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Immune Modulation of *B. terrestris* Worker (a Type of Bumblebee), Extract on CFA-induced Paw Edema in Rats

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To develop a composition for enhancing immunity, based on alcohol extracts of the bumblebee as an active ingredient, bumblebee ethanol extracts were evaluated for their protective effect in chronic models of inflammation, adjuvant induced rat arthritis. *B. terrestris* worker extract (SDIEX) and, *B. hypocrita sapporoensis* lava and pupa extract (SPDYBEX), significantly decreased paw edema in arthritic rats, at a dose 100 mg/kg, respectively. The cytokine levels related inflammation of COX-2, $sPLA_2$, VEGF, and TNF-α, were decreased, compared to positive control, indomethacin (5 mg/kg). Histopathological data demonstrated decreases inflammatory activity, hind paw edema, and repaired hyaline articular cartilage in DRG over a 2 wk administration. HPLC and GC-MS analysis of SDIEX and SPDYBEX revealed the presence of cantharidin.

***Key words*:** *B. terrestris* worker extract, *B. hypocrita sapporoensis* lava and pupa extract, Inflammation

INTRODUCTION

The medicinal and edible uses of honeybee and other hive products including honeybee larva, are well known (1). Bumblebees (e.g. *B. igitus*, *B. terrestris* and *B. h. sapporoensis*) are mass-produced worldwide for use as pollinators. The bumble bee, *Bombus terrestris,* is a primitively eusocial species with an annual life cycle and colonies headed by a single queen (2). The workers of the bumble bee *Bombus terrestris* were observed to affect the immune response, with regard to the dynamics of the phenoloxidase (PO) system (3).

We sought to make a safe and effective bumblebee alcohol extract, and tested its anti-inflammation activity by determining NO production in endothelial cells and phospholipase A2, COX-2, IL-6, VEGF, and TNF-α activity, Cantharidin, a phosphatase inhibitor and a 7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid derivative, has been reported as a natural toxin extracted from Spanish fly and blister beetles, such as Mylabris. It has been used as an active ingredient with anticancer activity from Mylabris and in the form of a synthetic demethylated analog, nor-cantharidin with a view to reducing its nephrotoxic side effects (4).

In this study, we identified *B. terrestris* worker extract and *B. hypocrita sapporoensis* lava and pupa extract that displayed anti-inflammatory properties, and may be useful as a potential articular cartilage-repairing agent in the form of a combined drug product.

MATERIALS AND METHODS

Materials. The dried *B. terrestris* worker extract (SDIEX) or *B. hypocrita sapporoensis* lava and pupa extract (SPDYBEX), were soaked and extracted three times with ethanol by ultrasonification for 30 min. The extracts obtained were dried using a rotary evaporator and were freeze-dried as an alcohol extracts of *terrestris* worker or *B. hypocrita sapporoensis.*

Preparation of bumblebee extract. Dried alcohol extracts of bumblebee product were homogenized in a blender to a powder, stored at 4°C, dissolved in phosphate-

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Abbreviation: SDIEX, *B. terrestris* worker extract; SPDYBEX, *B. hypocrita sapporoensis* lava and pupa extract; CFA, Complete Freund's adjuvant; SC, subcutaneously; SD: Sprague-Dawley; COX, cyclooxygense; IL, interleukin; PLA_2; VEGF, vascular endothelial growth factor; TNF-α, tumor necrosis factor-α; SDIEX, *B. terrestris* worker extract; SPDYBEX: *B. hypocrita sapporoensis* lava and pupa extract.

buffered saline (Sigma-Aldrich; St. Louis, MO, USA), and then administered orally to SD rats at doses of 10 mg/kg daily, over 8 days.

Animal model. Specific pathogen-free, SD rats (weighing 200 ± 20 g, males) were purchased from Samtako Co. Ltd. (Osan, Korea). Anti-inflammatory activity was measured using complete Freud ajuvant (CFA, sigma, 0.1 m*l*/rat)-induced rat paw edema. CFA was injected into the subplantar tissue of the right hind paw.

