



Assessment of in vitro bioactivities of Pis v 1 (2S albumin) and Pis v 2.0101 (11S globulin) proteins derived from pistachio (*Pistacia vera* L.)

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Received: 17 September 2019 / Accepted: 19 December 2019 / Published online: 24 December 2019
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Abstract

We studied the biological activity of Pis v 1 (2S albumin) and Pis v 2.0101 (11S globulin) against *Coxsackie* viruses (CV) B2, B3, B4 and B5 as well as their cytotoxicity against six cell lines. Results showed that the two allergens of pistachio significantly reduce the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) against four Gram-positive and four Gram-negative bacteria as well as two fungi. Moreover, they exerted significant in vitro cytotoxic activities against Human ovarian carcinoma (A2780), human colon carcinoma (HTC), human glioblastoma (U-87-MG), human breast adenocarcinoma (MCF-7), and human prostate cell lines (PC3 and DU-145). Our findings revealed that the highest antimicrobial effect of the allergens was found against *M. luteus* (MIC = 10 µg/mL). The lowest IC₅₀ value was found for Pis v 2.0101 allergen (11S globulin) against CV-B4 (30.20 ± 1.01 µg/mL). Furthermore, in vitro cytotoxicity assay showed IC₅₀ values ranging from 21.09 ± 0.55 to 34.52 ± 0.90 and 26.34 ± 0.88 to 38.63 ± 0.80 µg/mL for Pis v 2.0101 (11S globulin) and Pis v 1 (2S albumin), respectively. As these allergens presented considerable effects against biofilm-related infections, CV-B2-5, and several cell lines, they might be employed as alternative/complementary treatments after further preclinical and clinical studies.

Keywords Allergen protein · Antibacterial · Anticancer · Antifungal · Antiviral · *Pistachio vera* L.

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Introduction

Peptides or proteins are one of the primary “natural antibiotics” which exert considerable antimicrobial effects against a wide range of microorganisms, including Gram-positive and Gram-negative bacteria, yeast, filamentous fungi and to a lesser extent, protozoans and enveloped viruses. Some of these natural antibiotics may be hemolytic and cytotoxic to cancer cells [2]. Plants are valuable sources of compounds that can effectively combat bacterial diseases and tumors. Their role in case of viral disease is contained *Corona* virus, *Coxsackie* virus, Dengue virus, *Enterovirus*, herpes simplex, measles virus respiratory syncytial virus, influenza, human immunodeficiency, hepatitis B and C [36].

Considering chemotherapeutics failure and increasing trend of antibiotic and antiviral resistance, it is essential to search for novel/more efficient antibiotics. Extensive research has been done to discover new alternative antimicrobial and antiviral agents from natural sources [3, 34]. One of the phytochemicals possessing such activities, is proteins [6].

Genus *Pistacia* with approximately ten members, belongs to the family Anacardiaceae [32]. *Pistacia vera* L. is recognized as a delicious nut with several nutritional benefits [30]. Due to its highly favorable taste, nutritional value, and health promoting composition, it is consumed worldwide as a snack (fresh or roasted and salted), pistachio halva, pistachio milk, pistachio butter, etc. Furthermore, pistachio is normally used as an ingredient in cakes, confectionery products, biscuits, candies, ice creams and chocolates [28, 31]. Pistachio contains various essential nutrients that can contribute to a healthy diet [10]. It means that pistachio allergens are from protein families of 2S albumins (Pis v 1), legumins (Pis v 2 and Pis v 5), vicilins (Pis v3), and iron/ manganese superoxide dismutase (Pis v 4) [11]. It is obvious that certain allergenic proteins, typically those which are stable towards heat and digestion, are the primary cause of an immune response [18]. Characterization of pistachio allergens helps to explain the possible cross-reactivity among tree nut allergens and is the first step towards development of better diagnostic and therapeutic approaches [1].

The *Coxsackie* viruses of group B (CV-B1-6) is a positive-sense single-stranded RNA virus belonging to *Enterovirus* genus, and Picornaviridae family. CV-B1-6 is known as the most common viral cause of acute and chronic diseases such as human heart infections, and is involved in the development of type I diabetes, myocarditis and central nervous system pathologies, especially among new-born and infants [5, 26]. From another medicinal point of view, natural compounds have good cytotoxicity profile and their highly selective toxicity makes them promising candidates to be used as adjuvants to anticancer therapeutics to overcome multidrug resistance phenomenon [16].

Various methods have been used for detection of food allergens. The most common quantitative methods used for allergen detection in food, are enzyme-linked immunosorbent assay (ELISA) and real-time PCR. But, it is difficult to detect multiple allergens using an ELISA assay. By using more specific, more quantitative and more sensitive methodologies, trace levels of allergens were also detected in food. As a highly sensitive apparatus, Liquid chromatography-mass spectrometry (LC-MS) has been employed to detect multiple allergens in nuts [18, 19]. To identify peptides, we used a quadrupole time-of-flight (q-TOF) mass spectrometer which enabled us to purify multiple allergens in a short time.

As there are few reports on biological properties of allergenic proteins, we were motivated us to evaluate the (1) antimicrobial, (2) antiviral (against *Coxsackie* viruses) and (3) cytotoxic effects of allergenic proteins isolated from pistachio.

