

Antioxidant Effects of *Berchemia berchemiaefolia* in Nerve Pain Models

Gil-Hyun Lee¹, Kyung-Yae Hyun² and Seok-Cheol Choi^{3,†}

¹Department of Clinical Laboratory Science, Kyungwoon University, Gyungbuk 36190, Korea

²Department of Clinical Laboratory Science, DongEui University, Busan 47340, Korea

³Department of Clinical Laboratory Science, Catholic University of Pusan, Busan 46252, Korea

Berchemia berchemiaefolia (BB) are climbing plants or small to medium-sized trees that live in Africa, Asia and America. We performed the present study to investigate whether oral administration of *Berchemia berchemiaefolia* extract (BBE) protects SD rats from pain. The SD rat experimental groups were divided into four groups. Two of the animal model groups were fed on BBE (200 mg/kg or 100 mg/kg). We performed oral acute toxicity test to determine the optimal oral dose of BBE. To explore if BBE alleviated pain in the SD rat, we undertook the tail flick latency test and formalin test. Additionally, we conducted the anti-oxidative test. The findings of the present study suggest that *Berchemia berchemiaefolia* extract exhibits strong antioxidant and analgesic activities.

Key Words: Nociceptive action, *Berchemia berchemiaefolia*, Rat, Nerve pain, Tail flick latency test

INTRODUCTION

Pain has been defined as an uncomfortable sensory and emotional experience associated with tissue damage (Beltramo et al., 2006). The direct and indirect activity of chemical mediators, such as arachidonic acid metabolites, cytokines, nitric oxide, peptides, serotonin, among others are responsible for the multiplicity of events that occur during pain transmission in both the peripheral and central nervous systems (Cimanga et al., 2001; Cao et al., 2008; Bojovic et al., 2015). Reactive oxygen species (ROS) are involved in sensitization of dorsal horn neurons that plays a fundamentally important role in pain (Connor, 2009; Bojovic et al., 2015). Antioxidant substances are known to increase the threshold of pain perception (Bojovic et al., 2015; Evers,

2017). Accordingly, animal pain situation is classified into two groups: central nervous system and peripheral nervous system, which measurement of anti-nociceptive effect is limited to three stimulations as chemical stimulus, mechanical irritation, and temperature shock (Hogan et al., 2013; Kaulmann and Bohn, 2014). In this context, we conduct tail flick latency test to confirm anti-pain effect for central nervous system and formalin test to confirm it for peripheral nervous system (Kishore et al., 2011).

Berchemia berchemiaefolia is a deciduous tree distributed in Korea and Japan. According to recent studies, it is found to have anti-inflammatory and anti-cancer efficacy through animal experiments (Li et al., 2014). As a natural edible substance, it is expected to have less side effects than existing chemosynthetic drugs. This study was conducted under the hypothesis that the pain relief effect of *Berchemia berche-*

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†Corresponding author: Seok-Cheol Choi. Department of Clinical Laboratory Science, Catholic University of Pusan, 57Oryundae-ro, Bugok 3(sam)-dong, Geimjeong-gu, Busan 46252, Korea.

Tel: +82-51-510-0564, Fax: +82-51-510-0568, e-mail: scchoi@cup.ac.kr

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miaefolia is related to the antioxidant effect. To evaluate the impact of the antioxidant efficacy of *Berchemia berchemiaefolia*, this study used DPPH and xanthine oxidase assay.

MATERIALS AND METHODS

Chemicals and reagents

All reagents and standards were of analytical reagent grade unless stated otherwise. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and xanthine oxidase were purchased from Sigma Chemical Co. All other chemicals and solvents were of analytical grade.

Experimental animals

Thirty male Sprague-Dawley rats (4 weeks of age, 140 g average body weight) were purchased from Central Lab Animal Inc (Seoul, Korea). Each animal was individually housed in a cage under standard laboratory conditions of 12/12 hours light/dark cycle at 25°C and 60% humidity and was allowed to access food and water ad libitum for 2 weeks. All experiments were approved by the Ethics Committee of Dong-Eui University and were in accordance with the guidelines of the International Association for the Study of Pain (IASP). Control group and experimental group were categorized as follows; Group I: control group (n=8), Group II: vehicle, formalin induced stimulation, saline oral administration (n=8), Group III: formalin induced stimulation, *Berchemia berchemiaefolia* extract (BBE) oral administration (200 mg/kg rat weight) (n=8), Group IV: formalin induced stimulation, *Berchemia berchemiaefolia* extract (BBE) oral administration (100 mg/kg *ratweight*).

