

Major Components of Clinically used Bee Venom Pharmacopuncture[※]

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[Abstract]

Objectives : The purpose of this study was to analyze the components of the clinically used bee venom (BV) pharmacopuncture.

Methods : Two kinds of bee venom pharmacopuncture (BV–I and II), three kinds of separate purification BV (SPBV) pharmacopuncture (SPBV–I, II, and III), and apitoxin were investigated in this study. We performed a component analysis of melittin, apamin, and phospholipase A₂ using high–performance liquid chromatography (HPLC).

Results : 1. BV–I contained approximately 40% more melittin than BV–II did.

2. In the three separate purification BV pharmacopuncture, SPBV–I, SPBV–II, and SPBV–III, phospholipase A₂ content decreased remarkably.

3. The melittin content in SPBV–I increased by 5% compared to that in BV–I.

4. The amount of melittin in apitoxin was similar to that in SPBV–I.

Conclusion : The compositions of the BV pharmacopuncture and separate purification BV pharmacopuncture changed depending on the collection method and concentration. Therefore, it is necessary to choose the most suitable BV for each specific medical treatment target. Furthermore, research into the composition of BV may be needed for its safe and effective use.

Key words :

Venom (BV);
Melittin;
Apamin;
Phospholipase A₂
HPLC;
Korean Medicine

Received : 2017. 01. 24.

Revised : 2017. 02. 05.

Accepted : 2017. 02. 06.

On–line : 2017. 02. 20.

※ This paper was supported by the Semyung University Research Grant of 2015.

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I. Introduction

Bee venom (BV) pharmacopuncture is used to treat diseases and requires the extraction of venom from live bees. BV has been used for 2,500 years, and classic treatment involves injecting the venom of a live bee directly into the affected body area¹. Korean medical doctors mainly use extracted and purified BV for treatment. The BV is injected into the meridian points or disease-related areas according to Korean medical principles. This method combines the effect of the acupuncture with that of the components of the BV². BV treatments are currently used in the US, Russia, and Europe to treat patients in the form of formulations such as injections or ointments, mainly for arthritis, atopy, and rheumatic diseases. BV treatment is recognized as an area of alternative medicine in many countries³.

Chromatography is a method used to separate the components of a mixture using mobile and stationary phases. Among the various types, liquid chromatography is characterized by the use of a liquid as the mobile phase. The mobile phase in which the sample of the test chemical substance is dissolved is pushed by a pump at a constant flow rate under high pressure to facilitate its passage through a fixed phase column containing a filler. Then, the individual chemical substances in the test sample pass through the column at different times according to their affinity for the mobile and fixed phases. Thus, high-performance liquid chromatography (HPLC) is a method for quantifying a

particular chemical by measuring the magnitude of its separation over time⁴.

Previously, Lee⁵ has studied the main components of bee venom and Kwon^{6,7} has studied the composition and safe use of sweet bee venom. The study by Kang⁸ confirmed the stability of Sweet BV. An's⁹ study compared the melittin content of BV from domestic sources and foreign countries. All of these studies used HPLC for the analyses. There have been some studies on BV components, but no study has compared BV and separate purification BV (SPBV) pharmacopuncture methods, which are commonly used in clinical practice by Korean medical doctors. Therefore, we analyzed three components (melittin, apamin, and phospholipase A₂) in BV and SPBV using HPLC and the results are reported here.

II. Materials and Methods

1. Materials

BV is produced using electrical and microwave stimulation methods. The BV used for BV pharmacopuncture, mainly used clinically, was used as the analytical sample. Two kinds of BV pharmacopuncture (BV-I and II), three kinds of SPBV pharmacopuncture (SPBV-I, II, and III), and apitoxin were investigated in these experiments. The samples were diluted to the appropriate concentration using distilled water (Table 1).

Table 1. Abbreviation and Conditions about samples

Abbreviation	Conditions
BV-I	Bee venom pharmacopuncture using electrical stimulation (dilution 1:4000)
BV-II	Bee venom pharmacopuncture by microwave stimulation(dilution 1:4000)
SPBV-I	Separate purification bee venom pharmacopuncture using electrical stimulation (dilution,1:4000)
SPBV-II	Separate purification bee venom pharmacopuncture using microwave stimulation (dilution 1:4000)
SPBV-III	Separate purification bee venom pharmacopuncture using microwave stimulation (dilution 1:8000)

2. Reagents

Methanol, acetonitrile (both Sigma-Aldrich, USA), and distilled water (Welgene, Korea) were used for the HPLC analyses. Melittin, apamin, and phospholipase A₂ (all Sigma-Aldrich, USA) were used as standard products for the HPLC analysis.

