A study of analgesic effect of *Zanthoxylum bungeanum* Maxim pharmacopuncture

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[Abstract]

**Objectives:** This study was carried out to evaluate analgesic effects of *Zanthoxylum bungeanum* Maxim (ZM) pharmacopuncture on formalin-induced pains in Sprague–Dawley (SD) rats and ICR-mice.

**Methods:** The subjects were divided 8 weeks aged rats with constant pain sensitivity into five groups: normal (treated with normal saline at Taegye (K13) and before injected with normal saline at hindpaw), Con-1 (treated with normal saline at K13 before injected with formalin at hindpaw), Lido-1 (treated with lidocaine at K13), ZMWD-1 (treated with Hot water extraction pharmacopuncture of *Zanthoxylum bungeanum* Maxim at K13), ZMEG-1 (treated with ethanol extraction pharmacopuncture of *Zanthoxylum bungeanum* Maxim at K13). After 35 minutes, we measured ultrasonic vocalization (USV) and enzyme activities of both Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) in rat serum. In addition, Tail flick test is performed by injecting ICR mice at 5 weeks of age. And it classified into 4 groups (Con-2, Lido-2, ZMWD-2, ZMEG-2) according to the kind of drug (normal saline, lidocaine, ZMW, ZME). After each drug injection, we examined the reaction by placing the tail in water at 50°C.

**Results:** ZME had analgesic effects in the early and late phase of USV during the formalin test. There were no significant differences between ZMEG-1 and Lido-1 in early and late phase of USV. Also, No significant differences observed in serum AST and ALT activity in ZMWD-1 and ZMEG-1 compared with Con-1. For tail-flick test, analgesic effect on warmth significantly increased in Lido-2 and ZMEG-2 compare to that of Con-2.

**Conclusion:** ZME pharmacopuncture had analgesic effects on formalin-induced pain without liver toxicity. Also, tail-flick test suggest that ZME pharmacopuncture could be useful technique on analgesic effect on warmth and treatment of pains.

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I. Introduction

The International Association for the Study of Pain defined pain as "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or a state that can be described in terms of such damage." Pain occurs as a result of physical or chemical stimulation. Furthermore, these noxious stimuli affect the nerve fibers or neurons and therefore they release chemical substances such as histamine, serotonin, and substance P, among others, which each can cause pain.

In Korean medicine, it is said, "Passability is not pain; Impassability is pain". What this means is that pain is decreased by passing through the Qi. Recently various treatment methods have been used for pain treatment, such as pharmacopuncture, miniscalpel acupuncture and traditional acupuncture, as well as moxibustion and cupping. Pharmacopuncture is a new acupuncture technique that is based on traditional forms of acupuncture and herbal theory.

There have of course been various studies on pain, demonstrating that the activation of the spinal serotonergic receptor contributes to electroacupuncture analgesia in rat with chronic pain, that heterotopic electroacupuncture modulates formalin-induced pain via descending inhibition in rats, and that acupuncture, moxibustion and laser beam acupuncture can influence the alleviation of pain in mice. According to previous studies on pain treatment involving pharmacopuncture an experimental study with bee venom therapy was conducted to determine its anti-inflammatory and analgesic effects, and the effects of Scolopendra subspinipes mutilans L. Koch pharmacopuncture extract solution on analgesia and anticonvulsion were also explored; this research has been conducted to determine how best to relieve pain caused by inflammatory mechanisms.

Zanthoxylum bungeanum Maxim (ZM), which belongs to the Rutaceae family, was said by Cho-Phi and Je-Phi et al., that its branch has not only local anesthetic effects but also ingredients poisonous to fish, so it was used to catch fish such as Styrax japonicas Siebold & Zucc.

Zanthoxylum bungeanum Maxim is heat, poisonous, so it can dispel wind, dissipate cold, relieve pain, and kill worms. Previous studies have reported the antioxidant, anti-inflammatory, antithrombotic, and antimicrobial activities of ZM. However, a study on the analgesic effects of ZM has not yet been reported in Korea.

We therefore report on the significant results of ZM’s analgesic effects through Ultrasonic vocalization (USV) analysis and tail-flick test.