Anti-inflammatory rat experiment. CFA was used to induce rat paw edema on the first day (pre-treatment), except the 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 five groups ($n = 10$ per group): control group, CFA (100 mM)-only treated group (negative control), indomethacin (5 mg/kg on first day, 1 mg/kg at 2~14th days) as a positive control, and the sample groups (bumblebee ethanol extracts : ethanol extract of SDI and SPDYB, daily 100 mg/kg treatment intraperitoneally, over 14 days). Paw size was measured 1, 3, and 5 hr and thereafter every day for 14 days using digital calipers (digimatic, Mitutoyo, Co., Japan).

The test parameters were paw edema, COX-2, interleukin 6, VEGF, TNF-α production levels, secretory phospholipase A_2 activity, and histopathological findings in the dorsal root ganglia, articular cartilage, and bone of the paw edema rats. Paw size was measured at 1, 3, and 5 hr and then every day. After the third, 7th, and 14th days of treatment, blood was collected from the posterior vena cava for serum biochemical analyses. The LV dorsal root ganglia, including articular cartilage, were extracted and processed for a histopathological study.

Measurement of COX-2, VEGF and PGE_2 assay. To analyze Cox-2 inhibitory activity, paw tissues were washed with Tris buffer (pH 7.4), homogenized on ice in a 4-fold volume lysis buffer, PRO-PREP protein extraction solution (iNtRON, Busan, Korea), centrifuged (15,000 ×*g*, 10 min), and the supernatant from the homogenate was removed for the for COX-2 and prostaglandin E_2 assay. The concentrations of COX-2 in lysates of extract-treated rat paw tissues, were determined with an enzyme-linked immunoassay (EIA) according to the manufacturer's protocol (Cayman Chemicals, Ann Arbor, MI, USA).

The concentrations of PGE_2 in sera were measured at 405 nm using ELISA (Cayman Chemicals, Ann Arbor, MI, USA) (5).

Cytokine (TNF-α and IL-6) production measurements. TNF-α and IL-6 level of SDIEX and SPDYBEX-treated rat serum were measured using commercial ELISA kits (Quantikine, R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

Secretary phospholipase A_2 measurements. Secretory phospholipase A_2 ($sPLA_2$) levels in SDIEX and SPDYBEX-treated rat serum were measured by ELISA using a $sPLA_2$ assay kit (Cayman Chemicals, Ann Arbor, MI, USA) (6,7).

Endothelial VEGF assay. The level of VEGF production was measured in human umbilical vein endothelial cells (HUVECs, ATCC, Manassas, VA, USA), grown in endothelial cell basal medium (EBM)-2 with EGM-2 singlequots (Cambrex, Walkersville, USA) at 37°C in an atmosphere containing 5% CO_2. The levels of VEGF production were measured, using an ELISA kit (human VEGF Immunoassay, Quantikine, R&D Systems, Inc.) according to the manufacturer's protocol.

Histopathology. The lumbar V (LV) dorsal root ganglion, including articular cartilage and near the 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.

Identification of cantharidin. The results here indicated anti-inflammatory effects of ethanol extracts of SDIEX and SPDYBEX from the bumblebee. Thus, we compared cantharidin components in the ethanol extracts of bumblebee worker and larvae, SDIEX and *B. hypocrita sapporoensis* lava and pupa extract (SPDYBEX) by HPLC (Dionex summit HPLC with UV; Luna C_{18} column, 5 μm, 250 × 4.6 mm, Phenomex, USA) and GC-MS analysis (Agilent 5973 N mass selective detector with HP 5 MS capillary column, 5% PH ME siloxane, 30 m × 0.25 mm, 0.25 m, USA) (8).

Statistical analyses. Means and standard error values of all the studied parameters were determined for each group. Student's *t*-test was used to establish significant differences between the final biochemical levels in control and treated groups. p values < 0.05 were considered statistically significant.