This study was approved by the Animal Ethic Committee of the Catholic University of Pusan.

Preparation of *Berchemia berchemiaefolia* extract

Berchemia berchemiaefolia was purchased from oriental medicine store in Pusan. We rinsed its leaves clean, dried it in the shade for a week, chopped roots into small pieces by grinder, and chopped sources were lyophilized by freeze dryer. After filling 15 mL tube with 1 milligrams of lyophilized sample and 10 milligrams of 70% ethanol, tube whirled into rounded mixed machine for 18 hours. Only supernatant

was collected, vaporized in 40°C and also lyophilized. Yield of extracted material was 6.6%.

Acute toxicity test

The procedure is incorporated into the European Community Directive guidelines as the acute toxicity test (Li et al., 2014). The rats were subjected to toxicity test using the fixed-dose procedure, which is a sequential testing scheme that was proposed by the British Toxicology Society in 1984 as an alternative for the assessment of acute toxicity via estimation of the Lethal Dose 50 (LD₅₀). Briefly, an initial dose of 5, 50, 500, or 2,000 mg per kg of body weight can be selected to evaluate the toxicity of the substance being investigated. Either 5 or 2,000 mg per kg can serve as the starting dose. The procedure was terminated when either toxicity or death was observed.

Tail-flick test

Tail-flick test was applied as follows (Mfotie et al., 2017). Each animal was placed in a ventilated tube with the tail laid across a wire coil maintained at room temperature (23 ± 2°C). The coil temperature was then increased by the passage of an electric current, and the latency for the tail withdrawal reflex was measured. Heat was applied to a portion of the ventral surface of the tail between 4 and 6 cm from the tip. Tail-flick latency (TFL) was measured in 5-min intervals until a stable baseline was obtained over three or four consecutive trials. The apparatus was fixed to obtain a baseline TFL at approximately 3 or 9 seconds. Only rats showing stable baseline TFL after up to 6 trials were used in each experiment. Each trial was terminated after 6 or 15 seconds to minimize the possibility of skin damage.

Formalin test

Experiments were carried out according to previously described method (Mor et al., 2017). SD rat were orally treated with BBE. One hour later, 20 µl of 2.5% formalin was injected subcutaneously into the right hind paw of each rat. Each rat was immediately placed into a glass cylinder to observe the nociceptive behavior. The time spent in licking the injected paw (an index of nociception) was measured in the first phase (0~5 min) and second phase (15~30 min)

after formalin injection. The nociceptive behaviors including licking/biting durations of the injected paw were recorded in 5-min blocks for 60 min by video camera.

Animal sacrifice and sampling

The rats were fasted for 24 hours before sacrificing. The weight of the rats was measured and recorded before anesthesia, and they were placed in supine position on a rat operating table (Dong Sew Science, Seoul, Korea). The lower abdomen was incised and 7~8 mL blood was collected from the abdominal aorta. A proper amount of blood was taken into an EDTA tube and serum separating tube.

DPPH assay

The free radical scavenging activity of all the extracts was evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) according to the previously reported method (Palle et al., 2016). Briefly, an 0.1 mM solution of DPPH in methanol was prepared and 1 mL of this solution was added to 3 mL of the solution of all extracts in methanol at different concentration (10, 20, 40, 80, 160, 320 & 640 µg/mL). The mixtures were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 517 nm using a UV-VIS spectrophotometer. Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability of scavenging the DPPH radical was calculated by using the following formula.

DPPH scavenging effect (% inhibition) = $\{(A_0 - A_1)/A_0\} \times 100$

Where, A₀ is the absorbance of the control reaction, and A₁ is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

Inhibition of xanthine oxidase

As described by Cimanaga et al. (2001), the assay mixture, containing 40 mM phosphate buffer, pH 7.5, 0.2 mM ethylenediaminetetraacetic acid (EDTA), and 5~50 µM xanthine, was incubated in a quartz cuvette at 37°C for 2 min. The reaction was started by the addition of 10 mU xanthine oxidase (XOD) and the increase value in absorption at 290

nm was recorded at 5 s intervals for 5 min. The compounds were dissolved in DMSO to obtain various concentration of solutions. In any appropriate long period of time, absorbance was increased linearly with time, and the slope was reaction rate (dA/min). First, inhibition rate against XOD of all kinds of compounds was determined in the concentration of 100 µM, and then IC₅₀ values were further measured on the samples whose inhibition rate was more than 50%. The solution of 0.5 mL methanol, 1.3 mL phosphate buffer, 0.2 mL xanthine oxidase and 1.5 mL xanthine substrate is used as a control. Percentage of inhibition is calculated using the formula:

Inhibition of xanthine oxidase (%) = $[1-(A_s/A_c)] \times 100$ where A_s and A_c are the absorbance values of the test sample and control, respectively.

