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Evaluation of anticancer activity of α-defensins purified from neutrophils trapped in leukoreduction filters

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ABSTRACT

Aims: The α-defensins or human neutrophil peptides (HNP 1–3) that exist in azurophilic granules are found to have anticancer activity. The pattern of disulfide bonds in α-defensins is crucial for the functional properties. Therefore, synthesis using the chemical and recombinant approaches is a challenging. A safe source for the production of α-defensins can be the use of leukoreduction filters in blood banks that contain large quantities of neutrophils and are discarded after use. The aim of this study was to purify α-defensins from neutrophils trapped in leukofilters and to investigate its anticancer activity.

Materials and methods: Immunoprecipitation was performed to purify α-defensins and the presence of protein was confirmed by Western Blot. The Jurkat T-cell line was incubated with different concentrations (5, 10 and 15 μg/ml) of purified HNP1–3 for 16 h. Cell viability was measured using a WST-1 assay and apoptosis was analyzed for Annexin V/PI markers. Caspase-3/7 activity was determined using fluorescence assay. The effects of purified α-defensins were compared to commercial HNP 1–3.

Key findings: Purified HNP 1–3 decreased the viability at 10 and 15 μg/ml and commercial HNP 1–3 at 15 μg/ml concentrations. Following to the purified HNP1–3 treatment, the percentage of Annexin V positive population and caspase-3 activity were significantly increased compared to control ($p = 0.000$ and $p = 0.001$, respectively) and commercial HNP1–3 ($p = 0.034$ and $p = 0.018$, respectively).

Significance: Results indicated the anticancer activity of HNP1–3 which can be used as future chemotherapeutic drugs. Furthermore, leukofilters can be considered as economic source for purifying these peptides.

1. Introduction

Human α-defensins are small cationic antimicrobial peptides that play an important role in host defense [1,2]. Up till now, six α-defensins have been described in humans. They include the human neutrophil peptides (HNP) 1, 2 and 3 which present in large amounts in neutrophil azurophilic granules and differed from each other only in the first amino acid [3]. A fourth defensin, termed HNP-4, comprises < 1% of the total defensins in neutrophils and has a distinct sequence from HNP1–3 [4]. The other two, human defensin 5 and 6, are synthesized mostly by intestinal Paneth cells [5]. *Neutrophil defensins* (HNP 1–3) are 3.4 kDa peptides that are characterized by three disulfide bridges [6]. The pattern of disulfide bonds in the mature forms is crucial for the functional properties. Due to this structural feature, synthesis of defensins using the chemical and recombinant approach presents quite a challenge. Moreover, purification from the natural source can be very difficult because the large number of neutrophils is needed to obtain a sufficient amount of protein [7]. Recently leukoreduction filters have been introduced as a major source of producing natural HNP1–3 [8]. In blood banks, leukofilters are used to remove leukocytes from blood components in order to prevent some complications related to transfusion [9,10]. Leukofilters contain high numbers of normal human cells [8,11–17]. Since these filters are discarded after use, they represent an economic source of neutrophils for purifying α-defensins. The purified peptide is safe because each donation is screened for infectious agents. On the other hand, a growing number of studies have demonstrated that α-defensins display anticancer activity [18–21]. In recent years, the possibility of using HNP-1 in cancer therapy gained much attention

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[22,23]. The exact functional mechanism of this peptide against cancer cells has not yet been identified, but activity was confirmed for different tumor cells, with no cytotoxicity against normal cells [19,20,24]. Moreover, inhibition of tumor growth by HNP1 and inducing apoptosis in vivo have been reported [25,26]. Inhibition of tumor angiogenesis could be another potential mechanism for the antitumor effect by HNP1 [25,27]. Such studies have considerably enhanced the significance of these peptides. Therefore, the aim of the present study was to purify human α-defensins from neutrophils trapped in leukoreduction filters and to investigate its anticancer activity.

