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Isolation, functional characterization and biological properties of MCh-AMP1, a novel antifungal peptide from *Matricaria chamomilla* L.

Running title: *Novel antifungal peptide from M. chamomilla* L.

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Abstract

The antimicrobial activities of natural products have attracted much attention due to the increasing incidence of pathogens that have become resistant to drugs. Thus, it has been attempted to promisingly manage infectious diseases via a new group of therapeutic agents called antimicrobial peptides. In this study a novel antifungal peptide, MCh-AMP1, was purified by reverse phase-HPLC and sequenced by de novo sequencing and Edman degradation. The antifungal activity, safety, thermal and pH stability of MCh-AMP1 were determined. This peptide demonstrated an antifungal activity against the tested *Candida* and *Aspergillus* species with MIC values in the range of 3.33-6.66 µM and 6.66-13.32 µM, respectively. Further, Physicochemical properties and molecular modeling of MCh-AMP1 were evaluated. MCh-AMP1 demonstrated 3.65% hemolytic activity at the concentration of 13.32 µM on human red blood cells and 10% toxicity after 48 h at the same concentration on HEK293 cell lines. The antifungal activity of MCh-AMP1 against *Candida albicans* was stable at a temperature range of 30-50ºC and at the pH level of 7-11. The present study indicates that MCh-AMP1 may be considered as a new antifungal agent with therapeutic potential against major human pathogenic fungi.

Keywords: Matricaria chamomilla, Antifungal peptide, Physicochemical properties, Molecular modeling, *Aspergillus*, *Candida*

Introduction

The incidence of invasive fungal infections caused by *Aspergillus* and *Candida* species as the main causes of morbidity and mortality are increasing in parallel with the growing population of immunocompromised patients (Pluim et al., 2012; Ruangritchankul et al., 2015). Despite the developments of new antifungal treatments with enhanced potential effects against *Aspergillus* and *Candida* species, there are still increasing rates of resistance to the current

antifungal treatments resulting in their failure and worsening of clinical outcomes (Wiederhold, 2017). Therefore, the need for developing new alternative treatments against these fungal infections is highlighted (Tang et al. 2018). Among the various types of compounds introduced in this field, antimicrobial peptides are suitable substitutes for traditional antibiotic drugs (Gaiser et al., 2011).

Antimicrobial peptides (AMPs) as the molecules found in most living cells are relatively short length (12–50 amino acid residues), positively charged, and amphipathic. They provide a natural host defense mechanism against pathogens, including viruses, bacteria, and fungi. Due to their broad-spectrum antimicrobial activity, having multiple targets in the plasma membrane and intracellular components, low toxicity for mammalian cells, effectiveness in low concentrations, rapid killing of multi-drug resistant pathogens, and low propensity for the development of resistance by microorganisms, they can act as potentially promising candidates for treating many infectious diseases (Tang et al. 2018; Ciociola et al., 2016).

According to the recent evidence, plants serve as natural sources of novel antimicrobial compounds with numerous therapeutic potentials (Tang et al. 2013). *Matricaria chamomilla*, one of the well-known medicinal plants in the world, is from the Asteraceae (Compositae) family. It is a traditional medicinal plant that is commercially cultivated in different parts of Iran, especially in Kerman and Isfahan provinces. A varied range of pharmacological properties have been known for *M. chamomilla,* including antioxidant, antispasmodic, anti-inflammatory, antimicrobial, antiviral, antiseptic, and sedative properties (Razzaghi-Abyaneh et al., 2013). In recent years, some researchers have described the antifungal activities of this plant (Jamalian et al., 2012; Tolouee et al., 2010).

With this background and given that there have been no published data on the AMPs existing in *M. chamomilla*, this paper aimed to provide insights into the presence of a novel antifungal peptide, MCh-AMP1, in *M. chamomilla* flowers.

Materials and methods

Plant and fungal Strains

Fresh flowers of *M. chamomilla* cultivated in Esfahan province were purchased in April 2016.Voucher specimens were deposited in the herbarium of the Research Institute of Forests and Rangelands, Tehran, Iran. *Candida albicans* ATCC 10231, *Candida krusei* DSM 70079, *Candida glabrata* ATCC 90030, *Aspergillus fumigatus* Af293, *Aspergillus niger* ATCC 9029, *Aspergillus flavus* PFCC 100 were obtained from the Pathogenic fungi Culture Collection of the Pasteur Institute of Iran (http://fa.pasteur.ac.ir/MBankResult.aspx).

Peptide Extraction and Purification

First, 400 g of the plant materials (flowers) were frozen in to fine powder in liquid nitrogen. Total protein extraction was followed by using 2000 ml of an extraction buffer (100 mM KCl, 15 mM NaH_2PO_4, 10 mM Na_2HPO_4 and 1.5 % EDTA) pH 7.2, in a shaker at 4°Cfor 4 h (Games et al., 2008). After centrifugation at 2400 × g at 4ºC for 20 min, the resulting supernatant was filtered through Whatman filter paper.