3. Methods

An HPLC system (Agilent 1200, USA) was used for the analysis, and it was equipped with a Varian 9300, 510 pump, U6K injector, and ultraviolet (UV) absorbance detector as well as a computing integrator D520A device. An analytical HPLC column (Shiseido, Japan, 5- μ m, C18, 300 Å, 3.9 mm \times 250 mm) was used. The melittin, apamin, and phospholipase A₂ standard solutions were prepared at concentrations of 10, 20, 50, and 100 μ g/mL (Table 2).

III. Results

1. Analysis of main components of BV using HPLC

1) Construction of calibration curve of melittin standard solutions using HPLC

Melittin standard solutions were analyzed at concentrations of 10, 20, 50, and 100 μ g/mL. The correlation coefficient of the calibration curve was 0.9984, and the calibration curve had a linear function, $y = 13960x - 21479$ (Fig. 1). The retention time of melittin was 32.044 min.

2) Construction of calibration curve of apamin standard solutions using HPLC

The apamin standard solutions were analyzed at concentrations of 10, 20, 50, and 100 μ g/mL. The

Table 2. Analytical Conditions for measuring of Melittin, apamin, and Phospholipase A₂

Item	Condition
Column	Shiseido, μ , C18, 300 Å, 3.9 mm \times 250 mm
Mobile phase	A: 0.1% Trifluoroacetic acid (TFA) 2 L B: 0.1% TFA in acetonitrile:water (80:20)
Gradient	5% B-80% B for 40 min
Flow rate	1.5 mL/min
Wave length	220 nm
Sample volume	20 μ L

correlation coefficient of the calibration curve was 0.9991, and the calibration curve had a linear function, $y = 12704x - 113929$ (Fig. 2). The retention time of apamin was 11.437 min.

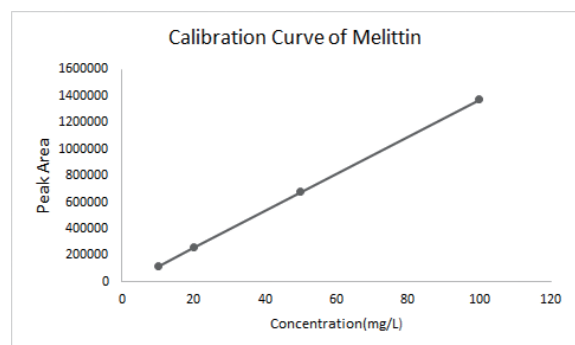


Fig. 1. Calibration Curve of Melittin ($y = 13960x - 21479$, $r^2 = 0.9984$)

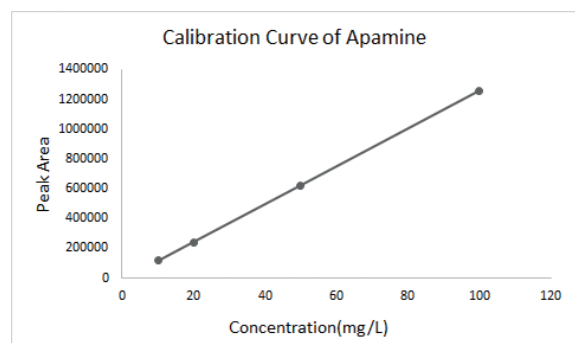


Fig. 2. Calibration Curve of apamin ($y = 12704x - 113929$, $r^2 = 0.9991$)

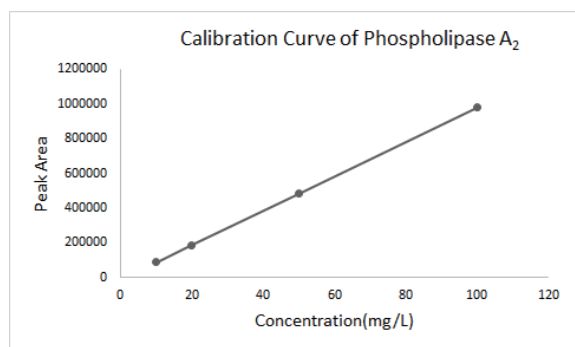


Fig. 3. Calibration Curve of Phospholipase A₂ ($y = 9931x - 12956$, $r^2 = 0.9987$)

3) Construction of calibration curve of phospholipase A₂ standard solutions using HPLC

The phospholipase A₂ standard solutions were analyzed at concentrations of 10, 20, 50, and 100 $\mu\text{g}/\text{mL}$. The correlation coefficient of the calibration curve was 0.9987, and the calibration curve had a linear function, $y = 9931x - 12956$ (Fig. 3). The retention time of phospholipase A₂ was 23.702 min.