2. Materials and methods

2.1. Leukoreduction filters

Blood bags from healthy donors were collected after written consent. All donors were screened for infectious diseases (HBV, HCV and HIV) and negative samples were included in the study. Blood bags (with a volume of 450 ml) with a half-life of < 24 h were filtered at 22 °C by gravity flow. Leukoflex LST-1 filters (Maco Pharma) are used by the Iranian Blood Transfusion Organization (IBTO) to prepare leukoreduced products. The cells were extracted from the filters by backflushing with a 70-mL syringe filled with cold phosphate buffered saline (PBS), pH 7.4, without MgCl2 and CaCl2 (Gibco), containing 5 mM EDTA and 2.5% sucrose. The PBS was homogenized with the filter content and then collected in a sterile tube. Granulocytes were separated from mononuclear cells by centrifugation through Ficoll (*Lymphodex*, *Inno-Train*, Germany) at 400 ×*g* for 30 min. After aspirating mononuclear cells along with the supernatant, granulocytes were collected. The cells were washed twice by low speed centrifugation (300 ×*g* for 10 min) in PBS 1× in order to remove contaminating erythrocytes. This left a pellet containing leukocytes, which consist mostly of neutrophils. To increase the number of cells, three to five LST1 filters were washed simultaneously and isolated neutrophils became pooled. Viability of cells was estimated by trypan blue dye exclusion.

2.2. Protein extraction

Isolated neutrophils resuspended in PBS 1× at a concentration of 1×10^7 cells/ml. For degranulation, cells were stimulated with 100 nM of formylmethionyl-leucyl-phenylalanine (fMLP; Sigma-Aldrich, Germany) for 5 min followed by stimulation with 10 μM of cytochalasin B (Sigma-Aldrich, Germany) for 5 min. Supernatant was collected by centrifugation at 200 ×*g* for 6 min.

2.3. Purification of α-defensins using Immunoprecipitation

For purification of HNP 1–3, immunoprecipitation was performed using μMacs Protein G Micro beads system (Milteny Biotec, Germany) according to the user's manual. Briefly, supernatant obtained from degranulation of neutrophils, was incubated with 2 μg of mouse monoclonal antibody to HNP 1–3 (Hycult Biotechnology), and followed by incubation for 30 min on ice with magnetically labeled protein G micro beads. The mixture was passed over a separation column placed in the magnetic field of a μMACS separator. Then the column was washed 4 times with “High Salt Washing Buffer”, then once with “Low Salt Washing Buffer”. The defensins were retained within the μMACS column while other proteins were removed by washing. This washing fraction that was unbound proteins (not HNP 1–3) collected and was used as the negative control in the next experiment. After that, the immunoprecipitated defensins was eluted from the μMACS column using elution buffer. The protein concentration was determined using the Bradford protein assay [28]. Anti-bacterial activity of purified HNP 1–3 was also investigated using a standard protocol [29].

2.4. Western Blot analysis

Purified HNP 1–3 obtained with each immunoprecipitation column was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by subsequent western blot to confirm the presence of protein. Purified HNP 1–3 were loaded on a 16% SDS-PAGE at 110 V for 2 h and were transferred to polyvinylidene difluoride (PVDF) membranes using a semi-dry system (Bio-Rad). The membrane was incubated with blocking buffer containing 5% skim milk in PBS overnight. After washing with PBS, the membrane was incubated with diluted (1:100) monoclonal antibody to HNP 1–3 (Hycult Biotechnology) at room temperature (RT) for 2 h. After an additional PBS washing step, diluted (1:3000) goat anti-mouse IgG horseradish peroxidase-conjugated antibody was added and the membrane was incubated for 1 h. The final protein band was detected using the substrate solution ECL (Abcam) and imaging with Chemi Doc (Bio-Rad, Hercules, USA). The washing fractions obtained in immunoprecipitation column that were unbound proteins (not HNP 1–3) used as the negative control. Commercial HNP 1–3 (Hycult Biotechnology) was used as the positive control.

2.5. Cell culture

Human T-cell acute lymphoblastic leukemia (Jurkat) was obtained from the Iranian Biological Resource Center (IRBC, Iran). The cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin and incubated at 37 °C in a humidified incubator with 5% CO2.

2.6. Cell viability assay

Jurkat T-cells were plated in 96-well polypropylene micro plates (2×10^4 cells/well) and treated with various concentrations (5, 10 and 15 μg/ml) of purified HNP 1–3 in serum-free medium. After incubation for 16 h, the viability of the cancer cells was measured using the WST-1 cell proliferation assay kit (Cayman Chemical Company) according to the manufacturer's protocol. The cytotoxic effects of purified α-defensins were compared to commercial HNP 1–3 (Hycult Biotechnology). Untreated cells were used as negative controls. Optical density was measured at 450 nm with a micro plate reader. The percentage of cell viability (%) was determined as: (OD treated group / OD control group) × 100.

