Journal of Asia-Pacific Entomology 22 (2019) 1129–1135

Contents lists available at ScienceDirect

Journal of Asia-Pacific Entomology

journal homepage: www.elsevier.com/locate/jape

Full length article

# Assessment of hemolytic activity of bee venom against some physicochemical factors

Yaser Yousefpoor[a,b], Amir Amani[a,c,*], Adeleh Divsalar[d], Seyyedeh Elaheh Mousavi[e], Yaser Eskandari Torbaghan[f], Omid Emami[f]

[a] Department of Medical Nanotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran
[b] Research Center of Advanced Technologies in Medicine, Torbat Heydariyeh University of Medical Sciences, Torbat Heydariyeh, Iran
[c] Natural Products and Medicinal Plants Research Center, North Khorasan University of Medical Sciences, Bojnurd, Iran
[d] Department of Cell & Molecular Biology, Faculty of Biological Sciences, Kharazmi University, Tehran, Iran
[e] Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[f] Khalil Abad Health Center, Mashhad University of Medical Sciences, Mashhad, Iran

ARTICLE INFO

*Keywords:*
Bee venom
Hemolytic activity
Physicochemical factors
Temperature
pH
UV radiation
Ultrasound waves

ABSTRACT

Bee venom (BV) is a biotoxin with biologically active peptides which have cell lysis and hemolytic activity properties. These properties can be affected under different storage conditions or during the production process. In present study, we investigated effects of a number of physicochemical factors, including temperature, pH, UV radiation, ultrasound waves and storage time on hemolytic activity of BV. Maximum absorption and melting temperature of BV solution were obtained as 280 nm and ~70 °C, respectively. Cell hemolysis 50 ($CH_{50}$) -concentration of BV that can lyse 50% of red blood cells- was determined as 0.94 μg/ml at ambient temperature. $CH_{50}$ was shown not to be importantly varied at temperature up to 60 °C, pH value 2 to 13 and under UV/ultrasound radiation. Storage at −20, 6 and 25 °C for 6 months made about 2.5, 35 and 1000 times increase in $CH_{50}$. From the results, it may be concluded that BV is a relatively resistant hemolytic agent and can be used in a variety of laboratory research and product manufacturing methods.

## Introduction

Bee Venom (BV) is a biotoxin, which is biosynthesized and secreted by a venom gland in abdominal cavity of bee's abdominal cavity, and used for protection from enemies. It is composed of at least 18 active components, including enzymes, peptides, biogenic amines and non-peptide components with an extensive pharmaceutical properties variety. BV could modify immune system functions in the body and increase production of cortisol (Son et al., 2007). Melittin, as main biologically active component of BV, could form 40% to 50% of dry weight of BV approximately, depending on bees species and their food (Mahmoodzadeh et al., 2013; Rady et al., 2017). Melittin is a water soluble, linear, cationic, hemolytic, cell lytic and amphipathic peptide with molecular weight of 2846.46 g/mol (Fidelio et al., 1984). It has several functions like anti-inflammatory (in very small doses), immunosuppressive and immunostimulatory, anti-microbial, cytotoxicity against cancer cells, anti-arthritic, anti-atherosclerosis, and could increase capillary permeability and blood circulation, along with decreasing blood pressure. Phospholipase A2, the second component in terms of abundance in BV (10–12%), is a hydrolyzing enzyme with immunomodulatory activity and has cytotoxic effects against cancer cells. The lipase is allergen and can reduce blood coagulation and also blood pressure (Bae et al., 2015; De Lima and Brochetto-Braga, 2003; Gauldie et al., 1976; Mizrahi and Lensky, 2013; Moreno and Giralt, 2015; Mousavi et al., 2012; Oršolić, 2012; Piek, 2013; Son et al., 2007; Urtubey, 2005). Bee venom therapy (BVT) has been applied as early as the second century BCE in Eastern Asia and specially in Korea (Lee et al., 2005) to treat a variety of diseases, like arthritis, rheumatism, back pain, tumors, and also for skin diseases (Son et al., 2007). Mukhtar et al. reported potential of melittin and its conjugates in cancer therapy (Rady et al., 2017). Anti-tumor mechanisms of melittin include inhibition of angiogenesis, tumor cell growth and migration along with as well as induction of apoptosis and lysis of tumor cells (Badria et al., 2017). In addition, properties like anti-nociceptive effects in arthritis (Baek et al., 2006), analgesic effects on knee osteoarthritis (Kwon et al., 2001), anti-nociceptive and anti-inflammatory effects on acute paw

* Corresponding author at: Department of Medical Nanotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran.
*E-mail address:* aamani@sina.tums.ac.ir (A. Amani).

https://doi.org/10.1016/j.aspen.2019.09.001
Received 12 May 2019; Received in revised form 15 August 2019; Accepted 2 September 2019
Available online 05 September 2019

edema of rat (Lee et al., 2001), attenuating neuro-inflammatory events (Yang et al., 2010), inhibition of replication of herpes simplex virus (Baghian et al., 1997) and HIV-1 (Wachinger et al., 1998) along with inhibition of mouse melanoma cells (Liu et al., 2002) have been reported for BV. However, to the best of our knowledge,still no study has been carried out on stability of BV and factors influencing it. This study aimed to examine the hemolytic activity of BV as a function of some physicochemical variables. These variables are temperature, ultraviolet (UV) radiation, alkaline/acidic conditions, ultrasound waves and storage at three different temperatures.

