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Synthesis and biological comparison of two antimicrobial peptides originated from the venom of *Vespa crabro* and *Polybia paulista*

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Abstract. Mastoparan C and **Polybia-MP1** are two well-known AMPs found in the European Hornet *Vespa crabro* and social wasåp *Polybia paulista*, respectively. Due to the potent antimicrobial activity toward a wide range of microbial pathogens, including Gram-negative, Gram-positive and fungal species, they are promising to tackle the global antimicrobial resistance crisis. Thus, understanding the structure and activity relationships (SARs) is essential for the development of potential therapeutic applications. In this study, we prepared **Mastoparan C** and **Polybia-MP1** and investigated the differences in their biological properties. Both AMPs were successfully synthesized and purified with more than 95 % purity, confirmed by high-performance liquid chromatography and mass spectrometry. Besides, the data indicated a decrease in antimicrobial activity but notably less hemolysis of **MP1** compared to **MPC**. Overall, our study suggests various important information regarding the structural requirements for the antimicrobial and hemolytic activity of antimicrobial peptides.

Keywords: Antimicrobial peptides, natural peptides, bee venom, antifungal agents, structural-activity relationships.

Classification numbers: 1.2.1, 1.2.4, 1.4.3.

1. INTRODUCTION

Traditional antibiotics and antifungal drugs have been playing a crucial role in global healthcare. However, though supporting the body in the management of bacterial infection, it is

also gradually increasing the threat of antimicrobial resistance (AMR). According to the report of the World Health Organization (WHO), the pipeline of newly developed antimicrobial drugs is dry and insufficient for the AMR challenge [1, 2]. In fact, only seven candidates were considered innovative among just 26 antibiotics in clinical development in 2020 [1]. Unfortunately, even though all of these were successful and entered the market, the new antibiotics will soon become ineffective. Notably, besides the antimicrobial drugs, host defense systems have been essential in combating infectious diseases for a very long time before the antibiotics era. The secretion of ribosomal antimicrobial peptides (AMPs) is considered a crucial role that is still irreplaceable to date. Interestingly, it is suggested that the chance of microbial pathogens becoming resistant to AMPs is significantly limited compared to traditional antibiotics [3, 4]. Hence, this important source has great potential in developing new therapeutic applications that can manage the AMR crisis.

