



Safety of essential bee venom pharmacopuncture as assessed in a randomized controlled double-blind trial



Yong-jun Ahn^a, Joon-Shik Shin^a, Jinho Lee^a, Yoon Jae Lee^a, Me-riong Kim^a, Ye-sle Shin^a,
Ki Byung Park^a, Eun Jee Kim^a, Min-Jeong Kim^a, Jae-woong Lee^a, Hwa Dong Lee^b, Yoonmi Lee^b,
SungGeun Kim^b, Hwa-Jin Chung^a, In-Hyuk Ha^{a,*}

^a Jaseng Spine and Joint Research Institute, Jaseng Medical Foundation, 858 Eonju-ro, Gangnam-gu, Seoul, Republic of Korea

^b National Development Institute of Korean Medicine, Gyeongsang-si, Gyeongbuk, Republic of Korea

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ABSTRACT

Ethnopharmacological relevance: While bee venom (BV) pharmacopuncture use is common in Asia, frequent occurrence of allergic reactions during the treatment process is burdensome for both practitioner and patient. **Aim of the study:** This study compared efficacy and safety in isolated and purified essential BV (eBV) pharmacopuncture filtered for phospholipase A2 (PLA2) and histamine sections, and original BV to the aim of promoting safe BV pharmacopuncture use.

Materials and methods: In vitro, we examined the effect of BV and eBV on nitric oxide (NO) production induced by lipopolysaccharide (LPS) in RAW 264.7 macrophages, and clinically, 20 healthy adults aged 20–40 years were randomly allocated and administered eBV 0.2 mL and BV pharmacopuncture 0.2 mL on left and right forearm, respectively, and physician, participant, and outcome assessor were blinded to treatment allocation. Local pain, swelling, itching, redness, wheals, and adverse reactions were recorded by timepoint.

Results: eBV and BV exhibited similar inhibitory effects on NO production. Also, in comparison between eBV and BV pharmacopuncture administration areas on each forearm, eBV displayed significantly lower local pain at 24 h post-administration ($P=0.0062$), and less swelling at 30 min ($P=0.0198$), 2 ($P=0.0028$), 24 ($P=0.0068$), and 48 h post-administration ($P=0.0253$). eBV also showed significantly less itching at 24 ($P=0.0119$), 48 ($P=0.0082$), and 96 h ($P=0.0141$), while redness was significantly less at 30 min ($P=0.0090$), 6 ($P=0.0005$), and 24 h ($P<0.0001$). Time-by-treatment interactions were statistically significant for itching and redness ($P<0.001$, and $P<0.001$, respectively), and all original BV pharmacopuncture administered regions showed a tendency toward more severe itching and redness in later measurements.

Conclusions: eBV and BV displayed comparable anti-inflammatory effects, and eBV pharmacopuncture presented less local allergic reactions.

1. Introduction

Bee venom (BV) is one of the most commonly utilized animal venoms and comprises various chemical agents that induce allergic responses (Czarnetzki et al., 1990). BV therapy, where BV is used as a medicinal intervention, is available worldwide, but is primarily used in Asia, Eastern Europe, and South America (Alqutub et al., 2011). In particular, BV pharmacopuncture is a type of applied acupuncture treatment that uses BV extracted from the venom sac of live honeybees (western species of worker bee, *Apis mellifera*), which is injected at appropriate dose at acupuncture points selected through syndrome differentiation as a pharmacopuncture preparation to the aim of simultaneous acupuncture and pharmaceutical BV stimulation

(Korean Pharmacopuncture Institute, 1999).

BV has pain-relieving anti-inflammatory effects (Koh, 1992; Kwon and Koh, 1998), affects the immune system (Kwon et al., 1997), and has been shown to possess significant therapeutic effects on degenerative knee (Lee et al., 2003c; Wang et al., 2002) and hip joint osteoarthritis (Kim et al., 2001), rheumatoid arthritis (Lee et al., 2003a, 2003b), and lumbar intervertebral disc herniation (Jun et al., 2003; Kim et al., 2005; Lee et al., 2004). BV pharmacopuncture is also recommended in the major clinical practice guidelines published by the Korea Institute of Oriental Medicine for lumbar intervertebral disc herniation treatment by Korean Medicine doctors (KMDs) (Evidence-based Korean Medicine Clinical Practice Guideline Development Committee for Lumbar Herniated Intervertebral Disc Korea Institute

* Corresponding author.

E-mail address: hanihata@gmail.com (I.-H. Ha).

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of Oriental Medicine and the Society of Korean Medicine Rehabilitation, 2014).

In addition, pathways involved in the various oxidative stress, allergy, and inflammation-related effects of BV are being studied. In mechanism studies on oxidative stress, BV inhibited lipid peroxidation (LPO) levels, which were used as a marker to indicate membrane damage (Rekka et al., 1990), and superoxide production (Somerfield et al., 1984). Regarding phosphatase activity, BV was shown to induce alkaline phosphatase (ALP) and acid phosphatase (ACP) expression in serum levels (Hoffman, 2006; Ishay et al., 1977). Also, BV has been reported to exert anti-inflammatory properties by blocking expression of inflammatory mediators such as NO, COX-2, TNF- α , and IL-1 in inflammation-related mechanism studies (Son et al., 2007).

