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Antioxidant activity and irritation property of venoms from *Apis* species

Suvimol Somwongin [a], Panuwan Chantawannakul [b, c], Wantida Chaiyana [a, d, *]

[a] Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Chiang Mai, 50200, Thailand
[b] Bee Protection Laboratory, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, 50200, Thailand
[c] International College of Digital Innovation, Chiang Mai University, Chiang Mai, 50200, Thailand
[d] Research Center of Pharmaceutical Nanotechnology, Chiang Mai University, Chiang Mai, 50200, Thailand

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ABSTRACT

Pharmacological effects of bee venom has been reported, however, it has been restricted to the bee venom collected from European honey bee (*Apis mellifera*). The aim of the present study was to compare the antioxidant activities and irritation properties of venoms collected from four different *Apis* species in Thailand, which includes *Apis cerena* (Asian cavity nesting honeybee), *Apis florea* (dwarf honeybee), *Apis dorsata* (giant honeybee), and *A. mellifera*. Melittin content of each bee venom extracts was investigated by using high-performance liquid chromatography. Ferric reducing antioxidant power, 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid), and 1, 1-diphenyl-2-picrylhydrazyl assay were used to determine the antioxidant activity, whereas, hen's egg test chorioallantoic membrane assay was used to determine the irritation property of each bee venom extracts. Melittin was the major constituent in all bee venom extracts. The melittin content in *A. dorsata*, *A. mellifera*, *A. florea*, and *A. cerena* were 95.8 ± 3.2%, 76.5 ± 1.9%, 66.3 ± 8.6%, and 56.8 ± 1.8%, respectively. Bee venom extract from *A. dorsata* possessed the highest antioxidant activity with the inhibition of 41.1 ± 2.2% against DPPH•, Trolox equivalent antioxidant capacity of 10.21 ± 0.74 mM Trolox/mg and equivalent concentration (EC_1) of 0.35 ± 0.02 mM $FeSO_4$/mg. Bee venom extract from *A. mellifera* exhibited the highest irritation, followed by *A. cerena, A. dorsata,* and *A. florea,* respectively. Melittin was the compound responsible for the irritation property of bee venom extracts since it could induce severe irritation (irritation score was 13.7 ± 0.5, at the concentration of 2 mg/ml). The extract from *A. dorsata* which possessed the highest antioxidant activity showed no irritation up to the concentration of 0.1 mg/ml. Therefore, bee venom extract from *A. dorsata* at the concentration not more than 0.1 mg/ml would be suggested for using as cosmetic ingredients since it possessed the highest antioxidant activity with no irritation. This study is the first report to compare the bee venom extracts from different *Apis* species and display their potential application of bee venom extracts in cosmetic products.

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1. Introduction

Bee venom (BV) can be produced by all species of honey bees, (e.g. *Apis mellifera*, *Apis cerena*, *Apis florea*, and *Apis dorsata*). Among these species, *A. mellifera*, the European honey bee, is the cosmopolitan species and extensively used in apiculture all over the world (Park et al., 2014a,b). Several researches have reported the potent pharmacological effects of BV from *A. mellifera*, including antibacterial (Issam et al., 2015), antiviral (Lee, 2016), anti-cancer (Son et al., 2007; Wang et al., 2009), anti-inflammatory (Im et al., 2016) and anti-coagulation effects (Zolfagharian et al., 2015). However, there is scarce study about other honey bee species. The present study is the first study to report antioxidant and irritation properties of *A. cerena* (Asian cavity nesting honeybee), *A. florea* (dwarf honeybee), and *A. dorsata* (giant honeybee) so as to evaluate their potential to be used in cosmetic products.

BV, also known as apitoxin secreted by bee venom glands, is composed of a complex mixture of various components, including proteins, peptides, and small molecules (Akhmerova et al., 2015; Lee et al., 2015a,b). However, the major component of BV is

* Corresponding author. Department of Pharmaceutical Science, Faculty of Pharmacy, Chiang Mai University, Chiang Mai, 50200, Thailand.
E-mail address: wantida.chaiyana@cmu.ac.th (W. Chaiyana).

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melittin, which is a large peptide with the molecular weight of 2840 Dalton. Melittin is a 26-amino acid peptide composing of 6 hydrophilic amino acids residue in the C-terminal region and 20 hydrophobic amino acids residue in the N-terminal region (Park et al., 2007; Dong et al., 2015; Chen et al., 2016). Therefore, melittin is highly aqueous soluble and play important roles in various applications, including pharmaceutical industries (Park et al., 2014a,b). However, BV could cause various adverse reactions, such as anaphylactic shock, edema, and allergy because melittin could form a stable complex with cell membrane of corneocyte and resulted in cytotoxic effect to the living cells (Lauterwein et al., 1979; Komi et al., 2017). Additionally, melittin has also been recognized for the prolonged painful stimulation after bee sting, while other polypeptides in BV contribute only to the early nociceptive response within 10–20 min after the sting (Lariviere and Melzack, 1996; Chen and Guan, 2017). The nociceptive effect of melittin was unique and different from that of the other compounds (Wheeler-Aceto et al., 1990; Hong and Abbott, 1994). Therefore, melittin plays a central role in the production of long-lasting pain, hyperalgesia, and local inflammation (Lariviere and Melzack, 1996; Chen and Guan, 2017; Akhmerova et al., 2015).