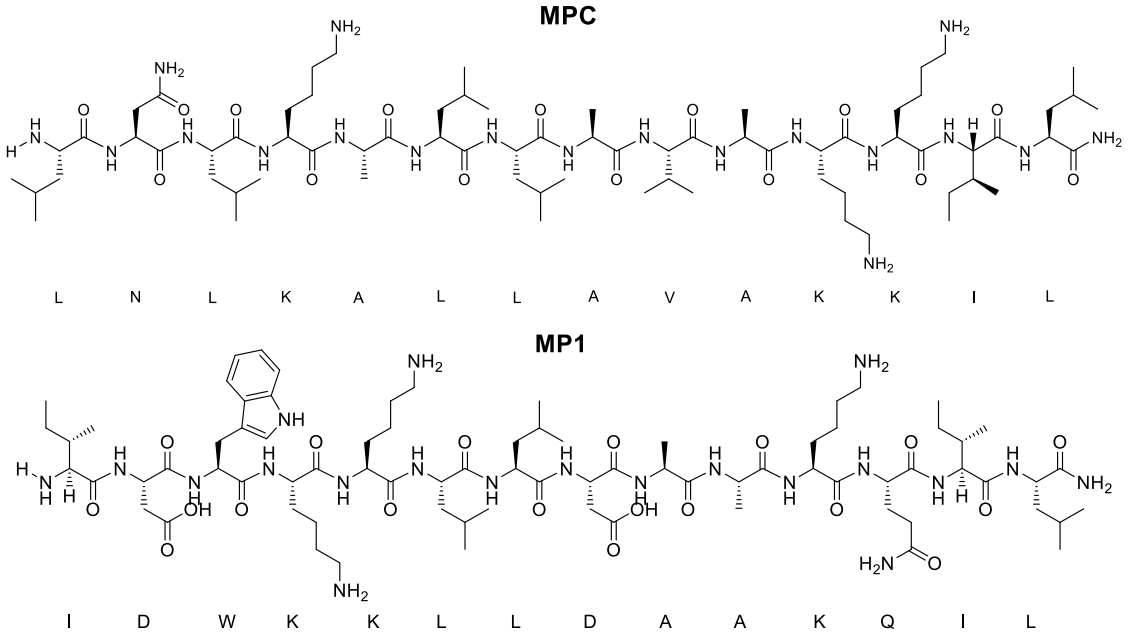

Figure 1. Structure of **MPC** (top) and **MP1** (bottom).

Recently, **Mastoparan C** (**MPC**) and **Polybia-MP1** (**MP1**) from bee venom were demonstrated to have dual antimicrobial and anticancer activity toward a wide range of bacteria, fungi, and cancer cells [5 - 12]. They shared some common structural properties with AMPs in the Mastoparan family, including the length of 14 residues, net positive charge with amidated C-terminus, and free amino group at N-terminus (Figure 1 and Table 1). In the membrane-mimicking solution, both peptides showed an α-helical structure, which allocates most of the hydrophobic residues on one side whereas the hydrophilic ones (including charged residues) on the opposite side of the helix [13, 14]. This hydrophilic face is essential for selective binding to negatively charged components on the microbial surface, and the hydrophobic face is essential for interaction with the hydrophobic interior [15]. Thus, the most common mechanism of action is supposed to be membrane perturbation, which leads to loss of function and, finally leakage of cytoplasmic components [16 - 18]. However, the drawback of most AMPs is the cytotoxicity of red blood cells. **MPC** was reported to cause hemolysis, which is 10 % of mouse red blood cells

at 64 µM [19] and 50 % of horse red blood cells at 40 µM [12]. Interestingly, **MP1** caused less than 50 % of human red blood cells at even 200 µM.

Hence, it is important to investigate the SARs and optimize their structure to improve their pharmacological properties. However, the difference between the testing conditions might affect the comparison between the two peptides, which makes it difficult to identify reliable structural-activity relationships. Therefore, in this study, we synthesized and compared their antimicrobial and hemolytic activity. Furthermore, the data was analyzed and interpreted based on the helical wheel model that represents the peptide secondary structure.

Table 1. Some structural properties of **MP1** and **MPC**

Properties	MP1	MPC
Origin	*Polybia paulista*	*Vespa crabro*
Chemical formula	$C_{78}H_{132}N_{20}O_{19}$	$C_{72}H_{135}N_{19}O_{15}$
Molecular weight	1654.04	1506.99
Length[a]	14	14
N-terminal	Free	Free
C-terminal	Amidation	Amidation
Polar residues	6	4
Non-polar residues	8	10
Retention time (min)	6.703	7.056
Net charge	+2	+4
Hydrophobicity[b]	0.489	0.634
Hydrophobic moment[b]	0.511	0.399

[a] Number of residues.

[b]Hydrophobicity (**H**) and Hydrophobic moment (**µH)** were predicted by HeliQuest server for standard α-helix [20].

2. MATERIALS AND METHODS

2.1. Materials

Commercially available solvents and reagents were used as received. All Fmoc-protected α-amino acids and 1-[(1-(cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-morpholino)]uranium hexafluorophosphate (COMU) were purchased from AK Scientific, United State of America. Rink Amide MBHA resin was purchased from NovaBiochem, Germany. Piperidine, dimethylformamide (DMF), *N*,*N*-diisopropylethylamine (DIEA), and trifluoroacetic acid (TFA) were purchased from Daejung, South Korea. Triisopropylsilane (TIS) was purchased from Sigma-Aldrich, Germany.