BV contains 40 main components, and of these constituents, peptides such as melittin, apamin, adolapin, and mast cell degranulating peptide (MCD peptide) play major roles in treatment. Melittin (a 26 amino acid peptide with the following sequence: Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gin-NH₂ which is the principal component of BV) takes up approximately 50% of dried BV, and is known to possess excellent pain-relieving and anti-inflammatory effects (Banks and Shipolini, 1986; Schmidt, 1986). However, phospholipase A₂ (PLA₂) and histamine are known to incur allergic reactions. Various allergic responses occurring during treatment impose a considerable burden on both practitioner and patient, and cases where the patient sustains anaphylactic shock from BV treatment as a systemic immediate hypersensitive response is the most severe type of hypersensitivity to BV (Heo, 1993; Hwang and Lee, 2000; Youn, 2005). To this aim, essential BV (eBV) was developed by removing macromolecular enzymes including PLA₂ and micromolecular substances including histamine to maximize active substance content and effectively block possible sources of allergic response for safe BV pharmacopuncture practice.

The basic research part of this study therefore investigated whether the original BV type and newly developed eBV differed in anti-inflammatory response at the same dose in vitro, and the randomized controlled trial (RCT) part compared eBV safety through local allergic response to promote wider clinical application of BV pharmacopuncture and Korean medicine.

2. Materials and methods

2.1. In vitro study

2.1.1. Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), sodium pyruvate, L-glutamine, antibiotics-antimycotics solution, and trypsin-EDTA were purchased from Invitrogen Co. (Grand Island, NY, USA). Lipopolysaccharide (LPS), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), bichinchonic acid (BCA), N-(1-naphthyl) ethylenediamine dihydrochloride, sulfanilamide, phosphoric acid, dimethyl sulfoxide (DMSO), melittin, histamine, PLA₂, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated.

Dried BV was commercially purchased (Chung Jin Biotech Co. Ltd., Ansan, Korea). For quantitative analyses, water, acetonitrile (ACN), and methanol (MeOH) of high performance liquid chromatography (HPLC) grades were purchased from JT Baker (Phillipsburg, NJ, USA). Trifluoroacetic acid (TFA) was purchased from Junsei Chemical (Tokyo, Japan).

2.1.2. Cell culture

Mouse macrophage RAW 264.7 cells obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 unit/mL penicillin, 100 μ g/mL streptomycin, and 0.25 μ g/mL amphotericin B.

Cells were incubated at 37 °C with 5% CO₂ in a humidified atmosphere.

2.1.3. Nitrite assay

To evaluate the inhibitory effect of BV and eBV on LPS-induced NO production, RAW 264.7 cells were plated in a 24-well culture plate (5×10^5 cells/mL), and incubated for 24 h. Cells were washed with PBS, fresh medium was added, and the cells were then incubated with 1 μ g/mL LPS in the presence or absence of BV or eBV. After 20 h of incubation, media was collected and analyzed for nitrite accumulation as an indicator of NO production by Griess reaction. Briefly put, 180 μ L of Griess reagents (0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in H₂O and 1% sulfanilamide in 5% H₃PO₄) was added to 100 μ L of each supernatant from LPS or BV or eBV-treated cells in 96-well plates. The absorbance was measured at 540 nm, and nitrate concentration was determined by comparison with a sodium nitrite standard curve. Percent inhibition was expressed as $[1 - (\text{NO level of BV or eBV} / \text{NO levels of vehicle treated control})] \times 100$. The IC₅₀ value, the sample concentration resulting in 50% inhibition of production, for BV, eBV, and melittin which was used as a positive control was determined using non-linear regression analysis (% inhibition versus concentration).

2.1.4. Cytotoxicity assay

To assess the cytotoxic effect of BV, eBV, and melittin in RAW 264.7 cells in the assay condition, MTT assay was performed. After the Griess reaction, MTT solution (final concentration of 500 μ g/mL) was added to each well and further incubated for 4 h at 37 °C. The media was discarded, and DMSO was added to each well to dissolve generated formazan. Absorbance was measured at 570 nm, and % survival was determined by comparison with the control (LPS only treated cells).

2.2. Clinical trial

2.2.1. Design and participants

This study was a participant, physician, and outcome assessor-blinded RCT. Subjects were healthy individuals aged 20–40 years and participant inclusion and exclusion criteria were as follows:

2.2.1.1. Inclusion criteria.

- 1) Individuals with no experience of previous BV pharmacopuncture administration.
- 2) Healthy male or female adults of age 20–39 years.
- 3) Individuals who give voluntary written consent to participate in the trial with no significant issues with expression of opinion.

2.2.1.2. Exclusion criteria.

- 1) Individuals taking conventional medicine for chronic disorders such as hypertension or diabetes.
- 2) Individuals with history of allergic dermatitis.
- 3) Individuals with sensory defect.
- 4) Individuals with psychopathic disorders.
- 5) Individuals with history of hypersensitivity to bee stings.
- 6) Individuals with fever symptoms (e.g. from colds).
- 7) Pregnant individuals.
- 8) Individuals who have apprehension regarding the trial or do not wish to participate.

Twenty participants were recruited through advertisements at Jaseng Hospital of Korean Medicine in Seoul, Korea, and the recruitment period was from January 21st to January 31st, 2015. Eligible individuals who gave voluntary and informed written consent to

participate received necessary examinations and tests according to the clinical trial protocol, and were included as study participants if they met inclusion and exclusion criteria and were deemed suitable for the clinical trial. Participants were informed of the assessment method (VAS, visual analogue scale) of subjective symptoms such as pain, discomfort, and itching from administration to 1 week post-administration prior to receiving pharmacopuncture.

Jaseng Hospital of Korean Medicine conducts 26,000+ sessions annually of the prescription (BV and eBV) employed in this clinical trial. This study followed guidelines of the Declaration of Helsinki and Tokyo and was approved by the Institutional Review Board at Jaseng Hospital of Korean Medicine (IRB approval no. KNJSIRB2015-1).