## Materials and methods

### Materials

BV was obtained by BV collector device (Asgharpoor Honey and Bee Products Company, Iran) by electrical stimulation using protocol suggested by Benton et al. (1963). It was collected from healthy and approved hives from north west areas of Iran, *Apis mellifera* medapersica strain. NaCl, Na2HPO4, KCl, KH2Po4 were purchused from Merck chemicals (Germany).

### Methods

#### Study of hemolytic activity of BV

Red blood cells (RBCs) hemolysis test was performed on human blood samples (Al-Badri et al., 2008) using Al-Badri's method in order to find Cell hemolysis 50 ($CH_{50}$). $CH_{50}$ is concentration of BV that can lyse 50% of RBCs. Heparin was used (20 IU in 5 ml blood) in order to prevent blood coagulation. The samples were incubated at 37 °C for 10 min. In addition, samples were centrifuged (10 min, 3000 RPM) in order to remove plasma and blood proteins. To wash the sediment containing RBCs, phosphate-buffered saline (PBS) was added, and the process was repeated 5 times until the time that supernatant was thoroughly clear. After that, supernatant was removed and RBCs were suspended by the use of PBS (2% final concentration). BV was dissolved in deionized distilled water (1000 μg/ml) and serial dilution was performed using tubes containing 1 ml PBS, and it was incubated for 2 h at 37 °C. Also, positive control (containing triton X-100) and negative control (without BV) were examined. Then, the tubes were centrifuged (10 min, 3500 RPM) and supernatants absorption were read at 540 nm by a photometer (Riele-5010, Robert Riele GmbH & Co KG, Germany) using PBS as blank. The tests were repeated for three times. Hemolysis percentage was then calculated using Eq. (1) as follows (Asthana et al., 2004; Zarrinnahad et al., 2018):

$$\text{Hemolysis percentage} = \left[\frac{\text{Sample absorption} - \text{Negative control absorption}}{\text{Positive control absorption} - \text{Negative control absorption}}\right] \times 100 \quad (1)$$

#### Spectrophotometery study of BV

Melting temperature ($T_m$) is a temperature at which both of the folded and unfolded proteins states are equally populated at equilibrium, and protein denaturation is at midpoint, which is often determined by the use of a thermal shift assay (Muñoz and Sanchez-Ruiz, 2004). Series of BV solutions (250, 500 and 1000 μg/ml) were prepared in PBS, and the samples were investigated using spectrophotometer (Cecil-7250CE, Cecil Instruments Ltd., ENGLAND) against PBS as blank. The wavelength spectrum was in UV–Visible range from 200 to 900 nm. In addition, BV solution with 500 μg/ml concentration was investigated by the use of a thermal spectrophotometer (Carry100Bio, Agilent Technologies, USA) in order to record changes in maximum absorbance (280 nm) along with increasing temperature. After that, using pace analysis $T_m$ was calculated (Noodeh et al., 2018).

#### Effect of temperature on hemolytic activity of BV

Eight tubes containing 100 μg/ml BV were prepared. As a next step, temperature was increased from 30 to 100 °C with intervals of 10 °C, and after that, samples were stored for 5 min at each temperature. A serial dilution was made from each tube and hemolysis test was applied as it was stated above earlier.

#### Effect of pH on hemolytic activity of BV

BV solution was added to 14 tubes containing PBS buffer having pH value of 1–14 (in order to obtain final concentration of 100 μg/ml). Then, serial dilution was performed with normal PBS, and hemolysis test was performed.

#### Effect of UV on hemolytic activity of BV

Six samples of 100 μg/ml BV solution were exposed to UV radiation by the use of UV lamp for 0, 5, 10, 15, 30 and 60 min. Then, hemolysis test was done with respect to the earlier protocol.

#### Effect of ultrasound waves on hemolytic activity of BV

Six samples of 100 μg/ml BV solution were sonicated using a probe sonicator (Scintz-UP400S, dijkstra vereenigde, Netherlands) for 0, 1, 3, 5, 7 and 10 min (continuous mode, power of 50 Hz). Samples were placed in an ice-bath in order to preventing them from warming up. After that, hemolysis test was performed according to the previous protocol.

#### Effect of storage temperature on hemolytic activity of BV

Six BV aqueous solution series with high concentration (1000 μg/ml) were stored for up to 6 months at different temperatures of −20, 6 and 25 °C, and also their $CH_{50}$ was determined at the end of each month, in order to measure the hemolytic activity. Samples were stored away from light condition and sealed to prevent from evaporation.

## Results

### Study of hemolytic activity of BV

Fig. 1 presents the effect of BV concentration on RBCs, and demonstrates that $CH_{50}$ can be calculated as 0.94 μg/ml. Moreover, it could be concluded that, BV does not have important hemolytic activity in concentrations lower than 0.625 μg/ml, while maximum activity is observed at concentrations of more than 1.25 μg/ml.

### Spectrophotometery study of BV

Fig. 2 indicates the BV absorption at 250, 500 and 1000 μg/ml concentrations in PBS. It is observed from the details that, BV solution has a maximum absorption at ~280 nm in spectrophotometry. In addition, no absorption is observed at 300 to 900 nm for BV, and this would make its solution colorless.