II. Materials and Methods

1. Materials

1) Animals

Sprague-Dawley rats (Hyochang Animal, Seoul, South Korea) weighing 270–300 g at the age of 7 weeks were used for the USV test. The rats were kept in the testing environment for a 12-hour light-dark cycle at room temperature (24°C), and with humidity set at 55%. After at least 1 week of an adaptation period, the animals were divergently selected for the experiments via randomized complete block design. ICR mice (Hyochang Animal, Seoul, South Korea) weighing 22.7±0.7 g at the age of four weeks were used in the tail-flick test. These experiments were in compliance with the protocols approved by the Institutional Animal Care and Use Committee at the Daegu Haany University (ICR mouse : DHU 2016–053, SD rat : DHU 2016–054).

2) Herbs

Branches of ZM were dried in the shade (Gyeongnam, South Korea) and were used in the manufacturing for pharmacopuncture.
2. Methods

1) Sample extraction and manufacturing for pharmacopuncture

(1) Sample extraction
The branches of ZM were extracted with 100% water and 80% ethanol, and 20 times of the solvent were added. Both hot water extraction and ethanol extraction were conducted for 8 hours after the temperature rose to 100℃, and these extracts were filtered with vacuum filtration on a Buchner funnel (Jeiotech, Daejeon, South-Korea) with Wattmann filter paper (No.2) (GE Healthcare Inc., Wisconsin, USA). After the first filtration process, these extracts were first concentrated in a vacuum concentrator COSMOS660 (Kyung Seo E&P, Incheon, South Korea). The first concentrate was centrifuged at 7000 rpm for 20 minutes to perform secondary filtration. Afterwards, 10.5 g (yield 3.5%) of a hot-water extraction ZM powder was obtained by lyophilization in a freeze dryer pilot (Kyung Seo E&P, Incheon, South Korea) from 300 g of materials, and 9.4 g (yield 3.13%) of ethanol extraction ZM powder was obtained by lyophilization from the same amount of materials.

(2) Manufacturing for pharmacopuncture
The powder samples obtained from each extraction method were dissolved in a normal saline solution, and the pH was adjusted to 6.8 ~ 7.0 using NaOH to manufacture 5% hot-water extraction for pharmacopuncture (ZMW) and 5% ethanol extraction for pharmacopuncture (ZME).

2) Von Frey test
This experiment was performed by modifying the method of Kim et al.13 and, Kim et al14, to measure the pain sensitivity of the experimental group before the USV testing. Before the experiment, the rats were placed in a glass bottle with mesh flooring for 40 minutes so that the animals could adapt. After that, when Von Frey hair was pierced into the center of the foot of the rat, it was marked as a positive reaction when lifting of the foot occurred. At the end of the test, results were quantified through a certain calculation procedures (Von Frey test), and rats with an average number of lifting the foot of more than 10 cycles were used in the experiment (Fig.1).

3) Group classification
(1) In the USV test and the hepatic toxic test, rats were classified into a normal group (Nor, n=11) and treated with normal saline only, and the pain-induced groups were treated with 5% formalin. The pain-induced groups

Fig. 1. Von frey test
(A) : Von frey hairs. 
(B) : When this pierces the rat, it will be bent. 
(C) : This shows how to pierce a rat’s foot with von frey hair.
were classified into four groups; Con-1 (treated with normal saline at KI3), Lido-1 (treated with lidocaine at KI3), ZMWG-1 (treated with ZMW at KI3), and ZMEG-1 (treated with ZME at KI3).

(2) In the tail-flick test, ICR mice were classified into 4 groups; Con-2 (treated with normal saline at the root part of the tail), Lido-2 (treated with lidocaine at the root part of the tail), ZMWG-2 (treated with ZMW at the root part of the tail), ZMEG-2 (treated with ZME at the root part of the tail).

4) Pharmacopuncture injection

During the USV test, rats were transferred to individual cages to minimize stress. To stabilize the rats, they were placed in a soundproof cage for at least 40 minutes after a 60-minute adaptation period outside the soundproof cage. Then, the baseline was measured for 20 minutes to confirm the stabilization. In the KI3, Nor, and Con-1, rats were injected with normal saline and, Lido-1, ZMWG-1 and ZMEG-1 were injected in amounts of 40㎕ for each drug (5% Lidocaine, 5% ZMW and 5% ZME) using a 0.5 cc syringe (31 gauge, BD Medical, France). The acupoint was determined by using the acupoint corresponding to the human body as determined by the proportional method based on the standard acupuncture points of the laboratory animals.

