Entomological Research

Entomological Research **51** (2021) 90–94

RESEARCH PAPER

Antioxidant and cytoprotective effects of enzyme-extracted constituents of *Protaetia brevitarsis seulensis* powder

In-Hag CHOI[1], Sung-Up CHOI[1], Jin-Sung SON[2], Woo-Whan JANG[3] and Tae-Ho CHUNG[1]

[1] Department of Companion Animal & Animal Resources Science, Joongbu University, Geumsan-gun, South Korea
[2] Hungnam Taean Agricultural Corporation, Taean-gun, Korea
[3] Department of Agricultural economics, Kyungpook National University, Daegu, Korea

Correspondence
Tae-Ho Chung, Department of Companion Animal & Animal Resources Science, Joongbu University, Geumsan-gun, 32713, South Korea.
E-mail: taehochung@daum.net

Received 8 May 2020;
accepted 6 August 2020.

doi: 10.1111/1748-5967.12484

In-Hag Choi and Sung-Up Choi contributed equally to this study as first author.

Abstract

This study was aimed to compare the antioxidant and cytoprotective effects of hot-water and enzyme-extracted constituents of *Protaetia brevitarsis seulensis* powder. The products of enzymatic extraction had 1,1-diphenyl-2-picrylhydrazyl (DPPH)-radical-scavenging activity, cytotoxic effects, and provided cytoprotection against H_2O_2 and intracellular reactive oxygen species. Enzymatic extraction improved DPPH-radical-scavenging activity relative to hot-water extraction ($P < 0.05$), with inhibition rates of 33% at 500 μg/mL and 35% at 1000 μg/mL. Cell viability ranged from 100 to 122%, and was higher for hot-water than for enzymatic extraction ($P > 0.05$). Hot-water extraction achieved greater cytoprotection against H_2O_2 than enzymatic extraction. However, enzymatic extraction achieved greater antioxidant and cytoprotective activity against reactive oxygen species than hot-water extraction. These results provide evidence of the antioxidant and cytoprotective effects produced by enzymatic extraction of the active ingredients from *Protaetia brevitarsis seulensis* powder.

Key words: antioxidant activity, cytoprotective effect, enzymatic extraction, hot-water extraction, *Protaetia brevitarsis seulensis* powder

Introduction

Insects provide food for humans and livestock at low environmental cost, and are an important source of nutrition (Anankware *et al*. 2015). They represent the largest and most diverse group of organisms on earth with a global biodiversity estimated at 80 to 90% (Im *et al*. 2018). Insects are an essential feed component in the livestock industry, because they are very rich in protein (39–64% by weight) and essential amino acids (Al-Qazzaz *et al*. 2016; Atteh & Ologbenla 2015). Insects are typically classified into four resource categories: (i) as industrial resources; (ii) as food and therapeutic resources; (iii) in forensic investigations; and (iv) as ecologically important organisms (Lokeshwari & Shantibala 2010).

Insects have been used therapeutically in traditional medicine for curing diseases such as arthritis, rheumatism, cancer, inflammatory diseases, and skin diseases (Lokeshwari & Shantibala 2010). *Protaetia brevitarsis seulensis* (Coleoptera: Scarabaeidae), the white spotted flower chafer, is a widely used medicinal insect. It is distributed primarily in Eastern Asia, and the larvae are used in traditional medicine (Kang *et al*. 2012; Suh & Kang 2012; Yoo *et al*. 2007). This species has been temporarily registered as a food resource by the KFDA (Food and Drug Safety of Korea) (Im *et al*. 2018). Further, other growth stages of this species have been reported to have high antioxidant effects (Suh & Kang 2012). Extracts from this beetle have also been used to improve the anti-inflammatory and whitening effects of cosmetic products (Sung *et al*. 2016).

The objective of this study was to evaluate the antioxidant and cytoprotective effects of enzyme-extracted constituents of *P. b. seulensis*, compared to those of hot-water extracted constituents.

