Macedonian pharmaceutical bulletin, 68 (Suppl 1) 315 - 316 (2022)
Online ISSN 1857 - 8969
DOI:10.33320/maced.pharm.bull.2022.68.03.152
Short communication

Characterization of honey bee venom from Spilje, Debar region, N. Macedonia

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Introduction

Honey Bee Venom (BV) has been used to treat many diseases since ancient times. In recent years some positive effects of BV usage have received scientific confirmation. BV has been widely used for the treatment of pain and inflammation, and in immune-related diseases such as rheumatoid arthritis and multiple sclerosis. Various scientific studies have reported that components of whole BV have numerous beneficial therapeutic effects, such as immune-stimulating, anticancer and radioprotective activities (Park et al., 2018).

BV is an intricate mixture of at least 18 active chemical compounds, including peptides (melittin and apamine), various enzymes (phospholipase A and hyaluronidase), amines and nonpeptide components with various pharmaceutical properties (carbohydrates, phospholipids and some volatile components) (Carpena et al., 2020). The main component of dry BV is the peptide melittin (40-50%), which is the most bioactive compound in BV with vast bioactivity usable in broad modern medical therapies (Ceremuga et al., 2020).

The aim of this study was to determine the quality and purity of the domestic BV collected from *Apis mellifera macedonica* by evaluating the total protein content, melittin content by high performance liquid chromatography (HLPC), structural and physical properties (X-ray diffraction (XRD), differential scanning calorimetry (DSC) and fourier transform infrared spectroscopy (FT-IR).

Materials and methods

BV as dry powder was collected by electric stunning, without harming honey bees during July 2021 (Spilje, Debar region, N. Macedonia) and was stored at -20 °C.

Moisture determination analysis was carried out at 105˚C with a Mettler Toledo Excellence HS153 (Mettler Toledo, Netherlands) moisture analyzer.

Total protein content in BV sample was determined utilizing the biuret method (540 nm, spectrophotometrically Cary 60UV-VIS, Agilent Instruments, USA). Distilled water with biuretic reagent was used as a blind test and bovine serum albumin (BSA) was used as the external standard.

A modified HPLC method (Rybak-Chmielewska and Szczêsna, 2004) was used for assay of melittin, apamin and phospholipase A2 in the BV sample. Melittin (Sigma, USA) was used as an external standard. Detection and assay determination was performed on Agilent 1100 Series, equipped with diode array detector, using C18 Zorbax 300 SB 150 mm x 4.6 mm; 5 µm; gradient elution with 0.1% trifluoroacetic acid (TFA) in water for liquid chromatography (mobile phase A) and 0.1% TFA in mixture of acetonitrile/water for liquid chromatography=80:20 (mobile phase B), were filtered through a 0.45-µm membrane filter and degassed via an ultrasonic water bath prior to use. The chromatographic conditions are: injection volume 20 µL, the column temperature was maintained at 40 ºC, detection was carried out at 220 and the flow rate is 2.5 ml/min. The

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gradient profile was optimized as follows: 0-2 min, isocratic 70% (*v/v*) A; 2-10 min, linear gradient 70-40% (*v/v*) A; 10-25 min, linear gradient 40-20% (*v/v*) A; 25-29 min, isocratic gradient 20% (*v/v*) A. The prepared mobile phase was degassed prior to use, under vacuum, by filtration through a PVDF filter. The sample preparation was centrifuged at 12,000 rpm for 10 min at 4 °C.

The identification and structure determination were done using an experimental powder XRD technique based on the use of X-rays and their diffraction (Benchtop X-ray diffractometer, MiniFlex 600C, Rigaku, Japan). Data were collected over an angular range comprised between 5 ° and 50 °, with a step size of 0.02 °. DSC was performed on the BV samples (2 mg) using differential scanning calorimeter (DSC 204 F1 Phoenix, NETZSCH, Germany). DSC was conducted from 25 to 250°C and backwards at a rate of 10°C/min. A FT-IR spectrometer (Varian 660 FT-IR, Varian Instruments, USA) was used to record the infrared spectra of the BV using the MIRAcle module with ZnSe crystal and micrometer clamp for low pressure recording under attenuated total reflection (ATR). The spectra were recorded in the range of 4000 - 550 cm^{-1}, with an average of 16 scans for each spectrum and a resolution of 4 cm^{-1}.

Results and discussion

Obtained BV appeared in the form of a typical powder with a light brown color, specific smell and 10.85%±0.12 moisture content.]

Total protein content of the BV sample was 56%. Using the stated HPLC-UV method, retention times were 23.08 minutes for melittin, 9.57 minutes for apamin and 16.705 minutes for phospholipase A2. Melittin content in the tested BV was 42.8%, suggesting a relatively good quality of the sample.

According to XRD analysis, BV showed regular crystal structure peaks at 2θ=9.9° and 21.3°. DSC thermogram of BV showed an endothermic peak at approximately 73.1°C, which is associated with the melting point of the specimen itself, and endothermic peak at 186°C which could be attributed to thermal degradation of the BV sample. On the other hand, on the DSC curve of the cooling segment, no phase transformations were observed, which confirms the decomposition of the sample.

The FT-IR spectra of the BV showed a broad medium-intensity absorption band in the spectral range from 3500 to 3100 cm^{-1} (with an absorption maximum at 3280 cm^{-1}), which is assigned to the N–H stretching vibrations (amide A band) of the peptide and protein secondary structures. Intensity absorptions observed at 2923 cm^{-1} and 2852 cm^{-1} correspond to CH_2 asymmetric stretching vibrations. The fingerprint region (spectral region between 1700 and 600 cm^{-1}) showed a series of absorption bands that are unique for the peptide/protein secondary structure. The most prominent vibrations arising at 1648 sm^{-1} and 1538 sm^{-1} are assigned to the amide I (C=O stretching) and amide II (N–H bending and C–N stretching vibrations) bands.

Conclusion

The chemical composition and quality of honey BV obtained directly from the primary producer (Debar region, N. Macedonia) was investigated in this study. The analyzed BV showed average quality properties. Further studies will be focused on determination of the quality of BV samples from other regions in N. Macedonia.

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