In the tail-flick test, 20㎕ of drug (normal saline, 5% Lidocaine, 5% ZMW and 5% ZME) was injected into the point 7cm from the root or tip of the tail of the selected mice with a 0.3 cc syringe (31 gauge, BD Medical, France).

5) Pain-induced formalin test

A formalin test was conducted to induce pain during the USV test. To stabilize the rats, they were placed in a soundproof cage for at least 40 minutes. Then, the baseline was measured for 20 minutes to confirm the stabilization with Avisoft Recoder program (Avisoft Bioacoustic Germany) and the rats were injected with 40㎕ of each drug (5% Lidocaine, 5% ZMW, and 5% ZME) into the KI3. After the drug was absorbed for 3 minutes and the rats were soaked, 40㎕ of normal saline were injected into the right hind paws of the rats in the Nor. Then, 40㎕ of 5% formalin was injected into the right hind paws in the rats of the Con-1, Lido-1, ZMWG-1 and ZMEG-1 to induce pain, and the USV was measured for 40 minutes to verify the effects of the injected drugs.

3. Assessment methods

1) USV analysis

If a rodent feels pain, a sound ranging from 18–30 Hz will be made, and the degree of pain can be estimated by analyzing the number of times the sound is made. In this experiment, each group was injected with normal saline, lidocaine, 5% ZMW, and 5% ZME and then pain was induced, and ultrasound sounds were recorded. Pulse train analysis was

Fig. 2. Ultrasonic vocalization program
This shows Ultrasonic vocalization amplifiers connected with computer installed analysis program.
performed by cutting at a wavelength of 18∼30Hz when the SD rats generally felt pain, Alexandre R. Oliveira et al. classified the early phase up to 5 minutes, the interphase from 10 minutes to 20 minutes, and the late phase after that. In this study, we defined the early phase as up to 20 minutes, and late phase as any point after that (Fig. 2).

2) Tail–flick test

This experiment was performed by modifying the method of Gilbert J et al. Mice were used after these were stabled throughout the course of a week. In the 5th day of the adaptation period, the mice were immobilized and kept 2 cm from the tip of the tail in a water bath at 50°C to exclude the group with low sensitivity, with a duration of less than 3 seconds. After 1 minute of injection, the 2 cm from the tip of the tail was immersed in a 50°C water bath, and the reaction was examined. Sensory block was regarded as onset when persistence was more than 4 seconds and repeated twice, and the time from every 5 minutes to 15 minutes to offset further injection was measured (Fig. 3) (Fig. 4).
3) Hepatic toxic analysis

AST and ALT activities related to hepatocyte injury were analyzed through the use of a spectrophotometer using the enzyme method and the colorimetric method; the method employed was similar to that used by Reitman18. (Fig. 5).

4) Statistical analysis

The results were calculated using the SPSS software program (Statistical Package for the Social Sciences, SPSS Inc., Chicago). One-way analysis of variance (ANOVA) was conducted for the significance test of the mean difference between the multiple groups. A Duncan’s multiple range test was used to compare the differences between the groups ($p<0.05$). A student’s $t$-test was used to confirm the significance between the normal group and the control group. All results were expressed in terms of the mean ± SE, (Standard error).

Fig. 4. Tail-flick test

(A) : Injection into root part of tail.
(B) : This shows putting the tail into hot water.
III. Results

1. USV analysis

For the USV analysis of Nor, 15.8 times were reported in the early phase, and 15.4 times in the late phase. In the USV analysis of the formalin-injected Con-1, 1347.6 times in the early phase and 920.2 times in the late phase were recorded. The difference between the Nor and Con-1 was observed, and it could be observed that the pain was normally induced (Fig.6). In the early phase, Lido-1, ZMWG-1, and ZMEG-1 were each recorded at 477.2 times, 1262.3 times, and 646.7 times. When the reports for Lido-1 and ZMEG-1 were compared with those of Con-1, it was observed that USV data was significantly lower than that of Con-1 (Fig.7), especially at 15 and 20 minutes (Fig.6).
Fig. 6. Ultrasonic vocalization observed in experimental groups