Materials and methods

Chemicals and plant material

The ripe fruits of pistachio (*P. vera*) ("Kaleghoochi" cultivar) were collected from a commercial orchard of Rafsanjan (at 30° 41' N and 55° 09' E; 1580.9 m above sea level), Kerman province, Iran. Kernels, hull and hard shells of fruits were separated and the kernels were air-dried at room temperature, ground and then stored at 4 °C until analysis. L-Glutamine, penicillin, streptomycin, nystatin, amphotericin B, fetal bovine serum (FBS), Eagle's Minimal Essential Medium (MEM), fetal calf serum (FCS), non-essential amino acids and trypsin were obtained from GIBCO. Hexane, TRIS/HCl, dibasic sodium phosphate, acetonitrile, trifluoroacetic acid (TFA), 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT), soybean casein digest agar (SCDA), normal saline (NS), Mueller-Hinton broth (MHB), 2,3,5-triphenyltetrazolium chloride (TTC), Sabouraud's dextrose broth (SDB), phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO) and RPMI 1640 medium were procured from Sigma Aldrich (Europe).

Protein isolation

Defatting pistachio powder

Pistachio kernel powder was extracted using hexane at 1:5 (v:v) ratio and stirred thoroughly for 1 h at room temperature. The lipid fraction was removed and the precipitate was air-dried in a draft chamber, overnight. The defatting process was repeated in order to produce a completely dried powder. The defatted pistachio kernel powder (DPKP) was stored at -80 °C [9].

Protein isolation

Protein extraction was performed using a slightly modified method that was previously described by Sealey-Voyksner et al. [18]. For this purpose, 30 mg of DPKP was extracted for 2 h at 50 °C using 1 mL of TRIS/HCl (50 mM, pH 7.5); other solvent used for this purpose were HCl (0.01 M, pH 2.5) and dibasic sodium phosphate (50 mM, pH 8). The DPKP extracts (with the exception of those subjected to enzymatic digestion) were centrifuged at 8000×g for 10 min and the supernatant was collected for analysis by LC-MS for the presence of proteins. For enzymatic digestion, we added trypsin to samples extracted with 50 mM TRIS/HCl (pH 7.5). Also, 25 µL of 12 mg/mL trypsin solution was dissolved in 50 mM sodium phosphate and the mixture was added to the samples and incubated at 38 °C for 2 h while

vortexed every 15 min; after this time, 200 μ L of acetonitrile with 0.2% TFA was added to the sample. The digested sample was centrifuged at 8000 \times g for 10 min and the upper 200 μ L was fractionated into a 96-well plate for LC–MS/MS analysis [18].

Liquid chromatography–mass spectrometry (LC–MS)

Liquid chromatography–mass spectrometry (LC–MS) for pistachio proteins and trypsin digest analysis, was carried out by an Agilent 1290 LC system (Agilent Santa Clara, CA, USA) coupled with an Agilent 6530 quadrupole time-of-flight (q-TOF) mass spectrometer to identify peptides that represent known pistachio proteins.

Protein analysis

The DPKP proteins were separated on an Agilent Poroshell 300 column (2.1 \times 75 mm, 2.7 μ m, 300 Å ; Agilent, Santa Clara, CA, USA), using a gradient of 0 to 40% of solution I for 70 min (solution I comprised of 95% water + 5% acetonitrile + 0.025% TFA) and solution II (solution II comprised of 5% water + 95% acetonitrile + 0.025% TFA), then from 40% solution II to 60% solution II at 80 min at a flow rate of 0.3 mL/min. The temperature of column was held at 30 $^{\circ}$ C for the analysis. The injection volume was 20.0 μ L.

For q-TOF–MS operation, the electrospray positive ion detection mode was set up in 2 GHz extended dynamic range mode. The duration of scanning was 0.5 s from m/z 300 to 2800. The electrospray characteristics were: drying gas 350 $^{\circ}$ C at 9 L/min, nebulizer gas (nitrogen; flow 10 L/min) and sheath gas 350 $^{\circ}$ C at 11 L/min, capillary voltage 4000 V, nozzle voltage 1000 V, and fragmentor voltage 160 V.

Peptide analysis

The digested sample was analyzed by an Agilent Poroshell 120 column (2.1 \times 50 mm, 2.7 μ m) using a gradient of 0–40% solution II for 70 min then 40–60% solution II for 80 min with a flow rate of 0.25 mL/min. The column temperature for the analysis was 30 $^{\circ}$ C and the injection volume was 20 μ L. The q-TOF–MS operation was conducted as in a same way as that done for protein analysis. The auto MS/MS was also operated for the most intense precursor ions that were selected via single MS before, by considering > 15,000 counts from 600 to 2100 m/z . For MS/MS, nitrogen was used as collision gas and the collision energy was 10 eV. The MS/MS spectrum was operated in the positive ion as follows: Agilent at 3 spectra/sec over 60–2100 m/z with capillary voltage 4000 V, nozzle voltage 1000 V, fragmentor voltage 160 V, and collision energy of 10 eV.