2. Analysis of main components of BV-I

The analysis results of BV-I are as follows. The retention times of melittin, apamin, and phospholipase A₂ were 31.256, 11.240, 23.880 min, respectively. The peak areas of melittin, apamin, and phospholipase A₂ were 47.4751, 2.9602, and 26.5647%, respectively (Fig. 4).

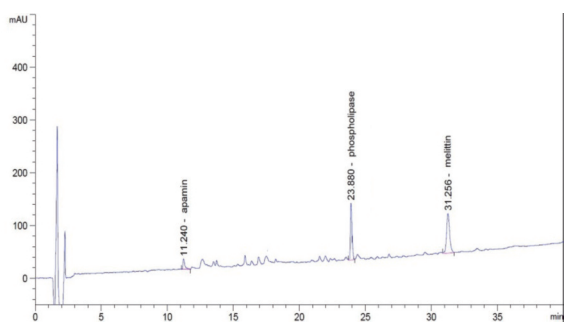


Fig. 4. HPLC chromatogram of BV-I

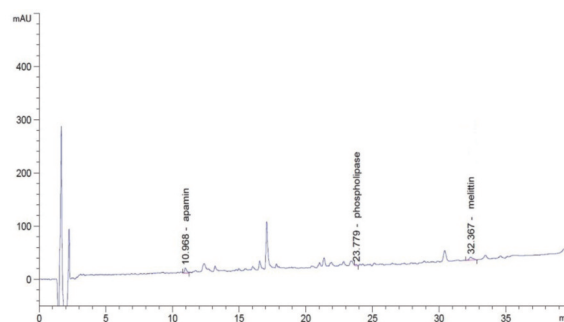


Fig. 5. HPLC chromatogram of BV-II

3. Analysis of main components of BV-II

The analysis results of BV-II are as follows. The retention times of melittin, apamin, and phospholipase A₂ were 32.367, 10.968, 23.779 min, respectively. The peak areas of melittin, apamin, and phospholipase A₂ were 5.7384, 3.6380, and 10.6236%, respectively (Fig. 5).

4. Analysis of main components of SPBV-I

The analysis results of SPBV-I are as follows. The retention times of melittin, apamin, and phospholipase A₂ were 31.981, 11.141, 23.729 min, respectively. The peak areas of melittin, apamin, and phospholipase A₂ were 52.1313, 0.0553, and 1.1427%, respectively. An unknown peak with an area of 38.6727% was observed at 11.763 min (Fig. 6).