** : p<0.01 compared with Nor by student’s t-test.
* : p<0.05 compared with Nor by student’s t-test.
a, b, c, d : Values with each superscript are significantly different by Duncan’s multiple range test, (p<0.05)
ZMW : Hot water extraction pharmacopuncture of Zanthoxylum bungeanum Maxim.
ZME : Ethanol extraction pharmacopuncture of Zanthoxylum bungeanum Maxim.
Nor : SD rats group to treat by normal saline at KI3, and inject normal saline into right hindpaw after 35 minutes.
Con-1 : SD rats group to treat by normal saline at KI3, and inject 5% formalin into right hindpaw after 35 minutes.
Lido-1 : SD rats group to treat by Lidocaine at KI3, and inject 5% formalin into right hindpaw after 35 minutes.
ZMWG-1 : SD rats group to treat by ZMW at KI3, and inject 5% formalin into right hindpaw after 35 minutes.
ZMEG-1 : SD rats group to treat by ZME at KI3, and inject 5% formalin into right hindpaw after 35 minutes.

Fig. 7. The early phase of ultrasonic vocalization observed in experimental groups

a, b, c : Values with each superscript are significantly different by Duncan’s multiple range test, (p<0.05)
ZMW : Hot water extraction pharmacopuncture of Zanthoxylum bungeanum Maxim.
ZME : Ethanol extraction pharmacopuncture of Zanthoxylum bungeanum Maxim.
Nor : SD rats group to treat by normal saline at KI3, and inject normal saline into right hindpaw after 35 minutes.
Con-1 : SD rats group to treat by normal saline at KI3, and inject 5% formalin into right hindpaw after 35 minutes.
Lido-1 : SD rats group to treat by Lidocaine at KI3, and inject 5% formalin into right hindpaw after 35 minutes.
ZMWG-1 : SD rats group to treat by ZMW at KI3, and inject 5% formalin into right hindpaw after 35 minutes.
ZMEG-1 : SD rats group to treat by ZME at KI3, and inject 5% formalin into right hindpaw after 35 minutes.
In the late phase, Lido-1, ZMWG-1, and ZMEG-1 were each recorded at 476.0 times, 1029.4 times, and 593.5 times. When the reports for Lido-1 and ZMEG-1 were compared with those of Con-1, it was observed that the data for USV was significantly lower than that of Con-1 (Fig. 8), especially at 25 and 35 minutes (Fig. 6).

2. Tail-flick test

In the tail-flick test, the Con-2 was maintained at 9.3 min compared with 60.3 min for the Lido-2, 34.3 min for the ZMWG-2, and 52.5 min for the ZMEG-2. In contrast to the results for the Con-2, ZMEG-2 relieved pain caused by warming similar to Lido-2 (Fig. 9).

Fig. 8. The late phase of ultrasonic vocalization observed in experimental groups

a, b, c : Values with each superscript are significantly different by Duncan’s multiple range test.
ZMW : Hot water extraction pharmacopuncture of Zanthoxylum bungeanum Maxim.
ZME : Ethanol extraction pharmacopuncture of Zanthoxylum bungeanum Maxim.
Nor : SD rats group to treat by normal saline at KI3, and inject normal saline into right hindpaw after 35 minutes.
Con-1 : SD rats group to treat by normal saline at KI3, and inject 5% formalin into right hindpaw after 35 minutes.
Lido-1 : SD rats group to treat by Lidocaine at KI3, and inject 5% formalin into right hindpaw after 35 minutes.
ZMWG-1 : SD rats group to treat by ZMW at KI3, and inject 5% formalin into right hindpaw after 35 minutes.
ZMEG-1 : SD rats group to treat by ZME at KI3, and inject 5% formalin into right hindpaw after 35 minutes.