Detection in database

The Agilent technology (The Spectrum Mill MS Proteomics, workbench Rev B.04.00127) was used to search the database. Searches were based on a mass tolerance window of 20 ppm for precursor ion and 50 ppm window for the product ions around the measured m/z . The search used trypsin digestion with up to two missed cleavages with no modifications. The LC–MS data from the tryptic digests of pistachio proteins were searched to identify peptide sequences from the major peaks in the LC–MS run and to match the identified peptide to a specific pistachio protein. A MS scoring algorithm was used to match ions present in the spectrum with the sequence based upon factors described by Kapp et al. [13]. Scores of 15 and above are well assigned fragmentation and correct interpretation. Scores that were greater than 10 were very good and usually right. Spectrum Mill was used to confirm the amino acid sequences based on the acquired MS spectra as a secondary confirmation of the postulated sequence. Spectrum Mill was also used to verify that these sequences were unique to the pistachio analyzed (based on the search sequence in the green plant NCBI nr database using MS Edman program) and would not be found in other nuts or green plants. The sequencing process was done at Department of Molecular Biochemistry, Faculty of Chemistry, University of Gdańsk, Poland [18].

Peptide standards

High resolution MS/MS was used to confirm the identity of the peptide. All the peptide standards (2S albumin MS Spec standard: CAT No. A8471; and 11S globulin Analytical Standard: CAT No. P8119) were purchased from Sigma Aldrich (Europe).

In order to make 1 mg/mL stock solution, each peptide was dissolved in water: acetonitrile (80:20 ratios) then, diluted to make the threshold standard of 0.1 ppm.

Protein content

Protein was quantified by Bradford assay. The assay calibration was set over the range of 25–2000 μ g/mL and bovin albumin was used as the standard protein.

Antimicrobial activity

Strains and growth condition

DPKP-isolated allergens' antimicrobial properties were examined against 4 Gram-positive [*Staphylococcus aureus* (PTCC (Persian Type Culture Collection) 1431), *Staphylococcus epidermidis* (PTCC 1435), *Micrococcus luteus* (ATCC (American Type Culture Collection) 9341) and

Bacillus cereus (PTCC 1247)], 4 Gram-negative [(*Pseudomonas aeruginosa* (PTCC 1074), *Salmonella typhi* (PTCC 1609), *Serratia marcescens* (PTCC 1267) and *Escherichia coli* (ATCC 1533)] bacteria and 2 fungi [(*Candida albicans* (ATCC 10231) and *Aspergillus niger* (ATCC 16404)]. A fresh culture media for each microorganism was prepared separately. For bacterial strains, the culture media was prepared through 24 h incubation at 37 °C on SCDA and the bacterial population was adjusted to 10⁶ CFU/mL using normal saline (NS 0.9%). Then, microorganisms were cultured at 25 °C for 48 h on SCDA, and adjusted to 10⁶ CFU/mL by sterile 0.9% NS to be tested against fungal strains [33].

Minimum inhibitory concentration and minimum bactericidal and fungicidal concentrations

Minimum inhibitory concentration (MIC) of DPKP-isolated allergens was determined by the micro-dilution method using 96-microwell culture plates. Accordingly, in order to prepare the first concentration (10% v/v) of each DPKP-isolated allergen, 650 µL of each allergen was added to 325 µL DMSO and the final volume of the mixture was adjusted to 6.5 mL using MHB. The other concentrations of DPKP-isolated allergens were made by using two-fold serial dilution method. A mixture of 200 µL of each concentration of DPKP-isolated allergens and 20 µL of bacterial suspension (10⁶ CFU/mL), was added to each well of a 96-well culture plate. For each bacterium, the experiment was done in duplicate. Also, MHB with DMSO and the microorganisms (with exception DPKP-isolated allergen) served as the negative control. After 24 h incubation at 37 °C, 20 µL of TTC (5 mg/mL) was added to each well for bacterial growth estimation. The lowest concentration of the DPKP-isolated allergens inhibiting the formation of red color in culture media was considered the minimum inhibitory concentration (MIC). In addition, the minimum bactericidal concentration (MBC) was measured by incubation of 20 µL of colorless content with Mueller–Hinton agar media, for 24 h at 37 °C. Gentamicin and vancomycin were applied as the positive control against Gram-positive and Gram-negative bacteria, respectively [29].

To investigate the anti-fungal activity of DPKP-isolated allergens, different concentrations of DPKP-isolated allergens were prepared as previously mentioned in the antimicrobial assay. The volume was adjusted to 1 mL by SDB. Using two-fold serial dilution technique, other concentrations of DPKP-isolated allergens were prepared. Then, 200 µL of cell suspension (10⁶ CFU/mL) was incubated for 48 h at 25 °C. Finally, the concentration with no visible microbial growth (i.e. MIC) and the concentration with no visible fungal growth [i.e. minimum fungicidal concentration (MFC)] were determined. In

this experiment, the negative and positive controls were treated with SDB and nystatin, respectively [22].