The crude extract was precipitated by using 85% ammonium sulfate ($(NH_4)_2SO_4$) and then continuously stirred by a magnetic stirrer at 4ºC for 24 h. The precipitated proteins were centrifuged at 17000 × g at 4°C for 20 min. The pellets were dissolved in distilled water. Desalting by dialysis was performed by using benzoylated membrane performance (MWCO 2000 Da) against several changes of the distilled water at 4ºC within 12 h to remove the residual $(NH_4)_2SO_4$. To remove the insoluble debris from the dialysate, the dialyzed suspension was then centrifuged at 17000 × g for 10 min and the supernatant was collected for peptide purification. To isolate low molecular weight peptides, the protein extract was passed through Amicon Ultra-15 ml 10,000 MWCO centrifugal filters and then lyophilized. To purify the antifungal peptides, the lyophilized extract was dissolved in distilled water and subjected to reverse phase HPLC column (C18 column, 7.8 mm × 300 mm; Tosoh, Tokyo, Japan) using the gradient of 5-65% (v/v) solution B (0.098% TFA in acetonitrile) and A (0.1% TFA in water) at a flow rate of 1 ml/min for 85 min (Asoodeh et al., 2012). Based on the absorbance at 220 nm, each peak was manually collected and then concentrated by lyophilization to determine the antifungal activity. To check the purity of the active peak, this peak was re-chromatographed in the same column with the same solvent system under similar conditions.

SDS-PAGE

Tricine Sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tricine- SDS-PAGE) was carried out to evaluate the molecular weight and further purity of the active peak as described by Schagger (Schägger and Von Jagow, 1987). After electrophoresis, the gels were detected through silver staining and protein ladder (2-250 KDa) was applied as the standard to determine approximate molecular mass.

Assay of antifungal activity

The antifungal activity was determined with a Radial Diffusion Assay (RDA) described by K. Wang et al (Wang et al., 2015). Conventional antifungal agent like Amphotericin B (AmpB) was considered as positive control in this research. Briefly, a volume of 1×10^6 CFU/ml of fungal cells was added to 100 ml of Sabouraud dextrose agar adjusted to 42ºC and then quickly dispensed into a Petri dish. The wells were punched and filled with the purified peptide at a 50 μM concentration. After incubation at 35°C for 48 h for *Candida* species and 72 h for *Aspergillus* species, the clear zones surrounding the wells were investigated. All the experiments were performed in triplicates.

The minimum inhibition concentration assay (MIC) of the purified peptide was determined according to Li et al. (2015) with some modifications. Briefly, two fold serial dilutions of the peptide were prepared in the 96 well plates up to a final volume of 100 μL. Conventional antifungal agent, such as AmpB was included in this study as control. The final concentrations of each peptide and AmpB had the range of 1.67-105.58 μM and 0.13-17.31 μM, respectively. Then, 100 μl of fungal suspension (10^5cell/ml) was added to each well followed by incubation at 35°C for 24-48 h. The pure broths alone and with the inoculum suspensions were included in this study as the negative and positive controls, respectively. The MIC was defined as the lowest concentration that inhibited fungal growth under experimental conditions.

The minimum fungicidal concentration assay (MFC) was measured by removing 20 μl of the cultures from any wells showing no visible fungal growths and plating on Sabouraud dextrose agar plates. Following incubation at 35ºC for 24-72 h, MFC was expressed as the lowest concentration required for killing at least 99.9% of the primary inoculums.

Amino acid sequence analysis

The active peak was lyophilized and subjected to amino acid sequence. The sequence and intact mass of the peptide was elucidated by nanoHPLC-ESI-MS/MS. The sample powder was re-suspended in water and an aliquot was directly analyzed by nanoHPLC-ESI-MS/MS. An Orbitrap XL (linear ion trap/fourier transform electrostatic ion trap hybrid) mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nano electrospray ion source was utilized for detection. De novo sequencing was done using PEAKS (Bioinformatics Solutions Inc., Waterloo, ON, Canada).

N-terminal amino acid sequence of the peptide was determined by Edman degradation. For this, an ABI Procise Edman Micro Sequencer (Model 492) was connected online to an ABI PTH Amino Acid Analyzer (Model 140C) (Proteome factory, Germany).

Bioinformatic and physicochemical analysis

The bioinformatic programs of ProtParam (ExPASy Proteomics Server: http://www.expasy.org/tools/protparam.html) were applied to analyze the physicochemical parameters. The Clustal W2 software (http://www.ebi.ac.uk/Tools/clustalw2) was employed to do the sequence alignments. Helical Wheel Projections were created using the online software (http://lbqp.unb.br/NetWheels/). The online prediction of the three dimensional structure was done by using I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). The model quality was evaluated by Accelrys, DS Visualizer ver. 1.7.