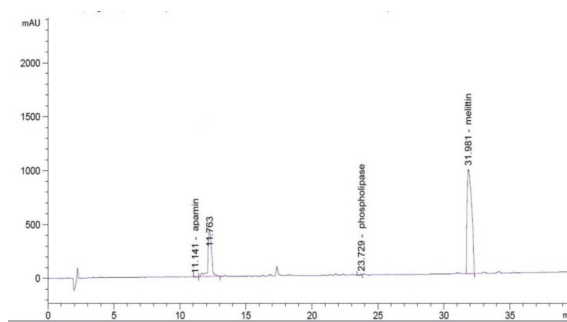


Fig. 6. HPLC chromatogram of BV-I

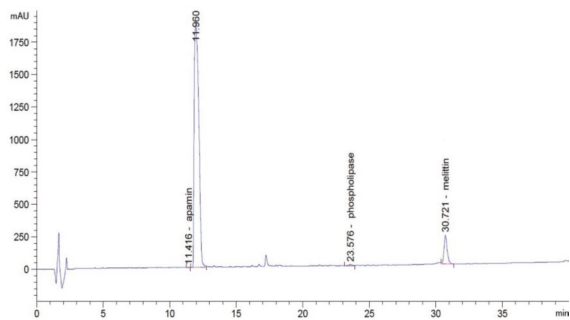


Fig. 7. HPLC chromatogram of BV-II

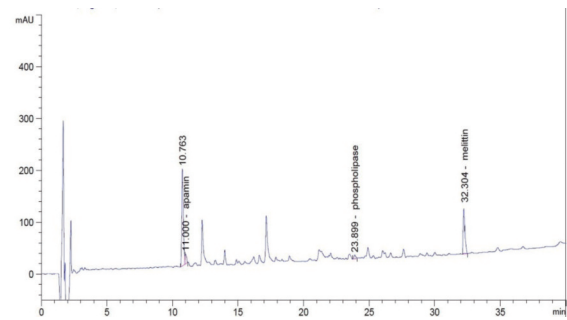


Fig. 8. HPLC chromatogram of BV-I

5. Analysis of main components of SPBV-II

The analysis results of SPBV-II are as follows. The retention times of melittin, apamin, and phospholipase A₂ were 30.721, 11.416, and 23.576 min. The peak areas of melittin, apamin, and phospholipase A₂ were 10.2250, 0.0208, and 1.4051%, respectively. An unknown peak with an area of 81.3537% was observed at 11,960 min (Fig. 7).

6. Analysis of main components of SPBV-III

The analysis results of SPBV-III are as follows. The retention times of melittin, apamin, and phospholipase A₂ were 32.204, 11.000, and 23.899 min, respectively. The peak areas of melittin, apamin, and phospholipase A₂ were 21.8047, 8.6487, and 0.9635%, respectively. An unknown peak with an area of 68.5831% was observed at 10.763 min (Fig. 8).

7. Analysis of main components of apitoxin

The analysis results of apitoxin are as follows. The retention time of melittin or apitoxin was 32.355 min while the peak area of melittin was 51.4417% (Fig. 9).

IV. Discussion

BV is a transparent liquid stored in venom sac. Following exposure to air, the water content evaporates and turns it crystalline. The specific gravity of BV is 1,1313 and, therefore, it is heavier than water while its pH is 5.2-5.5, which is slightly acidic. The effective amount of water removed from the BV is approximately 30%. Each bee has approximately 1 μ L of venom, which yields a dry weight of approximately 0.3 mg of crystals. Approximately 5,000-10,000 bees are required to produce 1 g of dried BV. BV is composed of approximately 40 main ingredients including peptides, enzymes, physiologically active amines, carbohydrates, lipids, and amino acids.

The major peptides are melittin, apamin, adolapin, and the mast cell degranulating (MCD) peptide. These peptides have anti-inflammatory, antibacterial, and antipyretic effects. Melittin is

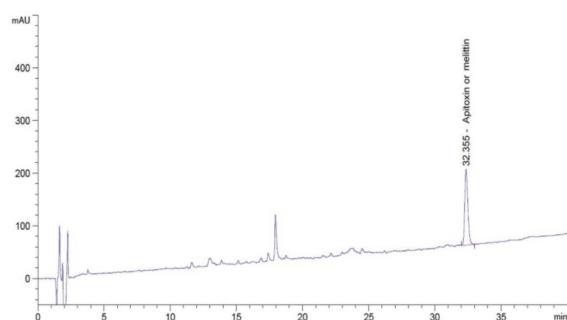


Fig. 9. HPLC chromatogram of Apitoxin

the most important component of BV, and it consists of 40–50% of the dry weight of BV. It has cell lysis and hemacytolysis properties, and induces histamine release and increases cortisone levels. Apamin is a representative neurotoxin among the BV components, which promotes nerve conduction and causes hyperactivity. An overdose of apamin causes muscle spasms and respiratory failure. Melittin and apamin stimulate axes including the hypothalamus, pituitary, and adrenal cortex. The action of both substances increases the secretion of adrenocortical hormones.

Phospholipase A_2 , which is the main enzyme in BV, is a polymer with a molecular weight $\geq 10,000$. Phospholipase A_2 constitutes 10–12% of the weight of dried BV and is a significant antigen of allergic reactions, which is the major cause of the systemic allergic reactions due to BV. Previous studies have shown that phospholipase A_2 induces immunoglobulin E (Ig E) synthesis¹⁰. It is also known that people who are allergic to BV have Ig E that responds to phospholipase A_2 ¹¹, which destroys cell membranes and causes cell damage.

