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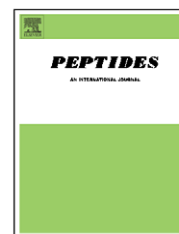

A novel and exploitable antifungal peptide from kale (*Brassica alboglabra*) seeds

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ABSTRACT

The aim of this study was to purify and characterize antifungal peptides from kale seeds in view of the paucity of information on antifungal peptides from the family Brassicaceae, and to compare its characteristics with those of published *Brassica* antifungal peptides. A 5907-Da antifungal peptide was isolated from kale seeds. The isolation procedure comprised affinity chromatography on Affi-gel blue gel, ion exchange chromatography on SP-Sepharose and Mono S, and gel filtration on Superdex Peptide. The peptide was adsorbed on the first three chromatographic media. It inhibited mycelial growth in a number of fungal species including *Fusarium oxysporum*, *Helminthosporium maydis*, *Mycosphaerella arachidicola* and *Valsa mali*, with an IC_{50} of 4.3 μM, 2.1 μM, 2.4 μM, and 0.15 μM, respectively and exhibited pronounced thermostability and pH stability. It inhibited proliferation of hepatoma (HepG2) and breast cancer (MCF7) cells with an IC_{50} of 2.7 μM and 3.4 μM, and the activity of HIV-1 reverse transcriptase with an IC_{50} of 4.9 μM. Its N-terminal sequence differed from those of antifungal proteins which have been reported to date.

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1. Introduction

Antifungal proteins are a family of proteins employed to combat pathogenic fungi which can cause diseases in plants and animals. Those proteins have been purified from a diversity of flowering plants [8,30–33,36,37], animals [3,28], bacteria [39], and fungi [34,35]. Plant tissues that produce antifungal proteins and peptides comprise seeds [1,2,4,6,11,30,31,36], bulbs [7,32], leaves [10,13], tubers [9], fruits [33], shoots [13], and roots [12]. Monocots [4,6,11,13,14,25,32,37], dicots [9,15,16,17,24,29,33,36,38,40], and gymnosperms [10,30] have been reported to produce antifungal proteins.

Plant antifungal proteins are classified, based on structure or activity, into various types [26]. The different types include chitinases and chitinase-like proteins [29], chitin-binding proteins [2,10], lipid transfer proteins [16,17], protease inhibitors [11,40], ribosome inactivating proteins [14,24], embryo abundant protein-like proteins [30], thaumatin-like proteins [33], and defensin-like peptides [38]. To date, only one antifungal peptide has been reported from *Brassica campestris* seeds [16,17] in spite of the presence of multiple *Brassica* species. In new of the structural diversity manifested by the various aforementioned antifungal proteins and the presence of different antifungal proteins even in the same species [9,29], the intent of the present investigation was to isolate an antifungal peptide from the seeds of kale (*Brassica alboglabra* L.H. Bailey), another *Brassica* species, and to compare it with the antifungal peptide from *B. campestris* seeds [16] and other plant antifungal proteins.

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2. Materials and methods

2.1. Materials

Seeds of kale (*B. alboglabra* L.H. Bailey) were purchased from a local vendor. The fungi were provided by Department of Microbiology, China Agricultural University, China. SP-Sepharose, Mono S and Superdex Peptide were from GE Healthcare (Sweden), Affi-gel blue gel was from Bio-Rad (USA). All chemicals were of the highest purity available.

2.2. Isolation of antifungal peptides

The crude extract of kale seeds was chromatographed on a 5 cm $\times$ 20 cm affinity chromatography column of Affi-gel blue gel (Bio-Rad) in 10 mM Tris–HCl buffer (pH 7.8). Unadsorbed proteins (fraction BG1) were eluted with the same buffer while adsorbed proteins (fraction BG2) were eluted with 10 mM Tris–HCl buffer (pH 7.8) containing 1 M NaCl. Fraction BG2 was subjected to cation exchange chromatography on a 2.5 cm $\times$ 20 cm column of SP-Sepharose (GE Healthcare) which had been equilibrated with and was then eluted with 10 mM NH_4OAc buffer (pH 4.5). After unadsorbed proteins had come off the column, the column was eluted with 10 mM NH_4OAc buffer (pH 4.5) containing 0.2 M, 0.5 M and 1 M NaCl to yield fractions SP1, SP2 and SP3. Fraction SP2 was further purified by FPLC on an anion exchange Mono S (GE Healthcare) column in 10 mM NH_4OAc buffer (pH 4.5). After elution of unadsorbed proteins the column was eluted with three linear NaCl concentration gradients (0–0.4 M, 0.4–0.7 M and 0.7–1 M) in the starting buffer to yield two adsorbed fractions S2 and S3. Fraction S2 was subjected to final purification on a Superdex Peptide gel filtration column (GE Healthcare). The main peak constituted purified antifungal peptide.