Due to appropriate absorption, amongst different investigated concentrations, 500 μg/ml was selected for thermal behavior study and determination of $T_m$. Thermal behavior of BV was investigated at 25 to 85 °C (as shown in Fig. 3). As the figure shows, absorption at 280 nm decreases gradually from 25 to 55 °C, then, a sharp increase is observed above 60 °C. With respect to the findings, $T_m$ was calculated as 70 °C.

### Effect of temperature on hemolytic activity of BV

Evaluating the hemolytic activity of BV was performed at various temperatures (up to 100 °C), and the results are given in Fig. 4. It shows that $CH_{50}$ is more or less similar (~0.94 μg/ml) at 30–70 °C. However, along with temperature increasing, $CH_{50}$ increases to 1.1 μg/ml for 80 °C and 1.37 μg/ml for 90 and 100 °C.

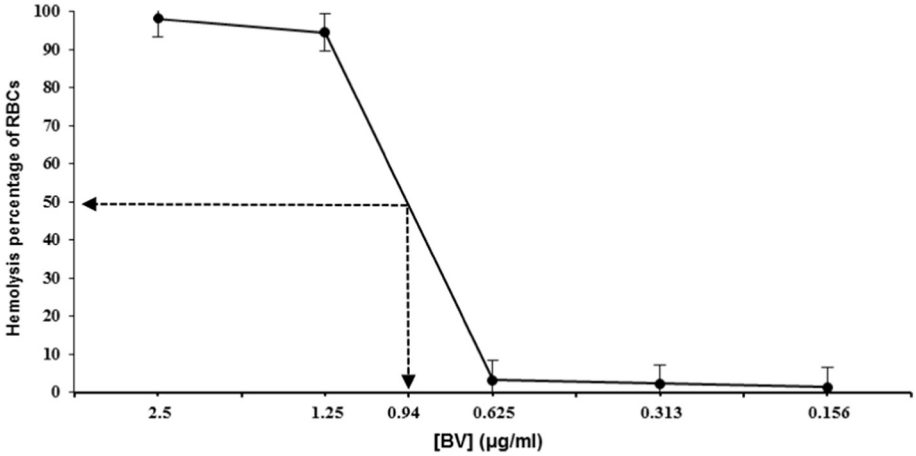


**Fig. 1.** Hemolytic activity of bee venom (BV) on human RBCs at different concentrations.

*Effect of pH on hemolytic activity of BV*

BV function was investigated against different pH values (from 1 up to 14) and results are indicated in Fig. 5. As details present, $CH_{50}$ is quite similar (i.e. ~ 0.94 µg/ml) for pH values of 2 to 13, but it is different for pH 1 (3.00 µg/ml) and pH 14 (1.56 µg/ml).

*Effect of UV on hemolytic activity of BV*

According to the changes in hemolytic activity of BV results, that were treated with various UV exposure times (see Fig. 6), $CH_{50}$ has no change and is fixed at around 0.94 µg/ml for 0–60 min UV exposure.

*Effect of ultrasound waves on hemolytic activity of BV*

Fig. 7 indicates the hemolytic activity for tubes containing similar BV concentration, which were treated with ultrasound waves with different durations of treatment. It is quite evident that there is no difference amongst $CH_{50}$ of BV treated at different treatment durations.

*Storage temperature effect on hemolytic activity of BV*

Hemolytic activity of BV was investigated at various incubation times (up to 6 months) at three different temperatures by measuring the $CH_{50}$ values. According to the results (Fig. 8), minimum increase in $CH_{50}$ was observed after 6 months at −20 °C (i.e. 2.5 times), while medium increase was detected at 6 °C (35 times). This value is maximum at 25 °C: in less than 3 months, the sample containing 1000 µg/ml BV indicated no hemolytic activity.

## Discussion

When manufacturing a BV-containing product, many physicochemical variables may render some effects on BV peptide structure. In this study, we used $CH_{50}$ as an indicator for assessing the structural/physiological changes of BV. $CH_{50}$ was obtained in this study more or less similar to that of others (e.g. 0.5 µg/ml (Mahmoodzadeh et al., 2015), 0.5 µg/ml (Zarrinnahad et al., 2018) and 2–3 µg/ml (Watala and Kowalczyk, 1990)). BV is known with its high lytic activity on human erythrocyte cells. This is because of its ability to permeate ions and hemoglobin release from RBCs (Tosteson et al., 1985). Melittin and phospholipase A2 (PLA2), two important ingredients of BV, have synergic effects on lipid membranes, which would lead to cell damage and lysis (Damianoglou et al., 2010). The hemolytic property can also be applied in order to determine the hemocompatibility of BV. On the other hand, because BV has various sources, it can have different percentages of melittin (Banks and Shipolini, 1986), which should be taken into account when changing the source of supply.