Peptide synthesis

The peptide was chemically synthesized by using *N*-(9-fluorenyl) methoxycarbonyl (Fmoc) according to the standard solid phase methods (Merrifield, 1986). The purity of the synthetic peptide was confirmed by using the RP-HPLC with purity of >85%. Its molecular weight was assessed by electrospray ionization mass spectrometry (ESI-MS).

Cytotoxicity assays

Human embryonic kidney cell line 293 (HEK293) (Pasture institute, Iran) was used for testing cytotoxicity through MTT assay. Briefly, the cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and incubated under 5% CO_2 at 37ºC. The cells were seeded in 96-well microtiter plates at a density of 1.0×10^5 cells per well and treated with serially diluted peptide (3.33-53.23 µM). The plates were incubated at 37ºC for 6, 24, and 48 h under 5% CO_2. After incubation, a total of 10 µl of the MTT (3-(4, 5 dimethyl thiazol-2 yl)-2, 5-diphenyl tetrazolium bromide) solutions (5 µg/mL) was added to each well, which was then incubated for additional 4 h under the same conditions. Finally, the medium of each well was removed and then, 100 µL of DMSO (dimethyl sulfoxide) was added to the wells. The optical density of each well was measured at 570 nm using an ELISA reader. Dead % = [1= ((OD test)/ (OD control)) ×100]. 1% Triton X-100 and PBS (phosphate-buffered saline pH: 7.4) were used as the positive and negative controls, respectively. All the tests were done three times.

Hemolysis assay

The hemolytic activity assay was performed according to the methods of Asoodeh et al. (Asoodeh et al., 2012). Briefly, human red blood cells (RBCs) were obtained from heparinized human whole blood by centrifugation at 2400 × g for 10 min, washed five times using sterile PBS, and centrifuged again at 2400 × g for 10 min. The obtained erythrocyte pellets were diluted in PBS (pH 7.4) to make a 4% (v/v) solution and then incubated with the peptide or AmpB concentrations of 1.67-105.58 μM at 37°C for 1 h. Hemoglobin release was determined by measuring the absorption at 567 nm with an ELISA reader. Hemolysis (%) = [OD of test −OD of negative control)/ (OD of positive control – OD of negative control)] × 100. The control samples of 0% (in PBS) and 100% lysis (in 1% Triton X-100) were used in the experiments. All the tests were done three times.

Thermal and pH stability determination

To evaluate temperature effects on the antifungal activity of the peptide, a solution of the peptide was incubated at various temperatures (20-100°C) for 30 min. The peptide not heated at different temperatures served as the control. To determine the pH-stability, the peptide solution was incubated in buffers at various pH values (2.0-14.0) for 1 h at 25°C. Antifungal activity was determined after adjusting the mixtures to pH 7.2 at room temperature (Chan et al., 2012). *C. albicans* ATCC 10231 was used as the indicator to detect the antifungal activity. The antifungal activity was measured by radial diffusion assay as mentioned above. The peptide dissolved in 100 μl of solution with a pH of 7.2 served as the control. All the tests were done three times.

Statistical analysis

All the data were obtained from at least three independent experiments. The results were presented as the means ± standard deviation. The statistical analyses of the experimental data were performed using GraphPad Prism 5 Statistical software (GraphPad Software, Inc., La Jolla, CA,USA).

Results

Peptide isolation and purification

The chromatographic profiles of the lyophilized protein extract from *M. chamomilla* flower yielding 18 fractions are presented in Fig. 1a. All the fractions were collected from the C18 RP-HPLC as an individual peak to be tested for the antifungal activity. The antifungal

activity analysis of the peaks showed that Peak16 had a good antifungal activity. For further purification, this active peak was applied again to the same column for rechromatography under the same elution conditions (inset to Fig. 1a).

Evaluation of purity of peptide

The electrophoresis pattern of the extracted peptides passed through an Ultra membrane with a 10 kDa cut-off and active peak purified from the RP-HPLC column on the SDS-PAGE was shown in Fig. 1b. The purified antifungal peptide exhibited a molecular weight of ~2400 Da.

Antifungal activity

As previously mentioned, the antifungal activities of all the peaks against *Candida* and *Aspergillus* species were evaluated according to RDA. Based on the results of RDA assay presented in Fig. 2a, the peak NO. 16 obtained from *M. chamomilla* flower displayed good antifungal activity against *Candida* and *Aspergillus* species when compared with AmpB as a conventional antifungal agent. After incubation, the inhibition zones for *Candida* and *Aspergillus* species were found to be 21-25 and 15-19 mm for the peptide, while they were 23–27 and 22-24 mm for AmpB, respectively (Fig. 2b). As shown in Fig. 2b the largest diameter of the inhibition zone is displayed by *C. glabrata* and *C. krusei,* while the lowest diameter was related to *A. flavus.* The antifungal activity of the peptide was further analyzed as MIC values through micro broth dilution assay against *Candida* and *Aspergillus* species. As to the control, AmpB showed the MIC range of 1.8-2.16 and 1.08-4.32 µM for *Candida* and *Aspergillus* species, respectively. As shown in Table 1, the isolated peptide has good activity against the yeasts and filamentous fungi within the MIC range of 3.33-6.66 and 6.66-13.32 µM, respectively. The highest activity against *C. glabrata* was found with the significant MIC value of 3.33 µM.