2.7. Annexin V/propidium iodide (PI) staining

Jurkat cells were exposed to appropriate concentration of purified and commercial HNP 1–3 in serum-free medium for 16 h and subsequently analyzed by flow cytometry (FACS Calibur, BD Biosciences). Apoptosis was determined using the Bio Legend's FITC Annexin V Apoptosis Detection Kit with PI according to the manufacturer's instructions.

2.8. Caspase 3 activity assay

Caspase-3 activity was measured using a fluorescence assay kit (Cayman Chemical Company) according to the manufacturer's instructions. Fluorescence was measured at excitation 485 nm and emission 535 nm using a micro plate reader (Synergy HT, Bio-Tek Instruments, USA).

2.9. Statistical analysis

Data was analyzed for statistical difference by the student's *t*-test for paired samples or one way ANOVA and differences were considered significant when $P < 0.05$. The data represents the mean ± SD of three independent experiments.

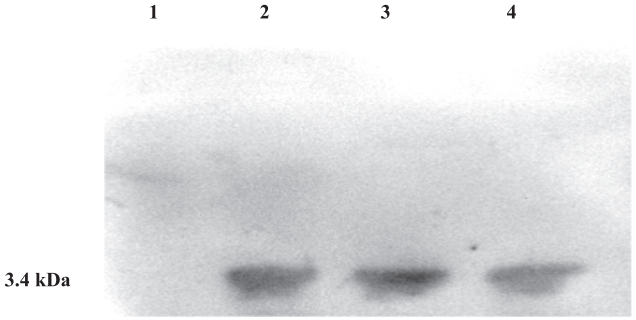

Fig. 1. Western blot analysis of purified HNP 1–3: The presence of defensins at the expected masses (3.4 kDa) was detected in the eluates (Lanes 2 and 4). The washing fractions obtained in immunoprecipitation column that were unbound proteins (not HNP 1–3) used as the negative control (Lane 1). Commercial HNP 1–3 (Hycult Biotechnology) was used as the positive control (Lane 3). 500 ng of protein were loaded for each lane.

3. Results

3.1. Purification of HNP 1–3 from LST-1 filters

After isolation of neutrophils from LST-1 filters, the purification of HNP 1–3 was performed by immunoprecipitation. Each obtained eluate was analyzed by Western Blot and the presence of the 3.4 kDa band was detected, that corresponded to the size of the HNP 1–3 (Fig. 1). The amount of obtained protein was differed according to the number of used filters and was approximately 7–15 μg/ml in different runs. Results also showed the purified HNP 1–3 had bactericidal activity.

3.2. HNP1–3 is cytotoxic to Jurkat cells

The cytotoxicity of purified and commercial HNP 1–3 (5, 10 and 15 μg/ml) on Jurkat cells was evaluated by WST-1 cell viability assays during 16 h. As shown in Fig. 2, purified HNP 1–3 decreased the viability at 10 and 15 μg/ml and commercial HNPs 1–3 at 15 μg/ml concentrations. In the subsequent experiments, we used a concentration that caused a percentage of cell death over 50%, corresponding to 15 μg/ml of purified and commercial HNP 1–3. For the rest of experiments we used this concentration for comparison of apoptotic effects.

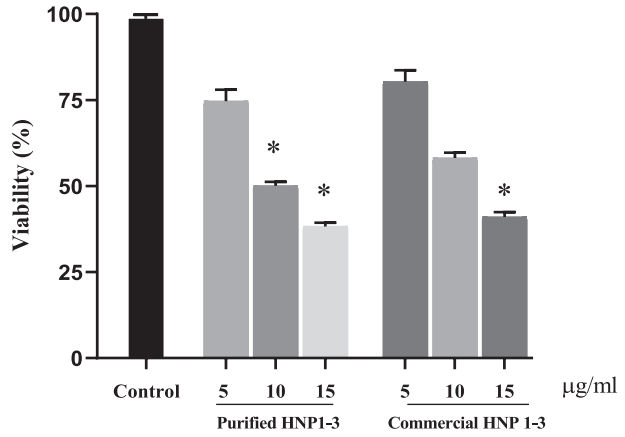

Fig. 2. The effect of purified and commercial HNP 1–3 on Jurkat cell viability. Cells were treated with different concentrations (5, 10 and 15 μg/ml) of HNP 1–3 for 16 h and cell viability was measured by the WST-1 assay. Data is presented as the means ± SD of three independent experiments. The statistical analysis was carried out using Student's *t*-test for paired samples and differences were considered significant when $p < 0.05$.