Antiviral activity

The *Coxsackie* viruses (CV) B2, B3, B4 and B5 were propagated in HEp-2 cells provided from National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). Then, they were maintained in MEM supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% (2 mM) L-Glutamine, 1% (50 µg/mL) streptomycin, 1% (50 IU/mL) penicillin, 1% non-essential amino acids and 1% (2.5 µg/mL) amphotericin B. The viruses titer was determined based on the 50% tissue culture infectious dose (TCID₅₀) on HEp-2 cells and all viruses were stored at – 80 °C until use. The antiviral activity of DPKP-isolated allergens against CV B2, B3, B4 and B5 was studied with respect to their inhibitory activity on virus-induced cytopathogenicity and cell death in HEp-2 cells. HEp-2 cells were seeded into 96-well plates at 10⁴ cells/well at 37 °C with 5% CO₂. After 24 h, the culture media was removed, and cells were washed two times with PBS before adding 100 µL of each DPKP-isolated allergen mixed with MEM 2% FCS (twofold dilutions, ranging from 3.9 to 1000 µg/mL). Cells were inoculated with 50 µL of 2% MEM containing 100 TCID₅₀ of each *Coxsackie* virus (B2–B5). The MTT assay was performed for investigation of cell death due to virus infection. The virus inhibition percentages were measured using the following equation:

$$\text{Virus inhibition(\%)} = \frac{(T - Vc)}{(Cc - Vc)} \times 100$$

where T is the optical density (OD) of cells treated with DPKP-isolated allergens, Vc is the OD of virus control, and Cc is the OD of cell control. The antiviral activity curve was then generated by plotting virus inhibition percentages against DPKP-isolated allergens concentrations. The concentration that could reduce 50% of cytopathic effect (CPE) with respect to the virus control (cells that were infected only with the virus, but not treated with the compound in the antiviral assays), was estimated from plots of the data and defined as 50% inhibitory concentration (IC₅₀). Another important issue was to determine the selectivity index (SI) towards the host cell. The SI was defined as the ratio of the maximum concentration causing either 50% inhibition of growth of normal cells (CC₅₀) to the minimum concentration at which 50% of the virus is inhibited (IC₅₀) [26].

Cytotoxicity assay

Cell culture

Human ovarian carcinoma (A2780), human colon carcinoma (HTC), human glioblastoma (U-87-MG), human

breast adenocarcinoma (MCF-7), and human prostate cell lines (PC3 and DU-145) were purchased from National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). All cells were maintained at 37 °C in humidified (90%) atmosphere with 5% CO₂ in RPMI 1640-supplemented with 10% (v/v) FBS, 100 IU/mL penicillin and 100 mg/mL streptomycin.

Cell viability assay

Cytotoxicity of DPKP-isolated allergens against human cancer cell lines was examined using MTT assay. Also, 3T3 was used as a normal cell line. Briefly, the cells (5×10^3 per well) were seeded in a 96-well plate; after 24 h, for adhesion, different concentrations of each DPKP-isolated allergens were added to wells and all experiments were done in triplicate. After 48 h incubation, 10 µL of MTT (5 mg/mL stock solution) was added to each well and the plates were incubated at 37 °C for 4 h. The medium was removed and the formazan blue crystals were dissolved by 200 µL DMSO. The absorbance was measured at 570 nm by an ELISA microplate reader (Awareness Technology, Inc., Palm City, FL, USA). Cytotoxicity of each DPKP-isolated allergen was presented as its concentration inhibiting cell growth by 50% (IC₅₀) and the percentage of viable cells was determined using the following equation ([21]):

$$\text{Viable cell}(\%) = \frac{(Atc - Ab)}{(Ac - Ab)} \times 100$$

where Atc, Ab, Ac are the absorbance of the treated cells, blank and control cells, respectively.

Statistical analysis

Statistical analysis was performed as a completed randomized design (CRD) in triplicate and data are presented as mean ± SD. One way analysis of variance (ANOVA) and LSD test were performed to determine statistical differences among groups by JMP 8 (SAS Campus Drive, Cary, NC 27513) and Sigma Plot 12 software. The IC₅₀ values were determined by GraphPad Prism 5.0 (GraphPad software, San Diego, CA, USA). Significant differences among mean values were determined by using LSD at the level of 0.05.

Results

Pistachio allergenic proteins purification

Method validation and protein content

Protein content of each fraction was estimated using the Bradford assay. As shown in Table 1, among three solvents

used (i.e. 50 mM TRIS/HCl, pH 7.5; 0.01 M HCl pH 2.5 and 50 mM dibasic sodium phosphate, pH 8), the highest yields of protein extraction was recorded for TRIS/HCl; however, the difference between TRIS/HCl and dibasic sodium phosphate was not statistically significant ($p < 0.05$). HCl could not completely extract the high molecular weight proteins. The Bradford assay indicated that protein contents of TRIS/HCl and dibasic sodium phosphate fractions were 77.4 and 70.2% with recovery of 90.2 and 89.3% of DPKP on a weight basis, respectively. Nevertheless, the HCl extract contained 33.5% total protein, and the percentage of its recovery represented 89.1% of DPKP on a weight basis.

Proteins purification and peptide markers

The DPKP mass spectra taken at different retention time points showed numerous homogenous peaks with distinctive multiply charged ions that could easily determine the molecular weight.

Using our multiplexed LC–MS/MS analytical method, two marker peptides were detected based on retention times, accurate mass (in a 10 ppm window) and the product ions. The initial step in peptide selection included identification of the peptides. Among different detected proteins, two allergens are shown in Table 2 and Figs. 1 and 2.