RESULTS

Anti-inflammatory effects. We showed that bumble bee alcohol extracts have potential efficacy in treating inflammation in SD rats, because they significantly reduced paw edema levels, in the following order: SDIEX > SPDYBEX, and repaired damaged dorsal root ganglia in CFA adjuvant arthritis (Fig. 1). The total mean changes in paw edema size (mm) from 1 hr to 14 days for each group were as follows: control (8.200 ± 0.514), CFA (9.86 ± 1.073), SDIEX (9.205 ±

Worker of *B. terrestris* Worker of *B. hypocrita sapporoensis*

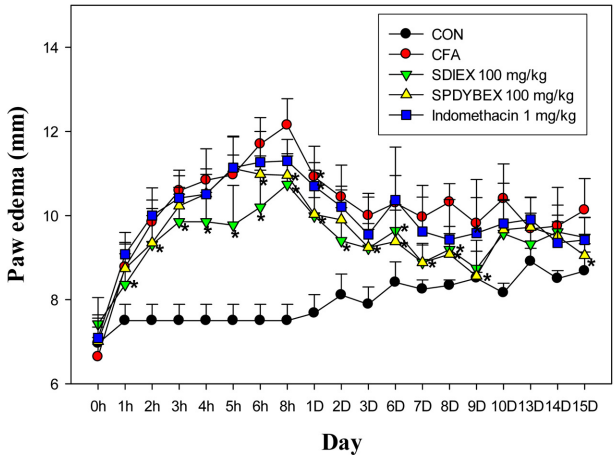

Fig. 1. Anti-inflammatory activity of SDIEX and SPDYBEX on Complete Freund's adjuvant (CFA)-induced paw edema in a rat arthritis model. CFA was used to induce rat paw edema on the first day (pre-treatment), except in the control group. Each extract (100 mg/kg) and indomethacin (5 mg/kg, 1st day, extra day 1 mg/kg) was treated over 14 days. SDIEX: *B. terrestris* (bumblebee) worker extract; SPDYB: *B. hypocrita sapporoensis* larva and pupa extract; IND: indomethacin. Values are means ± SD, # $p < 0.05$, vs. each vehicle (CFA).

0.656), SPDYBEX (9.17 ± 9.168), and IND (9.587 ± 0.891).

Inhibition of the COX-2 activity. In SDIEX or SPDYBEX 14 day-treated rat serum we measured COX-2 inhibitory activities using a COX II assay kit. The inhibitory effects of SDIEX and SPDYBEX on COX-2 were concentration-dependent (Fig. 2). Regarding the COX-2 experiment, *B. terrestris* worker extract (SDIEX) and *B. hypocrita sapporoensis* larva and pupa extract showed slight decreases, compared with the control value (control 11.62 ± 0.87, CFA 14.99 ± 1.90, SDIEX 11.73 ± 1.23; $p < 0.01$) and SPDYBEX (13.13 ± 7.38) and IND (12.74 ± 7.57). The inhibition of COX-2 activity by SDIEX was somewhat lower than that by SPDYBEX.

Inhibition of IL-6 activity. In SDIEX and SPDYBEX 7-day treated rat serum, we measured intereukin-6 inhibitory activities using an IL-6 assay kit. There is no difference in IL-6 levels between the groups. The no inhibitory levels on IL-6 of SDIEX and SPDYBEX were concentration-dependent on the 7th extract treatment day: control (11.72 ± 0.45), CFA (12.20 ± 0.11), SDIEX (11.78 ± 0.14),