2.3. Protein determination

Protein concentration was determined by the dye-binding method (Bio-Red) using bovine serum albumin as a standard.

2.4. Tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis (tricine–SDS–PAGE)

It was conducted according to the method of Schagger and von Jagow [27]. After electrophoresis using 18% acrylamide gel, the gel was stained with Coomassie Brilliant Blue. The molecular mass of the isolated antifungal peptide was determined by comparison of its electrophoretic mobility with those of

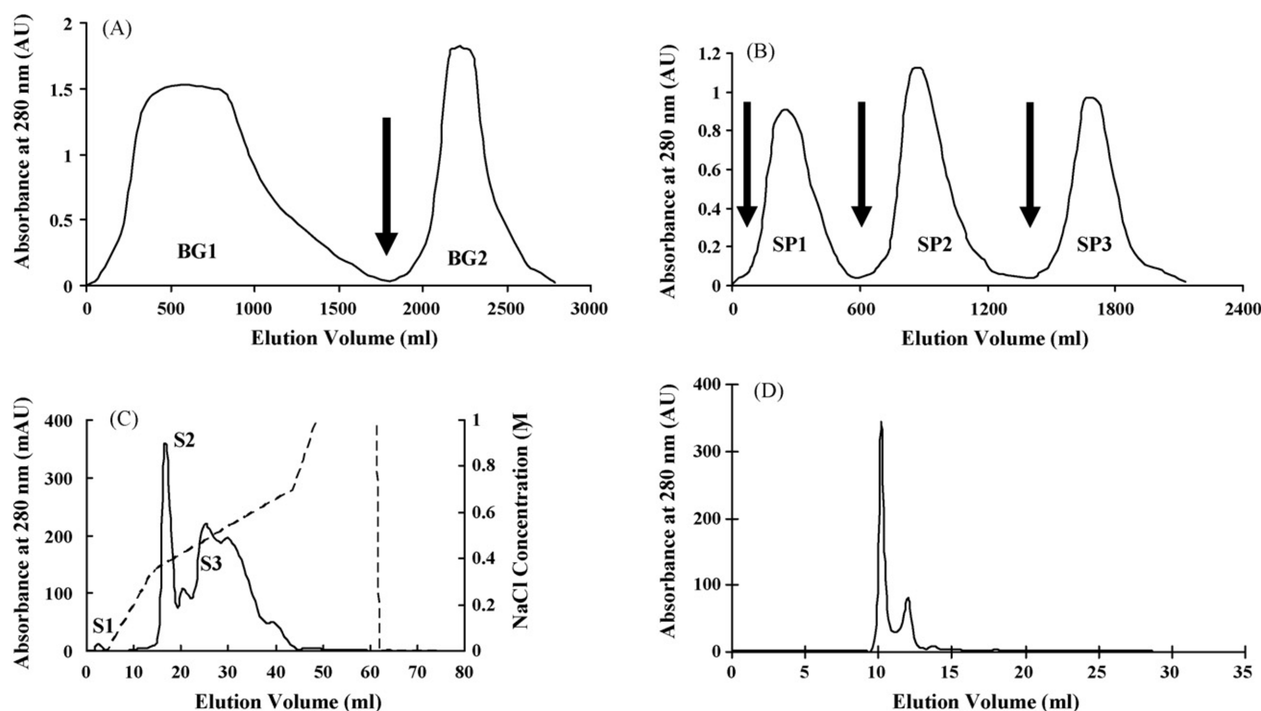

Fig. 1 – Purification of kale (*Brassica alboglabra* L.H. Bailey) antifungal peptide by chromatography on (A) Affi-gel Blue gel, (B) SP-Sepharose, (C) Mono S and (D) Superdex Peptide. In (A), the extract of kale seeds was applied on an Affi-gel Blue gel column (5 cm $\times$ 20 cm). Unadsorbed proteins (fraction BG1) were eluted with the same buffer while adsorbed proteins (fraction BG2) were eluted with 10 mM Tris–HCl buffer (pH 7.8) containing 1 M NaCl as indicated by the arrows. In (B), fraction BG2 from the Affi-gel Blue gel column was dialyzed and applied on an SP-Sepharose column (2.5 cm $\times$ 20 cm) in 10 mM NH_4OAc buffer (pH 4.5). After elution of unadsorbed proteins, the column was eluted stepwise with 0.2 M NaCl, 0.5 M NaCl and then with 1 M NaCl added to the buffer as indicated by the arrows. In (C), fraction SP2 from the SP-Sepharose column was loaded on a 1-ml Mono S column. Following elution of unadsorbed proteins with 10 mM NH_4OAc buffer (pH 4.5), adsorbed proteins were eluted sequentially, first with a 0–0.4 M NaCl gradient and then with a 0.4–0.7 M and 0.7–1 M NaCl gradient. In (D), fraction S2 from the Mono S column was subjected to gel filtration on a Superdex Peptide HR 10/30 column in 10 mM NH_4OAc buffer (pH 4.5).

molecular mass marker proteins from GE Healthcare including horse myoglobin peptides of different molecular masses: 16,949 Da, 14,404 Da, 10,700 Da, 8159 Da, 6214 Da, and 2512 Da.

2.5. Mass spectrometry

Mass spectrometric (MS) analysis of the antifungal peptide was performed on a Finnigan LCQ-MS, an instrument that essentially consists of an atmospheric pressure electrospray positive-ion source, attached to a triple-quadrupole mass analyzer. The purified peptide (100 pmol) was dissolved in water/methanol (50:50, v/v) containing 1% (v/v) acetic acid at a protein concentration of 5 μmol/l, and then applied on the MS instrument [16].

