



Anti-inflammatory Effect of Bumblebee Alcohol Extracts in CFA-Induced Rat Edema

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In this study, we prepared alcohol extracts of the larva, pupa, queen, and cocoon (clony) of *B. ignitus*, *B. terrestris*, and *B. h. sapporoensis*, and tested the anti-inflammatory activity of the extracts by using a rat model of adjuvant-induced edema. The extracts derived from the queen of *B. ignitus*, the queen of *B. terrestris*, and the cocoon of *B. ignitus* decreased hind paw edema after 1 day of *i.p.* administration. These extracts also induced vasorelaxation and NO production in calf pulmonary artery endothelial cells. These results suggest that bumblebee alcohol extracts has anti-inflammatory and vasorelaxant properties.

Key words: Edema, Bumblebee alcohol extract

INTRODUCTION

Inflammation is natural immune reaction of the body that results from an infection, injury, or illness. Inflammation has been shown to be one of the causes of coronary artery disease (Hansson, 2005) and arthritis. Nitric oxide has many functions in the body as a vasodilator and neurotransmitter (Nussler and Billiar, 1993), and is an important biomarker of the inflammatory response (Hussain *et al.*, 2008). Also, phospholipase A₂, the enzyme that cleaves membrane phospholipids, was found to be increased through inflammation and in the presence of various inflammatory diseases (Schoenberg *et al.*, 1997). The medicinal and nutritional uses of honeybee and other hive products including honeybee larva, are well known (Meda *et al.*, 2004). Bumblebees (e.g. *B. igitus*, *B. terrestris* and *B. h. sapporoensis*) are mass-produced worldwide for use as pollinators. We endeavored to make a safe and effective bumblebee alcohol extracts, and tested its anti-inflammation activity by determining NO production in endothelial cells and phospholipase A₂ activity, furthermore, various bumblebee extracts were applied to the animal inflamma-

tion model-paw edema experiment.

MATERIALS AND METHODS

Materials. The dried larvae (500 g) of *B. ignitus* (BIL) were soaked and extracted three times with ethanol by ultrasonification for 30 min. The extracts obtained were dried using a rotary evaporation and were freeze-dried as an alcohol extracts of *B. ignitus*. Also, *B. ignitus* pupae (BIP), clony shell of *B. ignitus* (BIC), clony of *B. h. sapporoensis* (BHSPL), BTQ (Queen of *B. terrestris*), and Queen of *B. ignitus* (BIQ) were soaked and extracted according to the method above, respectively. The extracts were obtained using a rotary evaporation and were freeze-dried as alcohol extracts of each bumblebee products.

Test preparation of bumblebee product extracts. Dried alcohol extracts of bumblebee product were homogenized in a blender to a powder, stored at 4°C, dissolved in phosphate buffered saline from Sigma-Aldrich (St. Louis, MO, USA), and were then orally administered to SD rats at doses of 10 mg/kg daily, over 8 days.

Analysis of mineral content and amino acid composition. Mineral contents were analyzed by atomic absorption spectroscopy and phosphorus contents were determined by colorimetric method, which utilizes ammonium molybdate, hydroquinone, and sodium sulfate (Kim *et al.*, 2001). Amino acid compositional analysis was carried out by derivatization of the first N-terminal amino acids with phenylisothiocyanate (PITC) followed by RP-HPLC (Williams *et al.*, 1988).

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Animals. Specific pathogen free SD rats (6 weeks old, weighing 204.6 ± 2.1 g, male), purchased from Samtako Co. Ltd. (Osan, Korea), were housed in an environmentally-controlled room with $23 \pm 1^\circ\text{C}$, relative humidity of $55 \pm 10\%$, air ventilation of 10~18 air changes/hr, a 12-hr light/dark cycle of 150~300 lux, and feed and water were available *ad libitum*. Rats were kept for one week under normal conditions above and were fed with a standard diet (Samtako Co. Ltd., Osan, Korea), before repeated-dose anti-inflammatory study testing began.

Anti-inflammatory rat experiment. Complete Freund's adjuvant (CFA, Sigma Co., USA) was used to induce rat paw edema at 1st day (pre-treatment) except control group in a chronic arthritis experimental model, and the anti-edema effect of individual solvent extracts (post-treatment) was compared.