3.3. HNP1–3 induces apoptosis in Jurkat cells

Following to the purified HNP1–3 treatment, the percentage of the Annexin V positive population (Annexin V^+/PI^- and Annexin V^+/PI^+) was significantly increased compared to control ($p = 0.000$) and commercial HNP 1–3 ($p = 0.034$). Furthermore, the percentage of Annexin V/PI positive population (dead cells) was increased after treatment by commercial peptide compared to control ($p = 0.000$) and purified HNP1–3 ($p = 0.01$) (Fig. 3).

3.4. HNP1–3 activates caspase-3/-7

Incubation of Jurkat cells with 15 μg/ml of purified HNPs 1–3 resulted in increased caspase-3/-7 activity after 16 h compared to control ($p = 0.001$) and commercial HNP 1–3 ($p = 0.018$) (Fig. 4).

4. Discussion

American Association of Blood Banks (AABB) standards define a *leukoreduced* product with residual leukocyte content $< 5 \times 10^6$ [30]. The different leukofilters are used in routine blood banking to prepare *these* products such as Compoflex T3908 (Fresenius Hemocare), Leukoflex LST-1 (MacoPharma), Leukotrap WBF-3 (Pall Medical), Optipure RZ 2000 (Baxter) and Immugard III (Terumo Penpol) [31–33]. Blood banks produce thousands of leukofilters that are discarded annually. Therefore, these filters can be used with no cost.

In the present study, we used LST-1 filters as the source of neutrophils for purification of α-defensins. The purified protein by immunoprecipitation method was analyzed by western blot and the presence of protein was confirmed. Then, to investigate its anticancer activity, we tested the effect of purified HNP1–3 on cell viability in Jurkat leukemia at concentrations of 5, 10 and 15 μg/ml. This concentration range was chosen, because is in line with the range of concentrations of HNP1–3 that used in different tumors to evaluate its anticancer activity [21,34,35]. Results indicated that purified HNP 1–3 decreased the viability at 10 and 15 μg/ml concentrations. Apoptosis was measured by flow cytometry to determine the percentage of cells in viable (Annexin V/PI double-negative), early apoptotic (Annexin V positive and PI negative), late apoptotic (Annexin V/PI double-positive), and necrotic (Annexin V negative and PI positive) stages. The percentage of apoptotic cells was determined by adding Annexin V positive cells (early apoptotic) to Annexin V/PI positive ones (late apoptotic) [36–38]. To verify the stages of apoptosis, additional methods such as caspase assays are necessary. Caspase 3 is the main effector caspase that is involved in both external and internal apoptosis pathways [38,39]. Our finding showed following to the purified HNP1–3 treatment, the percentage of Annexin V positive cells and activation of caspase-3/7 were significantly increased compared to control. In addition, we found that the anti-cancer activity of natural defensins is better than commercial peptide. To our knowledge, assessment of anticancer activity of purified HNP 1–3 from leukoreduction filters has not reported before. Therefore, we think purification of these peptides from LST-1 filters and evaluation its anticancer activity is our novelty.

A recent research in Franc has also shown that neutrophils trapped in leukofilters can be a good source for direct purification of the natural defensins [8]. *Leukocyte filtration* was performed using TACSI system (Terumo BCT) and the authors were reported a mean production of 10 μg of HNP 1–3 per filter with high antimicrobial activity in comparison to commercial peptide. TACSI system (Terumo Automated Centrifuge & Separator Integration) allows obtaining six leukoreduced pooled platelet concentrates (PPC) in one run from a pool of buffy coats [40]. Using this automated device, a large number of neutrophils are trapped. However, this device is not available in all blood centers. Each LST-1 filter that used in the present study, reduce leukocytes of a single unit of whole blood. Therefore, to obtain large numbers of neutrophils,