2.2. Methods

Peptide synthesis. Both **MP1** and **MPC** were prepared using Fmoc chemistry on Rink Amide MBHA resin with a loading capacity of 0.67 mmol/g [6]. The dry resin (358 mg, 240 µmol) was swelled in DCM for 1 min and DMF for 10 min before using. The Fmoc protecting group was removed by treatment with 20 % piperidine in DMF (2 x 10 min). Amino acids were

coupled for 30 min using an activating agent COMU (4.75 equiv.), 5 equiv. of Fmoc-protected amino acid, and 10 equiv. of DIEA in DMF. After each coupling or deprotection reaction, the resin was washed with dichloromethane (DCM) (1 × 2 min), DMF (1 × 2 min), DCM (1 × 2 min), and DMF (3 × 2 min). See Figure 2 for more information.

Purification. After the final Fmoc-deprotection reaction, resin was washed with NMP (2 × 2 min) and DCM (5 × 2 min) and dried *in vacuo* overnight. The peptides were deprotected and cleaved from the resin by treating them with a mixture of TFA/TIS/water (95/2.5/2.5) for 2 hours and precipitated by adding a 1:1 mixture of *n*-pentane and diethyl ether. The precipitate was collected by centrifugation, dissolved in a 1:1 mixture of acetonitrile and water, and filtered to remove the resin [21]. The products were purified through reverse phase high-performance liquid chromatography (RP-HPLC) using a Zorbax C18 column (Agilent, 5 µm, 9.4 × 250 mm), and then characterized by Agilent 6400 Series Triple Quadrupole LC/MS System (see Figure 2**).**

Antimicrobial assay. The antimicrobial assay was conducted against three Gram-positive strains (*Bacillus cereus* ATCC 11778, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212) and three strains of Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 13076). These strains of bacteria were pre-cultured with 5 ml lysogeny broth (LB) medium (10 g/L Polypeptone, 5 g/L Bacto-yeast extract and 10 g/L NaCl) overnight at 37 °C. The overnight cultures of bacterial cells were diluted with the fresh LB medium with 6.4 µM peptide to obtain OD_{600} of 0.01. Then, 200 µL of diluted broth were divided into 96-well microtiter plates. The cell growth was measured with OD_{600} at 24 h of incubation at 37 °C. The bacterial cells were cultivated with LB medium as a negative control. In the independent experiment, *Candida albicans* cells were cultured in YM medium (10 g/L glucose, 3 g/L malt extract, 5 g/L peptone, 3 g/L yeast extract) at 37 °C. The cell growth inhibition was calculated using the following formula:

$$Growth\ inhibition = \frac{\text{OD600 sample } \times\ 100\ \%}{\text{OD600 negative control}}$$

Hemolysis assay. Peptides were dissolved in PBS. 10 µl of serially diluted peptides (6.4 and 64 µM final concentration) was added to 190 µl of suspensions of human red blood cells (10 % v/v in PBS) and incubated for 30 min at 37 ^{o}C. After centrifugation, the supernatants were diluted with 10-fold PBS and the absorbance at 405 nm was measured for each solution. The blood suspension treated with 0.2 % Triton X-100 was used as a control for 100 % hemolysis [14]. The percentage of hemolysis was determined using the following equation:

$$\%\ Hemolysis = \frac{\text{OD405nm sample } \times\ 100\ \%}{\text{OD405nm positive control}}.$$

Statistical analysis. Experiments were performed in triplicate. The obtained data were statistically analyzed using Microsoft Office Excel 365 to determine the means of triplicates and the standard deviation. Data were presented as the mean values with standard deviation (± SD) and p–value < 0.05 were considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Peptide preparation

3.1.1. Peptide synthesis protocol

MP1 and **MPC** were synthesized with the protocol based on Fmoc strategy [6, 22, 23]. The coupling catalyst COMU is the third generation of uronium-type reagent with safer and more effective than benzotriazole based HDMA and HDMB [24, 25]. The synthesis procedure starts coupling amino acids from C-terminal to N-terminal, before the final cleavage to obtain raw peptide (Figure 2 for more details).