In spectrophotometery researches, BV solution indicated a maximum absorption at 280 nm due to existence of aromatic amino acids in BV. Most likely, melittin and PLA2 are considered as the most effective agents of this phenomenon, due to their abundance in BV peptides (about 50–70% in total). Melittin has a tryptophan group (indole ring) and PLA2 has 2 tryptophans, 8 tyrosines (phenol ring) and 5 phenylalanins (benzene ring) (Shipolini et al., 1974). Moreover, peptide bonds that are present in different BV compounds would lead to absorption at

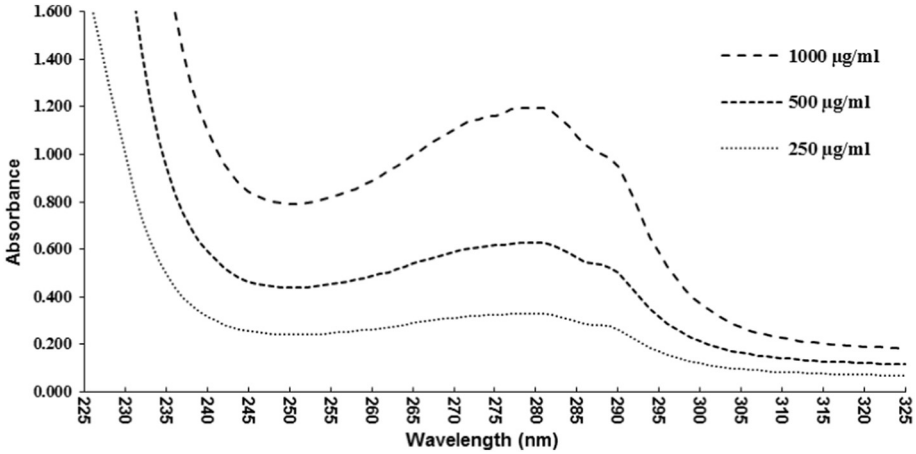


**Fig. 2.** Spectrophotometery study of bee venom (BV) solution in PBS (at concentration of 250, 500 and 1000 µg/ml).

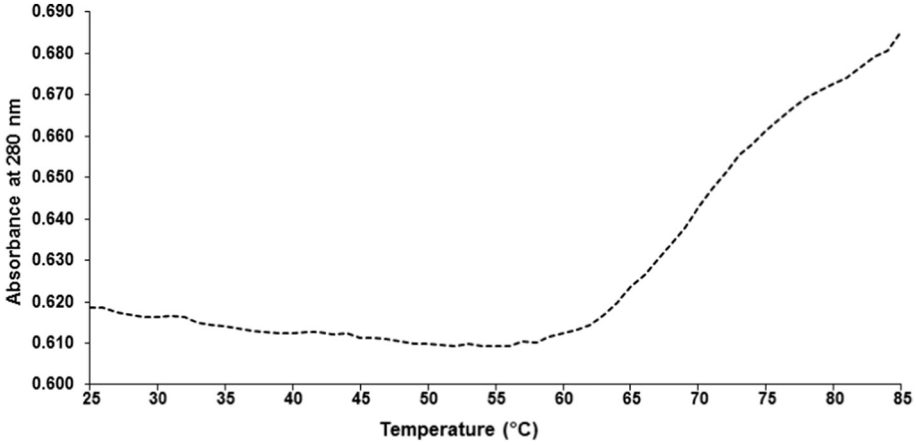


**Fig. 3.** Thermal spectrophotometery of bee venom (BV) (500 µg/ml).

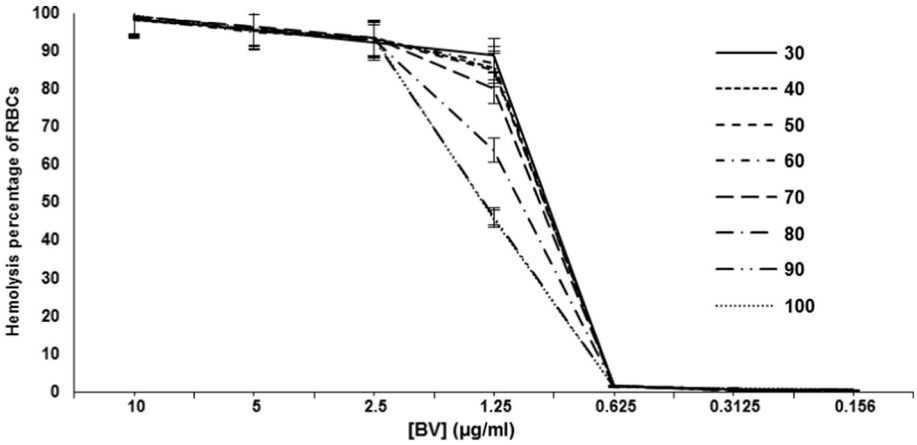


**Fig. 4.** Hemolytic activity of bee venom (BV) at temperatures 30 to 100 °C.

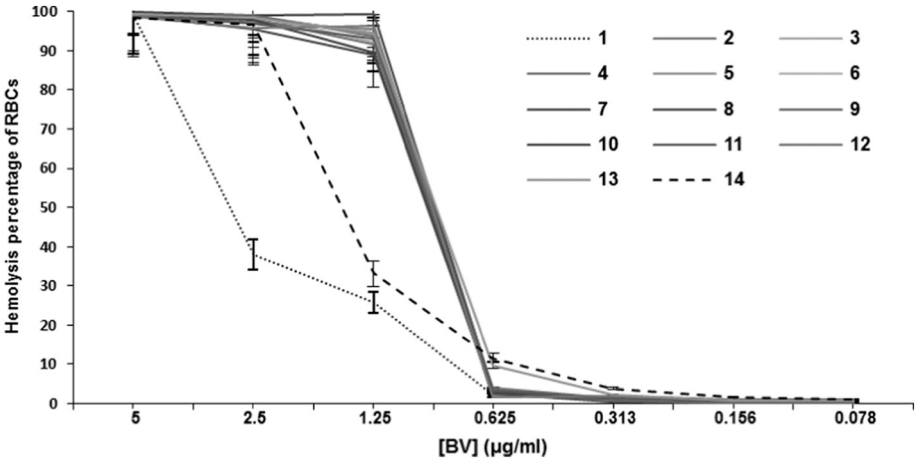


**Fig. 5.** Hemolytic activity of bee venom (BV) at pH values of 1–14.

200 to 225 nm in the spectrophotometry (Damodaran, 2008).

