

# The analgesic effect of *Berchemia berchemiaefolia* are mediated via suppression of p38 MAPK activation in rats

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## Abstract

**Aims:** We carried out the present study to demonstrate that oral treatment with *Berchemia berchemiaefolia* extract (BBE) protects SD rats from pain.

**Methods:** Animal experimental groups were divided by four groups and two animal model groups were fed on extract of BBE 200 mg/kg and 100 mg/kg. We carried out oral acute toxicity test to determine oral dose of BBE. To identify the pain relief of rat, we performed tail flick latency test and formalin test. Additionally we carried out monoclonal antibodies (p38 and Nrf2) of western blot.

**Conclusion:** Our study has demonstrated the BBE showed anti-nociceptive effects for pain animal model, which would be related to the inhibition of relevant signaling pathways such as p38 and Nrf2 controlling the production of nociceptive and inflammatory mediators.

**Keywords:** Pain, Nociceptive action, *Fomes fomentarius*, Tail flick latency test, Formalin test, p38, Nrf2

## 1. Instruction

*Berchemia* is a genus of plants in the family Rhamnaceae. They are climbing plants or small to medium-sized trees that occur in Africa, Asia and the Americas. *Berchemia berchemiaefolia* (BB), used in experiments, is Korea's natural monument. Traditionally BB has been used for food additives for hundreds of years. In Korea' traditional remedy and BB is believed to have anti-inflammatory effect. This plant was one of our novel natural drug candidates. Our ongoing study using

*Berchemia berchemiaefolia* showed anti-inflammatory effect for rheumat arthritis [1].

It is difficult to categorize animal pain situation into small groups in pain experiments on using animals on account of different environmental and biological conditions [2]. Accordingly animal pain situation classifies two way, 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. [3]. In this context, we conduct tail flick latency test to confirm anti-pain effect for central nerve system and formalin test to confirm it for peripheral nervous system [4]. Accumulating evidence shows that MAPK pathways contribute to pain sensitization after tissue and nerve injury via distinct molecular and cellular mechanisms [5, 6]. Activation (phosphorylation) of MAPKs under different persistent pain conditions results in the induction and maintenance of pain hypersensitivity via non-transcriptional and transcriptional regulation [7]. After nerve injury, ERK, p38, and JNK are differentially activated in spinal glial cells (microglia vs astrocytes), leading to the synthesis of proinflammatory/pronociceptive mediators, thereby enhancing and prolonging pain [8]. Inhibition of all three MAPK pathways has been shown to attenuate inflammatory and neuropathic pain in different animal models [7, 9]. In this experiment, we conduct p38 and Nrf2 of activation to identify anti-pain mechanism.

## 2. Materials and Methods

### 2.1. Animal Experiments

32 male Sprague-Dawley rats 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 *rat weight*)

### 2.2. 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 4.3%.

### 2.3. Surgical procedure

At the end of the experiments, each rat was anaesthetized with an intraperitoneal (*ip*) injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). Then brain and spinal cord were dissected. The tissues were stored at -80 °C before using.

### 2.4. Acute toxicity test

The procedure is incorporated into the European Community Directive guidelines as the acute toxicity test [10]. The rat 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 (LD<sub>50</sub>). Briefly, an initial dose of 5, 50, 500, or 2000 mg per kg of body weight can be selected to evaluate the toxicity of the substance being investigated. Either 5 or 2000 mg per kg can serve as the starting dose. The procedure is terminated when either toxicity or death is observed.

### 2.5. Tail-flick test

Tail-flick test was applied as follows [11]. 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.

### 2.6. Formalin test

Experiments were carried out according to previously described method [12]. 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.

### 2.7. Western blot

Segments of brain and spinal cord were homogenized using homogenizer (Intron Biotechnology, Gyeonggi-do, Korea) with lysis buffer (PRO-PREP™, Protein Extraction Solution). Homogenized samples were centrifuged 13,000 rpm for ten minutes. Protein absorbance of supernatant was measured using X-ma spectrophotometer (Human Cor. Korea) at 595 nm. Extracting about each tissues, equal amounts of protein (50 µg) were fractionated on 10% sodium dodecyl sulfate-polyacrylamide gels in running buffer (25 mmol/L Tris, 0.25mol/L glycine, 0.1% sodium dodecyl sulfate, pH 8.3) at 90 V and then electroblotted to nitrocellulose membranes. Nrf2 and p38 were detected by monoclonal antibodies (Santa Cruz Biotech, Inc. Santa Cruz, CA). Membranes were blocked at room temperature with 5% nonfat milk in Tris-buffered saline containing 0.05% Tween-20 and then incubated overnight at 4°C with the following primary antibodies: Histone monoclonal antibody (Cell Signaling Technology, INC. USA ; dilutions, each 1:2,000, 1:500, 1:20,000) Then the membranes were washed three times in Tween-20 and incubated with the corresponding secondary antibody (Santa Cruz Biochemicals; dilutions, each 1:8,000, 1:3,000, 1:10,000)