Materials and Methods

Preparation of extracts

All materials, including *P. b. seulensis* larvae powder, protease, *Bacillus natto* culture medium, and RAW 264.7 cells were obtained from Deeprootedfarm Co. (Taean, South Korea). Two methods, hot-water extraction and enzyme extraction, used to extract the active ingredients. For the hot-water extraction, 100 kg of *P. b. seulensis* larvae powder was mixed with 400 L of distilled water (DW), and, the active ingredients were extracted using reflux technology at 60°C for 15 h. The hot-water extracts were filtered, evaporated to dryness under a reduced-pressure hood, and freeze-dried until final yields were reached. For the enzymatic extraction, we added 3 kg of protease to 400 L of DW containing 100 kg of *P. b. seulensis* larvae powder.

DPPH-radical-scavenging activity

The 1,1-Diphenyl-2-picrylhydrazyl (DPPH)-radical-scavenging activity was measured as described by Brand Williams *et al*. (1995), with some modifications. The extracts obtained using both methods were dissolved in 50% ethanol. One hundred microliters of each sample was diluted to eight different concentrations (0, 15, 30, 60, 125, 250, 500, and 1,000 μg/mL) to 100 μL of 60 μM solution dissolved in 95% ethanol. After reacting for 30 min at room temperature, the absorbance was measured at 517 nm using a microplate reader. The DPPH IC_{50} (concentration required to inhibit 50% of free radical activity) was calculated using the following equation.

$$\text{DPPH radical scavenging}\ (\%) = \left[1 - \left(\frac{\text{sample} - \text{sample blank}}{\text{control} - \text{control blank}}\right)\right] \times 100$$

Cytotoxicity

Cytotoxicity was confirmed using an EZ-Cytox Cell Viability Assay Kit (Daeil Lab Service Co, Seoul, South Korea). In brief, RAW 264.7 cells were dispensed into a 96-well culture plate at 1×10^5 cells/well and attached for 4 h. The extracts were added at concentrations of 0, 25, 50, 100, 200, and 400 μg/mL, and the cells were incubated at 37°C for 24 h. After 24 h incubation, the medium was removed, and then 100 μL of CCK-8 reagent was added, followed by an incubation period of 3 h at 37°C. Absorbance was measured at 450 nm using the microplate reader.

Cytoprotection against H_2O_2

Cytoprotection against H_2O_2 was assessed as for cytotoxicity. After a 4 h incubation period at 37°C, the reaction was initiated by adding 2 mM of H_2O_2 to each well, followed by incubation for 1 h. Next, the medium was removed, and 100 μL of CCK-8 reagent was added, followed by incubation at 37°C for 3 h. **Absorbance** was **measured** at 450 nm using the microplate reader.

Intracellular reactive oxygen species (ROS) assay

The level of intracellular ROS was quantified using the ROS detection fluorescent probe DCFH-DA. Briefly, RAW 264.7 cells were plated in 96-well plates at a density of 1.0×10^5 cells per well, incubated for 24 h, then treated with the extracts at concentrations of 0, 25, 50, 100, 200, and 400 μg/mL. After 24 h incubation, the medium was removed and 2 mM H_2O_2 was added, followed by incubation for 1 h. The medium was again removed, and 10 μM of 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich Co.) was immediately added to the cells, followed by incubation in a CO_2 incubator for 30 min in the dark. The cells were then washed twice with PBS and resuspended in 50 μL of PBS. The intracellular fluorescence intensity was recorded at excitation and emission wavelengths of 490 nm and 525 nm, respectively, using the microplate reader.

Statistical analysis

The results expressed are the means ± SE of three replicate measurements. Analysis was carried out using SAS (2002) with the Student's *t*-test. The probability threshold for statistical significance was $p < 0.05$.

RESULTS AND DISCUSSION

The final extraction yields using the hot-water and enzymatic extraction methods were 15.8% and 17.4% (w/w), respectively (Table 1). The DPPH inhibition, which increased with the extract concentration, differed between the methods ($P < 0.05$), except at 0 μg/mL (Fig. 1). Enzymatic extraction resulted in greater antioxidant-scavenging capacity than hot-water extraction. For the highest tested extract concentrations (500 and 1,000 μg/mL), enzymatic extraction achieved inhibition rates of 33% and 35%, respectively. Antioxidants are considered to perform DPPH-radical scavenging via their hydrogen atom-donating ability. The DPPH is a stable free radical that easily accepts an electron or hydrogen radical, creating a stable diamagnetic molecule (Marjoni & Zulfisa 2017). IC_{50} is widely used to assess antioxidant activity (Sánchez-Moreno *et al*. 1998); higher