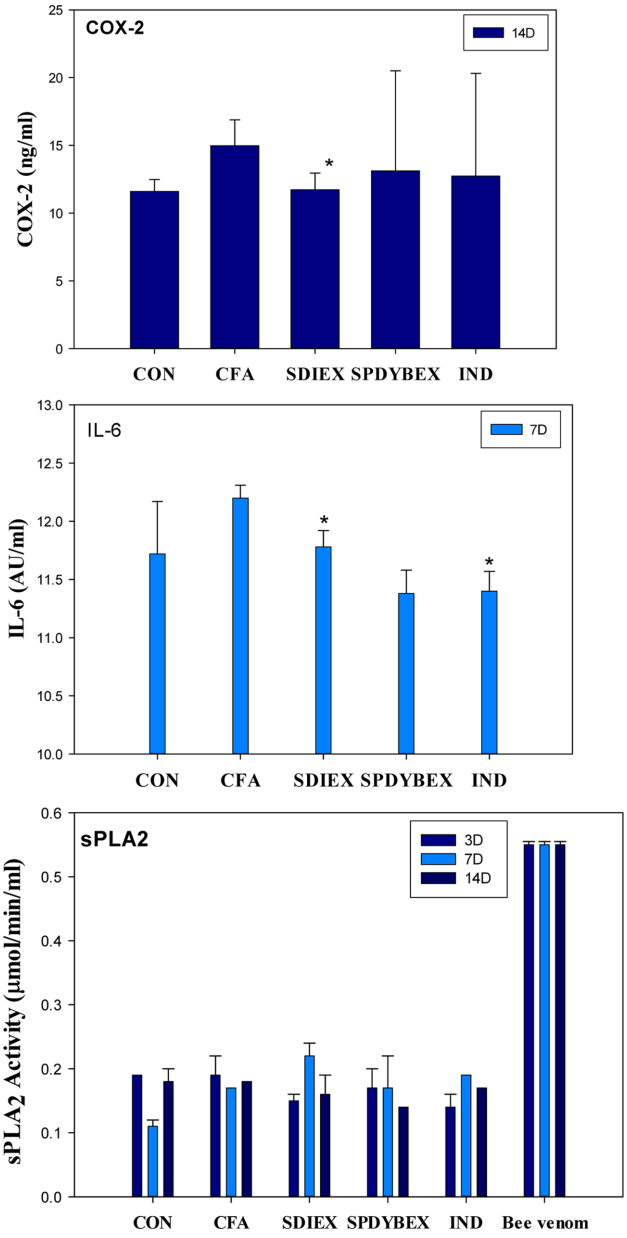

Fig. 2. COX, IL-6, and $sPLA_2$ activity of SDIEX and SPDYBEX treated CFA-induced paw edema rats. In each group, rats were treated intraperitoneally, daily over 3 days (3D), 7 days (7D), or 14 days (14D) at doses of 100 mg/kg SDIEX (*B. terrestris* worker extract), 100 mg/kg SPDYB (*B. hypocrita sapporoensis* larva and pupa extract), or 1 mg/kg IND (indomethacin). Values are means ± SD. # $p < 0.05$, vs. each vehicle (CFA).

SPDYBEX (11.38 ± 0.20), and IND (11.40 ± 0.17; Fig. 2).

Secretory PLA_2. These extracts had other actions, such as remarkable changes in secretory phospholipase A_2 activity at the 7th extract treatment day: control (0.110 ± 0.010), CFA (0.17 ± 0.0), SDIEX (0.22 ± 0.20), SPDYBEX (0.17 ± 0.05), IND (0.19 ± 0.0), and bee venom (positive control)

0.550 ± 0.0 (Fig. 2).

Articular cartilage destruction: repair by SDIEX and SPDYBEX. In the histological analysis, the LV dorsal root ganglion, including the articular cartilage and linked to the paw treated SDIEX and SPDYBEX, was repaired, when compared against the CFA-induced cartilage destruction. This was in contrast to the effects observed in the CFA-treated group, where there was destruction with erosion of the articular cartilage (Fig. 4). The CFA (no bumblebee extract treatment) group showed particular destruction of cartilage and an average abnormal cell nuclei number of 18/100, whereas abnormal cell nuclei numbers were much lower in the SDIEX group (2/100), the SPDYBEX group (5.7/100), and the indomethacin group (6/100) throughout the treatment period (14 days).

Histopathological finding in the lumbar (LV) dorsal root ganglion, including the articular cartilage linked to the paw treated with SDIEX and SPDYBEX for 14 days.

Cytokine (Prostaglandin E_2) production measurements in CFA-treated rat model. As another factor indicating anti-inflammatory effects, prostaglandin E_2 (PGE_2) levels were slightly decreased, suggesting anti-inflammatory action (data not shown).