conjugated to horseradish peroxidase at room temperature. Immunoreactive bands were visualized with the chemoluminescence kit (Santa Cruz Biochemicals) according to the manufacturer's instructions. Band intensities and molecular weight were quantified by using a Vision Works Image Software (UVP, Cambridge, UK).

### 2.8. Data analysis

Experimental values are presented as mean ± SD of triplicate cultures and representative of experiments performed on three occasions. Statistical significance was determined by Mann-Whitney *U* test or ANOVA with Bonferroni's post-hoc test using SPSS version 18. Values of  $P < 0.05$  were considered statistically significant,  $*P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$ . In analysis of Nrf2 and p38, not sharing a common letter were significantly different ( $p < 0.05$ ) when analyzed by ANOVA and Duncan's multiple range test.

## 3. Results

### 3.1. Data analysis

Table 1. Demographic characteristics in four groups

Variable	Group			
	Control	Vehicle	BBE 200mg/kg	BBE 100mg
Total number	8	8	8	8
Gender	Male	Male	Male	Male
Age (week)	4	4	4	4
BW before experiment (g)	141.88 ±4.11	141.71 ±5.09	143.10 ±6.11	142.76 ±5.18
BW after experiment (g)	148.13 ±7.15	149.45 ±9.34	150.12 ±7.81	149.48 ±7.99
Experimental period (week)	for 2	for 2	for 2	for 2

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.

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

### 3.2 Toxicity studies

Oral LD50 of BBE in rats was found to be >2000 mg/kg body weight as administration of BBE at a dose of 2000 mg/kg weight did not produce any mortality in the tested animals In chronic administration of BBE at a dose of 2000 mg/kg body weight for 28 days did not produce any pathological changes as compared to normal animals (data not shown).

### 3.3. Tail flick test

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 200mg/kg (Figure 1). A significant increase in the tail-flick latency was observed after 40~70 minutes of BBE 200 mg/kg ( $p < 0.05$ ).

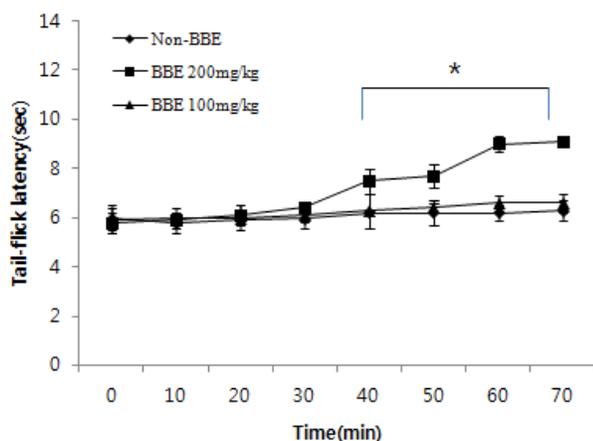


Figure 1. Inhibition effect of *Berchemia berchemiaefolia* Extract treatment on acute pain Abbreviation: Non-BBE

(Vehicle), no treatment group; BBE 200 mg/kg, 100mg/kg, *Berchemia berchemiaefolia* Extract treatment group 200 mg/kg or 100 mg/kg

### 3.4. Formalin test

In the formalin test (Table 2), formalin induced nociception in the vehicle rats versus to the sham operated group ( $p < 0.05$ ). 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 ( $p < 0.05$ ). BBE demonstrated a dose dependent relationship in the formalin-induced pain test.