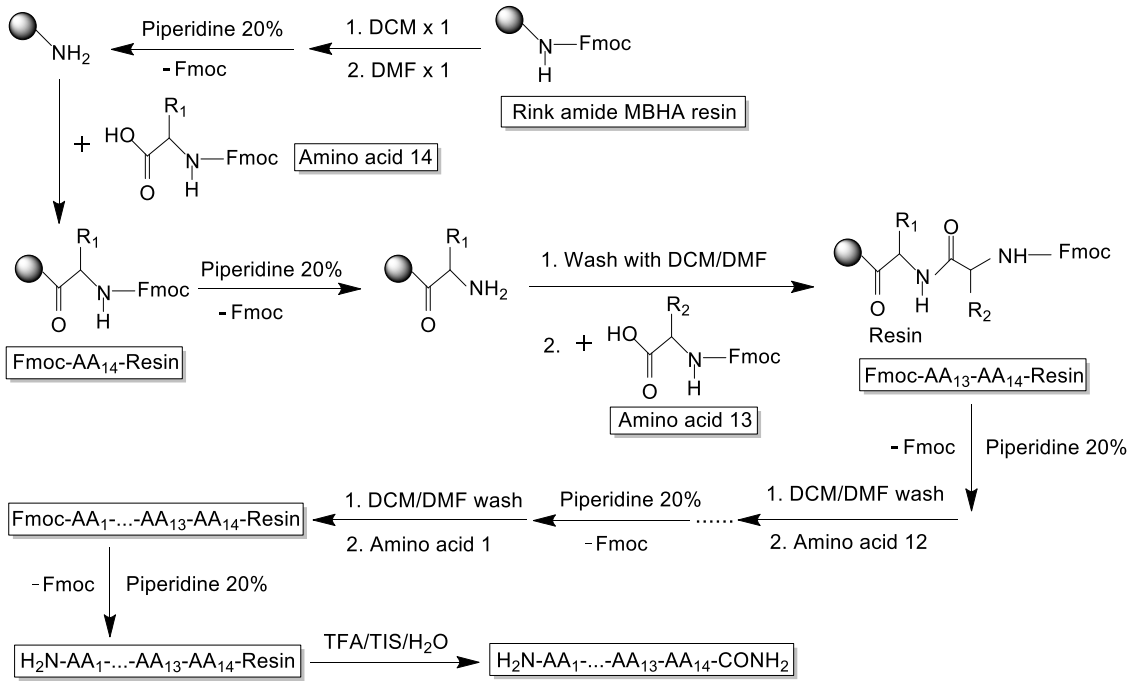

Figure 2. Schematic illustration of solid-phase peptide synthesis of **MP1** and **MPC**. (Both AMPs were prepared in a similar process, the only difference is the order in which amino acids were coupled to the peptide sequences).

3.1.2. RP-HPLC profiles

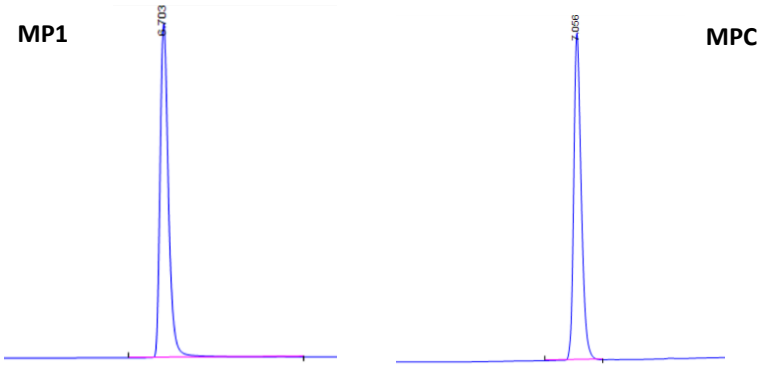

Figure 3. Purity profiles of **MP1** and **MPC** by RP-HPLC.
(Gradient condition: 5 - 100 % B for 0 - 8 min; 100 % B for 8-12 min; 100 - 5 % B for 12-14 min; 5 % B for 14 - 16 min. A: 0.1 % TFA in H_2O, B: 0.1 % TFA in Acetonitrile (ACN), flow rate 1mL/min).

Antimicrobial peptides in this study were synthesized and purified with more than 95 % purity checked by RP-HPLC. See Figure 3 below for the purity profiles of **MP1** and **MPC** (at wavelength 220 nm). Besides, the HPLC data indicated that **MPC** is slightly more hydrophobic than **MP1** as showing higher retention time. This result is also consistent with the calculated data, which suggested that **MPC** has a higher hydrophobicity value (0.634, Table 1) than that of **MP1** (0.489).