Fig. 9. The sustainment time in tail-flick test

*** : p<0.001 compared with Con-3 by student's t-test.
a, b, c : Values with each superscript are significantly different by Duncan’s multiple range test. (p<0.05)
ZMW : Hot water extraction pharmacopuncture of Zanthoxylum bungeanum Maxim.
ZME : Ethanol extraction pharmacopuncture of Zanthoxylum bungeanum Maxim.
Con-2 : Mouse group to inject normal saline into root part of tail.
Lido-2 : Mouse group to inject 5% formalin into root part of tail.
ZMWG-2 : Mouse group to inject ZMW into root part of tail.
ZMEG-2 : Mouse group to inject ZME into root part of tail.
3. Hepatic toxic analysis

AST activity was recorded as 81.7 Karmen/mL for the Con-1, which had significantly increased compared to the data for the administration of 60.9 Karmen/mL for the Nor. In even greater contrast, the Lido-1 and ZMWG-1 rates were measured as 74.1 Karmen/mL, and ZMEG-1 was measured as 73.6 Karmen/mL, but there was no significant difference in comparison to the the Con-1.

ALT activity was recorded as 27.9 Karmen/mL for the Con-1, which had significantly increased compared to the data for the administration of 10.7 Karmen/mL for the Nor. Again, in even greater contrast, the Lido-1 rate was measured as 20.9 Karmen/mL, ZMWG-1 was measured as 21.1 Karmen/mL, and ZMEG-1 was measured as 18.8 Karmen/mL, indicating a significant decrease compared to the Con-1 rates (Fig. 10).

IV. Discussion

Pain refers to the unpleasant sensations and emotional experiences caused by actual or potential tissue damage. Pain is generally classified into the categories of neuropathic pain, nociceptive pain, and unexplained pain, and it can also be divided into acute pain and chronic pain, the latter of which lasts for 6 months or more.

In order to treat pain effectively, nonsteroidal anti-inflammatory (NSAID) or opioids are regardless of the degree of pain or underlying disease. Aspirin and NSAIDs used in the treatment of acute pain have anti-inflammatory and analgesic effects by inhibiting the production of prostaglandin via cyclooxygenase (COX) blockage. However, taking NSAIDs for extended periods of time can stimulate the gastrointestinal tract and, at the same time, interfere with thrombogenesis, bleeding, or perforation. Opioid analgesics, on the other hand, activate pain-suppressing neurons...
and inhibit the neurons that transmit pain; therefore, they are the most definitive treatment for extensive and rapid-onset pain, but they frequently lead to nausea, vomiting, and constipation.

In oriental medicine, a variety of therapies such as herbal medicine, acupuncture, moxibustion, pharmacopuncture, cupping, and chu-na have been implemented to treat pain. Among them, pharmacopuncture is a treatment method in which a pharmacopuncture-solution prepared by extracting an apparently active ingredient of herbal medicine is injected into an acupoint, and then the solution is injected into a blood vessel related to the disease using a syringe. The advantage of this method is that it has a wide range of indications, has a fast therapeutic effect, and can be applied to patients who are unable to take the drugs. In addition, the effects of pharmacopuncture on alleviating joint pain, edema, inflammatory reactions and the side effects of pharmacopuncture on neuropathic pain have been reported in the literature.

Cho-phi tree is a broad-leaved tree of the Rutaceae, with a height of about 3m and a diameter of about 15cm. Its scientific name is Zanthoxylum bungeanum Maxim, and its herbal medical name is hwa-cho. It has been widely used for a variety of spices, medicines, and oil manufacturing purposes because it contains essential oils, fats, and aromatic ingredients in its leaves and roots. Its main ingredients are limonene, 1,8-cineole, myrcene, pinene and sabinene, etc. The chemical nature is pungent, heat, and toxin, which allows for it to treat wind and pain. The water or ethanol extract of hwa-cho reduces motor conduction as it limits the excitability of the sciatic nerves, as reported after a study on toad. Also, the branches are used to catch fish because they contain local anesthetic effects and fish toxicity. Still, there are few experimental and clinical studies on the analgesic effects of hwa-cho in Korea.

The formalin test, which is used to induce pain, is characterized in terms of early phases and late phases of pain: the early phase is mainly induced by the activation of the C nerve fibers through peripheral stimuli, whereas the late phase is induced when inflammation of the peripheral tissues and functional changes in the anterior spinal cord are combined. In other words, the early phase and the late phase have different characteristic results, and these have been proven to be a very useful methods for examining the pharmacologic and other ways of pain and the response of the nociceptors.