2.6. N-terminal amino acid sequence analysis

The N-terminal amino acid sequence of the purified peptide was performed by Edman degradation using a Hewlett-Packard amino acid sequencer [30].

2.7. Assay of antifungal activity

The assay for antifungal activity was executed using 100 mm × 15 mm petri plates containing 10 ml of potato dextrose agar. The fungal species tested included the following: *Fusarium oxysporum*, *Helminthosporium maydis*, *Mycosphaerella arachidicola* and *Valsa mali*. After the mycelial colony had developed, sterile blank paper disks (0.625 cm in diameter) were placed around and at a distance of 1 cm away from the rim of mycelial colony. An aliquot (8 μl containing 60 μg or 300 μg) of the purified peptide in 20 mM PBS buffer (pH 6.0) was introduced to a disk. The plates were incubated at 23 °C for 72 h until mycelial growth had enveloped peripheral disks containing the control (buffer) and had produced crescents of inhibition around disks containing samples with antifungal activity.

To determine the IC_{50} value for the antifungal activity of the isolated antifungal peptide, four doses of the peptide were added separately to four aliquots each containing 4 mil potato dextrose agar at 45 °C, mixed rapidly and poured into four separate small Petri dishes. After the agar had cooled down, a small amount of mycelia, the same amount to each plate was added. Buffer only without antifungal peptide served as a control. After incubation at 23 °C for 72 h, the area of the mycelial colony was measured and the inhibition of fungal growth determined. Inhibition of fungal growth = % reduction in area of mycelial colony = [(area of mycelial colony in absence of antifungal peptide − area in presence of antifungal peptide)/area in absence of antifungal peptide] × 100%. A graph plotting % reduction in area of mycelial colony caused by antifungal peptide against the concentration of antifungal peptide was then plotted. The concentration of the isolated antifungal peptide that brought about 50% reduction in the area of mycelial colony is the IC_{50} [30].

To investigate the thermal (0–100 °C) stability, pH (0–3 and 10–14) stability and effects of ions, the isolated antifungal peptide was pretreated accordingly and the antifungal assay was then conducted as mentioned above.