2.2.2. Preparation and formulation of BV and eBV

eBV was prepared using the following methods: 10 g of dried BV was dissolved in 20 mL of purified water and the solution was added to a sephadex G-25 column (diameter 6×height 90 cm) infused with purified water, and effluent was divided into 40 mL units. Subdivisions were analyzed using LC/MS (LCMS-2020, Shimadzu, Kyoto, Japan) and histamine-free units were collected. Collected units were filtered using Cogent M1 TFF System (Merck, Darmstadt, Germany) to eliminate substances, including PLA2, with a molecular weight of 10 kDa or higher. After freeze-drying the filtrate, any remaining moisture was removed (See Fig. A.1).

BV and eBV preparation concentrations were both set at 0.1 mg/mL for comparison of local allergic response, and were used after purification and preparation processing at the extramural herbal dispensary of Jaseng Hospital of Korean Medicine, which complies with K-GMP standards.

2.2.2.1. BV preparation method. Dried BV 1 g was dissolved in 10 L of purified water, and the prepared pharmacopuncture solution was sterile filtered and filled in 2 mL vials.

2.2.2.2. eBV preparation method. Dried eBV material 1 g was dissolved in 10 L of purified water to formulate pharmacopuncture solution. The prepared pharmacopuncture solution was sterile filtered and filled in 2 mL vials.

2.2.3. Pharmacopuncture administration procedure

Subjects received eBV on one forearm as the experimental material and BV on the other forearm as the control in this clinical trial. A clinical research coordinator familiar with the overall research procedure prepared the test eBV and control BV and a double blind test where neither the subject nor physician was unaware of BV type the was conducted following the supervision of the research coordinator for unbiased execution. The pharmacopuncture was administered by an experienced KMD who received 6 years of regular Korean medicine university education courses and 4 years of specialty training at a medical institution accredited by the Korean Ministry of Health and Welfare.

2.2.3.1. Randomization and allocation concealment. The participants of this clinical trial were randomly allocated into 2 groups: One group who received test eBV on the right forearm and control BV on the left, and a second group who received test eBV on the left forearm and control BV on the right. Random allocation was conducted by block randomization generated by a statistician blinded to study purpose and contents for even allocation of subjects to each group. Block size was set as 2 (physician and outcome assessor were blinded to block size). SAS version 9.1.3 statistical package (SAS Institute, Cary, NC, USA) was used to generate random numbers of uniform distribution, and a random allocation code was created using a program at a ratio of 1:1 to

the 2 groups. A research coordinator that did not participate in pharmacopuncture administration or assessment prepared the test and control BV types to be administered to the participants' left and right forearms in order of enrollment according to a code file with the random allocation code.

2.2.3.2. Blinding. Participants and physician were blinded to BV type administered to left and right arm as the research coordinator who did not participate in pharmacopuncture administration or assessment prepared the BV and eBV pharmacopuncture upon confirming the random number. The physician did not take part in outcome assessment. All assessment data was recorded in excel files and delivered to the statistician with no reference to random allocation. Therefore, the statistician was also blinded to administered BV type.

2.2.3.3. Intervention. The research coordinator filled 29G×1/2" (Shinchang medical Co., Ltd, Gumi, Korea) injection needles with 0.2 mL of BV or eBV, and handed the syringes to be injected to the left and right arms of the participant to the physician following the random allocation code. The physician injected the pharmacopuncture solution to left and right arm of the participant in compliance with the coordinator's provision. The administration site on each participant was designated as 5 cm below the middle of the elbow crease on the palmar side of both arms which is convenient for observation and has high responsiveness, and pharmacopuncture sessions were conducted in the morning which has relatively higher responsiveness (Rihoux, 1990).

2.2.4. Outcome measures

2.2.4.1. VAS. Levels of pain, swelling, and itching at the administration site was self-recorded using VAS immediately after administration, and 30 min, and 2, 6, 24, 48, 72, 96, 120, and 144 h post-administration. VAS uses a 10 cm line with labels for anchors at each end (Huskisson, 1974). Participants are asked to mark a point on the line that corresponds to their pain severity on the VAS pain scale, and the anchor labels at each end were assigned 'no pain' and 'pain as bad as it could be' (labels may be termed differently by study). Anchor labels for swelling were 'no swelling' and 'swelling as bad as it could be', and those for itching were 'no itching' and 'itching as bad as it could be'. As scores are measured in millimeters, overall range is 0–100 mm (Kersten et al., 2014). As severity may differ at rest and when active, patients were instructed as follows to minimize potential error: "Please indicate the level of pain/swelling/itching you experience during daily activities."

2.2.4.2. Measurement of wheals and redness size. The outcome assessor covered the participants' arms with clear adhesive cellophane large enough to cover the wheal and redness area immediately after administration, and the assessor marked the outline of the wheal and redness area with a nonerasable namepen-X marker (Monami, Gimpo, Korea) at 30 min, and 6, 24, and 72 h post-administration. The cellophane was removed from the participants' arms, and the diameter was calculated as the average of the sum of the lengths of the line connecting the 2 farthest points measured using a 0–200 mm dial caliper (Bluebird, Korea) (measurement unit: mm) (A) and the perpendicular line at its midpoint (B) to the nearest hundredth millimeter. The measurement process was repeated 3 times for each parameter and the average from the triplicate measurements was used (Fig. 1).

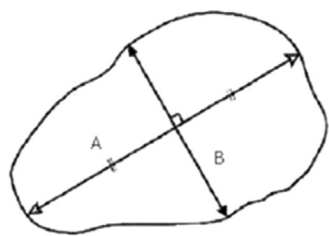


Fig. 1. Size calculation method for wheals and redness $((A+B)/2)$. Wheal and redness diameters were measured as the average of the sum of the lengths of the line connecting the 2 farthest points (mm) (A) and the perpendicular line at its midpoint (B) to the nearest hundredth millimeter. The average from triplicate measurements was used.