Table 1 Enzyme extract conditions and yield of PB in each time

Time	PBx powder[a] (kg)	Distilled water (L)	Enzyme (kg)	Fermented extract temperature (°C)	Fermented extract time (h)	Culture medium of *Bacillus natto* (kg)	Fermented extract temperature (°C)	Fermented extract time (h)	Drying	Freezing dry (kg)	Extract yields (%)
1	100	400	3	60	6				X	10.2	10.2
2	100	400	3	60	15				X	11.3	11.3
3	100	400	3	60	24				X	11.5	11.5
4	100	400	3	60	15	20	40	15	X	14.1	14.1
5	100	400	3	60	15	20	40	24	X	15.5	15.5
6	100	400	3	60	15	20	40	36	X	15.8	15.8
7	100	400	3	60	15				O	13.3	13.3
8	100	400	3	60	15				O	13.1	13.1
9	100	400	3	60	15				O	13.3	13.3
10	100	400	3	60	15				O	13.2	13.2
11	100	400	3	60	15	20	40	24	O	17.5	17.5
12	100	400	3	60	15	20	40	24	O	17.5	17.5
13	100	400	3	60	15	20	40	24	O	17.4	17.4

[a]PB powder, *Protaetia brevitarsis seulensis* powder.

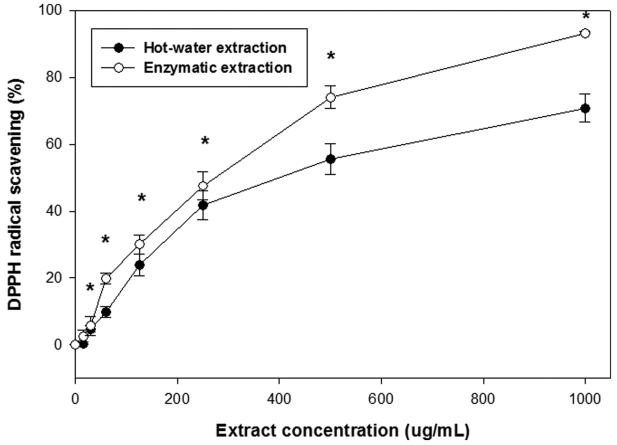

Figure 1 DPPH-radical-scavenging activity of *Protaetia brevitarsis seulensis* powder extracts obtained using hot-water and enzymatic extraction. Data are expressed as SEM. $*P < 0.05$ (Student's t-tests).

antioxidant ability corresponds to a lower IC_{50} value. The results in this study are consistent with the findings of Sim *et al*. (2018), who studied the products of fermentation of *P. b. seulensis* larvae by various micro-organisms. The possible mechanism of antioxidant activity of the extracts obtained using enzymatic extraction is via their bioactive compounds such as total phenols. For instance, Sim *et al*. (2018) found that the phenolic and flavonoid contents of the aqueous extract of *B. subtilis*-fermented *P. b. seulensis* powder provided antioxidant effects.

No cytotoxicity was observed, and there was no significant difference ($P > 0.05$) in cytotoxicity between the products of the two extraction methods (Fig. 2). Compounds are considered to be non-cytotoxic if cell viability remains above 80% (Kim & Son 2012). Using both methods, cell viability ranged from 100 to 122%, and tended to be higher using the products of hot-water extraction. Lee (2018) reported that aqueous-extraction products of *P. b. seulensis* larvae were not cytotoxic: cell viability ranged from 93 to 103%.

In terms of cytoprotection against H_2O_2, cell viability was significantly lower ($P < 0.05$) at 50, 100, and 200 μg/mL following enzymatic extraction than following hot-water extraction (Fig. 3). However, the methods did not produce significant differences ($P > 0.05$) in cell viability at concentrations of 0, 25, and 400 μg/mL. Cell viability following both methods initially decreased from 0 μg/mL to 25 μg/mL, then increased abruptly to 50 μg/mL (for hot-water extraction) and slowly to 100 μg/mL (for enzymatic extraction) (Fig. 3). Hot-water extraction resulted in greater cytoprotection against H_2O_2 than enzymatic extraction.

