Master's Thesis of Molecular Medicine, Biopharmaceutical Science

Molecular Mechanisms of Dicentra Formosa

Analgesic effect

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Abstract

Surgeons, Native Americans and Eclectics have all sworn to the multiple healing properties including anthelmintic, analgesic, and anti-cancer effects that Dicentra Formosa possess. However, none of the above provided any scientifically proven data to conclude the healing properties of Dicentra Formosa as fact. Here, I've examined the analgesic effect of Dicentra Formosa in two pain models and also identified the molecular target for the analgesic effect of Dicentra.

In the chronic pain model of carrageenan-induced inflammation and sciatic nerve ligation, I observed that the threshold for thermal and mechanical nociception was increased in Dicentra-treated mice compared to the vehicle-treated group. As TRP channels, especially TRPV1 and TRPA1 are known to play a crucial role in the hypersensitivity to thermal and mechanical stimuli, we proceeded to test the analgesic effect of Dicentra Formosa on TRPV1, TRPA1 channel activities. Calcium imaging and Patch Clamp recordings revealed Dicentra Formosa to have an antagonistic effect on the TRPA1 channel comparable to the known TRPA1 antagonist, HC-030031. However, Dicentra Formosa showed no inhibitory effect on the TRPV1 channel.

Therefore Dicentra Formosa shows a substantial analgesic effect in inflamed and neuropathic pain, which is presumably mediated by the inhibition of TRPA1 channel. These findings are the first scientific report for analgesic

effect of Dicentra Formosa, which provides potential therapeutic promise of Dicentra Formosa for pain relief.

Key words: Dicentra Formosa, Analgesic, Pain, Thermal / Mechanical nociception, TRPA1, TRPV1

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Introduction

Analgesic refers to the classes of drugs sold through a pharmacy under the direction of a physician; used for moderate to severe pain [1]. Pain in its most benign form warns us that something is not quite right, at its worst, pain robs us of our productivity and well being [1]. Pain is a complex perception that differs enormously among individual patients, even those who appear to have identical injuries or illnesses [1]. Presently a vast major of people suffering from pain, use herbal alternatives for pain relief. 80% of the population of Asia / Africa presently use herbal medicine for primary health care [2]. Herbal remedies have long history, including opium, aspirin, digitalis, and quinine [2]. 25% of modern drugs used in the United States have been derived from plants [3]. At least 7,000 medical compounds in the modern pharmacopoeia are derived from plants. However, only 156 had clinical trials published in a 2010 survey of the most common 1000 plant-derived compounds, while 12% of the plants had "no substantial studies" although available in the Western market [3].

Dicentra Formosa is also known as ‘Pacific bleeding heart’, ‘금낭화’ belongs to the Fumariaceae family, a cousin of the opium poppy is used as an anthelmintic, topical analgesic (for toothache) and to make hair grow and anticancer remedies though the exact nature of use was not examined [4]. Dicentra Formosa contains several isoquinoline alkaloids including corydine, isocorydine, bulbocapnine and dicentrine, etc [5]. However the therapeutic effect of Dicentra Formosa including the analgesic effect have not been examined so far.

The (TRPA1) channel is believed to be involved in many forms of acute and chronic hyperalgesia. TRPA1 agonist AITC (Allyl isothiocyanate) creates cold hypersensitivity in TRPA1 activation under inflammatory conditions. Nerve Growth Factor (NGF) regulates chronic inflammatory hyperalgesia by controlling gene expression in sensory neurons, including genes involved in inflammatory hyperalgesia [6]. TRPA1 is considered as an attractive pain target based on the fact that TRPA1 knockout mice showed near complete attenuation of formalin-induced pain behaviors [7][8]. TRPA1 antagonists such as HC-030031 are effective in blocking pain behaviors induced by inflammation [7][8].