2.2.5. Adverse events

Participants were carefully monitored for adverse events (AE) by the outcome assessor after pharmacopuncture administration. Participants were monitored for any itching, heat sensations or flushing, nausea, or vomiting symptoms immediately after administration and any pain, headache, dizziness, or fatigue at 6, 24, and 72 h timepoints.

Spilker's criteria was used to assess AE severity as mild, moderate, or severe (mild (1): no additional intervention necessary and no significant impediment to daily activity (function) of participant; moderate (2): significant impediment to daily activity (function) of participant, and may need intervention with consequent recovery; and severe (3): advanced intervention necessary for serious AE, leaving sequelae). AE type was classified into 3 categories: systemic reaction (SR), skin problem (SP), or other (nonspecific response, symptom, or sign which was neither SR nor SP). In occurrence of SR as an AE, a modified 5-category grading scale modeled after the 4-category Mueller classification method with large local reaction (large local reaction, grade I, grade II, grade III, and grade IV) was used (Mueller, 1966). The causal relationship between AE and BV was also assessed based on the WHO-UMC causality scale (World Health Organization, 2005).

2.2.6. Statistics

Sample size was arbitrarily set based on clinical feasibility as this trial was a preliminary study with the main aim of exploring and comparing local reaction to BV and eBV pharmacopuncture.

Outcome measures were analyzed by intention-to-treat using PASW Statistics 18.0 (IBM, Armonk, NY, USA). Missing values were handled by way of last observation carried forward (LOCF). Baseline characteristics are presented as mean $\pm$ standard deviations (SDs), and adverse events are given as frequencies. For between-group comparison of baseline characteristics, Wilcoxon rank sum test was used. Repeated measures analysis of variance was conducted to test for trends by timepoint (time-by-treatment interactions).

3. Results

3.1. In vitro study

In order to evaluate whether eBV affects NO production, production of nitrite, which is a stable metabolite of NO and therefore frequently used as an indicator of NO production, was measured in cultured LPS-induced RAW 264.7 macrophage cells. As shown in Fig. 2, treatment with LPS (1 μ g/mL) markedly increased NO production from baseline level of 1.35 ± 0.1 μ g/mL to 27.0 ± 0.7 μ g/mL following 20 h incubation. eBV and BV treatment with LPS inhibited NO production in a concentration-dependent manner with IC_{50} values of 2.6 and 2.5 μ g/mL, respectively. The inhibitory effect of eBV on NO production was similar to that of BV, and NO inhibition by eBV and BV was not statistically different by timepoint. In the same assay conditions, melittin, which was used as a positive control, exhibited an IC_{50} of 2.1 μ g/mL.

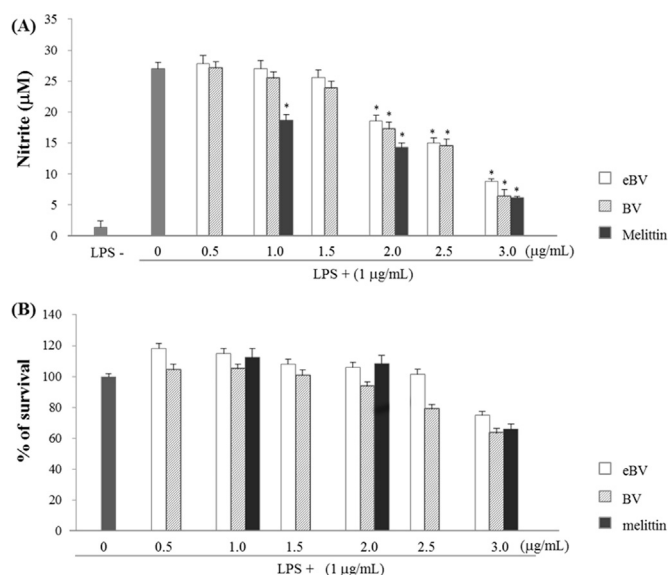


Fig. 2. Inhibitory effect of eBV, BV, and melittin on LPS-induced NO production in macrophage cells. (A) RAW 264.7 cells were stimulated with LPS (1 μ g/mL) in the presence or absence of eBV, BV, and melittin, respectively. After 20 h, cultured media was collected and nitrite concentration was analyzed using Griess reaction. Data are expressed as mean $\pm$ SD of triplicate tests. * $p < 0.01$ was considered statistically significant. (B) Cell viability was measured by MTT method as described in the Materials and methods section. LPS, lipopolysaccharide; BV, bee venom; eBV, essential bee venom.

Cell viability was determined by MTT assay, and no significant cytotoxic effect was observed ($> 100\%$ cell survival for eBV at 2.5 μ g/mL; $> 79\%$ cell survival for BV at 2.5 μ g/mL; $> 100\%$ cell survival for melittin at 2.0 μ g/mL), indicating that inhibition of NO production by eBV and BV were not mediated by cytotoxic effect (Fig. 2).