Antimicrobial activity

MIC and MBC values

The antimicrobial activity of each DPKP-isolated allergen, namely Pis v 1 (allergen 2S albumin) and Pis v 2.0101 (allergen 11S globulin), was examined against eight bacteria and two fungi (Table 3). According to the results, Pis v 1 and Pis v 2.0101 had similar MIC and MBC values of 16 and 12 µg/mL, respectively against *S. epidermis*. Also, it was observed that the most sensitive strain to these allergens was *S. epidermis*. Moreover, both allergens had similar MIC and MFC values of 12 and 10 µg/mL, respectively against *C. albicans*. In terms of antifungal activity, significant differences were observed between the allergens (Table 3). Based on our results, MIC and MFC values against *C. albicans* and

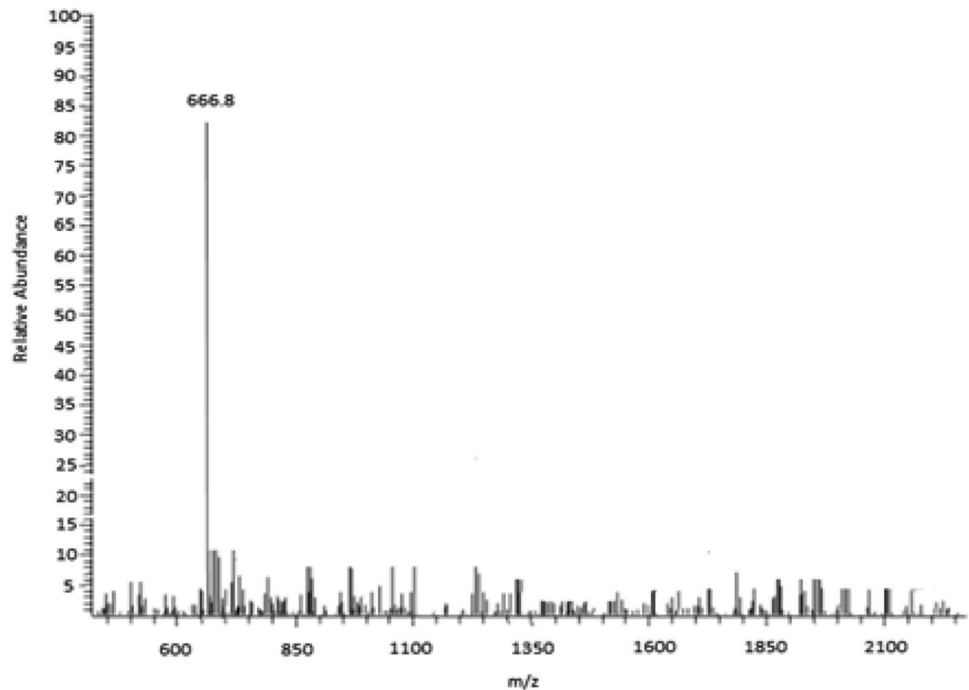
Table 1 Total protein content and percentage of recovery in different fractions

Fractions	Protein by weight on dry basis (%)	Recovery (w/w%)
TRIS/HCl	77.4 ± 2.1	90.2 ± 1.5
HCl	33.5 ± 1.9	89.1 ± 1.5
Dibasic sodium phosphate	70.2 ± 2.1	89.3 ± 1.3

Table 2 Pistachio allergenic proteins profiles

Peptide marker	Sequence	RT ^a (min)	Accurate mass (m/z)	MS/MS sequence	MS/MS mass	Protein name	Allergen content (mg)	Protein MW (KDa)
pis2	LQELYETASELPR	25.5	774.9005	19.4	1064.334	Pis v 1 allergen 2 s albumin	1.2 ± 0.03	17.300
Pis1	TNGLSQTSQLAGR	13.0	666.8486	19.8	1307.123	Pis v 2.0101 allergen 11 s globulin	2.3 ± 0.03	56.470

^aRetention time

Fig. 1 Mass spectrum of Pis v 2.0101 allergen 11 s globulin

A. niger for Pis v 2.0101 were similarly 10 and 50 µg/mL, respectively (Table 3).

Antiviral activity

Inhibition of *Coxsackie* viruses (CV-B2-5) infectivity by DPKP-isolated allergens

The antiviral activity of different concentrations of the two allergens was evaluated against *Coxsackie* viruses (B2-B5) at 37 °C (Fig. 3). The virus assay examined the inhibitory effects of the compounds on virus-induced pathogenicity in HEp-2 cells. Our results proved that both of these allergens are active against *Coxsackie* viruses (B2-B5) and the lowest IC₅₀ values for both allergens were observed for CV-B4. IC₅₀ values calculated for Pis v 2.0101 were significantly lower than those of Pis v 1 against all cell lines (Table 4). The selectivity indexes (SI) (CC₅₀/IC₅₀) of both allergens

for all CVs were > 3. Generally, 50% cytotoxic concentration (CC₅₀) is considered a useful marker in cytotoxicity. Based on our results, the CC₅₀ of Pis v 2.0101 against all cell lines was 710.30 ± 1.03 (Table 4). A CC₅₀ of 100–1000 µg/mL shows moderate cytotoxicity. It seems that the antiviral activity is appropriate when the samples tested have an IC₅₀ values below 100 µg/mL [4].