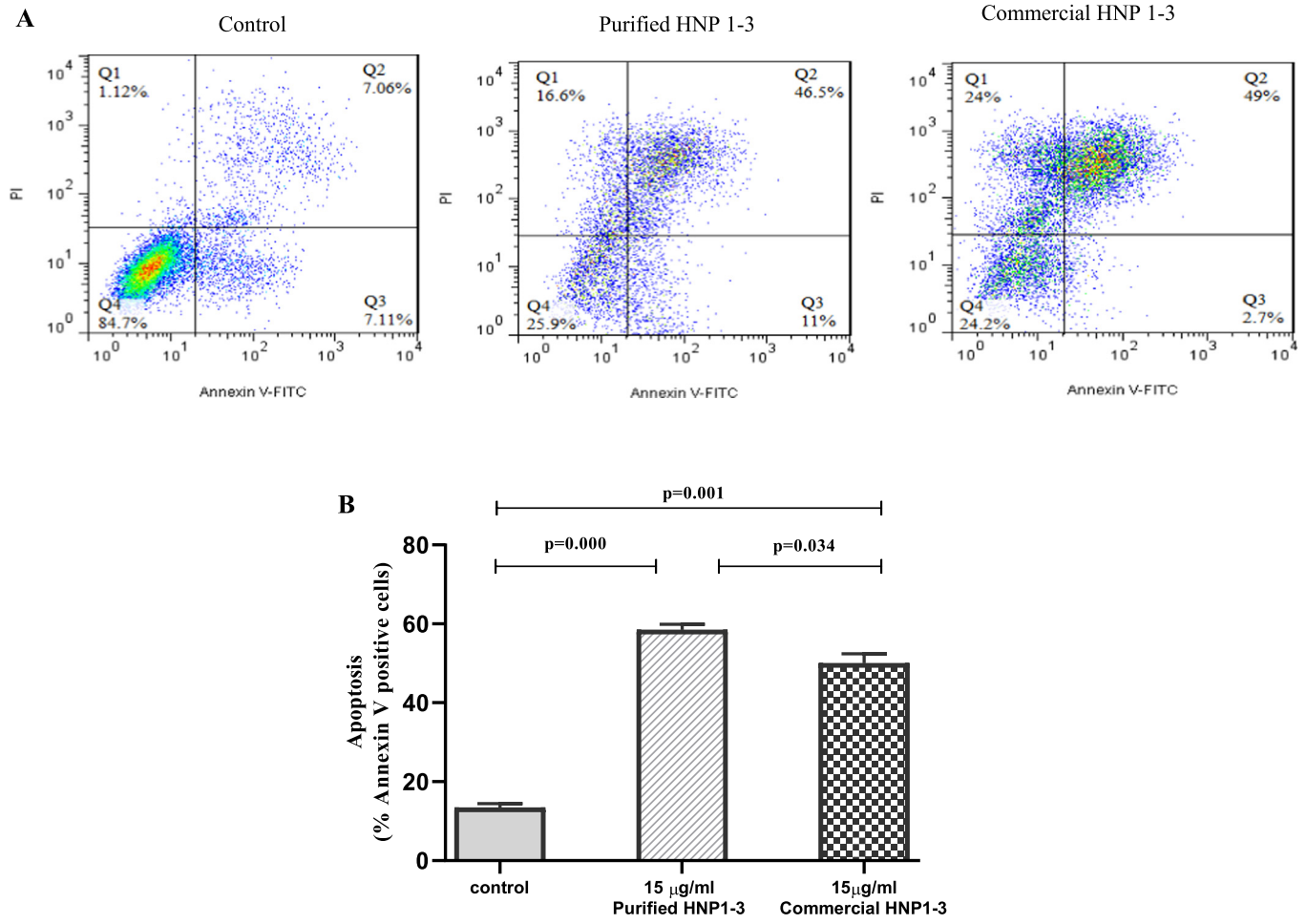

Fig. 3. The effect of purified and commercial HNP 1–3 on the induction of apoptosis in Jurkat cells after 16 h of treatment by 15 μg/ml of HNP1–3, as determined by flow cytometry using annexin V-FITC/PI staining (A), Percentage of apoptotic cells (early + late apoptotic cells) following to the treatment with HNP 1–3 in serum-free medium (B). The statistical analysis was carried out using one-way ANOVA test followed by Tuckey's multiple comparisons test. Values are compared between treated (by purified HNP1–3 or commercial HNP1–3) and untreated cells and each bar represent the mean ± SD of experiments. Differences were considered significant when $p < 0.05$.