The thermal spectrophotometery results indicated increasing in absorption at 280 nm along with increasing the temperature increasing to more than 60 °C. Possible reason for this finding is changing conformations of BV peptides, particularly melittin. Melittin has two forms: monomer and tetramer. Two different conformations are frequently observed for the peptide: α-helical and random coil (Drake and Hider, 1979). The α-helical form gives an amphiphilic structure to the peptide: the inner part is hydrophobic in which tryptophan ($Trp_{19}$) is located and the outer surface of it is hydrophilic, while no specific structure could be detected in the random coil. Melittin has monomeric form with 90% of peptide structure being in the random coil conformation at low concentration. At high concentration, melittin becomes tetrameric in which $Trp_{19}$ is placed mostly inside in this situation (Gauldie et al., 1976). Tetramer consists of nearly 65% α-helical. Therefore, in the monomer form, $Trp_{19}$ is more accessible to light in the spectrophotometer. As heat is a cause for changing tetrameric structure into monomer and α-helical to random coil, the change in absorbance is also

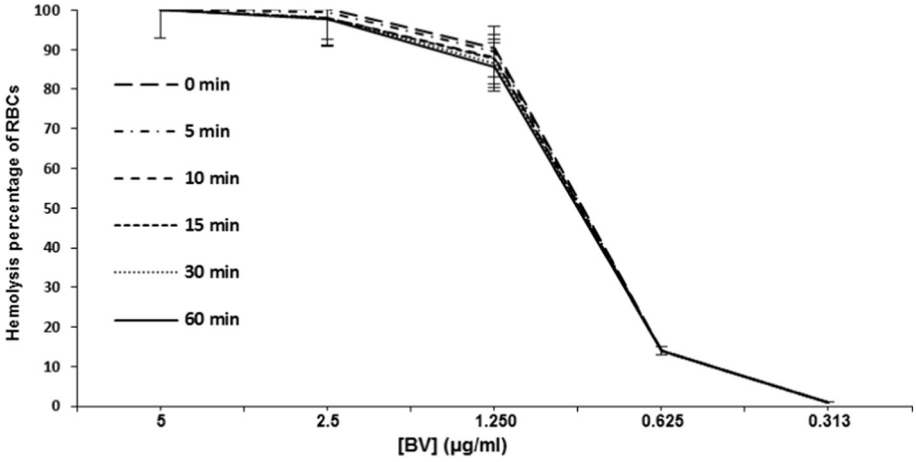


**Fig. 6.** Hemolytic activity of bee venom (BV) under different UV exposure times.

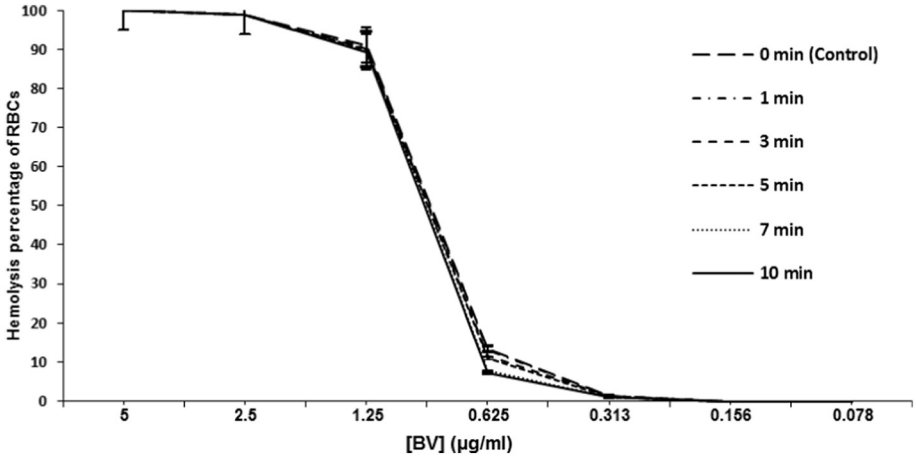


**Fig. 7.** Hemolytic activity of bee venom (BV) treated with different times of ultrasound waves.

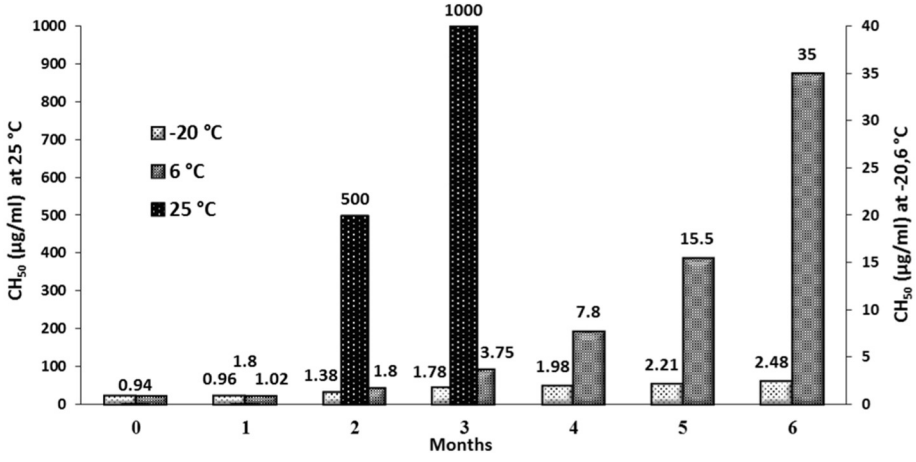


**Fig. 8.** Hemolytic activity of bee venom (BV) as a function of storage time at −20, 6 and 25 °C.

expected as a function of heating (Banks and Shipolini, 1986; Dill and Shortle, 1991; Park and Russell, 2000).

Thermal examination for hemolytic activity measurement of BV indicates that along with increasing the temperature, up to 60 °C, $CH_{50}$ is not changing, but after that, it changes by increasing to 70 upwards, which is the $T_m$ value that was obtained in this study. However, this change in $CH_{50}$ is not considerable (i.e. from 0.94 at ambient temperature to ~1.37 µg/ml at 100 °C). Thermal denaturation of proteins and peptides could be the reason for the loss biological activity in here (Park and Russell, 2000). In some cases, returning to normal conditions renaturates the proteins to regain their activity. In an interesting report, boiling BV for 5 min significantly decreased the paw edema that was caused by subcutaneous injection of BV (Calixto et al., 2003). This indicated that biological stability of BV should be studied with caution when considering different properties of BV, as in our case, boiling did not make any important change in hemolytic activity of BV.

Solvents with various pH values can be used in process of manufacturing pharmaceutical or cosmetic products. This study results demonstrated that BV is resistant to acidic and alkaline conditions; consequently, $CH_{50}$ had no significant changes for pH 2 to 13. Electrostatic interactions are one type of forces involved in process of folding polypeptides and stability of protein structure (Damodaran, 2008). Strong alkaline or acidic solutions can modify these interactions, and as a result, lead to polypeptides deformation (Dill and Shortle, 1991). The lack of change in hemolytic activity of BV as it was observed in this study could be due to lack of a complex molecular structure in BV peptides.