3.2. Clinical studies

Local pain at administration site was not significantly different between BV and eBV at all timepoints except at 24 h post-administration ($P=0.0062$) where eBV was significantly lower. Local swelling at administration site was significantly less in eBV at 30 min ($P=0.0198$), 2 ($P=0.0028$), 24 ($P=0.0068$), and 48 h post-administration ($P=0.0253$). Itching at administration site was significantly less in eBV at 24 ($P=0.0119$), 48 ($P=0.0082$), and 96 h ($P=0.0141$) post-administration. Wheals at administration site did not show significant difference across all timepoints. Redness at administration site was significantly less in eBV at 30 min ($P=0.0090$), 6 ($P=0.0005$), and 24 h post-administration ($P < 0.0001$) (Fig. 3, Table A.1).

eBV and BV displayed significant interactions in swelling, itching and redness (swelling $p=0.007$, itching $p=0.023$, and redness $p < 0.001$). Pain, swelling, itching, wheals, and redness were additionally assessed for between-group interactions in eBV and BV by timepoint. While time-by-treatment interactions for itching and redness were statistically significant ($P < 0.001$, and $P < 0.001$, respectively), itching and redness exhibited trends towards increasing severity for BV in later measurements (Table 1).

Two AEs requiring further intervention were observed. One participant displayed a delayed type local allergic response including swelling and redness across the forearms around the pharmacopuncture administration area that grew more severe with time, which subsided within 24 h of chlorpheniramine maleate 1 mL injection at 48 h post-pharmacopuncture administration. However, local response of swelling and redness in the arm that was administered eBV in this participant appeared at 24 h post-pharmacopuncture administration and resolved a short while after. The other participant showed an immediate systemic allergic response including chest discomfort,

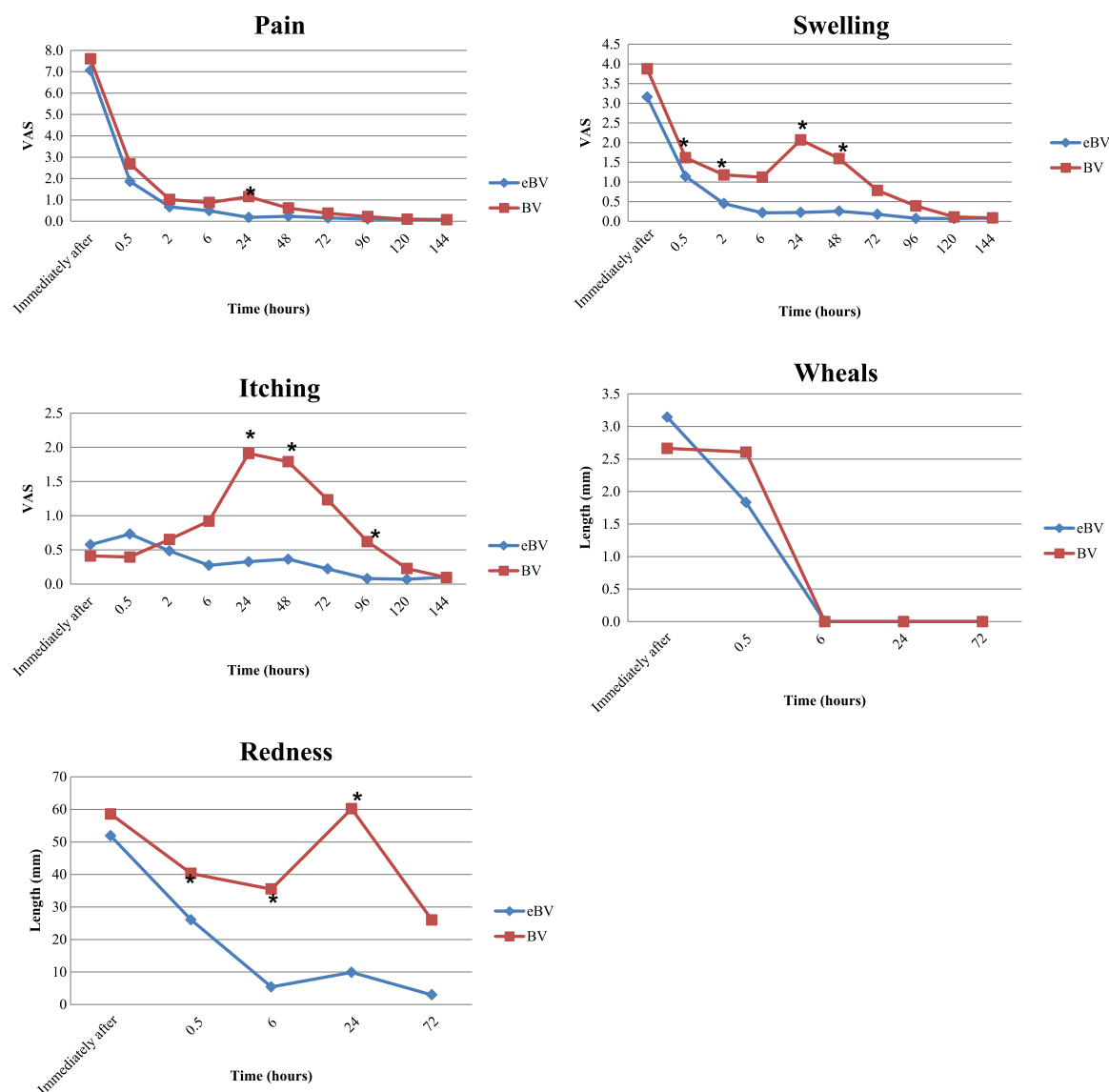


Fig. 3. Pain, swelling, itching, wheals, and redness in eBV and BV groups by timepoint. * Significant difference between eBV and BV at specific timepoint ($p < 0.05$). VAS, visual analogue scale; BV, bee venom; eBV, essential bee venom.

Table 1

Pain, swelling, itching, wheals, and redness analysis results from repeated measures two factor analysis of variance.

	Group differences		Time by group interactions	
	F	p value	F	p value
Pain	3.257	0.079	0.960	0.473
Swelling	8.311	0.007	1.900	0.051
Itching	5.618	0.023	4.156	0.000
Wheals	0.011	0.917	0.285	0.887
Redness	15.657	0.000	5.348	0.000

Table 2

Adverse events associated with bee venom pharmacopuncture.