Rats were divided into 9 groups (n = 7 per group): con-

Table 1. Yield and chemical composition of various bumblebee extracts used in this study

Sample	BIC	BHSPL	BTQ	BIQ
Yield (%)	3.8	17.6	2.4	7.5
Fat (%)	14.19	4.05	4.80	14.19
Preotein (%)	12.61	10.78	17.23	35.13
Ash (%)	2.37	2.45	5.63	7.05
Ca (%)	0.02	0.03	0.03	0.05
P (%)	0.42	0.42	1.37	1.65
K (%)	0.65	0.65	1.60	2.15
Na (%)	0.1	0.1	0.16	0.24
Mg (%)	0.05	0.05	0.03	0.04
Fe (ppm)	31.40	31.40	88.88	171.17
Mn (ppm)	2.19	2.19	2.98	8.87
Zn (ppm)	33.20	33.20	51.34	53.31
Cu (ppm)	9.35	9.35	12.30	28.07
Amino acid composition (%)				
Cys	0.094	0.063	0.094	0.120
Met	0.032	0.098	0.232	0.427
Asp	0.273	0.339	0.329	1.523
Thr	0.123	0.207	0.230	0.868
Ser	0.155	0.226	0.247	0.761
Glu	0.478	0.960	1.469	3.479
Gly	0.261	0.378	0.451	1.335
Ala	0.195	0.387	0.704	1.456
Val	0.189	0.360	0.316	1.045
Ile	0.114	0.221	0.250	0.935
Leu	0.198	0.383	0.414	1.690
Tyr	0.055	0.211	0.064	0.359
Phe	0.069	0.177	0.137	0.616
Lys	0.103	0.213	0.380	1.505
His	0.052	0.143	0.161	0.317
Arg	0.058	0.141	0.408	0.726
Pro	0.629	0.900	0.562	1.422

BIC: clony shell of *B. ignitus* extract; BHSPL: clony of *B. h. sapporoensis* extract; BIQ: Queen of *B. ignitus* extract; BTQ: Queen of *B. terrestris* extract.

rol group, CFA (100 mM) only treated group (negative control), indomethacin (5 mg/kg at 1st day, 1 mg/kg at 2~8th day) as a positive control, sample groups [bumblebee ethanol extracts (each ethanol extract of BIL, BIP, BIC, BHSPL, BTQ, and BIQ)] daily treated intraperitoneal, over 8 days. Paw size was measured 1, 3, 5 hr and thereafter every day for 14 days using a digital caliper (digimatic, Mitutoyo, Co., Japan).

Seven male rats in each group were weighed and were administered with bumblebee alcohol extracts at a dose of 10 mg/kg or its vehicle a consecutive dose, over a 8 days. The test parameters were paw edema, cytokine interleukin 6 production level, secretory phospholipase A₂ activity and histopathological findings of dorsal root ganglias, articular cartilage and bone of paw edema rats.

Nitrite assay. The production of NO was measured as the nitrites that accumulated in the culture medium after colorimetric reaction with Griess reagent according to the manufacturer's manual (Cayman Chemicals, Ann Arbor, MI, USA). In brief, samples (200 mg/ml, 20 μl , dilution factor 10) were collected 24 hr after treatment with cultured bovine vascular endothelial (CPAE) cells.

The absorbance at 540 nm was measured with a VERSAmax microplate reader (Molecular Devices, Menlo Park, CA, USA). The cytotoxicities of the purified fractions were tested against the CPAE cell line using XTT {sodium 3'-[1-