Tumor necrosis factor-α production measurements. SDIEX and SPDYBEX did not increase the level of TNF-α, showing regulation to inflammation: 7th day, control (49.45 ± 6.39), CFA (169.21 ± 42.72), SDIEX (54.450 ± 11.61), SPDYBEX (80.14 ± 46.18), and IND (12.07 ± 14.00; Fig. 2).

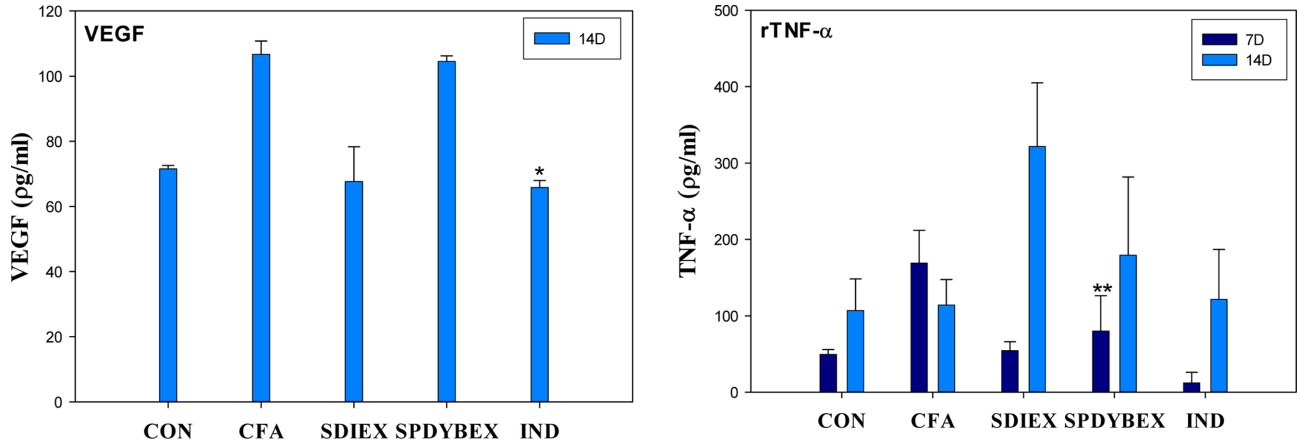

Fig. 3. (A) Effects on VEGF and TNF-α by SDIEX and SPDYBEX-treated CFA-induced paw edema rats. Values are means ± SD. # $p < 0.05$, vs. each vehicle (CFA).

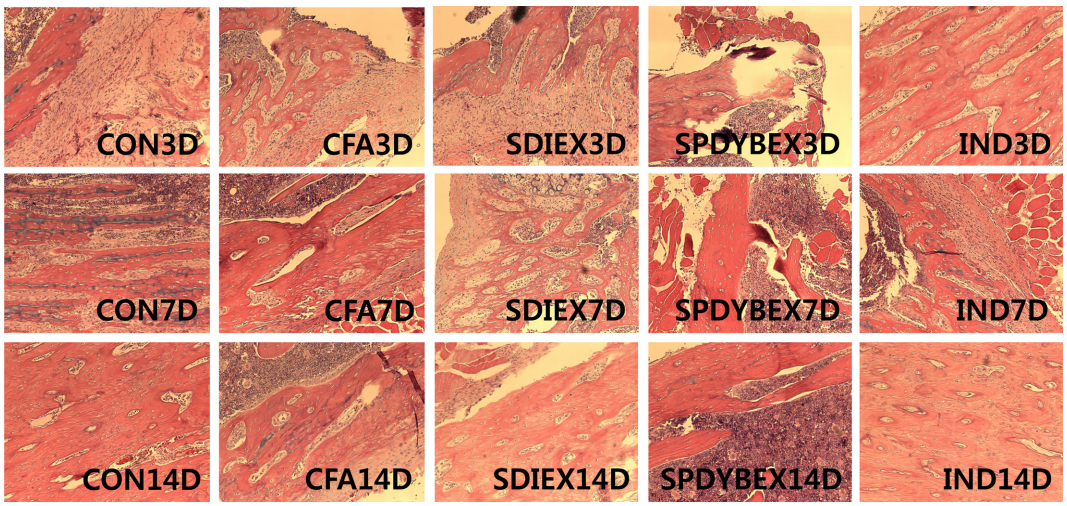

Fig. 4. Histopathological finding in the lumbar (LV) dorsal root ganglion, including the articular cartilage linked to the paw treated with SDIEX and SPDYBEX for 14 days. CON3D, CON7D, CON14D indicate the control group over a 3-, 7-, or 14-day period. The CFA group, SDIEX, SPDYBEX, and IND groups were named in the same manner as the control group (so, e.g., CFA3D, CFA7D, CFA14D).