Cytotoxicity

Cytotoxicity of the two isolated allergens was investigated using MTT method against six human cancer cell lines (Table 5). Doxorubicin was used as the positive control. The results are summarized in Table 5. Both allergens had potent cytotoxic effects toward all cancer cell lines at all tested concentrations (IC₅₀ values < 100 µg/mL). Comparison made between the two allergens showed that IC₅₀ values of Pis v

Fig. 2 Mass spectrum of Pis v 1 allergen 2 s albumin

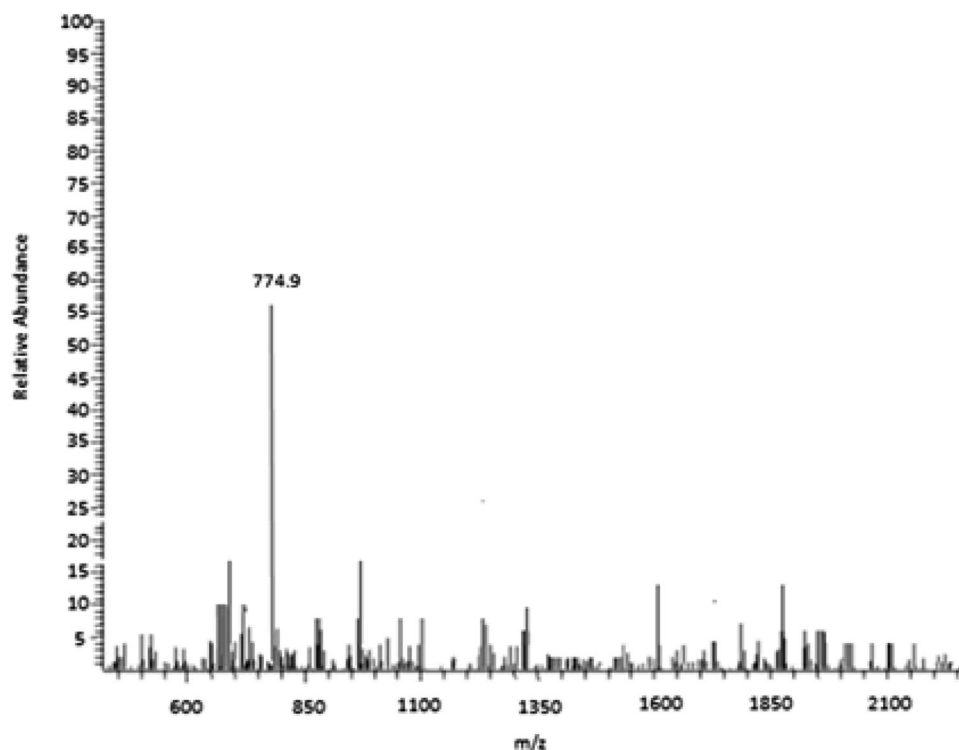


Table 3 MICs and MBCs of Pis v 1 and Pis v 2.0101 against different bacterial and fungal strains

Microorganism	Pis v 1		Pis v 2.0101		Gentamicin		Vancomycin		Nystatin	
	MIC ^a	MBC ^b	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Bacterial strains										
<i>S. aureus</i> (PTCC 1431)	50	105	12	25	–	–	4	32	–	–
<i>S. epidermis</i> (PTCC 1435)	16	16	12	12	–	–	2	24	–	–
<i>B. cereus</i> (PTCC 1247)	80	140	6.25	13.5	–	–	4	80	–	–
<i>M. luteus</i> (ATCC 9341)	16	16	10	22	–	–	2	40	–	–
<i>E. coli</i> (ATCC 1533)	90	90	50	112	8	8	–	–	–	–
<i>P. aeruginosa</i> (PTCC 1074)	120	250	80	145	16	16	–	–	–	–
<i>S. typhi</i> (PTCC 1609)	92	180	60	90	16	16	–	–	–	–
<i>S. marcescens</i> (PTCC 1267)	120	120	60	73.2	8	8	–	–	–	–
Fungal strains										
<i>C. albicans</i> (ATCC 10231)	12	12	10	10	–	–	–	–	4	4
<i>A. niger</i> (ATCC 16404)	20	30	50	50	–	–	–	–	8	8

^aMinimum inhibitory concentration (µg/mL)

^bMinimum bactericidal concentration (µg/mL)

^cMinimum fungicidal concentration (µg/mL)

2.0101 were significantly lower than those of Pis v 1 against all cell lines (Table 5).