A solution of the isolated antifungal peptide (1 mg/ml) was incubated with an equal volume of trypsin or pepsin (1 mg/ml)

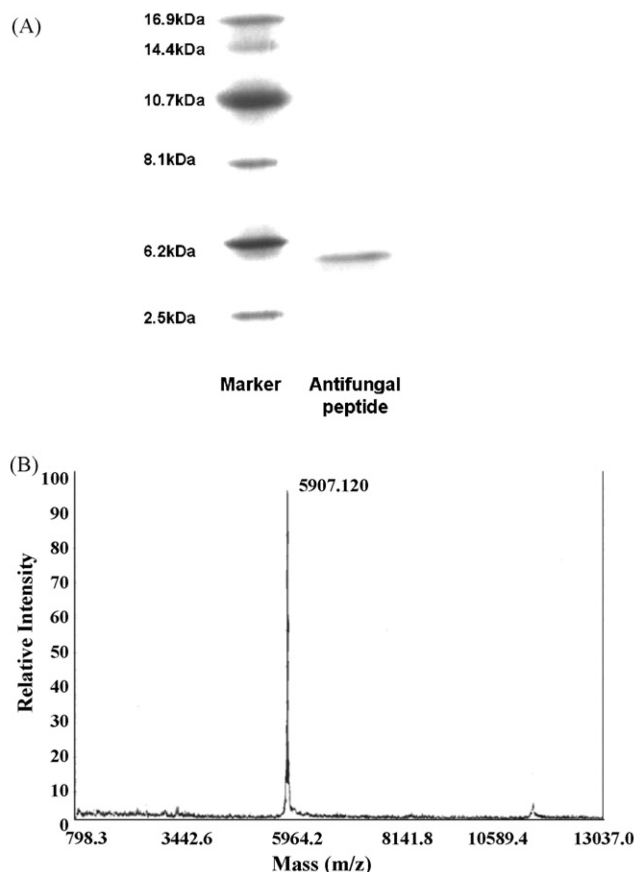

Fig. 2 – (A) SDS–PAGE of kale antifungal peptide. (B) Mass spectrometric analysis of kale antifungal peptide.

Table 1 – N-terminal sequence of kale antifungal peptide in comparison with other *Brassica* peptides

	N-terminal sequence	Reference
Kale (*Brassica alboglabra*) antifungal peptide	^{1}PEGPFQGPKATKPGDLAXQTWGGWXGQTPKY^{31}	This study
Brassica campestris antifungal peptide	^{1}ALSCGTVSGNLAACAGYV^{18}	[17]
Brassica napus trypsin inhibitor	^{1}SECLKEYGGDVGFGFCAPRIYPSFCVQRC^{29}	[5]
B. alboglabra napin-like polypeptide 4.3-kDa subunit	^{1}PAQPFRIKK^{9}	[21]
B. alboglabra napin-like polypeptide 7.2-kDa subunit	^{1}RQGPFERP^{8}	[21]

^{1}P and Y^{31} indicate P and Y being the 1st and 31st residue, respectively.
Only the kale and *B. campestris* antifungal peptides [17] exhibit antifungal activity. The remaining two proteins [5,21] manifest trypsin inhibitory activity. The napin-like polypeptide also possesses antibacterial and antiproliferative activities [21].

at 37 °C for 1 h. At the end of the incubation, the reaction mixture was examined for antifungal activity.

2.8. *Assay of antiproliferative activity on tumor cell lines*

Breast cancer MCF-7 cell line and hepatoma HepG2 cell line were suspended in RPMI medium and adjusted to a cell density of 2×10^4 cells/ml. A 100 μl aliquot of this cell suspension was seeded to a well of a 96-well plate, followed by incubation for 24 h. Different concentrations of the antifungal peptide in 100 μl complete RPMI medium were then added to the wells and incubated for 72 h. After 72 h, 20 μl of 5 mg/ml [3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide] [MTT] in phosphate buffered saline was spiked into each well and the plates were incubated for 4 h. The plates were then centrifuged at $324 \times g$ for 5 min. The supernatant was carefully removed, and 150 μl of dimethyl sulfoxide was added in each well to dissolve the MTT-formazan at the bottom of the wells. After 10 min, the absorbance at 590 nm was measured by using a microplate reader [38].