As shown in Table 1, the MFCs of the peptide against the fungal strains are 2 to 4 times higher than its MICs. No synergistic activity was observed for MCh-AMP1 and AmpB in checkerboard assay (Data are not shown in details)

Peptide Identification

An aliquot of the sample was dissolved and subjected to nanoHPLC- ESI-MS spectrometry in order to obtain the accurate molecular mass of the peptide, which was found to be 2402.369 as displayed in Fig. 1c. The result was in close agreement with the relative molecular weight indicated by Tricine- SDS-PAGE. An MS/MS spectrum of the doubly protonated peptide is

portrayed in Fig. 1d. The obtained data were analyzed by de novo sequencing. De novo sequencing of the mass spectrometric data gave a sequence with a 23-amino acid peptide, which was in agreement with the measured intact mass: LSVKAFTGLQLRGVCGLEVKARG.

As the amino acids of leucine and isoleucine were isobaric, they could not be discerned by mass spectrometry. The confirmed sequence was LSVKAFTGIQLRGVCGIEVKARG. The results are not shown in the document.

Bioinformatic and physicochemical analysis

The key physicochemical parameters of the short active isolated peptide were analyzed by the ProtParam tool from the ExPASy server, where the chain length, net charge, hydrophobic residue, protein-binding potential (Boman index), and pI were equal to 23, +3, 47%, 0.68, and 10.05, respectively. The sequence of the acquired peptide showed no complete sequence homology to any reported AMPs. Thus, this peptide was a novel AMP, which was named as MCh-AMP1 according to the isolated source.MCh-AMP1 was compared to the plant AMPs presented in the APD database, with the results suggesting that (Fig. 3a) MCh-AMP1 shared 26.9%, 20.7%, and 20% similarity with Plantaricin CS, Ac-AMP1, and Ar-AMP, respectively (Broekaert et al., 1992; Lipkin et al., 2005; Zare-Shehneh et al., 2014). Structure prediction with generation of helical wheel projection (Fig. 3b) revealed that some hydrophobic residues such as two valine, isoleucine, and phenylalanine have trended in the same moiety of the peptide, causing hydrophobic tendency of the peptide to the same direction. Positive residues such as lysine and arginine also leant to the same moiety of the peptide. A three-dimensional model of MCh-AMP1 revealed a reliable model (Fig. 3c). The C-score is a confidence score for estimating the quality of predicted models by I-TASSER (in the range of -5.0 to 2.0). The expected C-score of MCh-AMP1 was −1.58 indicating a correct global topology for the model.

Cytotoxicity of peptide on HEK293 cells

The cytotoxic effects of AmpB and MCh-AMP1 towards HEK293 cells is shown in Fig. 4a, b. AmpB exhibited a significant toxicity at the concentration of 13.23 μM, and over 50% of cytotoxicity was observed at 26.64 μM. In the case of MCh-AMP1, cytotoxicity at the concentrations of 13.23 and 26.64 μM were 10% and 23% after 48 h, respectively, and its toxicity was significantly lower than AmpB at the exact tested concentrations.

Hemolytic assays

As shown in Fig. 4b, MCh-AMP1 induced 10.6% of hemolytic activity at the highest tested concentration of 105.58 μM, which was 2-4 times higher than the MICs for fungal strains. This new peptide demonstrated 3.65% hemolytic activity at the concentration of 13.32 μM. This result was compared to that of AmpB which could more potentially induce hemolytic activity at the tested levels of concentration.

Effect of temperature and pH on antifungal activity

As depicted in Fig. 5a, MCh-AMP1 exhibited remarkable activity against *C. albicans* in the temperature range of 30-50ºC. At 60ºC and 70ºC, about 38–46% loss of activity was observed. More ever, MCh-AMP1 revealed the antifungal activity in the pH range of 7-11, while the activity significantly decreased when the pH values were less than 5 or greater than 12 (Fig. 5b). The results indicated that the antifungal activity was higher in neutral and basic conditions than in acidic conditions.

Discussion

The increasing prevalence of multidrug resistance to the available antifungal drugs has limited the relevant therapeutic choices of treatment which is now becoming a public health concern. Hence, antimicrobial peptides (AMPs) are a promising alternative to conventional antimicrobial agents (Nawrot et al., 2014).

The importance of the study lies in the fact that it is the first report on the isolation of a novel antifungal peptide, MCh-AMP1, from *M. chamomilla* flowers. The data obtained from the present survey confirmed the importance of MCh-AMP1 for its remarkable inhibitory effects on *Candida* and *Aspergillus* species with MIC values within the range of 3.33-6.66 and 6.66-13.32 μM, respectively.