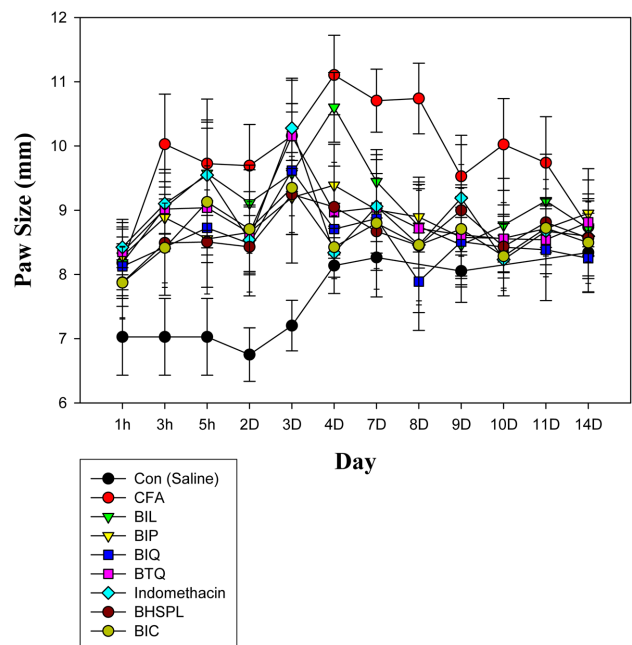


Fig. 1. Anti-inflammatory effect of various bumble bee extract treatment on CFA-induced paw edema in rats. *B. ignitus* pupae extract (BIP), clony shell of *B. ignitus* extract (BIC), clony of *B. h. sapporoensis* extract (BHSPL), Queen of *B. terrestris* extract (BTQ), and Queen of *B. ignitus* extract (BIQ), 10 mg/kg, respectively.

(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate} kit solution (Boehringer Mannheim), as described previously (Ahn *et al.*, 2009).

Secretory phospholipase A₂ measurements in CPAE cells. Phospholipase A₂ cleaves membrane phospholipids to release arachidonic acid, and is the precursor to a large family of pro-inflammatory eicosanoids (Reid, 2005). The secretory phospholipase A₂ (sPLA₂) levels in these 24 hr-incubated bumblebee extracts were measured by ELISA using a sPLA₂ assay kit purchased from Cayman Chemi-

cals (Ann Arbor, MI, USA).

Histopathology. The lumbar V (LV) dorsal root ganglion, including articular cartilage and near leg bones, were dissected from the rats and were fixed in phosphate-buffered formalin. The spinal cords of the rats, including the bone and articular cartilage, were also excised and fixed. After paraffin embedding, they were stained with hematoxylin and eosin, and were analyzed with microscopy.

Statistical analysis. Mean and standard errors of all parameters were determined for each of the 8 rats. The Stu-

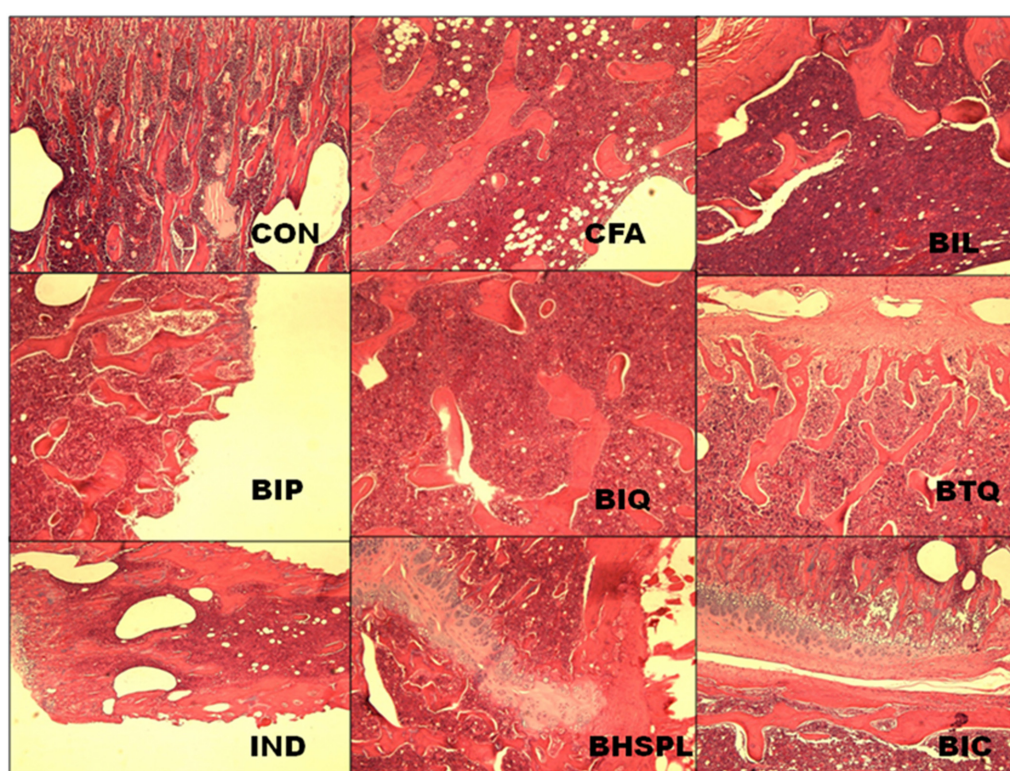


Fig. 2. Histopathological data of dorsal root ganglion treated with bumblebee extracts.