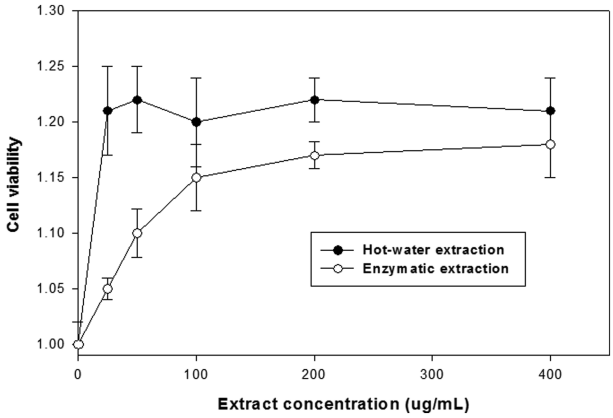

Figure 2 Cytotoxic effect of *Protaetia brevitarsis seulensis* powder extracts obtained using hot-water and enzymatic extraction. Data are expressed as SEM.

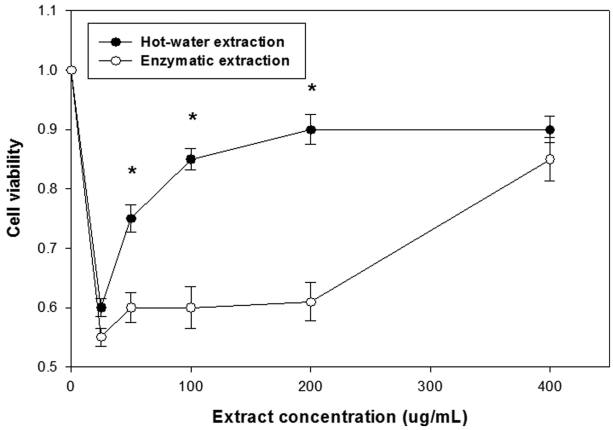

Figure 3 Cytoprotective effects against free radicals, by extracts obtained from *Protaetia brevitarsis seulensis* powder using hot-water and enzymatic extraction. Data are expressed as SEM. * $P < 0.05$ (Student's *t*-tests).

For both hot-water and enzymatic extraction, ROS scavenging increased significantly from 0 μg/mL to 25 μg/mL. After 25 μg/mL, ROS generation declined to the same level as in the control at 50 μg/mL (for enzymatic extraction) and at 100 μg/mL (for hot-water extraction). The methods did not differ at 0, 200, or 300 μg/mL, but differed significantly at 400 ug/mL (hot-water extraction achieved lower ROS generation; $P < 0.05$) (Fig. 4). Although this is not entirely consistent with the cytoprotective effects described above, the findings show that both methods have antioxidant activity and provide cytoprotection against ROS. In particular, ROS, including superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl ($OH^{\cdot}$) radicals are generated when ATP is produced in the mitochondria. ROS are signaling substances that regulate gene expression and cell activity, but when generated in excess, they are a major cause of aging and various diseases—they damage DNA, cause cell death and abnormal proliferation, and thereby damage tissues and organs (Sung *et al*. 2016). The antioxidant and cytoprotective effects of insect extracts have been confirmed by Lee (2018) and Sim *et al*. (2018), who studied the biological activity of aqueous extracts of *P. b. seulensis* larvae. In conclusion, enzymatic extraction achieved a noticeable antioxidant effect on free radical scavenging, which improved with increasing concentration. Although enzymatic extraction achieved less effective cytoprotection than hot-water extraction, it nonetheless achieved some cytoprotection against H_2O_2 and ROS. The enzyme-extracted constituents of *P. b. seulensis*, which have antioxidant and cytoprotective effects, may be an effective way to reduce damage caused by ROS. In terms of insect industry and marketing point of view, it is essential to select insects as nutritious alternatives to feed and food

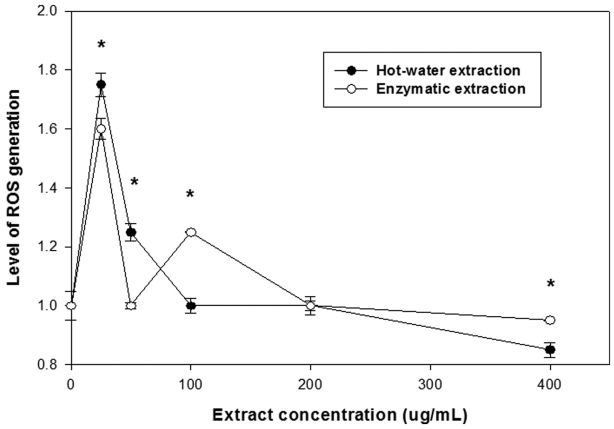

Figure 4 Effects of *Protaetia brevitarsis seulensis* powder extracts on reactive oxygen species (ROS) generation, comparing extracts obtained using hot-water and enzymatic extraction. Data are expressed as SEM. * $P < 0.05$ (Student's *t*-tests).

resources or the development of functional foods because of the increasing demands for treating diverse diseases. In addition, an analysis of existing policies and regulations on the insect industry and marketing should be followed.