The percentage of hydrophobic residues, net charge, and Boman Index of MCh-AMP1 were 47%, + 3, and 0.68 respectively. Hydrophobicity is one of the important parameters for the antimicrobial activity of AMPs which facilitates the interaction between AMPs and cell-membranes (Kim et al., 2013; Chen et al., 2007). The positive net charge residues of the AMPs promoted their electrostatic attraction towards anionic phospholipids in fungal membranes (Van Der Weerden et al., 2013; Wang et al., 2014). Boman Index provides an estimation of potential AMPs for binding to other proteins where the index value of ≤1 can be indicative of higher antimicrobial activity of AMPs with few side effects (Boman, 2003).

The results of the sequencing structure alignment revealed that MCh-AMP1 has the greatest homology to the antimicrobial peptides of Plantaricin CS, Ac-AMP1, and Ar-AMP (Zare-Shehneh et al., 2014; Broekaert et al., 1992; Lipkin et al., 2005;). Plantaricin CS was isolated from *Coriandrum sativum* and displayed a broad spectrum of antimicrobial activity against the bacteria such as *S. aureus, K. pneumoniae, P. aeruginosa,* fungi including *P. lilacinum* and *A. niger* (Zare-Shehneh et al., 2014). The net charge and percentage of hydrophobic residue of Plantaricin CS were +4 and 26%, respectively. Despite the clear identity shared with Plantaricin CS (Fig. 3a), MCh-AMP1 exhibited different physiochemical properties such as hydrophobicity (47%) and net charge (+3). Interestingly, despite the low hydrophobicity of Plantaricin, this peptide indicated an antimicrobial activity, possibly due to the high net charge (+4). Other antimicrobial peptides which shared sequence similarity with MCh-AMP1 were Ac-AMP1 and Ar-AMP, isolated from *Amaranthus caudatus* and *Amaranthus retroflexus* seeds, respectively. They showed an effective activity against Gram-positive bacteria and saprophytic fungi (Broekaert et al., 1992; Lipkin et al., 2005). Ac-AMP1 and Ar-AMP had +3 net charges with a hydrophobic residue percentage of about 33% and 34%, respectively. Despite the similarities in the net charge of MCh-AMP1, Ac-AMP1, and Ar-AMP, their percentages of hydrophobic residues are different.

Several approaches have been characterized for antifungal action modes of AMPs including disruption of fungal cell membrane, formation of reactive oxygen species (ROS), inhibition of DNA, RNA and protein synthesis, and inhibition of protein folding (Matejuk et al., 2010). Structure prediction by the helical wheel projection (Fig. 3b) illustrated that some hydrophobic residues such as two valine, isoleucine and phenylalanine, accumulated in the same moiety of the peptide, causing hydrophobic tendency of the peptide to the same direction. This may lead to facilitate penetration of hydrophobic amino acids in the peptide in to microbial cell membrane. Positive residues such as lysine and arginine have also leant to the same moiety of the peptide, which have an important role in placement of peptide toward the negative charge of microbial cell membrane. According to the results of 3D analysis (Fig. 3c), one folding structure in N-terminal of MCh-AMP1 led to proximity of some hydrophobic residues towards each other such as leucine1, valine3, phenylalanine6, isoleucine9, leucine11 and valine14 in response to the band between sulfur of cysteine residue (Cys15) and N-terminal residues. This structure position in folded motif may affect its activity, especially antifungal activity. Furthermore, the presence of three glycine residues (Gly8, Gly13 and Gly16) in the center of the peptide seems to confer greater flexibility to its structure. Further, chain flexibility is an important factor of AMPs to improve the antimicrobial activity (Gauri

et al., 2011). Phenylalanine 6 is an aromatic residue which has a large aromatic ring in which seems to be involved in membrane penetration and reinforcement of antifungal activity (Weibing et al., 2017). It seems that MCh-AMP1 interacts with the microbial membrane surface through its hydrophilic parts destroying the microbial membrane integrity through its hydrophobic parts such as leucine1, valine3, phenylalanine6, isoleucine9, leucine11, and valine14.

As a conventional antifungal agent, AmpB helps manage any systemic fungal infections. Despite its recorded effectiveness in life-threatening cases of systemic fungal infections, discontinuation of this type of treatment would sometimes become inevitable due to its worsening toxicity and side effects. Thus, there is an urgent need to find alternative drug candidates with the same efficacy as that of AmpB with no or fewer detrimental toxic effects (Laniado-Laborín and Cabrales-Vargas, 2009). The known cytotoxicity of AmpB was detected at the concentration of 13.32 μM where about 46% of HEK293 cells were seen to be killed after 48 h. In contrast, MCh-AMP1 proved to be less toxic to HEK293 than to AmpB with a cytotoxicity of 10% at the same concentration and with similar time duration (Fig. 4a, b).

In a complementary toxicity study on HEK293 cells, the hemolysis profiles of the peptide and AmpB were compared using the absorbance values of the released hemoglobin as depicted in Fig. 4c. MCh-AMP1 revealed a negligible hemolytic activity against the tested RBCs within the studied concentration range of MCh-AMP1. MCh-AMP1 demonstrated approximately 11.6% hemolysis at the concentration of 105.5 μM.