Table 2. Histopathological finding of the LV dorsal root ganglion (DRG) including articular cartilage treated various bumblebee extracts (10 mg/kg) on 14th day

Group	Pathologic finding
1 Con	
2 CFA	Induced injury without treatment
3 BIL	Induced injury with treatment
4 BIP	Induced injury with treatment
5 BIQ	Induced injury with treatment
6 BTQ	Induced injury with treatment
7 IND	Induced injury with treatment
8 BHSPL	Induced injury with treatment
9 BIC	Induced injury with treatment

B. ignitus pupae extract (BIP), clony shell of *B. ignitus* extract (BIC), clony of *B. h. sapporoensis* extract (BHSPL), Queen of *B. terrestris* extract (BTQ), and Queen of *B. ignitus* extract (BIQ).

Table 3. Histopathologic finding of the spinal cord, including articular cartilage and bones, treated with various bumblebee extracts in CFA-induced arthritis rat model on 14th day

Group		Pathologic finding	
1. Control		Normal	
2. CFA	Control	Induced injury without treatment	Repair incomplete with residual damaged area
	Control	Opposite site without treatment	Partial destruction of articular cartilage
3. BIL	Treat	Induced injury with treatment	Repair
	Control	Opposite site without treatment	Partial destruction of articular cartilage
4. BIP	Treat	Induced injury with treatment	Repair, incomplete with residual damaged area
	Control	Opposite site without treatment	Mildly damaged articular cartilage, focal
5. BIQ	Treat	Induced injury with treatment	Repair, incomplete with residual damaged area
	Control	Opposite site without treatment	Mildly damaged articular cartilage, focal
6. BTQ	Treat	Induced injury with treatment	Repair
	Control	Opposite site without treatment	No significant alteration
7. Indomethacin	Treat	Induced injury with treatment	Repair
	Control	Opposite site without treatment	Destruction of articular cartilage
8. BHSPL	Treat	Induced injury with treatment	Repair, incomplete with residual damaged area
	Control	Opposite site without treatment	Partial destruction of articular cartilage
9. BIC	Treat	Induced injury with treatment	Repair
	Control	Opposite site without treatment	Partial destruction of articular cartilage

*Bumblebee ethanol extracts (10 mg/kg), *Treat: the paw portion with induction of inflammation and sample treatment; Control: the paw portion without induction of inflammation (opposite paw).

dent's *t*-test was used to establish the significances of differences between the control and treatment groups. $p < 0.05$ was considered statistically significant.

RESULTS

Anti-inflammation. We showed that bumblebee alcohol extracts have potential efficacies in treating inflammation in SD rats, as they significantly reduced paw edema levels as the following order: BIQ > BIC > BTQ > BIL, and repaired damaged dorsal root ganglia of CFA adjuvant arthritis.

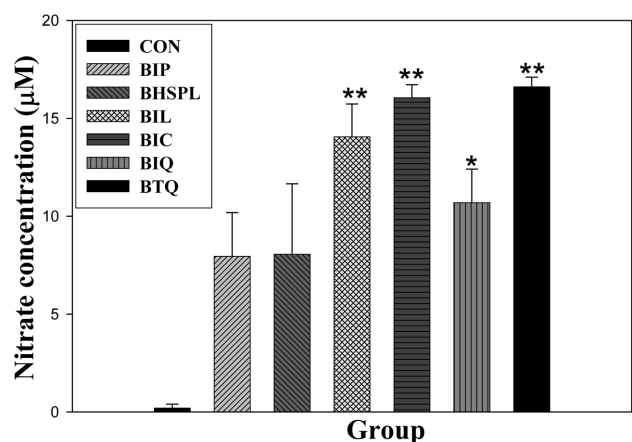
Six total alcohol extracts, of BIL, BIP, BIQ, BTQ, BHSPL and BIC, were treated to the peritoneum (10 mg/kg) of paw edema induced rats, and the effect of individual extracts was in the following order: BIQ > BIC > BTQ > BIL (Fig. 1). The mean changes in paw edema size (mm) from 1 hr to 14 days for each group were as follows: control (7.54 ± 0.21), CFA (9.86 ± 0.23), BIL (9.11 ± 0.18), BIP (8.81 ± 0.091), BIQ (8.53 ± 0.43), BTQ (8.87 ± 0.13), IND (8.19 ± 0.70), BHSPL (7.96 ± 0.67), BIC (7.95 ± 0.67).