Sex	Age	AE symptoms	AE severity ^a	AE type ^b	Mueller classification ^c	Diagnosis	Causality ^d
M	30	Localized edema, pain and erythema	Moderate	SP	–	Late onset reaction	Probable
F	28	Chest discomfort, nausea, generalized pruritus, urticaria, erythema and fever	Moderate	SR	Grade II	Hypersensitivity	Probable

^a AE severity was assessed using Spilker's criteria: mild, moderate, and severe.

^b AE type was classified into 1 of 3 categories: systemic reaction (SR), skin problem (SP), and other.

^c Mueller classification: if a systemic reaction occurred as an AE, it was classified into 1 of 5 categories: large local reaction, grade I, grade II, grade III, and grade IV.

^d Causality was determined using the WHO-UMC causality scale: certain, probable, possible, unlikely, conditional, and inaccessible.

nausea, generalized itching, wheals, redness and fever starting 20 min post-administration, and was injected chlorpheniramine maleate 1 mL and dexamethasone 1 mL within 30 min of pharmacopuncture administration. Symptoms subsequently subsided within 24 h. Although it cannot be ascertained which type of BV caused the systemic reaction, the arm that was administered BV showed more severe local allergic response such as pain, redness, swelling, and itching compared to the arm administered eBV (Table 2).

4. Discussion

BV and eBV displayed similar inhibitory effects on LPS-induced NO production at the same dose in *in vitro* studies. Moreover, eBV exhibited statistically significantly less pain, swelling, itching, and redness in the forearm area compared to BV in a physician, subject, and assessor-blinded RCT. These results imply that eBV induces less allergic responses while producing comparable medicinal effect.

Enzymes, especially PLA2, are known to play an important role in allergic response to BV. PLA2 contains mainly enzyme elements, and IgE antibody is found in almost 90% of individuals with hypersensitivity to BV. Also, an essential substance for the antigen-antibody response in allergic reaction to BV is histamine. Histamine has a chemical formula of C₅H₉N₃ and low molecular weight. It may cause allergic responses such as wheals from intake (e.g. food), and may lead to allergies when released in the body through antigen irritation entailing consequent basophil and mast cell stimulation. A hypersensitivity reaction may occur when histamine bonds with IgE of the cellular membrane (Beaven and Baumgartner, 1996). The results of this study suggest that use of isolated and purified bee venom filtered for PLA2 and histamine sections such as eBV used in this study may reduce occurrence of such unnecessary allergic reactions.

Recent studies on BV pharmacopuncture safety include a systematic review that used 12 databases from their inception to June 2014 without any language restrictions (Park et al., 2015a). Of the 6 audits and cohort studies on BV acupuncture included in this study, 3 studies had subject populations of 300 or more, and incidence of AEs were 48/374 (12.83%; local reaction n=48) (Choi et al., 2010), 11/32000 (0.03%; systemic reaction n=11) (Hwang and Lee, 2000), and 361/2765 (13.00%; systemic reaction n=361) (Kwon and Koh, 2000). Also, meta-analysis of AE occurrence in 4 RCTs comparing BV safety with normal saline showed a 261% increased relative risk for occurrence of AEs in BV acupuncture. The wide range of AE occurrence for BV in these studies appears to be due to difference in study design or definition of AE in BV pharmacopuncture. AE occurrence for BV is markedly higher than that for general acupuncture as exemplified in a large patient population study on general acupuncture conducted in Germany (Witt et al., 2009). BV concentration was set at 0.1 mg/mL in this clinical trial. This concentration was determined in accordance with BV related clinical studies and BV products currently marketed in Korea (Koh et al., 2013; Park et al., 2012). Also, in actual clinical practice, 0.1 cc of 0.1 mg/mL BV is used in skin tests, of which the dose is gradually increased in BV treatment up to 1–2 cc. Considering that the LD₅₀ of Korean BV is 5.25 mg/kg (Jang et al., 2005), this is a minimal dose.

The largest strength of this study is that it is the first RCT to inject different types of BV in each arm of the same subject and monitor the difference to minimize individual responsiveness differences to BV. Immune response to BV treatment shows wide individual difference, ranging from minor skin reactions that subside over the course of several days to serious responses such as anaphylaxis that may be life-threatening (Park et al., 2013; Valentine et al., 1990). Therefore, investigating effectiveness and safety of different BV types in different subject groups may not be an effective method for accurately determining difference in responsiveness. While review of comparative studies on BV pharmacopuncture yield 4 previous studies investigating therapeutic effects of sweet bee venom (SBV) with BV as active control (Kim et al., 2008a, 2008b; Lee et al., 2008; Na et al., 2007), these studies all assign different individuals for the experimental and control groups and consequently cannot adjust for individual differences in sensitivity to BV. Although this study is also limited in that it was unable to clearly discern whether systemic reaction was caused by BV or eBV, taking into consideration the severity of local redness and wheals around the administration area, it can be tentatively inferred that the systemic reaction was related to BV as the arm injected with BV exhibited more redness, wheals and pain. Also, this trial was