The TRPV1 channel is researched and targeted for the development of analgesics of inflammatory pain relating to its distribution and function [9]. TRPV1 channels show an increased response in preclinical models of inflammation and are critical in inflammatory pain signaling [10][11]. TRPV1 agonist such as capsaicin; treat pain with the excitation of sensory neurons followed by a refractory state of desensitization [12]. TRPV1 antagonist, provide additional knowledge of TRPV1 channels in inflammation conditions. TRPV1 antagonist such as capsazepine, are effective at blocking thermal hypersensitivity to many inflammogens [13-15]. These findings suggest that TRPV1 channels are a logical mechanism in a variety of pain conditions.

In the 1990's many scientist began to study the effects of tissue injury on the development of persistent or chronic pain. In most models animals are awake and mimic human clinical conditions [16]. When studying the mechanism of persistent pain in animal models that mimic human clinical pain conditions, an injection of an inflammatory agent (carrageenan) is injected into the mouse

hind paw. A paw withdrawal latency or threshold is measured when the mouse withdraws their limb [17]. In subcutaneous Inflammation, the injection of carrageenan into the footpad produces persistent pain and hyperalgesia [17]. However, persistent pain models showing neuropathic pain, the mechanical and thermal hyperalgesia and allodynia have been noted to occur within 4 days of injury, which persist for several weeks (up to 6 months) post- injury [18][19]. This form of pain is important because it allows simultaneous investigation of changes in primary sensory neurons and unharmed sensory neurons [20][21]. Neuropathic pain models based on ligation are more commonly used due to the clinical setup being simple.

Materials and Methods

Animals

Six week old, C57BL/6 mice (Nara Biotech, South Korea) were used as sources of sensory ganglion neurons and for behavioral tests. Experiments were carried out according to the ethical guidelines issued by the International Association for the Study of Pain. Animal protocols used, have been approved by the Institute of Laboratory Animal Resources at Seoul National University.

Pain Models

Hyperalgesia – To induce inflammation, carrageenan was injected subcutaneously into the plantar surface of the left hind paw of mice. Carrageenan 1.1g mixed with 500 ml D.W (Distilled Water) was used to induce inflammation. A BD ultra fine needle filled with 5ml of carrageenan was injected into the left hind paw. Mice were placed into cubicle sections while waiting 1hr for the left paw to become inflamed. Vehicle (45% Glycerol, 20% D.W, 20% Ethanol) or Dicentra Formosa was injected in the left paw, after waiting 1 hour for Carrageenan to cause inflammation. 5ml Dicentra Formosa or Vehicle was injected into the left hind inflamed paw, 1 hour passed before performing behavioral experiments. T-test statistics were derived using the Duncan's Multiple Range Test for RESPONSE.

Spared nerve injury- Spared nerve injury was performed after mice were anesthetized by 3ml intraperitoneal injection of pentobarbital. Reflexes were checked pinching paws using a pincette. The left leg was shaved from the foot to thigh; afterwards the leg was rubbed with an alcohol swab. Next I touched the leg with a pincette to find the femur bone. A 1.5 inch incision was made parallel to the femur bone cutting into the skin. Injury was induced in mice through cutting the tibial nerve and common peroneal nerve branches among three branches of the sciatic nerve, also known as the L4 & L5 regions of the sciatic nerve. Lastly, the muscle tissue was stitched, followed by the skin. One week after inducing Neuropathic Pain, behavioral experiments were performed. In the control experiments, the mouse had the same incisions on the right leg as the left but the Sciatic nerve was not cut. 10ml Dicentra Formosa or Vehicle (45% Glycerol, 20% D.W, 20% Ethanol) was intraperitoneally injected for pain relief, 1 hour before behavioral experiments were performed. T-test statistics were derived using the Duncan's Multiple Range Test for RESPONSE.

Calcium Imaging

The Hek-293 cell line was established by transformation of embryonic kidney cells with fragmented human Adenovirus DNA (Graham et al., 1977) HEK293 cells were passaged in 2-3 day cycles into fresh media. For splitting, media was removed and cell debris and dead cells were discarded by PBS washing from the cultured flasks. The remaining cells attached to the flask surface were washed 3 times with warmed Trypsin and incubated in the 37