Assessing the hemolytic activity of BV against UV light and ultrasound waves indicated no important change in $CH_{50}$. A number of methods use UV light for different purposes. For instance, as initiator in polymerization (Mark, 2007) and disinfection of products. In addition, sonication could be used as a method for dispersing the particles, for instance, for emulsification. However, several reports have detailed destruction of peptides/genes when sonicated (Brown-Skrobot et al., 2011; Hillman, 1983; Nguyen and West, 2002). This study indicates the potential of BV to be manipulated by UV/sonication. In a report, BV was loaded in PLGA/PVA nanoparticles by the use of a water-in-oil-in-water emulsion using a microtip probe sonicator (Jeong et al., 2009). Loading BV into PLGA nanoparticles (Lee et al., 2014) or chitosan-coated-PLGA nanoparticles using sonication have also been described (Lee et al., 2016).

To be economically acceptable, BV products should have shelf-life of minimum two years, similar to other medicines. Currently, majority of BV products in the market have powder form and have such shelf life. However, no liquid form of BV with acceptable shelf life could be found at this moment. Our results show that keeping BV in aqueous form for up to 6 months would diminish its hemolytic activity during the time of storage at room temperature, while this drop activity is substantially improved when it was frozen at −20 °C. Therefore, it may be suggested that short-term storage of BV (up to a month) could be possible at −20 °C.

## Conclusion

Evaluation of hemolytic activity of BV against temperature variation up to 100 °C, pH changes of 1–14, UV and ultrasound waves indicated that its activity has no significant changes. Our preformulation studies showed that BV has nearly resistant peptides, which make it useful for therapeutic applications in a wide variety of industrial/laboratory manufacturing methods.

## Declaration of Competing Interest

None.

## Acknowledgment

We would like to extend our sincere thanks to those who collaborated including Mr. Asgharpoor (Honey and Bee Products Company, Ardabil, Iran). This project was supported by a grant from Tehran University of Medical Sciences (grant No. 96-04-87-37262).

## References

Al-Badri, Z.M., Som, A., Lyon, S., Nelson, C.F., Nüsslein, K., Tew, G.N., 2008. Investigating the effect of increasing charge density on the hemolytic activity of synthetic antimicrobial polymers. Biomacromolecules 9, 2805–2810.

Asthana, N., Yadav, S.P., Ghosh, J.K., 2004. Dissection of antibacterial and toxic activity of Melittin a leucine zipper motif plays a crucial role in determining its hemolytic activity but not antibacterial activity. J. Biol. Chem. 279, 55042–55050.

Badria, F., Fathy, H., Fatehe, A., Elimam, D., Ghazy, M., 2017. Evaluate the cytotoxic activity of honey, propolis, and bee venom from different localities in Egypt against liver, breast, and colorectal cancer. J. Apither. 2, 1–4.