In a previous study, three major components showed various effects. A study by Ahn¹² investigated the relationship between the efficacy of melittin and lung carcinoma while Kang¹³ reported the anti-inflammatory efficacy of melittin. The study by Han¹⁴ was on the effect of melittin on synovial cells. A study by Kim¹⁵ investigated the anticancer effect of apamin while the study by Kwon¹⁶ was on the anticancer effect of melittin and apamin. The study by Kim^{17,18} was on the neuroprotective effect of phospholipase A_2 . Possible methods for isolating BV components include dialysis, dissolution, filtration, and HPLC⁹. In particular, HPLC allows the precise analysis of small samples⁴ and has been used to measure purity after separating pure peptides from BV.

Reversed-phase (RP) HPLC is an important method of evaluating BV tablets¹⁹. Therefore, we analyzed two kinds of BV pharmacopuncture (BV-I and II), three kinds of SPBV pharmacopuncture (SPBV-I, II, and III), and apitoxin using HPLC.

Most of the previous component analysis studies have been on melittin and apamin. In this study, we analyzed phospholipase A_2 , which causes allergic reactions. And this study used two concentrations samples that most commonly used. The analysis results of BV-I revealed that the retention time of melittin was 31.256 min and its composition ratio was 47.4751%. The retention time of apamin was 11.240 min, and its composition ratio was 2.9602%. Melittin was detected at a concentration approximately 16 times higher than that of apamin. The analysis results of BV-II showed that the retention time of melittin was 32.367 min and its composition ratio was 5.7384%. The retention time of apamin was 10.968 min, and its composition ratio was 3.6380%. These values are lower than the average melittin and apamin values for BV. Similar results were also observed in the study of Kwon⁵.

We found that BV-I had more melittin than BV-II did at the same concentration. The analysis results of SPBV-I showed the retention time of Melittin was 31.981 min, and its composition ratio was 52.1313%. The retention time of apamin was 11.141 min, and its composition ratio was 0.0553%. Furthermore, the retention time of phospholipase A_2 was 23.729 min, and its composition ratio was 1.1427%. The unknown peak observed with a retention time of 11.763 min had a composition ratio of 38.6727%.

The analysis results of SPBV-II showed that the retention time of melittin was 30.721 min and its composition ratio was 10.2250%. The retention time of apamin was 11.416 min, and its composition ratio was 0.0208%. Furthermore, the retention time of phospholipase A_2 was 23.576 min, and its composition ratio was 1.4051%. The unknown peak observed with a retention time of 11.960 min had a composition ratio of 81.3537%.

The analysis results of SPBV-III revealed that the retention time of melittin was 32.204 min and its composition ratio was 21.8047%. The retention time of apamin was 11.000 min, and its composition ratio was 8.6487%. Furthermore, the

retention time of phospholipase A_2 was 23.899 min, and its composition ratio was 0.9635%. The unknown peak observed with a retention time of 10.763 min had a composition ratio of 68.5831%.

In the three SPBV pharmacopuncture analysis, phospholipase A_2 decreased remarkably. The analysis results of SPBV-I revealed that the melittin content of SPBV-I increased by 5% compared to that of BV-I. All three SPBV pharmacopuncture types showed an unknown peak. In addition, similar results were obtained in repeated experiments. It would be necessary to conduct further research studies on this. The analysis results of apitoxin showed that the retention time of melittin was 32.355 min and its composition ratio was 51.4417%. The amount of melittin in apitoxin is similar to that in SPBV-I. This study had a limitation in that we did not randomly test BV pharmacopuncture at various concentrations. Therefore, further studies using varying concentrations are needed.

V. Conclusion

We analyzed the components of BV pharmacopuncture, which is widely used in clinical practice, and arrived at the following conclusions.

1. BV-I contained approximately 40% more melittin than BV-II did.
2. In the three SPBV pharmacopuncture types (SPBV-I, SPBV-II, and SPBV-III), the phospholipase A_2 level decreased remarkably.
3. The melittin content in SPBV-I increased by 5% compared to that in BV-I.
4. The amount of melittin in apitoxin was similar to that in SPBV-I.

Therefore, we conclude that BV pharmacopunc-

ture and SPBV pharmacopuncture are suitable for clinical treatment. However, their compositions change depending on the collection method and concentration. Therefore, it is necessary to choose the most suitable BV for each medical treatment target. In addition, further research is needed to evaluate the safety and effectiveness of BV use.

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