Table 2. Inhibition effect of *Berchemia berchemiaefolia* extract treatment on pain behavior

Animal groups	Paw licking time(sec)	
	Early phase	Late phase
Vehicle	118.16±12.21	289.18±22.51
BBE 100mg/kg	129.45±15.84	225.65±15.18 *
BBE 200mg/kg	125.54±16.34	202.11±23.61 *

Abbreviation: BBE 200 mg/kg, 100 mg/kg, *Berchemia berchemiaefolia* Extract treatment group 200 mg/kg or 100 mg/kg

### 3.5. Western blot results

Protein expression of Nrf2 and p38 was evaluated by Western blot analysis of spinal cord (Figure 2.A). Figure 2.A shows that formalin evoked nociception strongly induced expression of Nrf2 and p38 compared with control group (no formalin injection). Treatment with BBE 100(0.16), 200(0.63) mg/kg significantly reduced the expression of Nrf2 and p38 in SD rat compared with

vehicle (1.7) (Figure 2.A). Figure 2.B shows Protein expression of Nrf2 and p38 evaluated by Western blot analysis of brain. There were no significant differences of expression of nrf2 and p38 among three groups except BBE 100 mg/kg group of Nrf2.

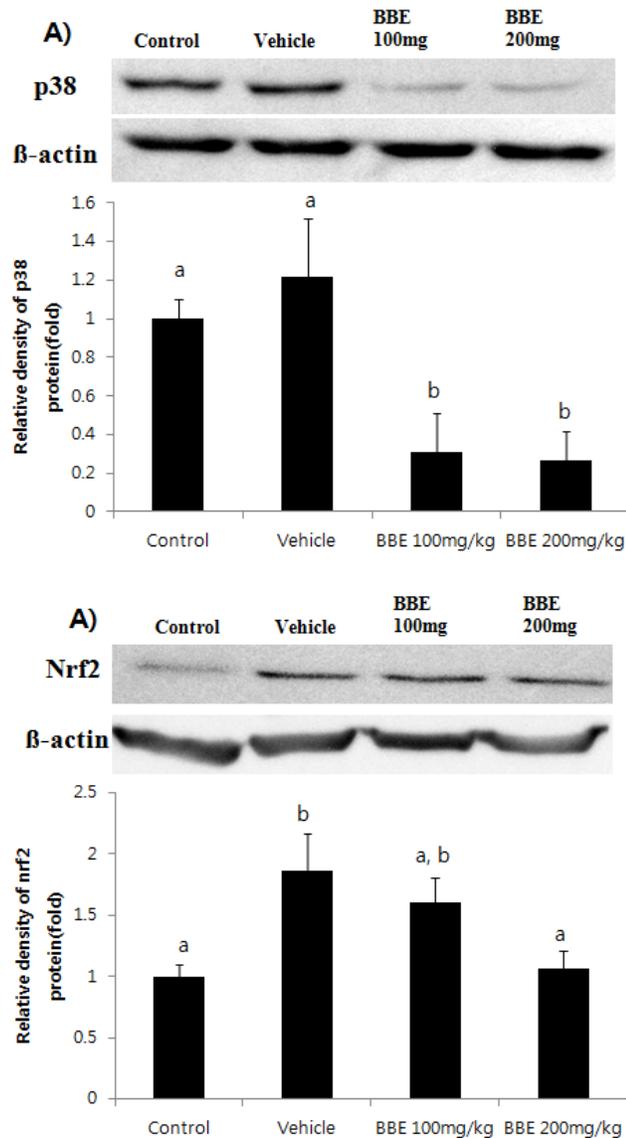


Figure 2 A. The expression of Nrf2 and p38 in spinal cord  
Abbreviation: Vehicle, no treatment group; BBE 200 mg/kg, 100mg'kg, *Berchemia berchemiaefolia* Extract treatment group 200 mg/kg or 100 mg'kg.

Means not sharing a common letter were significantly different ( $p < 0.05$ ) analyzed by ANOVA and Duncan's multiple range test.