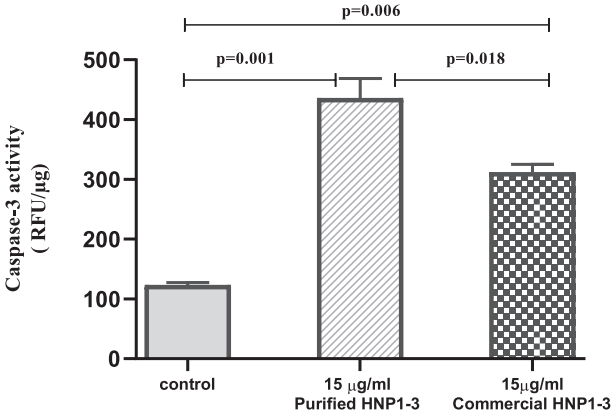

Fig. 4. The effect of HNP1–3 on caspase-3/-7 activation in Jurkat cells after 16 h of treatment by 15 μg/ml of HNP1–3. Data represented as relative fluorescence unit (RFU) per microgram of protein (RFU/μg protein). The statistical analysis was carried out using one-way ANOVA test followed by Tuckey's multiple comparisons test. Values are compared between treated (by purified HNP1–3 or commercial HNP1–3) and untreated cells and each bar represent the mean ± SD of experiments. Differences were considered significant when $p < 0.05$.

required for purification of α-defensins, we used several filters concomitantly and isolated neutrophils became pooled. Moreover, available commercial leukoreduction filters differ in physical design, the technology used to trap leukocytes and reduction efficiency [41,42]. In another study, Mattar et al. [24] revealed that natural α-defensins (HNP 1 to 4) purified from human neutrophils have strong anti-HCV activity while synthetic defensins can reach similar anti-viral potential at higher concentration. These differences in activity between natural and commercial types can be due to several reasons such as the method used for purification, the presence of nonspecific contaminants with the commercial peptides and the requirement of the presence HNP 1, 2 and 3 as a mixture [8].

Previous studies have also reported that HNP1–3 is cytotoxic to a variety of tumor cells which is consistent with the results of the present study [18,19,21]. In a study by Aarbiou et al. [18] purified HNP 1–3 from purulent sputum neutrophils of a patient with chronic obstructive pulmonary disease (COPD) was induced cell death at 25 μg/ml concentrations after incubation for 16 h in serum-free medium. PI/Annexin V double positive cells and caspase-3/-7 activity were increased at 50 μg/ml concentrations. In a study by Lu et al. [43] Jurkat cells were exposed to the synthetic human Defensin 5 (HD-5) at concentrations of 5, 10 or 25 μg/ml in serum-free medium. Results showed that HD-5 induces apoptosis at 25 μg/ml concentration after 16 h in a caspase-8-dependent manner. Liu et al. [21] found apoptotic changes in the lung epithelial cells with treatment of moderate and high concentrations (10

and 20 μg/ml) of synthetic α-defensin-1 and this apoptotic process was caspase-3-dependent. The findings of a study by Xu et al. [25] indicated that cell apoptosis mediated by HNP1 may provide a potential use of this peptide as a sensitizer for cancer chemotherapy by promoting penetration of chemotherapeutic drugs into the tumor cells. Furthermore, LI et al. [26] found that intratumoral expression of HNP1 can significantly improve the therapeutic efficacy of doxorubicin in breast cancer.

In conclusion, these peptides with anticancer activity can be used for clinical application in the future. The development of defensins as therapeutic products requires access to a steady supply of neutrophils. Our results indicated that LST-1 filters are economical source for purifying α-defensins.

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Disclosures

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. All authors contributed extensively to the work presented in this paper.

Conflict of interest

The authors declare that there are no conflicts of interest.

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None.

Author contributions

1- Study concept and design: Ali Akbar Pourfathollah.
2- Data acquisition: Shirin Ferdowsi, Fatemeh Amiri, Mohammad Hessam Rafiee, and Afsaneh Aghaei.
3- Analysis and interpretation of data: Shirin Ferdowsi and Fatemeh Amiri.
4- Drafting of the manuscript: Ali Akbar Pourfathollah and Shirin Ferdowsi.
5- Critical revision of the manuscript for important intellectual content: Ali Akbar Pourfathollah, and Shirin Ferdowsi.

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