Nitric oxide assay

Animal serum was mixed with an equal volume of Griess reagent [0.1% N-(1-naphthyl) ethylenediamine] in distilled water and 1% sulfanilamide in 5% phosphoric acid, 1:1 ratio). After a 10 min incubation period, absorbance was measured at 540 nm on a micro-plate reader.

Data analysis

Experimental values are presented as mean ± SD. Statistical significance was determined by Mann-Whitney test or ANOVA with Bonferroni's post-hoc test using SPSS version 18. $P < 0.05$ were considered to be statistically significant ($* < 0.05$; $** < 0.01$; $*** < 0.001$). Different letters are significantly different at $P < 0.05$, according to the Fisher's least significant difference (LSD) analysis.

RESULTS

Demographic characteristics

Thirty-two male Sprague-Dawley rats (4 weeks age, 140 g averaged body weight) were used in experiment. Each group had no meaningful differences among three before and after experiments (Table 1).

Toxicity studies

Oral LD₅₀ of BBE in rats was found to be >2,000 mg/kg

Table 1. Demographic characteristics in four groups

Variable	Group			
	Control	Vehicle	BBE 200 mg/kg	BBE 100 mg/kg
Total number (n)	8	8	8	8
Gender	Male	Male	Male	Male
Age (week)	4	4	4	4
BW before experiment (g)	138.18±4.58	139.15±11.21	140.15±7.68	136.61±6.88
BW after experiment (g)	151.21±5.55	153.09±6.81	153.81±8.15	149.58±6.65

Data are expressed as mean ± standard deviation (SD).

Abbreviation: Control, normal group; Vehicle, no treatment group; BBE 200 mg/kg, 100 mg/kg, *Berchemia berchemiaefolia* extract treatment group 200 mg/kg or 100 mg/kg; BW, body weight.

Table 2. Analgesic effect of BBE on the tail flick test

Animal groups	Tail-flick latency (sec)		
	10 min. after BBE oral administration	30 min. after BBE oral administration	50 min. after BBE oral administration
Non-BBE	5.82 ± 0.82	5.98 ± 0.56	6.01 ± 0.48
BBE 100 mg/kg	6.11 ± 0.75	6.05 ± 0.48	6.21 ± 0.62
BBE 200 mg/kg	5.98 ± 0.58	6.18 ± 0.78	6.36 ± 0.59*

Abbreviation: Non-BBE (Vehicle), no treatment group; BBE 200 mg/kg, 100 mg/kg, *Berchemia berchemiaefolia* extract treatment group 200 mg/kg or 100 mg/kg

body weight as administration of BBE at a dose of 2,000 mg/kg weight did not produce any mortality in the tested animals. In chronic administration of BBE at a dose of 2,000 mg/kg body weight for 28 days did not produce any pathological changes as compared to normal animals (not shown data).

Tail flick test

Thermal perception threshold was manifested by tail-flick latency. The hot tail flick test results showed that the mean reaction time increased in a dose related manner reaching a peak at 40 minutes for dose 200 mg/kg (Table 2). A significant increase in the tail-flick latency was observed after 50 minutes of BBE 200 mg/kg ($P < 0.05$).

Formalin test

In the formalin test (Table 3), formalin induced nociception in the vehicle rats versus to the sham operated group ($P < 0.05$, $P < 0.01$). BBE (100 mg/kg, 200 mg/kg) could not significantly inhibit the first phase, but was active in the later phase of formalin-induced pain ($P < 0.05$, $P < 0.001$). BBE

Table 3. Inhibitory effect of BBE treatment on the formalin test

Animal groups	Paw licking time (sec)	
	Early phase	Late phase
Non-BBE	104.81 ± 9.58	295.15 ± 15.68
BBE 100 mg/kg	108.19 ± 11.58	241.51 ± 18.51*
BBE 200 mg/kg	105.12 ± 10.81	171.54 ± 10.80**

Abbreviation: Non-BBE (Vehicle), no treatment group; BBE 200 mg/kg, 100 mg/kg, *Berchemia berchemiaefolia* extract treatment group 200 mg/kg or 100 mg/kg

demonstrated a dose dependent relationship in the formalin-induced pain test.