rigorously conducted as a double blind study, and outcomes were reported using such validated tools as VAS, and Spilker's criteria, Mueller classification, and WHO-UMC causality scale for AE assessment. However, this study also holds the following limitations. A major limitation of this study is the assumption regarding the consistency of therapeutic effect of eBV and original BV. While the current results demonstrated that the inhibitory effects in LPS-induced NO production at the same dose was comparable, BV has been shown to possess various effects such as a) inhibitory effect on Freund's complete adjuvant (FCA) induced-polyarthritis (Park et al., 2004; Saad Rached et al., 2010), b) hydroxyl radical scavenging activity (Rekka et al., 1990), c) superoxide anion scavenging activity (Somerfield et al., 1984), and d) effect on PGE2 production by COX-2 (Park et al., 2004; Saad Rached et al., 2010). The fact that PLA2, which is removed from eBV, has been reported to possess various positive effects such as immune function (Park et al., 2015b; Ye et al., 2016), is also of special interest. Further studies are warranted to more comprehensively address the agreement between the various effects of eBV and BV. The method of nitrite assay may also be a subject of debate. The authors opted for simultaneous treatment of BV, eBV, and melittin with LPS, and performed additional tests comparing NO production inhibition in treatment with experimental and control compounds after pre-incubation with LPS for 12 h, and treatment with LPS following pre-incubation with compounds for 12 h. While the inhibitory effect on NO generation may be conjectured to be variable by LPS treatment duration time, order, concentration, and drug treatment time, of the 3 different conditions, results indicated that NO production inhibition was most effective in simultaneous treatment of compounds with LPS (data not shown).

In the 2 cases of AE, the local site administered with eBV showed almost no local response, while BV displayed severe local reaction, and the systemic reaction may be an interactive result from the 2 types of BV pharmacopuncture. This may partially explain the reason why the AE occurrence rate of 2 out of 20 participants is much higher than previous reports.

Complementary and alternative medicine (CAM) is currently used widely throughout the world, and though CAM is generally viewed to be a safe method of treatment, the number of safety studies is disproportionately lacking considering the multitude of efficacy and effectiveness studies. The significance and implications of safety studies are great, especially with regard to determining preventive measures in clinical treatment. Despite the small number of cases restricting generalizability of eBV safety, the results of this study hold relevance as the first study to simultaneously investigate safety of the 2 BV types in healthy adults based on the premise that the anti-inflammatory effects were equivalent. Future prospective large-scale studies on AE occurrence of eBV with education and management guidelines for eBV related AEs are required to further establish safety, and as definition of AEs may differ by study, need for systematic reporting using such standards as adverse reaction terminology is indicated.

5. Conclusions

BV and eBV produced similar inhibitory effects on LPS-induced NO production at the same dosage in *in vitro* studies. In addition, physician, participant, and outcome assessor-blinded RCT results showed that eBV displayed statistically significantly less local pain, swelling, itching, and redness compared to BV in the forearm area. These results suggest that eBV possesses comparable efficacy to BV, and entails less local allergic response.

Author contributions

YJA, JSS, JHL, YJL and IHH drafted the study, and YJA, MRK, KBP, HJC, MJK, JWL and IHH wrote the final manuscript. JSS, JHL, YJL, HDL, YML and SGK contributed to the study design and made

critical revisions. YJA, YJL, MRK, YSS, HJC, MJK, JWJ, and IHH performed the experiments and contributed to acquisition of data. All of the authors have read and approved the final manuscript.

Declaration of interest

None.

Acknowledgement

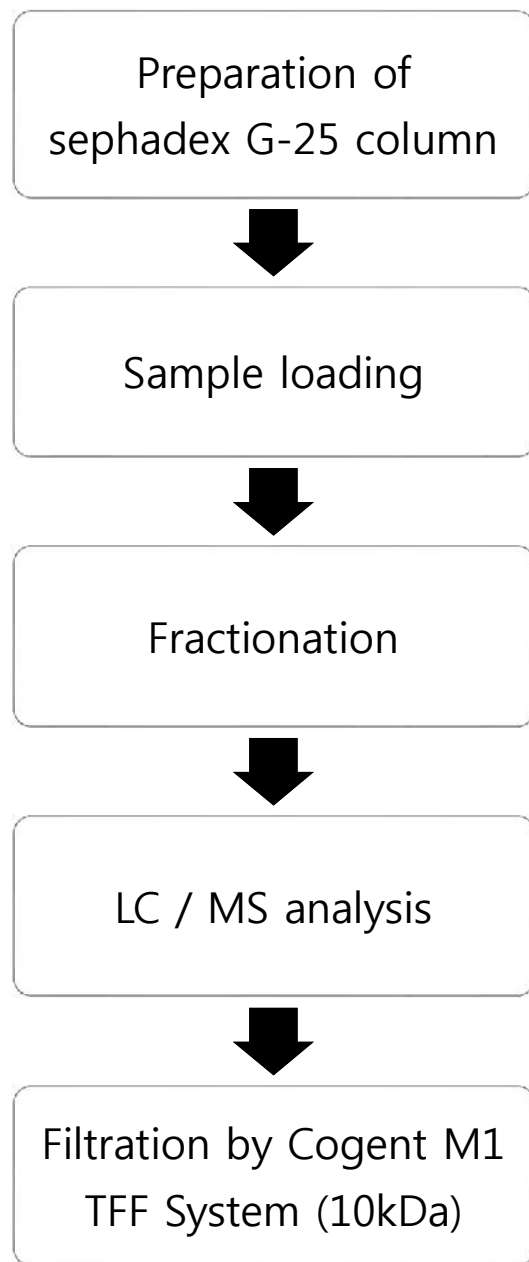
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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.jep.2016.11.012>.