Acknowledgments

This research was supported by the Ministry of Trade, Industry & Energy (MOTIE), Korea Institute for Advancement of Technology (KIAT) through the Encouragement Program for The Industries of Economic Cooperation Region.

REFERENCES

Al-Qazzaz MFA, Ismail D, Akit H *et al*. (2016) Effect of using insect larvae meal as a complete protein source on quality and productivity characteristics of laying hens. *Revista Brasileira de Zootecnia* **45**: 518–523.

Anankware PJ, Fening KO, Osekre E *et al*. (2015) Insects as food and feed: A review. *International Journal of Agricultural Research and Review* **3**: 143–151.

Atteh J, Ologbenla F (2015) Replacement of fish meal with maggots in broiler diets: effects on performance and nutrient retention. *Nigerian Journal of Animal Production* **20**: 44–49.

Brand Williams W, Cuvelier ME, Berset C (1995) Use of a free-radical method to evaluate antioxidant activity. *Food Science Technol-Lebens Wissens Technology* **28**: 25–30.

Im AR, Yang WK, Park YC *et al*. (2018) Hepatoprotective effects of insect extracts in an animal model of nonalcoholic fatty liver disease. *Nutrients* **10**: 1–19.

Kang M, Kang C, Lee H *et al*. (2012) Effects of fermented aloe vera mixed diet on larval growth of *Protaetia brevitarsis seulensis* (Kolbe) (Coleopteran: Cetoniidae) and protective effects of its extract against CCl4-induced hepatotoxicity in Sprague- Dawley rats. *Entomological Research* **42**: 111–121.

Kim YJ, Son DY (2012) Inflammatory mediator regulation of the *Zizyphus jujube* leaf fractions in the LPS-stimulated Raw264.7 mouse machrophage. *Korean Journal of Food Preservation* **21**: 114–120.

Lee YD (2018) Properties of aqueous extract of *Protaetia brevitarsis* larva and mountain ginseng fermented by *lactobacillus brevis*. *Journal of Food Hygiene and Safety* **33**: 369–374.

Lokeshwari RK, Shantibala T (2010) A review on the fascinating world of insect resources: reason for thoughts. *Psyche* **2010**: 1–11.

Marjoni MR, Zulfisa A (2017) Antioxidant Activity of Methanol Extract/Fractions of Senggani Leaves (*Melastoma candidum* D. Don). *Pharmaceutica Analytica Acta* **8**: 1–6.

Sánchez-Moreno C, Larrauri JA, Saura-Calixto F (1998) A procedure to measure the antiradical efficiency of polyphenols. *Journal of Agricutural and Food Chemistry* **76**: 270–276.

SAS Institute Inc (2002) SAS/STAT User's Guide: Version 8.2, Cary, NC, USA.

Sim SY, Ahn HY, Seo KI *et al*. (2018) Physicochemical properties and biological activities of *Protaetia brevitarsis* seulensis larvae fermented by several kinds of micro-organisms. *Journal of Life Science* **28**: 827–834.

Suh HJ, Kang SC (2012) Antioxidant activity of aqueous methanol extracts of *Protaetia brevitarsis* Lewis (Coleoptera: Scarabaedia) at different growth stages. *National Product Research* **26**: 510–517.

Sung GA, Kim MH, Park SN (2016) Anti-inflammatory and whitening effects of *Protaetia brevitarsis seulensis* extracts by oriental conversion methods. *Journal of Society of Cosmetic Scientists of Korea* **42**: 421–432.

Yoo YC, Shin BH, Hong JH *et al*. (2007) Isolation of fatty acids with anticancer activity from *Protaetia brevitarsis* larva. *Archives of Pharmacal Research* **30**: 361–365.