As displayed in Fig. 5a, MCh-AMP1 revealed greater efficacy within the temperature range of 30-50ºC. The need for the specific conformation of the peptide to function properly was supported by these results. Any changes to the mentioned conformation could result in the loss of its functions, including antimicrobial activity (Jabeen and Khanum, 2017). As evidenced from the results, the specific conformation of MCH-AMP1 required for its antimicrobial activity might have changed at high and low temperatures.

The environmental pH can influence the activity and amphipathic structure of the AMPs as well as their interaction with the cell membrane of microorganisms (Xu et al., 2017). Our results also revealed pH dependent activity of MCH-AMP1 (Fig. 5b), which was seen to become stable within the narrow pH range of 7–11.

Conclusion

We isolated and characterized a novel antifungal peptide, MCH-AMP1, from *M. chamomilla* flowers by using reverse phase-HPLC, de novo sequencing, and Edman degradation techniques. MCH-AMP1 had remarkably antifungal activity at low concentrations with no considerable hemolytic and toxicity effects. These results indicate that MCh-AMP1 can be a promising candidate as a potential antifungal agent to combat pathogens responsible for invasive fungal infections. Nonetheless, further work needs to be done to investigate the mode of action and structure-activity relationship of MCh-AMP1.

Conflicts of interest

Authors declare that they have no conflict of interest.

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Table 1. MIC and MFC values (µM) of MCh-AMP1and AmpB for pathogenic fungi belonging to the genera *Aspergillus* and *Candida*

Fungal strain	**MIC (µM)[a]**		**MFC (µM)[a]**	
	MCh-AMP1	**AmpB**	**MCh-AMP1**	**AmpB**
C. albicans ATCC 10231	6.66 ± 0.61	2.16 ± 0.20	13.32 ± 1.0	2.16 ± 0.25
C. glabrata ATCC 90030	3.33 ± 0.45	1.08 ± 0.15	26.64 ± 2.67	4.32 ± 0.55
C. krusei DSM 70079	6.66 ± 0.40	1.08 ± 0.11	13.32 ± 0.85	1.08 ± 0.12
A. flavus PFCC 100	13.32 ± 1.52	4.32 ± 0.34	26.64 ± 2.15	4.32 ± 0.70
A. niger ATCC 9029	6.66 ± 0.36	1.08± 0.05	13.32 ± 1.59	1.08 ± 0.15
A. fumigatus Af293	6.66 ± 0.58	2.16 ± 0.38	26.64 ± 1.70	4.32 ± 0.33

[a] Results are given as the mean ± SD of three independent experiments

Figure legends

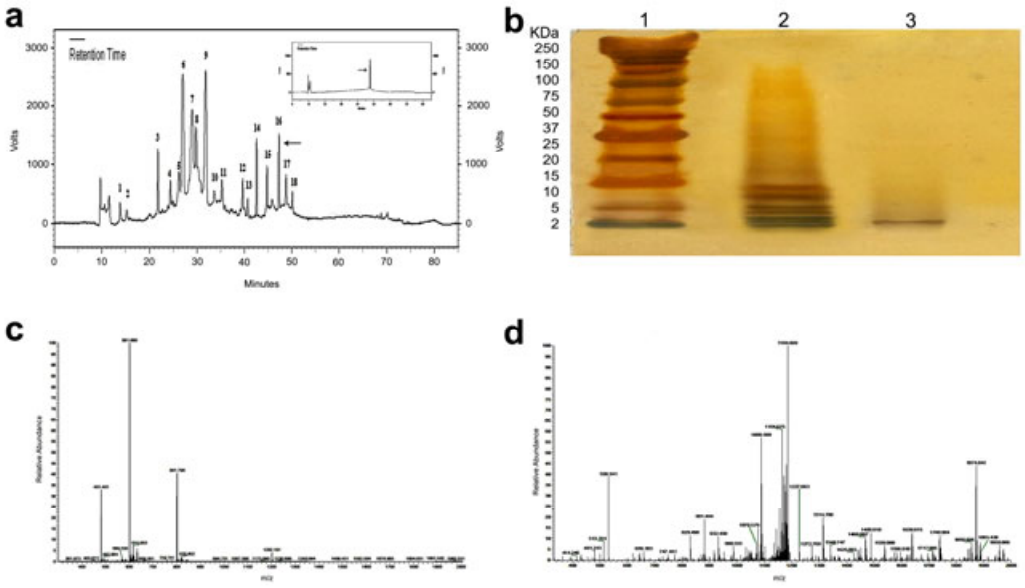
Fig. 1. (a) RP-HPLC purification of peptides from *M. chamomilla* flowers. The active peak 16 indicated by an arrow. The inset of the figure indicates the purity of the labeled peak. **(b)** Tricine-SDS-PAGE profile with silver staining of (1) Molecular weight markers 2 kDa to 250 kDa, (2) peptide extract passed through an ultra-membrane with a 10-kDa cutoff, and (3) the peak 16 purified from RP-HPLC column**. (c)** Mass spectrum of peptide showing multiply protonated ions. Note that not the first (monoisotopic) signals are annotated, but the most abundant one where ^{13}C is incorporated. **(d)** MS/MS spectrum of peptide (obtained by fragmentation of doubly protonated form).