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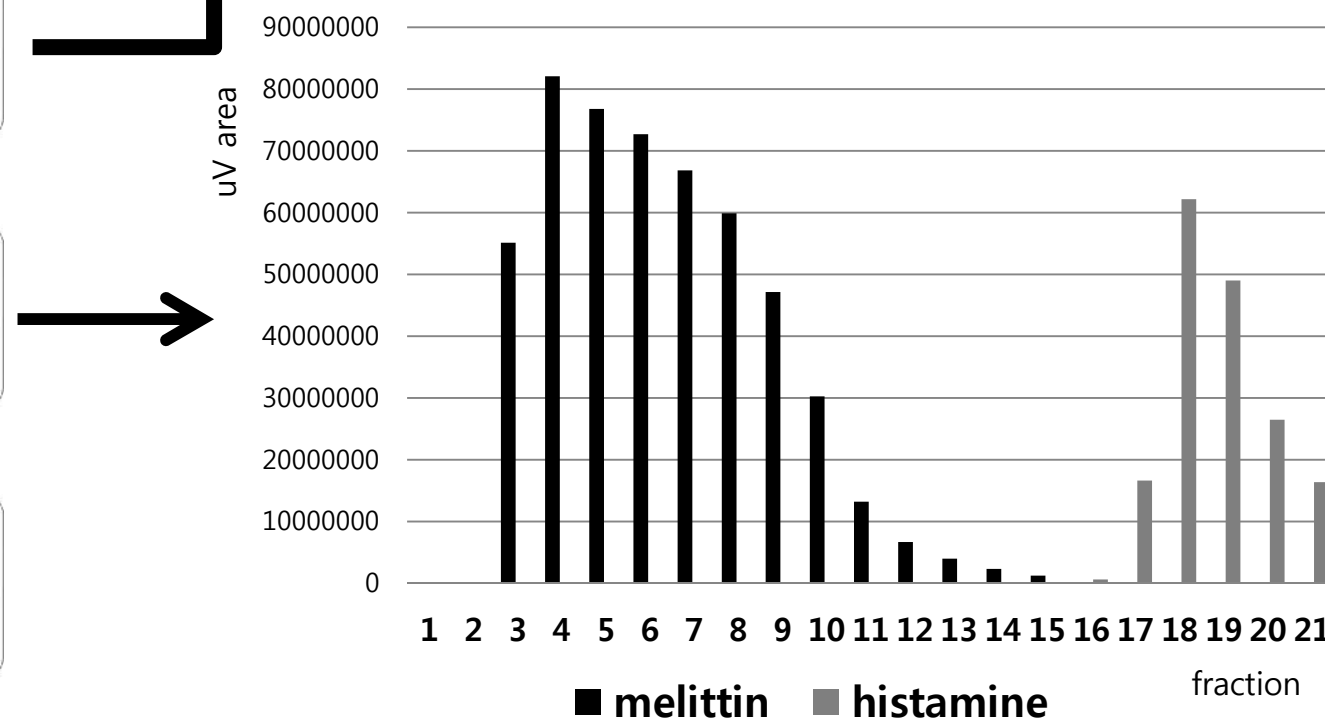
Bee Venom (10 g)

Sephadex G-25 column chromatography
using purified water

1 2 3 ~ 13 14 15 16 ~ 21

**melittin
fraction**

**histamine
fraction**



1 **Table A.1. Pain, swelling, itching, wheals, and redness in eBV and BV groups by timepoint**

	n	Pain			Swelling			Itching			Wheals			Redness		
		eBV	BV	p value	eBV	BV	p value	eBV	BV	p value	eBV	BV	p value	eBV	BV	p value
		Mean±sd	Mean±sd		Mean±sd	Mean±sd		Mean±sd	Mean±sd		Mean±sd	Mean±sd		Mean±sd	Mean±sd	
Baseline	20	7.06±1.93	7.61±2.18	0.2110	3.16±2.91	3.88±2.91	0.4450	0.58±1.45	0.41±0.51	0.8201	3.14±2.36	2.66±2.38	0.5831	51.84±13.54	58.56±10.27	0.0763
30 minutes	19	1.86±2.16	2.68±1.69	0.0797	1.14±1.72	1.62±1.17	0.0198	0.73±1.55	0.39±0.70	0.8853	1.83±3.30	2.60±7.26	0.7291	26.04±12.33	40.31±18.08	0.0090
2 hours	19	0.67±1.31	1.02±1.35	0.1629	0.45±1.11	1.18±1.13	0.0028	0.48±1.38	0.65±1.43	0.5250						
6 hours	19	0.49±1.28	0.87±1.24	0.1458	0.22±0.43	1.12±1.77	0.0533	0.27±0.73	0.92±1.69	0.4016	0.00±0.00	0.00±0.00	1.0000	5.42±9.38	35.48±43.35	0.0005
24 hours	19	0.18±0.51	1.15±1.51	0.0062	0.23±0.57	2.07±2.44	0.0068	0.33±0.89	1.91±2.10	0.0119	0.00±0.00	0.00±0.00	1.0000	9.89±15.97	60.19±47.46	0.0000
48 hours	19	0.23±0.62	0.62±1.19	0.3249	0.26±0.70	1.59±2.22	0.0253	0.36±0.96	1.79±2.06	0.0082						
72 hours	19	0.15±0.42	0.37±0.70	0.4523	0.18±0.52	0.78±1.40	0.0850	0.22±0.58	1.23±1.69	0.0612	0.00±0.00	0.00±0.00	1.0000	2.98±7.46	25.99±49.15	0.0612
96 hours	19	0.10±0.22	0.22±0.42	0.6441	0.07±0.14	0.39±0.73	0.1629	0.08±0.16	0.62±0.69	0.0141						
120 hours	19	0.08±0.20	0.09±0.18	0.9540	0.07±0.15	0.11±0.22	0.7509	0.07±0.15	0.23±0.27	0.1301						
144 hours	19	0.07±0.17	0.07±0.16	0.8626	0.08±0.17	0.08±0.17	0.8626	0.10±0.21	0.09±0.22	1.0000						

2 Analyzed using Wilcoxon rank sum test