Nowadays, BV extracts are recently used as primary ingredients in various cosmetic formulations, including facial creams, balms, masks, and serums according to its protective properties against bacterial and inflammation on the skin (Han et al., 2013; Lee et al., 2015a,b). However, the precaution needs to be taken over since high amount of BV incorporated into cosmetic formulations may lead to adverse effects of melittin. Therefore, the aims of the present study were to compare the melittin content of BV extracts form four bee species, including *A. mellifera*, *A. cerena*, *A. florea*, and *A. dorsata*. Moreover, antioxidant activity and irritation properties of various BV extracts were also investigated.

2. Materials and methods

2.1. Bee venom materials

A. mellifera was obtained from bee farm at Department of Biology, Faculty of Science, Chiang Mai University, Thailand. *A. cerena*, *A. dorsata*, and *A. florea* were naturally occurred in different parts of Chiang Mai, Thailand, including Saraphi District, Muang District, and Mae Rim District, respectively. The worker bees were captured and directly displaced in liquid N_2 and stored at −80 °C (Park et al., 2014a,b). The BV extracts from all bee species were manually collected and then lyophilized using a freeze dryer (Alpha 1–2 LD model Christ, Germany) and stored at −20 °C until further analysis.

2.2. Chemical materials

Acetonitrile, acetic acid, ascorbic acid (vitamin C), trifluoroacetic acid (TFA), dimethyl sulfoxide (DMSO), 2, 4, 6 tripyridyl-s-triazine (TPTZ), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH), potassium persulfate ($K_2S_2O_8$), ferrous sulfate ($FeSO_4$), ferric chloride ($FeCl_3$), hydrochloric acid, potassium chloride, sodium chloride, disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), sodium lauryl sulfate (SLS), and sodium acetate trihydrate ($C_2H_3NaO_2 \cdot 3H_2O$) were purchased from Sigma Aldrich (St. Louis, MO, USA). Methanol and ethanol were of analytical grade and obtained from Merck (Darmstadt, Germany). Melittin from *A. mellifera, A. cerena, A. florea*, and *A. dorsata* with the purity of 95.05%, 96.43%, 95.43%, and 98.25%, respectively, were obtained from Synpeptide Co., Ltd. (Shanghai, China).

2.3. Melittin content determination by high-performance liquid chromatography (HPLC) technique

The HPLC analysis was performed using a HP1100 system with a thermostatically controlled column oven a UV detector set at 280 nm (Hewlett-Packard, Palo Alto, CA, USA) instrument, operated at ambient temperature, consisting of an automatic autosampler system. The column used was a C18 column (Zobrax, 150 × 4.6 mm, 5 μm particle size, Agilent, CA, USA). The mobile phase, consisted of 0.1% TFA in DI water and 100% of acetonitrile in a ratio of 60:40, were used for sample elution at ambient temperature with the flow rate of 1 ml/min (Dong et al., 2015). Each BV extracts was dissolved in PBS (pH 5.5) at concentration of 1 mg/ml and filtered through a 0.45 μm membrane filter prior to use. Injection volume was 20 μl. Commercial melittin from each bee species were used as a marker for the quantitative analysis (Zhou et al., 2010).

2.4. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay

The free radical scavenging effects of BV extracts were evaluated by DPPH assay (Brem et al., 2004; Chaiyana et al., 2017). Briefly, 20 μl of various concentration the BV extracts (0.25, 0.5 and 1 mg/ml) in PBS (pH 5.5) was mixed with 180 μl of DPPH• solution in methanol. After kept in the dark for 30 min, the absorbance of the mixture was measured at 520 nm. The scavenging activities reported as percentage of inhibition which were calculated using the following equation: %inhibition = [(C-S)/C] × 100, where C is the absorbance of the mixture solution containing the sample and S is the absorbance of the mixture solution containing DPPH• and sample solution. Ascorbic acid was used as a positive control. The experiments were done in triplicate.

2.5. Ferric reducing/antioxidant power (FRAP) assay

The reducing properties was determined based on the capacity of ferric ion reducing activities (Saeio et al., 2011; Chaiyana et al., 2017). Briefly, 20 μl of 1 mg/ml of each BV extract in PBS (pH 5.5) was mixed with 180 μl of FRAP solution, which was previously prepared by mixing 5 ml of 10 mM TPTZ solution in 40 mM HCl and 5 ml of 20 mM $FeCl_3$ in 50 ml of 0.3 M acetate buffer (pH 3.6). After kept at room temperature for 5 min, the absorbance of the mixture was measured at 595 nm. Ascorbic acid was used as a positive control. The experiments were done in triplicate.

2.6. 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

The free radical scavenging effects of BV extracts were evaluated by ABTS assay (Tachakittirungrod et al., 2007; Chaiyana et al., 2017). Briefly, 20 μl of the extracts in PBS (pH 5.5) was mixed with 180 μl of ABTS solution. After kept in the dark for 16 h, the ABTS free radical ($ABTS^{\bullet +}$) solution, which was previously prepared by mixing 7 mM of ABTS solution and 2.45 mM of potassium persulphate ($K_2S_2O_8$) solution in a ratio of two to three, was diluted with 20-fold ethanol to obtain absorbance of 0.7 ± 0.1 units at 750 nm. The free radical-scavenging activity was reported as Trolox equivalent antioxidant capacity (TEAC) which represent the amount of Trolox that equivalent to 1 mg of the test BV extract. Ascorbic acid was used as a positive control. The experiments were done in triplicate.

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