2.9. *Assay of 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 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 molecule, which is freshly synthesized by the reverse transcriptase (RT). The detection and quantification of synthesized DNA as a parameter for RT activity follows a sandwich ELISA protocol. Biotin-labeled DNA binds to the surface of microtiter plate modules that have been precoated with streptavidin. 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 enzyme catalyzes the cleavage of the substrate, producing a colored reaction product. The absorbance of the sample 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 antifungal peptide [32,33].

2.10. *Assay of ability to inhibit HIV-1 integrase*

The assay was conducted as described in Ref. [19]. The ribosome inactivating protein trichosanthin was used as a positive control [19].

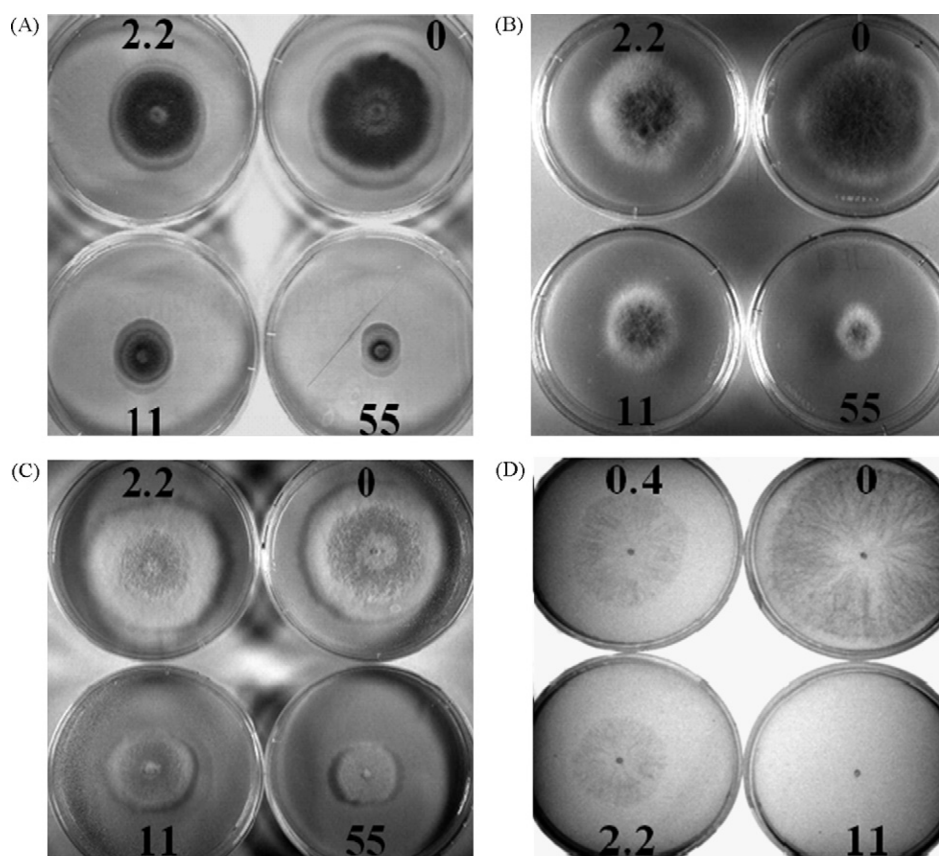

Fig. 3 – The IC_{50} of antifungal activity of kale antifungal peptide toward (A) *Mycosphaerella arachidicola*, (B) *Fusarium oxysporum*, (C) *Helminthosporium maydis*, and (D) *Valsa mali* was 2.1 μM, 4.3 μM, 2.4 μM, and 0.15 μM, respectively. The numbers on the plates represent the concentrations of the antifungal peptide in μM.

2.11. Screening for inhibitory effect on severe acute respiratory syndrome (SARS) coronavious (CoV) protease

The assay was conducted as described by Leung et al. [15].

2.12. Assay of trypsin-inhibitory activity

Trypsin activity was determined by using N-α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) as the substrate. A similar assay was conducted using casein as substrate instead of BAEE [22,23,40].

2.13. Assay of mitogenic activity

The assay was carried out as described earlier. Con A was used as positive control and bovine serum albumin as a negative control [38].

