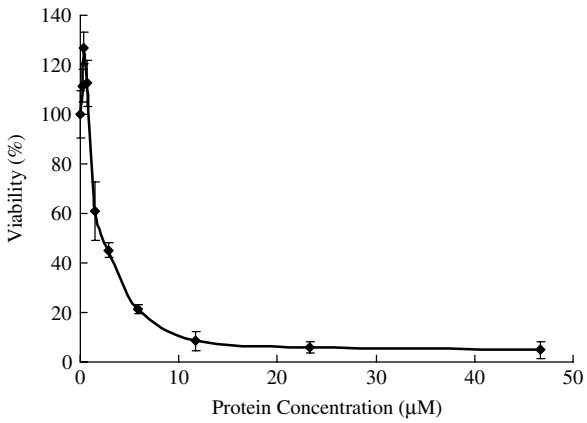

Figure 6 Inhibitory effect of buckwheat antifungal peptide on proliferation of L1210 (leukemia) cancer cells. Cell proliferation was determined by MTT assay (Data represent means ± SD, $n = 3$).

Buckwheat antifungal peptide suppresses proliferation in a variety of tumor cells including hepatoma, leukemia, and breast cancer cells. In this aspect it resembles chive chitinase-like antifungal protein [5] and defensin-like antifungal peptides from leguminous plants [27, 31]. In contrast to the pinto bean antifungal protein, which stimulates nitric oxide production by macrophages [28], and some antifungal proteins that elicit a mitogenic response from splenocytes [7, 14, 16, 27, 31], buckwheat antifungal peptide is devoid of these activities. Nevertheless, buckwheat antifungal peptide is capable of inhibiting HIV-1 reverse transcriptase, as has been shown for antifungal proteins of different origins [7, 14, 27, 29, 31, 32]. Its potency is fairly high compared with other anti-HIV natural products [33]. Overall, it seems that buckwheat antifungal peptide is an exploitable protein by virtue of it being a peptide and its fairly thermostable and pH-stable antifungal activity.

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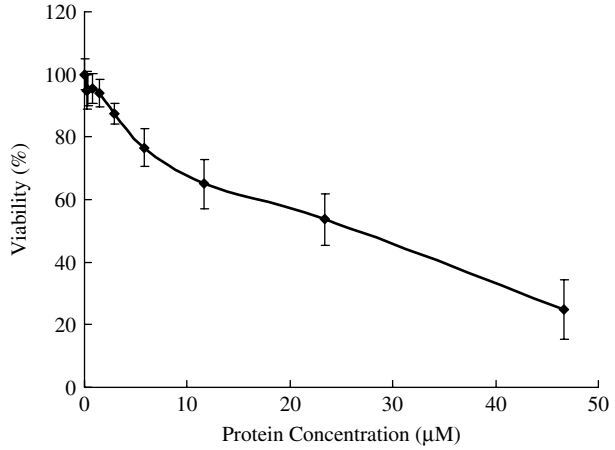

Figure 7 Inhibitory effect of buckwheat antifungal peptide on proliferation of MCF-7 (breast) cancer cells. Cell proliferation was determined by MTT assay (Data represent means ± SD, $n = 3$).

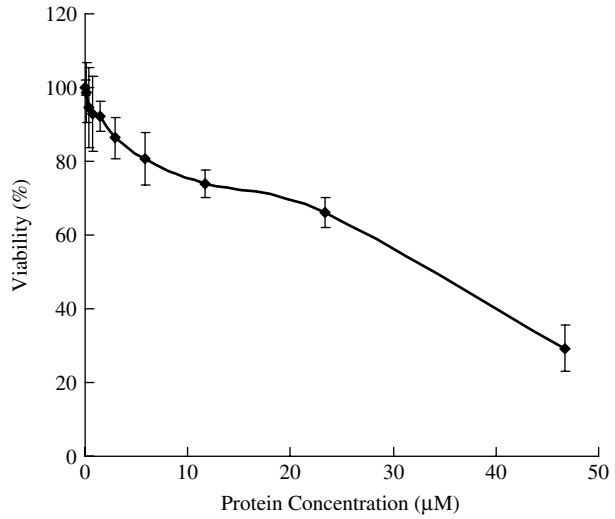

Figure 8 Inhibitory effect of buckwheat antifungal peptide on proliferation of HepG2 (liver) cancer cells. Cell proliferation was determined by MTT assay (Data represent means ± SD, $n = 3$).

Buckwheat antifungal peptide has a glycine-rich *N*-terminal sequence. Its full sequence possesses eight cysteine residues and is abundant in glycine residues. Fujimura *et al.* [25] have noted some similarities between buckwheat antifungal peptide and plant defensins. It is perhaps noteworthy that a number of proteins abundant in glycine or both glycine and cysteine residues manifest antifungal and/or antibacterial activities [34, 35].

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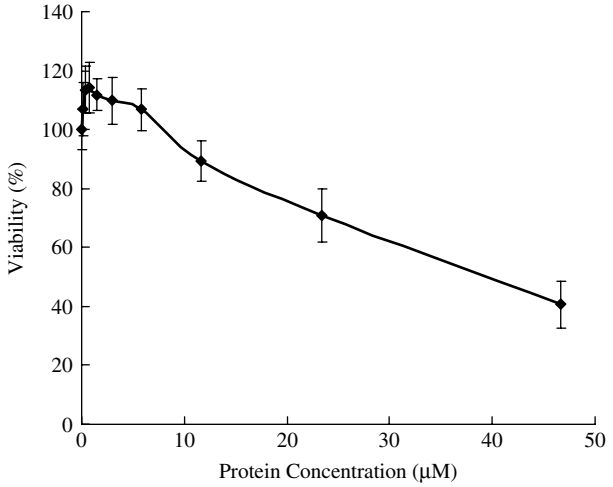

Figure 9 Inhibitory effect of buckwheat antifungal peptide on proliferation of WRL68 (liver) embryonic cells. Cell proliferation was determined by MTT assay (Data represent means ± SD, $n = 3$).

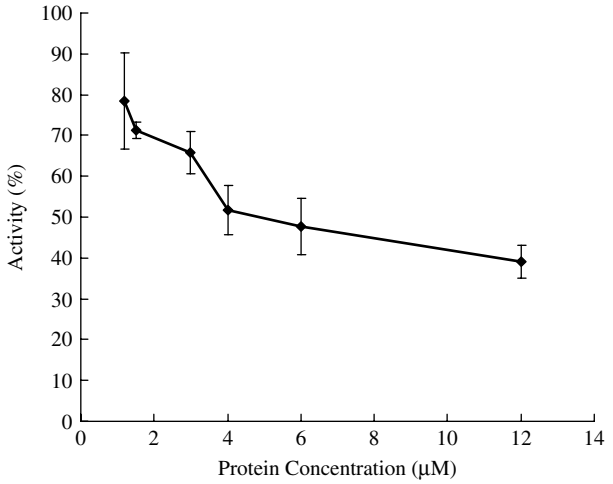

Figure 10 HIV-1 reverse transcriptase inhibitory activity of buckwheat antifungal peptide (Data represent means ± SD, $n = 3$).

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