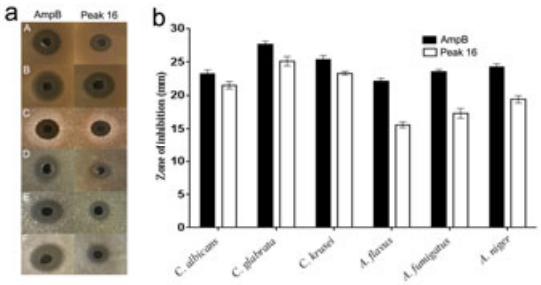
Fig. 2. (a,b) Antifungal activity of peak 16 against *C. albicans* ATCC 10231 (A), *C. glabrata* ATCC 90030 (B), *C. krusei* DSM 70079 (C), *A. flavus* PFCC 100 (D), *A. fumigatus* Af293 (E), *A. niger* ATCC 9029 (F) was determined by radial diffusion assay. **(a)** The results are given as the mean ± SD of three independent experiments.

Fig. 3. (a) Sequence alignment of MCh-AMP1against other antifungal peptides from different plant sources. Colored residues correspond to the conserved residues. Antibacterial activity of the peptide was not tested. (**b)** Three-dimensional structure model of MCh-AMP1. Yellow color indicates the hydrophobic residue, green color for aromatic residues (Phe6), and blue color for polar residues, respectively. **(c)** Helical wheel projection of MCh-AMP1.

Fig. 4. Cytotoxicity of AmpB **(a)** and MCh-AMP1 **(b)** against HEK293 cells. **(c)** Hemolytic activity of MCh-AMP1 and AmpB against human erythrocytes. The results are given as the mean ± SD of three independent experiments.

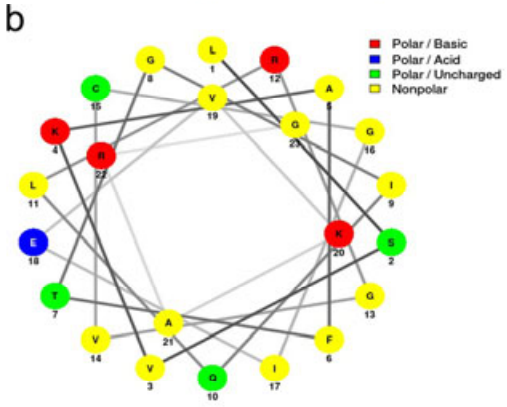
Fig. 5. The effects of temperature (**a**) and pH (**b**) on the antifungal activity of MCh-AMP1 against *C. albicans* ATCC 10231 cells. The results are given as the mean ± SD of three independent experiments.