3. Results

The crude extract was fractionated on Affi-gel blue gel into a larger unadsorbed fraction (BG1) eluted by the starting buffer and a smaller adsorbed fraction (BG2) eluted by starting buffer containing 1 M NaCl (Fig. 1A). Antifungal activity resided only in fraction BG2, which was then resolved on SP-Sepharose into an unadsorbed fraction and three adsorbed fractions (SP1, SP2 and SP3) of approximately equal size which were desorbed with 0.2 M NaCl, 0.5 M NaCl and 1 M NaCl, respectively (Fig. 1B). Antifungal activity was concentrated in fraction SP2. Fraction SP2 was separated on Mono S into a tiny unadsorbed fraction (S1) and two adsorbed fractions (S2 and S3) (Fig. 1C). Antifungal activity was detected in the sharp adsorbed fraction S2 eluted soon after application of the 0.3–0.6 M NaCl gradient. Final purification of S2 on Superdex Peptide resulted in a major and a tiny absorbance peak, the former representing purified antifungal peptide (Fig. 1D) with a molecular weight of 5.9 kDa in SDS–PAGE (Fig. 2A). Its molecular weight as determined by mass spectrometry was 5907 Da (Fig. 2B). The yields of the chromatographic fractions with antifungal activity from 500 g seeds are as follows: crude extract (13765 mg), fraction BG2 (6648 mg), fraction SP2 (1023 mg), fraction S2 (342 mg) and purified antifungal peptide (32 mg). It differed from known *Brassica* antifungal proteins, napins and trypsin inhibitors in N-terminal sequence (Table 1). It lacked trypsin inhibitory activity toward casein and BAEE when tested up to 10 μM and 50 μM, respectively (detailed data not shown). The peptide exerted antifungal activity against various fungal species including *F. oxysporum* (A), *H. maydis* (B), *M. arachidicola* (C), and *V. mali* (D) with the ranking of potency being D > C > B > A. The IC_{50} values were respectively 4.3 μM (A), 2.4 μM (B), 2.1 μM (C), and 0.15 μM (D) (Fig. 3). The antifungal activity of the peptide was retained after exposure to temperatures in the range 20–80 °C for 10 min (Fig. 4A) and to the pH ranges 2–3 and 10–11 for 30 min (Fig. 4B). It inhibited proliferation of HepG2 cells (Fig. 5A) and MCF7 cells (Fig. 5B) with an IC_{50} of 2.7 μM and 3.4 μM, respectively, and reduced the activity of HIV-1 reverse transcriptase with an IC_{50} of 4.9 μM (Fig. 5C). It was devoid of mitogenic activity, HIV-1 integrase inhibitory, and SARS proteinase inhibitory activities (data not shown).