DPPH scavenging effect

DPPH scavenging activity assay is widely used to evaluate the ability of compounds to scavenge-free radicals or donate hydrogen/electron, and determine the antioxidant activity in foods. At all concentrations, ascorbic acid was shown higher inhibition than BBE fractions (Fig. 1). Among of BBE frac-

tions, H₂O fraction was higher antioxidant activity (48.9% 640 µg/mL).

Inhibition of xanthine oxidase

The impact on XOD activity of compounds was showed in Table 4. Among these compounds, BBE H₂O was the strongest in inhibiting XOD activity whose IC₅₀ value (45.15 µM) is similar to positive reference substance quercetin (15.9 µM), while four substance showed almost no inhibitory activity.

Nitric oxide assay

Initially, to assess the anti-oxidant and anti-inflammatory

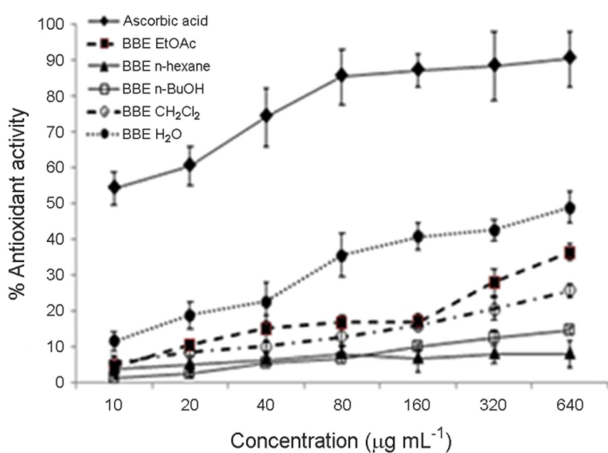


Fig. 1. DPPH scavenging effect of BBE fractions. BBE H₂O fraction was higher antioxidant activity among BBE fractions. Positive control was ascorbic acid. Data represent the mean ± SD of three independent experiments; n-hexane, methylene chloride (CH₂Cl₂), ethyl acetate (EtOAc), n-butanol (n-BuOH).

effect of BBE, the impact of BBE on NO production in experimental serum was analyzed. After formalin treatment, the production of NO was dramatically increased. However, treatment with BBE markedly reduced NO production (Fig. 2). There was around 27.44% and 41.86% reduction in serum NO production at 100 and 200 µg/kg BBE oral administration compared with NO production of Vehicle.

DISCUSSION

We conducted this research into effect of anti-pain for *Berchemia berchemiaefolia* (BBE). The present study demonstrated that oral treatment with BBE protects SD rats from

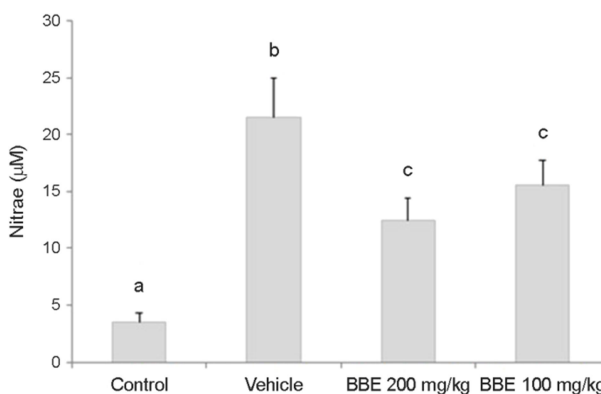


Fig. 2. Production of nitrate oxide in formalin animal model. There was around 27.44% and 41.86% reduction in serum NO production at 100 and 200 µg/kg BBE oral administration compared with NO production of Vehicle. Data represent the mean ± SD of three independent experiments; values with different letters are significantly different at $P < 0.05$, according to the Fisher's least significant difference (LSD) analysis.

Table 4. Inhibitory effects of the compounds on xanthine oxidase activity

Sample (Fraction)	% Inhibition (100 µM)	IC ₅₀ (µM)
	0	—
BBE n-hexane	0	—
BBE CH ₂ Cl ₂	0	—
BBE EtOAc	11.81 ± 1.90 ^a	—
BBE n-BuOH	25.38 ± 0.85 ^a	—
BBE H ₂ O	58.15 ± 1.48 ^c	45.15 ± 2.45
Quercetin	78.99 ± 3.51 ^c	15.9 ± 1.78

Data represent the mean ± SD of three independent experiments; values with different letters are significantly different at $P < 0.05$, according to the Fisher's least significant difference (LSD) analysis, n-hexane, methylene chloride (CH₂Cl₂), ethyl acetate (EtOAc), n-butanol (n-BuOH)

formalin induced pain. We have expected that BBE reduces level of pain because BBE has anti-oxidant feature.