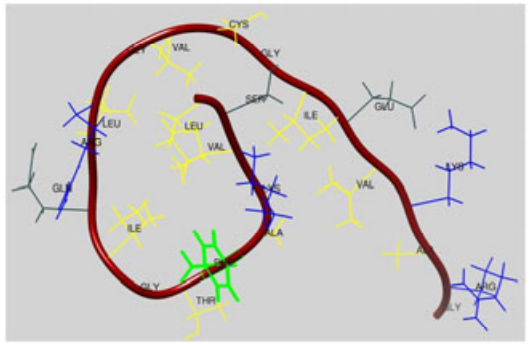


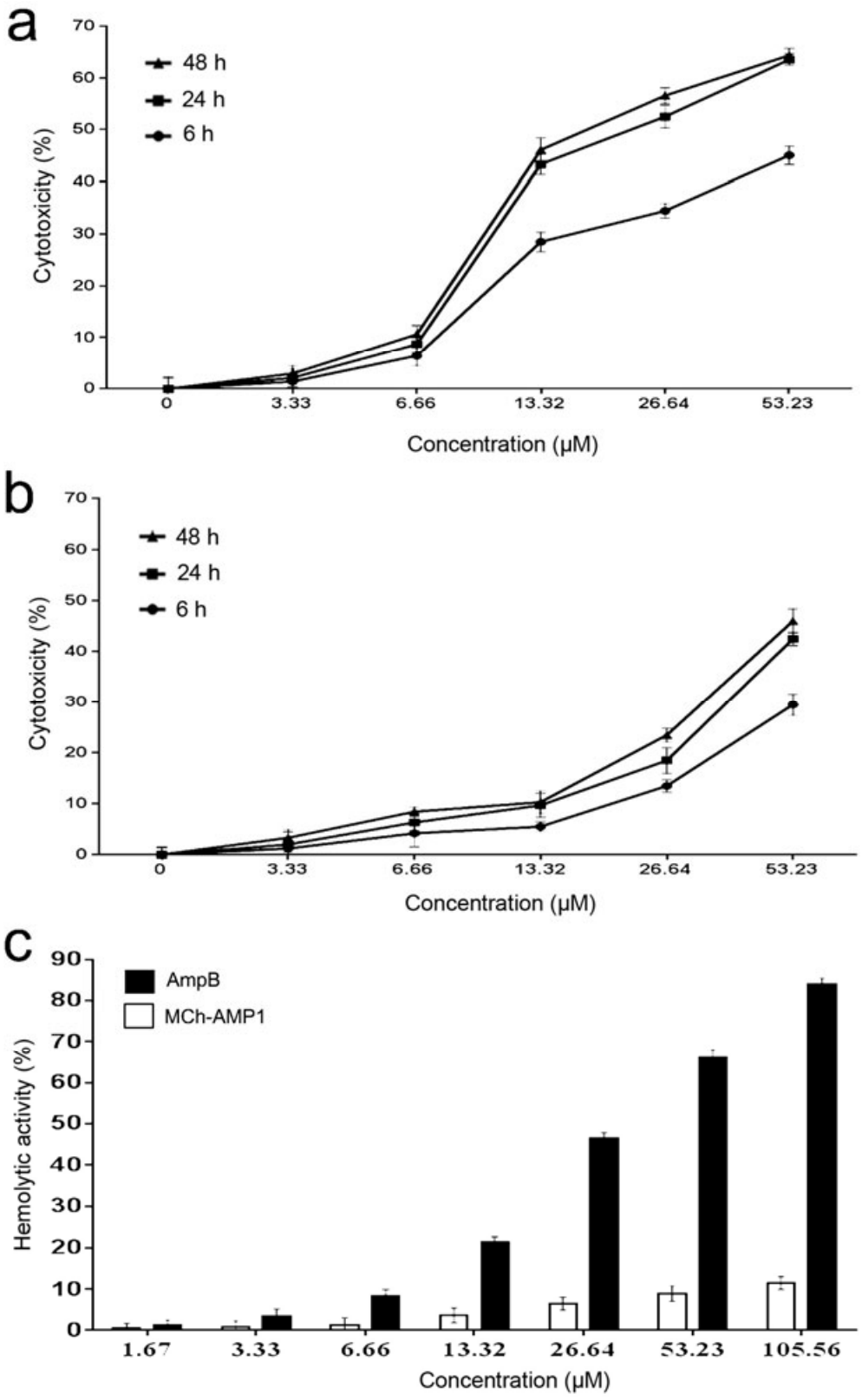


a

Name	Sequence	Activity	Source
MCH-AMP1	LSV----KAFTGIQLR--GVCGIEVKARG-	Antifungal	*Matricaria chamomilla*
Ac-AMP1	VGECVRGRCPSGMCCSQFGYCGKGPKYCG-	Antifungal,Antibacterial	*Amaranthus caudatus*
Ar-AMP	AGECVQGRCPSGMCCSQFGYCGRGPKYCGR	Antifungal	*Amaranthus retroflexus*
Plantaricin CS	GGY----KNFYGSALRKGFYEGEAGRAIRR	Antifungal, Antibacterial	*Coriandrum sativum*







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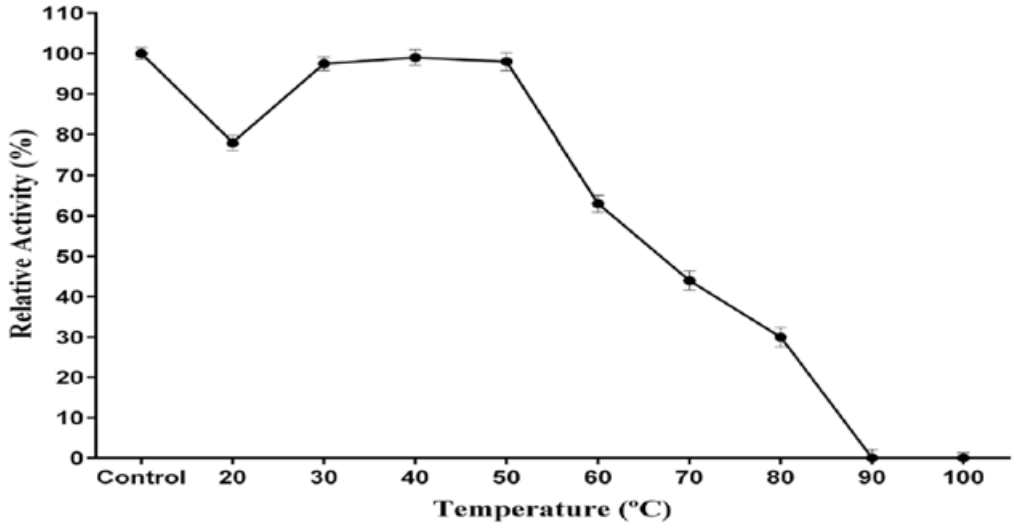

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