3.1.3. Mass spectrometry

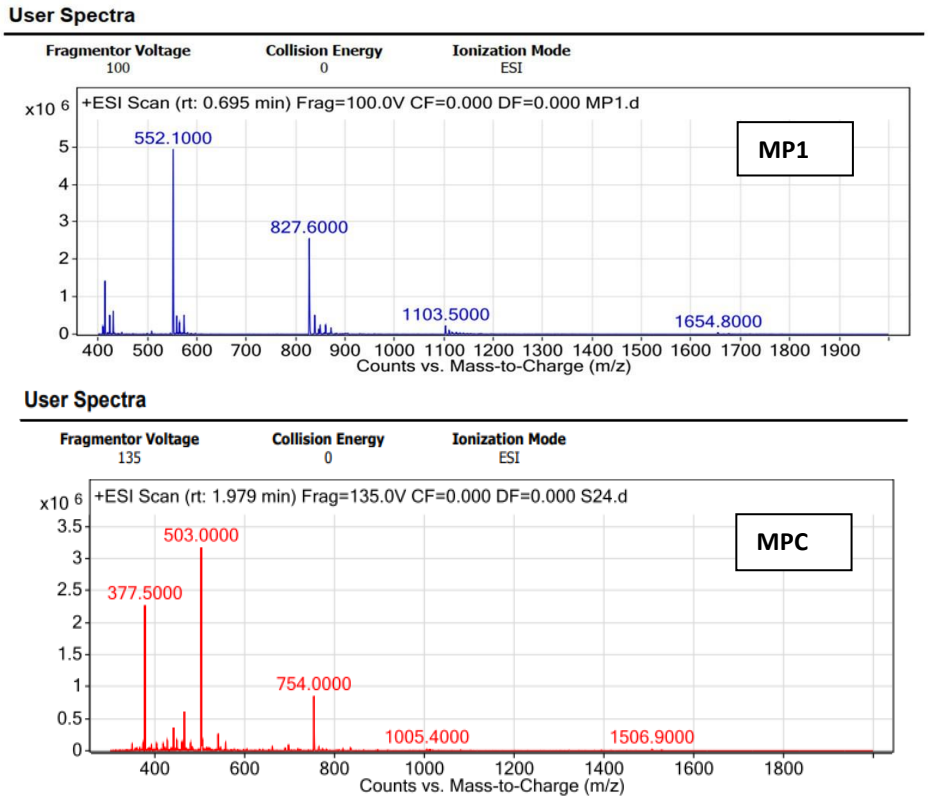

Figure 4. Mass spectrum of **MP1** and **MPC**.

Table 2. Mass spectrometry analysis of MP1 and MPC.

		MP1	**MPC**
[M+H]	*Calculated*	1654.0057	1507.0465
	Found	1654.8000	1506.9000
[M+2H]/2	*Calculated*	827.5062	754.0272
	Found	827.6000	754.0000
[M+3H]/3	*Calculated*	552.0071	503.0207
	Found	552.1000	503.0000

Mass spectrometry analysis at a positive ionization mode of the two purified peptide was shown in Figure 4. (Gradient condition: 50 - 100 % B for 0 - 2 min; 100 - 50 % B for 2-4 min. A: 0.1 % TFA in H2O, B: 0.1 %TFA in Acetonitrile (ACN), flow rate 1mL/min). The observed results agree with calculated data (Table 2).

3.2. Structural analysis by helical wheel projection

In aqueous environments, **MP1** and **MPC** are mainly random coils, however, when reaching the target microbial membranes, they gradually fold to an amphipathic helical conformation [8 - 10]. Therefore, a helical wheel projection was created to visually represent the properties of AMP structures. The amino acids in the peptide sequence are plotted in a wheel where the angle between every two consecutive residues is 100°.