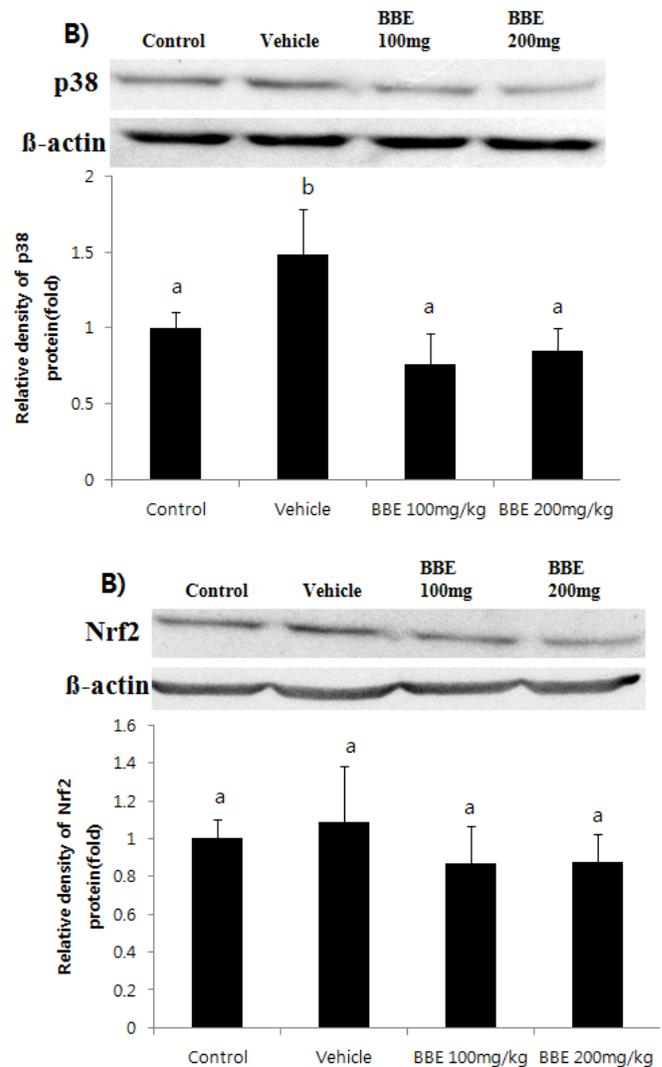


Figure 2 B. The expression of nrf2 and p38 in brain  
Abbreviation: Vehicle, no treatment group; BBE 200 mg/kg, 100mg'kg, *Berchemia berchemiaefolia* Extract treatment group 200 mg/kg or 100 mg'kg.

Means not sharing a common letter were significantly different ( $p < 0.05$ ) analyzed by ANOVA and Duncan's multiple range test.

#### 4. Discussion

First of all, we carried out acute toxicity test of BBE in SD rat to determine the fixed-dose [17, 20]. The results were that the LD<sub>50</sub> of BBE is more than 2000 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 stated above, we conduct tail flick latency test to confirm anti-pain effect for central nerve system and formalin test to confirm it for peripheral nervous system. 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. The formalin-induced test is believed to resemble clinical pain more exactly in comparison with thermal or mechanical stimuli [14, 15]. The first phase results from chemical stimuli of nociceptive afferent fibers, which can be suppressed by morphine [16, 17]. 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 [18]. 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.[19]. Nrf2 is a major regulator of oxidative response [20]. Most of the Nrf2 target genes encode antioxidant proteins and

detoxifying enzymes [21]. One of our interesting findings was Nrf2 reduction of BBE treatment groups (Figure 2). In this result, we suggest that BBE have antioxidant effects and inhibit acceleration of pain in SD rat. Previous studies have demonstrated that intrathecal delivery of inhibitors of p38 mitogen-activated protein kinase attenuate hyperalgesia including that produced by intraplantar carrageenan or formalin-induced pain [22, 23]. It is well known that intraplantar injection of formalin can induce biphasic spontaneous nociceptive responses. 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 [24]. 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 [25]. 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 [26]. Our study has found that BBE down-regulated the protein expression of p38 *in vivo* (Figure 2). These studies suggested that MAPKs signaling may mediate the role of BBE in preventing the inflammatory pain. Pre-treatment with BBE prevents pain behavior and spinal MAPKs expression induced by formalin. These findings indicated that MAPKs pathway was involved in the role of BBE in preventing formalin-induced nociceptive response. These results suggest that BBE could be useful to suppress pain in the second phase

of the formalin test through modulating Nrf2 and p38, but more pharmacological investigations are needed for finding the exact mechanism of action.

## 5. Conclusion

This study demonstrates that oral admission of BBE prevents the inflammatory pain induced by formalin through a MAPKs-mediated mechanism in rat. These findings may have important implications for exploring the roles and mechanisms and for understanding the molecular basis of BBE in analgesia. Therefore, our study suggested that BBE would be useful in treatment of inflammation pain as an analgesia drug.

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