Bae, H., Ye, M., Lee, C., Chung, H.-S., Shim, I., 2015. Neuroprotective effects of bee venom phospholipase A2 mediated by the suppression of neuroinflammatory responses in the 3xTg AD mouse model of Alzheimer's disease (THER2P. 967). J. Immunol. 194 67.18-67.18.

Baek, Y.H., Huh, J.E., Lee, J.D., Park, D.S., 2006. Antinociceptive effect and the mechanism of bee venom acupuncture (Apipuncture) on inflammatory pain in the rat model of collagen-induced arthritis: mediation by α2-Adrenoceptors. Brain Res. 1073, 305–310.

Baghian, A., Jaynes, J., Enright, F., Kousoulas, K., 1997. An amphipathic α-helical synthetic peptide analogue of melittin inhibits herpes simplex virus-1 (HSV-1)-induced cell fusion and virus spread. Peptides 18, 177–183.

Banks, B.E., Shipolini, R.A., 1986. Chemistry and pharmacology of honey-bee venom. In: Venoms of the Hymenoptera: Biochemical, Pharmacological and Behavioural Aspects, pp. 329–416.

Benton, A.W., Morse, R.A., Stewart, J.D., 1963. Venom collection from honey bees. Science 142, 228–230.

Brown-Skrobot, S.K., Ebel, J.A., Enns, J.B., Hill, G.A., Kimble, A.W., 2011. Method and apparatus of sterilization using monochromatic UV radiation source. Google Patents.

Calixto, M., Triches, K., Calixto, J., 2003. Analysis of the inflammatory response in the rat paw caused by the venom of Apis melifera bee. Inflamm. Res. 52, 132–139.

Damianoglou, A., Rodger, A., Pridmore, C., R Dafforn, T., A Mosely, J., M Sanderson, J., R Hicks, M., 2010. The synergistic action of melittin and phospholipase A2 with lipid membranes: development of linear dichroism for membrane-insertion kinetics. Protein Pept. Lett. 17, 1351–1362.

Damodaran, S., 2008. Amino acids, peptides and proteins. Fennema's Food Chem. 4, 425–439.

De Lima, P., Brochetto-Braga, M., 2003. Hymenoptera venom review focusing on Apis mellifera. J. Venom. Anim. Toxins Trop. Dis. 9, 149–162.

Dill, K.A., Shortle, D., 1991. Denatured states of proteins. Annu. Rev. Biochem. 60, 795–825.

Drake, A., Hider, R., 1979. The structure of melittin in lipid bilayer membranes. Biochim. Biophys. Acta 555, 371–373.

Fidelio, G.D., Maggio, B., Cumar, F.A., 1984. Interaction of myelin basic protein, melittin and bovine serum albumin with gangliosides, sulphatide and neutral glycosphingolipids in mixed monolayers. Chem. Phys. Lipids 35, 231–245.

Gauldie, J., Hanson, J.M., Rumjanek, F.D., Shipolini, R.A., Vernon, C.A., 1976. The peptide components of bee venom. Eur. J. Biochem. 61, 369–376.

Hillman, L., 1983. Ultraviolet apparatus for disinfection and sterilization of fluids. Google Patents.

Jeong, I., Kim, B.-S., Lee, H., Lee, K.-M., Shim, I., Kang, S.-K., Yin, C.-S., Hahm, D.-H., 2009. Prolonged analgesic effect of PLGA-encapsulated bee venom on formalin-induced pain in rats. Int. J. Pharm. 380, 62–66.

Kwon, Y.-B., Kim, J.-H., Yoon, J.-H., Lee, J.-D., Han, H.-J., Mar, W.-C., Beitz, A.J., Lee, J.-H., 2001. The analgesic efficacy of bee venom acupuncture for knee osteoarthritis: a comparative study with needle acupuncture. Am. J. Chin. Med. 29, 187–199.

Lee, J.-H., Kwon, Y.-B., Han, H.-J., Mar, W.-C., Lee, H.-J., Yang, I.-S., beitz, A.J., Kang, S.-K., 2001. Bee venom pretreatment has both an antinociceptive and anti-inflammatory effect on carrageenan-induced inflammation. J. Vet. Med. Sci. 63, 251–259.

Lee, J.-D., Park, H.-J., Chae, Y., Lim, S., 2005. An overview of bee venom acupuncture in the treatment of arthritis. Evid. Based Complement. Alternat. Med. 2, 79–84.

Lee, J.-A., Jung, B.-G., Kim, T.-H., Kim, Y.-M., Park, M.-H., Hyun, P.-m., Jeon, J.-w., Park, J.-k., Cho, C.-W., Suh, G.-H., Lee, B.-J., 2014. Poly d,l-lactide-co-glycolide (PLGA) nanoparticle-encapsulated honeybee (Apis melifera) venom promotes clearance of *Salmonella enterica* serovar Typhimurium infection in experimentally challenged pigs through the up-regulation of T helper type 1 specific immune responses. Vet. Immunol. Immunopathol. 161, 193–204.