Discussion

Since synthetic antibiotics have become less effective or even ineffective, peptides have been considered for development of new antibiotics. Many peptides are known to play important roles in immunomodulation, promotion of wound

healing during the clearance of infection, and antiviral activity against HIV-1. They can incorporate into the eukaryotic cells and interact with the members of mitochondria inducing membrane depolarization and tumor cell death via activation of mitochondrial apoptotic pathways [20]. In the present work, we found that these allergens have considerable antimicrobial effects against Gram-positive, Gram-negative and fungal as well as *Coxsackie* viruses and possess A2780, HTC, U-87-MG, MCF-7, PC3, DU-145 cytotoxic properties. It was previously shown that melittin isolated

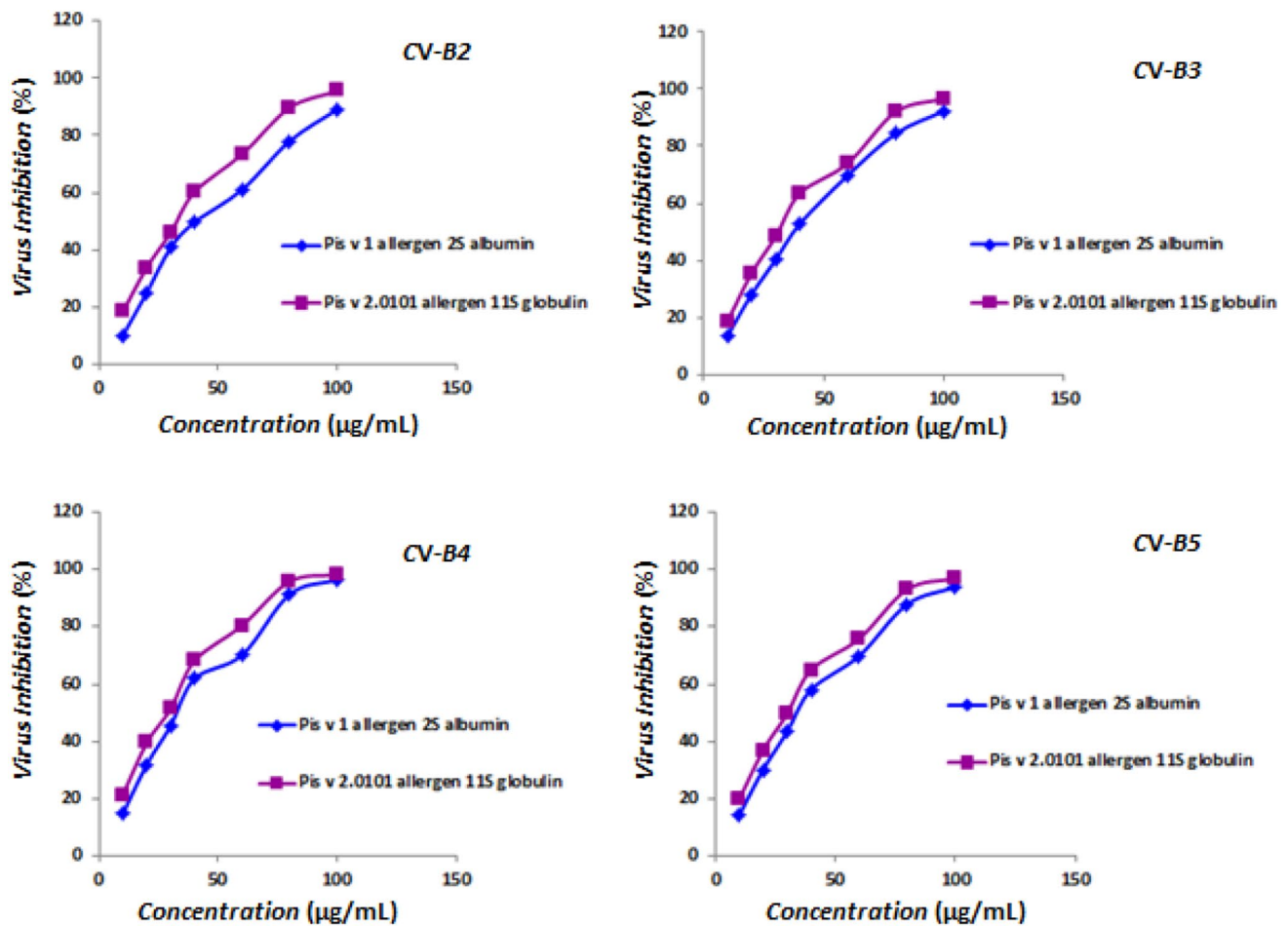


Fig. 3 Antiviral activity of Pis v 1 allergen 2S albumin and Pis v 2.0101 allergen 11S globulin against **a** *Coxsackie virus B2*; **b** *Coxsackie virus B3*; **c** *Coxsackie virus B4* and **d** *Coxsackie virus B5*

Table 4 Virucidal activity of Pis v 1 and Pis v 2.0101

<i>Coxsackie virus</i>	Pis v 1			Pis v 2.0101		
	CC ₅₀ ^a	IC ₅₀ ^b	SI ^c	CC ₅₀	IC ₅₀	SI
<i>CV-B2</i>	352.20 ± 1.03	48.05 ± 0.90	7.33	648.75 ± 1.02	37.50 ± 1.01	17.30
<i>CV-B3</i>	516.25 ± 1.05	43.75 ± 1.01	11.80	649.88 ± 1.01	35.61 ± 1.03	18.25
<i>CV-B4</i>	633.88 ± 1.01	38.77 ± 1.02	16.35	710.30 ± 1.03	30.20 ± 1.01	23.52
<i>CV-B5</i>	586.73 ± 1.02	41.29 ± 1.03	14.21	648.42 ± 1.03	31.77 ± 1.02	20.41