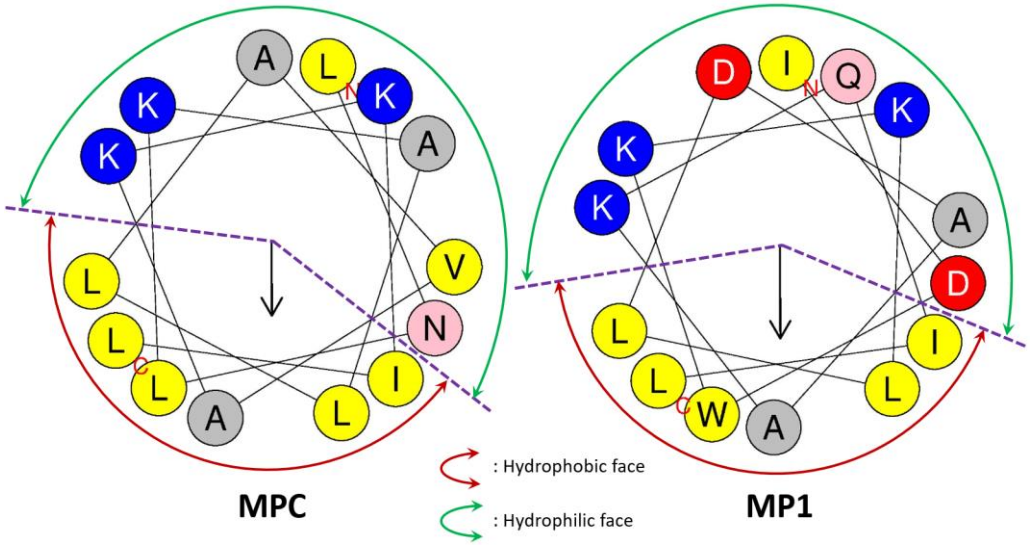

Figure 5. Helical wheel projection of **MP1** and **MPC**. The arrow represents the length and the direction of the hydrophobic moment µH. This helical wheel was created by HeliQuest webserver [20]. The helical wheel represents the peptide sequence when looking through its helical structure from N-terminus to C-terminus.

In Figure 5, the hydrophilic side of MPC is covered from Asparagine (N) at position 2 to Lysine (K) at position 10 while the rest is the hydrophobic face. Similar to **MPC**, the hydrophilic side of **MP1** is covered from aspartic (D) at position 2 to Lysine (K) at position 10. Notably, asparagine (in **MPC**) and aspartic acid (in **MP1**) at position 2 are highly hydrophilic residues, thus further broaden the hydrophilic face in their helical wheel. Besides, one of the common designs in AMPs from the Mastoparan family is the location of hydrophobic residues at position 1 and on the hydrophilic side of the helix [7, 26]. It should be noted that both **MP1** and **MPC** have the free amino group at N-terminal. Therefore, this design provides +1 unit to the overall cationic charge, but it still slightly interrupts the hydrophilic face, whereas moderately decreases the gathering of hydrophobic residues on the other side of the helix.

3.3. Antimicrobial activity

MP1 and **MPC** were demonstrated to have potent and broad-spectrum activity against various types of bacteria and *Candida albicans* [7, 10, 14]. However, the examinations were not

in the same conditions, thus it is unable to compare their potency. Thus in this study, the antimicrobial experiment was conducted against several microbial pathogens (Table 3).

Table 3. Antimicrobial activity of **MP1** and **MPC** at 6.4 µM against selected microbial pathogens.

Microbial pathogens		**MP1**	**MPC**
Gram-positive bacteria	*Staphylococcus aureus*	2.4 ± 0.5 %	12.7 ± 2.1 %
	Bacillus cereus	25.7 ± 1.8 %	99.6 ± 0.2 %
	Enterococcus faecalis	35.4 ± 5.9 %	99.1 ± 2.7 %
Gram-negative bacteria	*Escherichia coli*	34.2 ± 4.4 %	99.7 ± 0.6 %
	Pseudomonas aeruginosa	5.6 ± 1.3 %	5.9% ± 1.5 %
	Salmonella enterica	22.2 ± 2.5 %	99.8 ± 0.4 %
Fungal	*Candida albicans*	16.6 ± 2.2 %	37.3 ± 3.9 %

The results suggested that **MPC** showed higher activity than **MP1** against almost all the tested microbial pathogens, except in the case of *Pseudomonas aeruginosa* which both of them showed nearly inactive. The more potent in the activity of **MPC** can be explained by two reasons: Firstly, the net charge of MPC (+4) is twice more than **MP1** (+2), thus making it have more efficient binding to the microbial membrane. Secondly, compared to **MP1**, the higher levels of hydrophobicity in **MPC** increase the interaction with hydrophobic interior of bacterial and fungal membrane.