Lee, J.-A., Kim, Y.-M., Kim, T.-H., Lee, S.-H., Lee, C.-A., Cho, C.-W., Jeon, J.-w., Park, J.-k., Kim, S.-K., Jung, B.-G., Lee, B.-J., 2016. Nasal delivery of chitosan-coated poly (lactide-co-glycolide)-encapsulated honeybee (Apis mellifera) venom promotes Th 1-specific systemic and local intestinal immune responses in weaned pigs. Vet. Immunol. Immunopathol. 178, 99–106.

Liu, X., Chen, D., Xie, L., Zhang, R., 2002. Effect of honey bee venom on proliferation of K1735M2 mouse melanoma cells in-vitro and growth of murine B16 melanomas in-vivo. J. Pharm. Pharmacol. 54, 1083–1089.

Mahmoodzadeh, A., Morady, A., Zarrinnahad, H., Bagheri, K.P., Ghasemi-Dehkordi, P., Mahdavi, M., Shahbazzadeh, D., Shahmorady, H., 2013. Isolating melittin from bee venom and evaluating its effect on proliferation of gastric cancer cells. Basic Clin. Cancer Res. 5, 26–32.

Mahmoodzadeh, A., Zarrinnahad, H., Bagheri, K.P., Moradia, A., Shahbazzadeh, D., 2015. First report on the isolation of melittin from Iranian honey bee venom and evaluation of its toxicity on gastric cancer AGS cells. J. Chin. Med. Assoc. 78, 574–583.

Mark, J.E., 2007. Physical Properties of Polymers Handbook. Springer.

Mizrahi, A., Lensky, Y., 2013. Bee Products: Properties, Applications, and Apitherapy. Springer Science & Business Media.

Moreno, M., Giralt, E., 2015. Three valuable peptides from bee and wasp venoms for therapeutic and biotechnological use: Melittin, apamin and mastoparan. Toxins 7, 1126–1150.

Mousavi, S.M., Imani, S., Haghighi, S., Mousavi, S.E., Karimi, A., 2012. Effect of Iranian honey bee (Apis mellifera) venom on blood glucose and insulin in diabetic rats. J. Arthropod. Borne Dis. 6, 136.

Muñoz, V., Sanchez-Ruiz, J.M., 2004. Exploring protein-folding ensembles: a variable-barrier model for the analysis of equilibrium unfolding experiments. Proc. Natl. Acad. Sci. 101, 17646–17651.

Nguyen, K.T., West, J.L., 2002. Photopolymerizable hydrogels for tissue engineering applications. Biomaterials 23, 4307–4314.

Noodeh, M.F., Divsalar, A., Seyedarabi, A., Saboury, A.A., 2018. Antiproliferative and interaction studies of a synthesized palladium (II) complex with human hemoglobin. J. Mol. Liq. 249, 265–271.

Oršolić, N., 2012. Bee venom in cancer therapy. Cancer Metastasis Rev. 31, 173–194.
Park, Z.-Y., Russell, D.H., 2000. Thermal denaturation: a useful technique in peptide mass mapping. Anal. Chem. 72, 2667–2670.
Piek, T., 2013. Venoms of the Hymenoptera: Biochemical, Pharmacological and Behavioural Aspects. Elsevier.
Rady, I., Siddiqui, I.A., Rady, M., Mukhtar, H., 2017. Melittin, a major peptide component of bee venom, and its conjugates in cancer therapy. Cancer Lett. 402, 16–31.
Shipolini, R.A., Callewaert, G.L., Cottrell, R.C., Vernon, C.A., 1974. The amino-acid sequence and carbohydrate content of phospholipase A2 from bee venom. Eur. J. Biochem. 48, 465–476.
Son, D.J., Lee, J.W., Lee, Y.H., Song, H.S., Lee, C.K., Hong, J.T., 2007. Therapeutic application of anti-arthritis, pain-releasing, and anti-cancer effects of bee venom and its constituent compounds. Pharmacol. Ther. 115, 246–270.
Tosteson, M., Holmes, S., Razin, M., Tosteson, D., 1985. Melittin lysis of red cells. J. Membr. Biol. 87, 35–44.
Urtubey, N., 2005. Apitoxin: From Bee Venom to Apitoxin for Medical Use. Termas de Rio Grande Santiago del Estero, Argentina.
Wachinger, M., Kleinschmidt, A., Winder, D., von Pechmann, N., Ludvigsen, A., Neumann, M., Holle, R., Salmons, B., Erfle, V., Brack-Werner, R., 1998. Antimicrobial peptides melittin and cecropin inhibit replication of human immunodeficiency virus 1 by suppressing viral gene expression. J. Gen. Virol. 79, 731–740.
Watala, C., Kowalczyk, J.K., 1990. Hemolytic potency and phospholipase activity of some bee and wasp venoms. Comp. Biochem. Physiol. C 97, 187–194.
Yang, E.J., Jiang, J.H., Lee, S.M., Yang, S.C., Hwang, H.S., Lee, M.S., Choi, S.-M., 2010. Bee venom attenuates neuroinflammatory events and extends survival in amyotrophic lateral sclerosis models. J. Neuroinflammation 7, 69.
Zarrinnahad, H., Mahmoodzadeh, A., Hamidi, M.P., Mahdavi, M., Moradi, A., Bagheri, K.P., Shahbazzadeh, D., 2018. Apoptotic effect of Melittin purified from Iranian honey bee venom on human cervical cancer HeLa cell line. Int. J. Pept. Res. Ther. 24, 563–570.