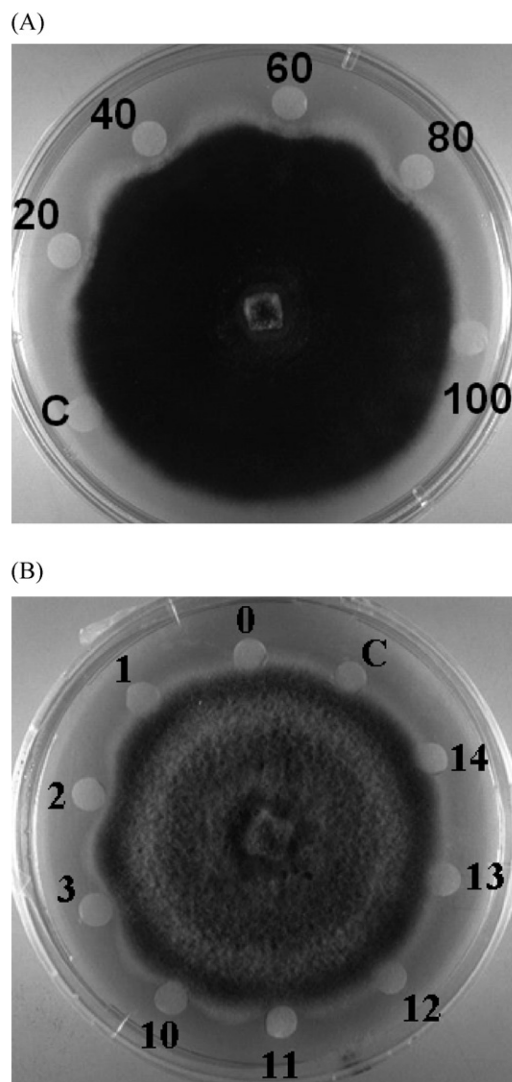

Fig. 4 – (A) Thermostability and (B) pH stability of antifungal activity of kale antifungal peptide. The same amount (2 μg) of peptide was added to each paper disk (except the control disk labeled as (C). The numbers in panel (A) (40–100) and in panel (B) (0–3 and 10–14) near the paper disks represent the various temperatures (panel A) and pH's (panel B) at which the antifungal peptide introduced to the disk had been pretreated for 10 and 30 min, respectively. The antifungal activity of the peptide was found to be stable after exposure to temperatures in the range 20–80 °C for 10 min and to the pH ranges 2–3 and 10–11 for 30 min.

4. Discussion

A 9-kDa nonspecific lipid transfer with antifungal activity has been isolated from *B. campestris* seeds [16]. The present study constitutes the second report on the isolation of an antifungal

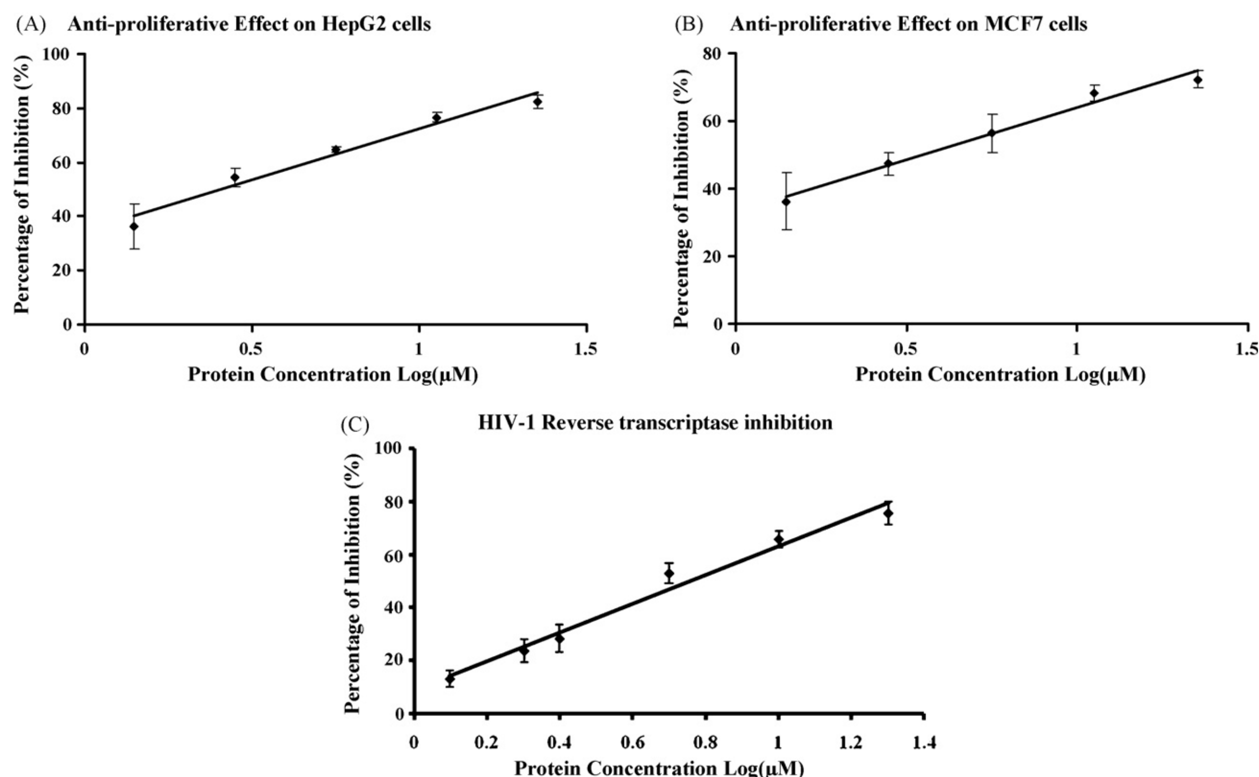

Fig. 5 – Kale antifungal peptide demonstrated antiproliferative activity (A and B), and HIV-1 reverse transcriptase inhibitory activity (C).