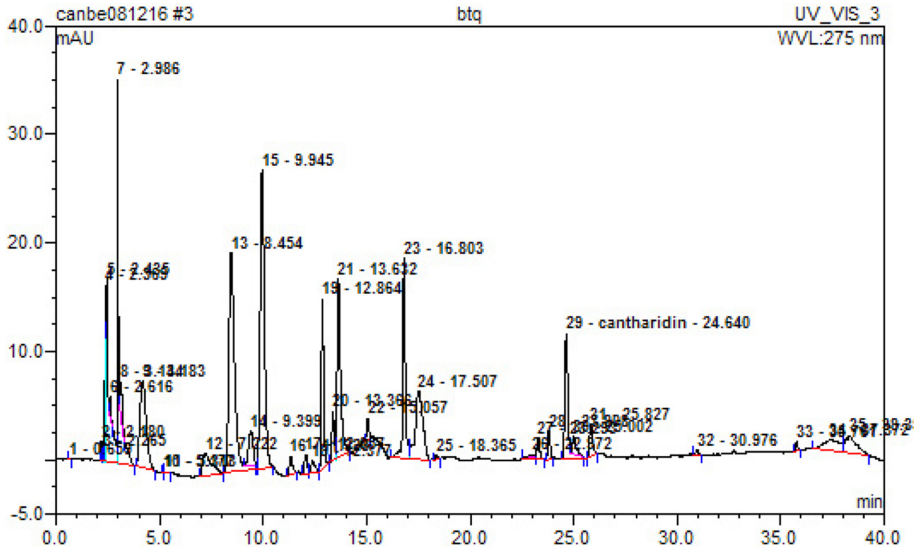

Fig. 5. Chromatogram of bumblebee extract using RPC-HPLC on a Luna C_{18} column.

Vascular endothelial growth factor (VEGF). In this study, there was regulation of VEGF production in the SDIEX and SPDYBEX- treated rat sera, suggesting virtually no relationship with angiogenesis associated with the cancer progenesis state: at the 14th extract treatment day, control (71.58 ± 1.160), CFA (106.73 ± 4.05), SDIEX (67.67 ± 10.65), SPDYBEX (104.52 ± 1.72), and IND (65.82 ± 2.14; Fig. 3).

Identification of cantharidin in SDIEX and SPDYBEX. HPLC and GC-MS analysis of SDIEX and SPDYBEX revealed the presence of cantharidin showing fragment: *m/z* 96 and *m/z* 128, and cantharidinimide fragment: *m/z* 70, 96 and 128 (Fig. 5). Further analysis will be needed to determine the active substance(s) in these extracts in the future.

DISCUSSION

The bumblebees *B. terrestris* and *B. hypocrita sapporoensis* belong to Hymenoptera, family Apidae, Subfamily Apinae, and are adapted as bumblebees for the pollination of natural vegetation and agricultural plants (9). Recently, *B. terrestris* workers have been abundant from the summer through the winter season because of mass artificial bumblebee production. *B. terrestris* workers and *B. hypocrita sapporoensis* larva and pupa can be used to prepare drugs, either as a crude insect extract or through a manufacturing process. We sought to make a bumblebee preparation with more efficacy and safety; we used an alcohol extract and studied its anti-inflammatory activity, assessing cytokine production and PLA_2 activity. That is, to identify the 'best' bumblebee extracts for the control of inflammatory reactions, we made alcohol extracts of *B. terrestris* workers and the larva and pupa of *B. h. sapporoensis*, and tested their medicinal activities.