First of all, we carried out acute toxicity test of BBE in SD rat to determine the fixed-dose. The results were that the LD₅₀ of BBE is more than 2,000 mg/kg, which indicates that BBE can be categorized as 'unclassified'. BBE may have fewer adverse effects in our experimental animals considering absence of animal death, disease, and organ abnormality. As mentioned above, we conduct tale flick latency test to confirm analgesic effect for central nerve system and formalin test to confirm it for peripheral nervous system (Palle et al., 2016). In the tail flick test, the groups that received 200 mg/kg of BBE had shown a significant increase in tail flick latency time. The increase in reaction time of the rats shows that BBE at the doses given has analgesic activities. The anti-nociceptive effects of BBE might be contributed partly by its action on opioid receptors in the spinal cord (Planjar-Prvan et al., 2004). The formalin-induced test is believed to resemble clinical pain more exactly in comparison with thermal or mechanical stimuli (Rosa et al., 2005; Ray et al., 2015). The first phase results from chemical stimuli of nociceptive afferent fibers, which can be suppressed by morphine (Russo et al., 2016; Siahposht-Khachaki et al., 2017). In the second phase, inflammatory mediators in peripheral tissues induce functional changes in the neurons of the spinal dorsal horn. In this model, BBE inhibited the licking duration of rat in the second phase (Table 2), suggesting BBE exerts anti-nociceptive effect related to peripheral mechanisms.

As a result of our tail flick test, the analgesic properties of BBE could be contributed by its antioxidant property (Srinivasan et al., 2010). Reports have shown the role of oxidative stress in the development of pain / hyperalgesia, and vitamin C, one of the antioxidants, has been shown to inhibit nociceptive transmission by interacting at the level of glutamate receptors in the central nervous system (Straub, 2001). In general, the first-phase response is due to the high level of activity in the primary afferents induced by formalin, and the second phase was considered to be a tonic response resulting from the inflammation factor (Taherian et al., 2010). Also release of a broad range of sensitizers from inflammatory tissue, acting on the related receptors and signaling

pathways on the peripheral terminal of nociceptors, induces a process of peripheral sensitization, which is an important neuronal mechanism underlying primary hyperalgesia at the site of injury or inflammation (Toyama et al., 2017). MAPKs, including p38, ERK, and JNK, are a family of serine protein kinases that transduce extracellular stimuli into intracellular posttranslational and transcriptional responses. It is well established that the MAPKs activation may be involved in the modulation of nociceptive information and peripheral and central sensitization produced by intense noxious stimuli through various routes (Umar et al., 2017).

For this reason, therapeutic approaches for treating pain include applications of antioxidant agents, as well as anti-inflammatory agent for symptomatic relief (Wang et al., 2016). Suppression of XO activity is one of the therapeutic strategies to reduce pain such as gout. A few XO inhibitors (allopurinol and febuxostat) have been clinically used (Ye et al., 2016). A variety of other antioxidants have also been used as analgesic drug candidates (Zhou et al., 2017). This experimental results show that the water fraction of this material has a strong antioxidant capacity (Fig. 1, Table 4). From this point of view, the analgesic effect of this substance is probably related to the antioxidant capacity of the substance itself. In addition, it is considered that the decrease of the serum NO concentration was involved as the basis of the other anti-pain. It is known that there is a strong relationship between the anti-inflammatory effect and antioxidant ability (Zuntini et al., 2017). However, further studies on the direct mechanism of BBE on antioxidant and anti-inflammatory action are needed.

In conclusion, this study demonstrates that oral admission of BBE prevents the inflammatory pain induced by formalin through antioxidant ability in rat. These findings may have important implications for exploring the roles and mechanisms and for understanding the molecular basis of BBE in analgesia. These results suggest that BBE could be useful to suppress pain in the second phase of the formalin test. It is anticipated that the strong antioxidant activity of BBE caused an anti-inflammatory action, resulting in an analgesic effect. Therefore, our study suggested that BBE would be useful in treatment of inflammation pain as an analgesia drug.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

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