^aThe 50% cytotoxic concentration for target cells (µg/mL)

^bThe concentration of the sample required for 50% inhibition (µg/mL)

^cSelectivity index

from the venom of Iranian honey bee, possesses broad antimicrobial spectra as well as anticancer properties [20]. By the advent of molecular biology techniques, many studies examined antibacterial, antifungal, antiviral, and anticancer activities of proteins isolated from different sources such as plants, mammals, marine invertebrates, and insects. For example, the peptide obtained from the wheat endosperm, purothionin, showed antibiotic activities [15]. The

cytoplasmic membranes of microbes are lipid-rich and the antimicrobial proteins can easily interact with the cytoplasmic membrane of pathogens using their charged- hydrophobic structure [17]. Based on our antimicrobial assessments, the most susceptible strains were Gram-positive bacteria. Biological evaluations revealed that it is easier for antimicrobial proteins to enter Gram-positive peptidoglycan layer compared to Gram-negative hydrophilic lipopolysaccharides

Table 5 Cytotoxic activity (IC₅₀) of Pis v 1 and Pis v 2.0101

Cell line	Pis v 1	Pis v 2.0101	Doxorubicin
MCF-7	25.71 ± 0.94	28.11 ± 0.88	0.78 ± 0.05
A2780	30.06 ± 0.71	33.95 ± 0.42	0.61 ± 0.02
PC3	31.74 ± 0.63	34.05 ± 0.42	0.44 ± 0.05
DU-145	34.52 ± 0.90	38.63 ± 0.80	0.98 ± 0.05
U-87-MG	21.09 ± 0.55	26.34 ± 0.88	0.39 ± 0.05
HTC	25.71 ± 0.94	28.11 ± 0.88	0.78 ± 0.05

Data are shown as mean (±SD)

IC₅₀: the concentration required for 50% inhibition (µg/mL)

(LPS) which show higher degree of resistance towards hydrophobic antimicrobial compounds [23].

Recently, many viral diseases were treated with a combination of synthetic drugs which improve sustained virological response, but also produce adverse side effects including anemia and gastrointestinal symptoms [14]. In addition, antimicrobial proteins can exhibit antitumor and antiviral effects [27]. It has been reported that two allergens of pistachio inhibited several group B *Coxsackie* viruses. Pharmacological activities of pistachio allergens were attributed to their antiviral potential. Our data showed that both allergens, markedly affected CV-B2-5 viability. Viral membrane fusion inhibitory activity of these proteins may be related to their lipopeptide-based structure [37]. It was indicated that tryptophan-rich, C8 (Ac-Trp-Glu-Asp-Trp-Val-Gly-Trp-Ile-NH₂) plays a major role against the membrane-proximal external region of the feline immune deficiency virus (FIV) [8]. It seems that the inhibitory action is related to adsorption of the peptide on the plasma membrane of the target cell, where it can inhibit interactions between virus fusion glycoprotein [7]. Adsorbed peptide supports the cell from the fusion glycoprotein of the virus and it is directly due to the fundamental peptide; however, small peptides may exhibit no antiviral activity because of their conformational instability [8]. In another study using FTIR spectroscopy, a secondary structure consisting of an N-terminal beta-sheet along with a turn and C-terminal beta-sheet was introduced as a new class of anti-HCV compounds [14].

Proteins have been regarded as candidates for treatment of cancer or inflammatory diseases and were shown to trigger apoptosis and prevent angiogenesis. Anticancer proteins are able to stimulate host defense system cells and tissues via direct and indirect interaction with host's genes and proteins [27].

In our study, pistachio allergens produced potent cytotoxicity in a concentration-dependent manner against all six cell lines. Interestingly, HTC cells were found to be more sensitive to the cytotoxic effects of both allergens compared to the other cell lines. In a previous study, R-lycosin-I modified by amino acid substitution from lycosin-I, showed

anticancer activity and inhibited cell proliferation [12]. In another research, fatty acids with different chain lengths were introduced to the N-terminal of R-lycosin-I; this modification increased cytotoxic activity of these lipopeptides against A549 cells [12]. Also, Tag7 was reported to be an innate immune protein that is involved in the antibacterial and antitumor effects [24]. Moreover, Tag7 complex with Hsp70 showed cytotoxic effects in many tumor cell lines. It seems that Tag7 can activate cytotoxic lymphocyte subpopulations [25]. Cytotoxicity of proteins may be exerted through different mechanisms including disruption of cell membrane integrity via depolarization, reducing the activity of membrane-bound enzymes, modification of the mevalonate pathway of metabolism and/or induction of apoptosis [35].

Conclusion

The two allergens isolated from pistachio in this study, presented considerable effects against biofilm-related infections, CV-B2-5, and several cell lines. These observations could be used as a basis for future experiments to further evaluate potential therapeutic applications of these proteins.

Acknowledgements The authors kindly thank Pharmaceutical Research Center, Pharmaceutical Technology Institute, Mashhad University of Medical Sciences, Mashhad, Iran.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

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