That is, to identify the best bumblebee extracts for the control of inflammatory reactions, we made alcohol extracts of *B. terrestris* worker and the larva and pupa of *B. h. sapporoensis*, and tested their medicinal activities. There are previous reports regarding effects of extracts from workers of the bumble bee *Bombus terrestris* on the dynamics of the phenoloxidase (PO) system, antibacterial activity (3), and in workers, antibacterial activity was higher in challenged groups than in controls (10). Here, we show that constitutive immune defense is enhanced by the sexual offspring of the bumblebee *Bombus terrestris*. The alcohol extracts of the larva, pupa, queen, and cocoon of *B. ignitus*, *B. terrestris*, and *B. h. sapporoensis* showed anti-inflammatory activity in an adjuvant-induced edema model in rats. The queen of *B. ignitus*, queen of *B. terrestris*, and cocoon of *B. ignitus* decreased hind paw edema after 1 day of *ip* administration and also produced vasorelaxation and NO production in CPAE cells (11). *B. terrestris* worker extract (SDIEX) and *B. hypocrita sapporoensis* lava and pupa extract (SPDYBEX) significantly decreased the paw edema in arthritic rats and levels of cytokines and other factors related to inflammation - COX-2, IL-6, $sPLA_2$, VEGF, and TNF-α were decreased, compared with indomethacin. The COX-2 levels with SDIEX were lower than with indomethacin, and it had anti-inflammatory actions in repairing articular cartilage destruction. SDIEX may be useful as a potential articular cartilage-repairing agent in the form of a combination drug product.

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REFERENCES

1. Meda, A., Lamien, C.E., Milogo, J., Romoto, M. and Nacoulma, O.G. (2004) Therapeutic uses of honey and honey bee larvae in central Burkian Faso. *J. Ethnopharmacol.*, **95**, 103-107.
2. Erler, S., Popp, M. and Lattorff, H.M. (2011) Dynamics of immune system gene expression upon bacterial challenge and wounding in a social insect *(Bombus terrestris*). *PLoS One*, **6**, e18126.
3. Korner, P. and Schmid-Hempel, P. (2004) In vivo dynamics of an immune response in the bumble bee Bombus terrestris. *J. Invertebr. Pathol*., **87**, 59-66.
4. Cheng, C., Zhu, Y., Tang, X. and Tao, W. (2011) The anti-proliferative effects of norcantharidin on human HepG2 cells in cell culture. *Mol. Biol. Rep*., **38**, 163-169.
5. Kwon, J.H., Kim, J.H., Choi, S.E., Park, K.H. and Lee, M.W. (2010) Inhibitory effects of phenolic compounds from needles of *Pinus densiflora* on nitric oxide and PGE_2 production. *Arch. Pharm. Res*., **33**, 2011-2016.
6. Schoenberg, M.H., Mayer, J.M. and Beger, H.G. (1997) [Phospholipase A_2-from basic research to clinical reality]. *Chirurg*, **68**, 1112-1118.
7. Reid, R.C. (2005) Inhibitors of secretory phospholipase A2 group IIA. *Curr. Med. Chem*., **12**, 3011-3026.
8. Nikbakhtzadeh, M.R., Dettner, K., Boland, W., Gäde, G. and Dötterl, S. (2007) Intraspecific transfer of cantharidin within selected members of the family Meloidae (Insecta: Coleoptera). *J. Insect Physiol*., **53**, 890-899.
9. Yoon, H.J., Lee, K.Y., Kim, M.A., Ahn, M.Y. and Park, I.G. (2013) Optimal cold temperature for the artificial hibernation of *Bumbus ignitus* queen bumblebee. *Int. J. Indust. Entomol*., **26**, 124-130.
10 Moret, Y. and Schmid-Hempel, P. (2001) Immune defence in bumble-bee offspring. *Nature*, **414**, 506.
11. Ahn, M.Y., Han, J.W., Yoon, H.J., Hwang, J.S. and Young, Y.E. (2012) Anti-inflammatory effect of bumblebee alcohol extracts in CFA-induced rat edema. *Toxicol. Res*., **28**, 249-253.