peptide from the seeds of a *Brassica* species. The two *Brassica* antifungal peptides differ in N-terminal sequence. Both exhibit pronounced thermostability and pH stability, HIV-1 reverse transcriptase inhibitory activity, antiproliferative activity toward tumor cells, and antifungal activity toward *F. oxysporum*, *M. arachidicola* and a *Helminthosporium* species. The antifungal peptide from kale (*B. alboglabra* L.H. Bailey) seeds did not exhibit trypsin inhibitory activity and also did not resemble *B. napus* trypsin inhibitor in N-terminal sequence. This is noteworthy in view of the fact that some of the trypsin inhibitors demonstrate antifungal activity [40] and that trypsin inhibitors and napins are produced by *Brassica* seeds. The two *Brassica* antifungal peptides were also isolated by using similar protocols. Ion exchange chromatography on Q-Sepharose, affinity chromatography on Affi-gel blue gel, ion exchange chromatography on Mono S, and gel filtration on Superdex Peptide were used for isolation of *B. campestris* antifungal peptide, the only difference from the present protocol for kale antifungal peptide being replacement of SP-Sepharose by Q-Sepharose.

The antifungal peptide from kale seeds had a smaller molecular mass (5.9 kDa) than that (9 kDa) of *B. campestris* antifungal peptide. Its yield (64 mg/kg) was lower than that of *B. campestris* antifungal peptide (175 mg/kg) [16]. Its HIV-1 reverse transcriptase inhibitory activity (IC_{50} = 4.9 μM) was similar to that of *B. campestris* antifungal peptide (IC_{50} = 4 μM), but more potent than many anti-HIV-1 natural products [18]. Its antiproliferative activity toward HepG2 cells and MCF-7 cells (IC_{50} = 2.7 μM and 3.4 μM, respectively) was also analogous to that of *B. campestris* antifungal peptide (IC_{50} = 5.8 μM and 1.6 μM, respectively). Both kale and *B. campestris* antifungal peptide were devoid of mitogenic activity toward mouse splenocytes. It has previously been demonstrated that some [7,38,40] but not other [17] antifungal proteins/peptides exhibit mitogenic activity. Neither kale nor *B. campestris* antifungal peptide demonstrated HIV-1 integrase inhibitory and SARS proteinase inhibitory activities, in line with the observation on French bean defensin-like antifungal peptide [15].

Napin-like polypeptides with trypsin-inhibitory activity but devoid of antifungal activity have been purified from seeds of various *Brassica* species [20–23]. A trypsin-inhibitor has also been isolated from *Brassica napus* seeds [5]. These proteins demonstrate N-terminal amino acid sequences distinctly different from those of the antifungal peptide from kale. The isolation of this antifungal peptide adds to the literature on proteins from *Brassica* seeds.

The chromatographic behavior of kale antifungal peptide on ion exchanger and Affi-gel blue gel is similar to non-Brassica antifungal proteins [30–36,38,40]. Its molecular mass is similar to those of some antifungal peptides from non-Brassica plants [24,30]. Its remarkable thermostability and pH stability resemble those of leguminous defensins [38]. Its broad spectrum of antifungal activity is interesting in view of the observation that shallot and asparagus antifungal proteins are activity toward only one out of several fungal species tested [31,32]. The antifungal activity of kale antifungal peptide is more potent than that of the antifungal proteins

[31,32,40]. Its potent antiproliferative and HIV-1 reverse transcriptase inhibitory activities are noteworthy since not all antifungal proteins have been reported to possess these potentially exploitable activities.

In summary, the antifungal peptide isolated from kale (*B. alboglabra* L.H. Bailey) seeds has potentially exploitable activities such as stable and broad-spectrum antifungal activity, HIV-1 reverse transcriptase inhibitory activity and antiproliferative activity toward tumor cells. In contrast, some antifungal proteins like mungbean chitinase [17] lack the last two activities. There are very few reports on the presence of defense proteins such as antifungal proteins, antiviral proteins, antibacterial proteins and lectins from *B. alboglabra* except for the demonstration of a napin-like peptide with antiproliferative, antibacterial and translation-inhibitory activities [23]. The antifungal peptide isolated in this study would add to the existing literature.

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