Articular cartilage destruction repair by bumblebee extracts. In the histopathological analysis, bumblebee extracts treatment repaired the LV dorsal root ganglion linked to the edematous paw, including the articular cartilage, against CFA induced cartilage destruction, respectively. This was in contrast to the effects observed in the CFA treated group, where the articular cartilage was destroyed by erosion (Fig. 2

and Table 2). In the histopathological finding of pathological spinal cord, the bumblebee extract groups, especially BIQ (Queen of *B. ignitius*) extract group, showed anti-edema effects compared to the CFA only treated group (Table 3).

Pro-inflammatory cytokines (secretory PLA_2 and NO).

Furthermore, these extracts also had multiple actions such as remarkable NO production in endothelial cells related to vasorelaxation and secretory phospholipase A_2 activity (Fig. 4). The productions of nitric oxide in the treated groups

**Fig. 3.** Effects of the various bumble bee extract on nitric oxide production activity in CPAE cells.

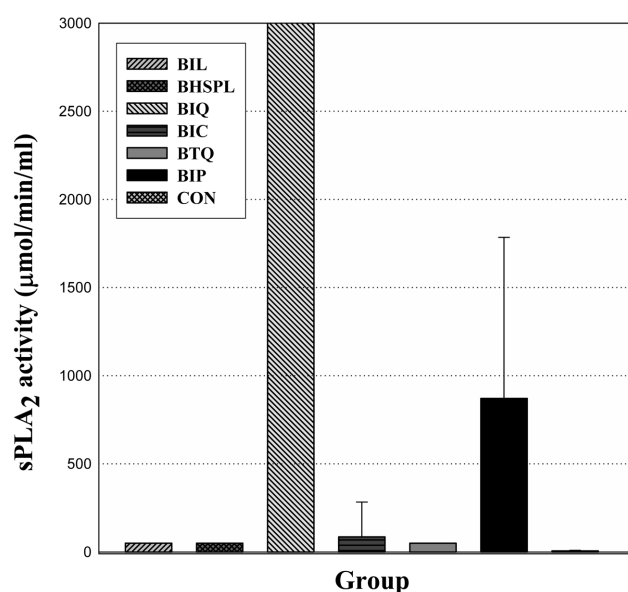


Fig. 4. Secretory phospholipase A₂ activity of various bee alcohol extract. * Sample Conc.: 200 mg/ml.

were also inhibited according to vasorelaxation of blood vessel in the alcohol extract of BTQ, BIC, BIL and BIQ (Fig. 3). There was also no cytotoxicity observed for the bumblebee extracts in XTT assay (data not shown).

DISCUSSION

In order to identify the best bumblebee extracts for the control of inflammation reaction, we made alcohol extracts of the larva, pupa, queen and cocoon of *B. ignitus* and *B. terrestris*, and *B. h. sapporoensis*, and tested their medicinal activities. The anti-inflammatory activity of Hymenoptera, bumblebee alcohol extract was examined by using adjuvant-induced edema and arthritis in rats. In the previous report, *B. ignitus* alcohol extracts did not show acute toxicity at 0.04, 0.2, 1 or 5 g/kg (Ahn *et al.*, 2009). Each alcohol extract from *B. ignites* queens, *B. terrestris* queens and *B. ignites* cocoons, decreased the hind paw edema after 1 day of *ip* administration. Also, the extracts contributed to vasorelaxation on NO production increases in CPAE cells. These results suggest that bumblebee alcohol extract has potential as a crude ant-inflammatory drug and vasorelax-

ation agent. As commercial bee venom, the secretory phospholipase A₂ levels of queen of *B. ignitus* extract was higher than those of other bumblebee extracts, and had anti-inflammatory activity and articular cartilage destruction repair, this is deemed to be the most effective agent for this purpose.

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