3.3. Hemolytic activity

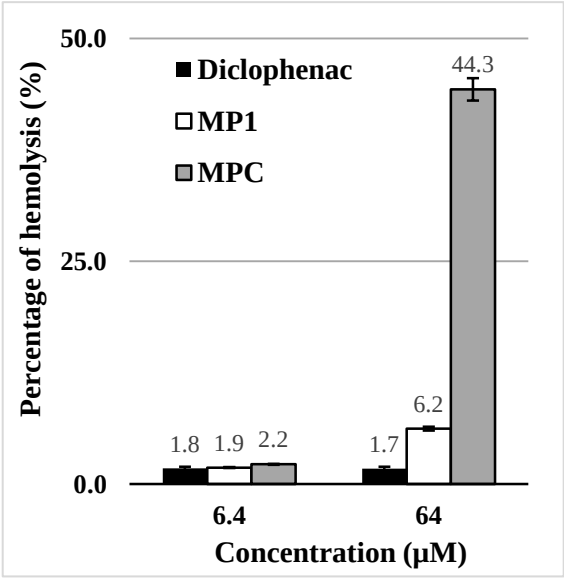

Figure 6. Hemolytic activity of **MP1** and **MPC**.

In the hemolysis assay, diclofenac was used as a negative control, which is considered to

not affect the human red blood cells. In contrast, 0.2 % Triton X–100 was a positive control for 100 % hemolysis. The data suggested that both peptides showed almost no hemolytic activity at low concentration (6.4 µM). However, at 10-fold concentration (64 µM), the difference become significant: **MPC** caused 44.5 % while **MP1** only caused only 6.2 % of hemolysis (see Figure 6). It can be observed from the helical wheel projection in Figure 6 that both peptides have six hydrophobic residues in the hydrophobic face, however, the hydrophilic face of MPC has 4 out of 8 positions are hydrophobic residues – more than that of **MP1** with only 2 out of 8. Therefore, the elevated toxicity toward red blood cells of **MPC** first comes from the higher hydrophobicity. Besides, previous studies also suggested that the two negative residues of Aspartic acid (D, Asp) at positions 2 and 8 showed an important role in decreasing hemolytic activity [27-29]. Hence, though resulting in less potent antimicrobial activity, the design of **MP1** sequence is more effective in minimizing the side effects on normal cells.

4. CONCLUSIONS

In summary, two antimicrobial peptides **MP1** and **MPC** have a lot of structural similarities, such as length, helical conformation, cationic charge, and amphipathicity. However, the appearance of two negatively charged residues (Aspartic acid) along with the decreased net charge and hydrophobicity are the main reasons for the reduction in antimicrobial activity but significantly less hemolysis of **MP1** compared to **MPC**. Further studies are underway to understand better the effect of structural differences on the biological properties of these two peptides. Further studies are underway to better understand the effect of structural differences (the helical conformation, the hydrophobicity, net charge, etc.) on the biological properties (i.e., antimicrobial and anticancer activity) of these two peptides.

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CRediT authorship contribution statement. Le Huy Binh and Bui Thi Phuong Hai: Peptide preparation, hemolysis and antimicrobial assay. Do Hai Yen: Peptide synthesis. Tran Thi Kim Anh: Data analysis. Nguyen Hong Minh: Antimicrobial and hemolysis assay. Nguyen Thi Thanh Binh: Peptide preparation. Luong Xuan Huy and Vu Dinh Hoang: Data analysis, Supervision. All the authors discussed the results and commented on the manuscript. [#]Le Huy Binh and Bui Thi Phuong Hai contributed equally.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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