Since, the ultrasonic waves emitted by rodents cannot be heard outside of audible human frequencies, USV analysis widely relies upon experimental methods to analyze emotions, states, and behaviors based on the these waves. The frequency of emotional, state and behavioral changes depending on the type of rodent and its age. In particular, mature rats emit ultrasonic waves on average within the 22 kHz range when external stimuli or unavoidable pain on the soles occurs, although there are differences among individuals.

In this study, only rats with an average rate of more than 10 times in the Von Frey test were used to adjust the sensitivity to pain after adapting the rats to the cage before the USV experiment. The acupoint for injecting pharmacopuncture was the KI 3 in the kidney meridian, which was located in the posterior of the malleolus internus. The KI 3 is a region where the tibial nerve and saphenous nerve pass through, and it is associated with the malleolus internus and the sole of the foot.

As a result of analyzing the frequency ranges of 18–30 kHz, and through considering the individual differences of rats in the USV analysis, it was observed that the USV of the Con–1 was significantly higher in comparison to that of the Nor. This means that the pain was normally induced by formalin. The USV figures were significantly lower for the Lido–1 and ZMEG–1 when compared to the Con–1 in the early and late phases. On the other hand, the ZMWG–1 showed no significant difference in the early phase and late phase when compared to Con–1. This suggests that ZME has an analgesic effect on nociceptive pain caused by pe-
The tail-flick test is the most widely used method for pain experiments in rats and mice. Because the tail-flick reaction is not blocked at the spinal cord transverse section above the lumbar level, this method is useful for measuring pain reflexes at the level of the spinal cord below the lumbar spine. Furthermore, it is very useful for experiments on general pain involving radiation pain or the pharmacological investigation of anesthetic.

In this experiment, Lido-2, ZMWG-2, and ZMEG-2 were found to be statistically significant compared to Con-2, indicating that hwa-cho pharmacopuncture significantly relieved pain caused by warmth. In particular, ZME had similar analgesic effects to Lidocaine.

Assessment of drug toxicity is one of many sensitive issues in medicinal research. AST and ALT concentrations were measured in order to confirm the level of acute drug-induced liver injury after formalin testing. As a result, AST activity was decreased in the Lido-1, ZMW-1, and ZMEG-1 without a significant difference being reported in the Con-1 injected with saline. ALT activity significantly decreased in the Lido-1, ZMW-1 and ZMEG-1 compared to the Con-1. As a result, there may be a small amount of AST and ALT elevation due to stress caused by formalin-induced pain. Still, it is important to note that AST and ALT levels had decreased after lidocaine, ZMW, and ZME injection, which suggests that there is no substance-related hepatotoxicity.

The USV test confirmed that ZME had a significant analgesic effect on formalin-pain-induced rat models administered hwa-cho pharmacopuncture injection of KI3, and ZMW and ZME were unlikely to cause drug-induced hepatotoxicity. In the tail-flick test, hwa-cho pharmacopuncture was found to have analgesic effect on pain caused by warmth. In particular, ZME showed significantly similar effects to lidocaine in both experiments, suggesting that the analgesic effects are superior. Therefore it is maintained that the use of ZME for clinical purposes will increase in the future. Therefore, further studies on the mechanisms of the tail-flick test and a study to clarify the exact substances showing such analgesic effect will be needed in the future.

V. Results

In the investigation of the effects of hwa-cho pharmacopuncture as an analgesic and the effects depending on the extraction method through the USV analysis, AST and ALT concentration analysis on the rat model with formalin-induced pain, and the tail-flick test analysis on mice model, the following conclusions were obtained:

1. In USV test, significant analgesic effect of hwa-cho pharmacopuncture were observed in both the early phase and late phase. Also, ZME had better analgesic effect than ZMW, and showed similar effects to lidocaine.
2. In the tail-flick test, analgesic effects of hwa-cho pharmacopuncture on pain from warmth in all the experimental groups was observed as significant, and ZME showed similar effect to lidocaine.
3. In hepatic toxic test, AST was not significantly different in terms of its results from the control group, but ALT demonstrated significantly lower than those of the control group.

VI. Reference

14. Kim SJ, Choi YH, Yu YC, Lee BY, Jo SM. Decrease in Zinc Concentration in the Rat Spinal Gray Matter Induced by Peripheral Nerve Lig-