degree CO^2 incubator for 1 minute. Afterwards the generated cell suspension was diluted 1:10 for passaging or adapted to a working concentration of 2000 cells 100 μl. 200 μl of Poly-L lysine was used to coat each well in the 8 well. After coating the 8 well with Poly–L lysine, the 8 well was incubated in the 37°C CO^2 incubator for 30 minutes. Afterwards the Poly-l lysine coating solution was removed from each 8 well. Finally the 8 well was incubated in the 37°C CO^2 incubator for 1 hour to dry out the remaining Poly-l lysine coating solution. After incubating for one hour, 50 μl of the working concentration of HEK 293 cells was injected into 2 ml of FBS media. After pipetting, 200 μl of the working concentration of the HEK 293 cells; 2 ml of media was injected into each 8 well and incubated for 24 hours. On the second day after 24 hours the HEK 293 cells proliferation was checked using a microscope. After checking HEK 293 cells proliferation the 8 well was placed back into the 37°C CO^2 incubator, Opti-MEM media was placed into a water bath for heating for 30 minutes. For transfection one e-tube was used. 150 μl of Opti-MEM media was injected into the e-tube, next a ration of 3 to 1 was used to calculate how much of the TRPA1 gene was needed for maximum transfection. Example (1 mg/ reaction1000 ng TRPA1 650 ng/ 1 μl = 650/1000 = 1.5 μl of TRPA1) Stored at 4 degrees lipofectamine was used and 10 μl of lipofectamine plus 2.5 μl of TRPA1 genes were pipetted into the 150 μl of Opti-MEM media, afterwards the e-tube was incubated in the 37°C CO^2 incubator for 15 minutes. After 15 minutes the 8 well and e-tube were removed from the CO^2 incubator and 20 μl of the TRPA1 genes were injected into each of the 8 wells. Afterwards the 8 well was placed

back in the 37°C CO^2 incubator for 24 hours. On the third day after 24 hours most genes are transfected. Two e-tubes were injected with 1 μl of F-127, 1 μl of Fluo- 3 am, (calcium indicator) and 1 ml of Na-Hepes + Ca^2 buffer and pipetting. Removed the 8 well from the 37°C CO^2 incubator and remove media from each well in the 8 well. Washed out the 8 plate with the Na-hepes + Ca^2 buffer twice. Afterwards 200 μl of the buffer mixed with 1 μl of F-127 and 1 μl Fluo-3am was injected into each well of the 8 well. Afterwards the 8 well was placed into the 37°C CO^2 incubator for 30 minutes. Four reagents were diluted for positive and negative control, testing the antagonist possibilities of Dicentra Formosa. 1st 2 μl AITC 100 mm stock was diluted 1/100 and injected into 98 μl of the Na-Hepes + Ca^2 buffer in one e-tube for a positive control. 2nd 2 μl AITC 100 mm stock was diluted 1/100 and injected into 96 μl of the Na-Hepes + Ca^2 buffer + 2 μl HC-030031 for a negative control. 3rd 80 μl of Dicentra formosa which is (45% ethanol, 20% glycerol, 20% H20 and 15% of the Dicentra formosa tincture V/V / V/W = 15 g/100 μl= 0.015 g or 1 ml/100 = 1.5% of the extract is Dicentra Formosa) was injected with 2 μl AITC 100 mm stock diluted 1/100 into 18 μl of the Na-Hepes + Ca^2 buffer into 1 e-tube. 4th 2 μl AITC 100 mm stock diluted 1/100, was injected into 88 μl of the Na-Hepes + Ca^2 buffer into 1 e-tube. 5th 80 μl of the Dicentra Formosa which is (45% ethanol, 20% glycerol, 20% H20 and 15% of the Dicentra Formosa tincture V/V / V/W = 15 g/100 μl= 0.015 g or 1 ml/100 = 1.5% of the extract is Dicentra Formosa) was injected with 2 μl AITC 100 mm stock diluted 1/100 and injected into 19 μl of the Na-Hepes + Ca^2 buffer into 1 e-tube. The dilutions from e-tubes 4-5 were used

as a double injection in the 4th well of the 8 well. The dilutions from e-tubes 1-3 were used for the first 3 wells of the 8 wells. Lastly the cells were imaged using a Fluorescence Microscope, logging into the Meta Morph program, starting a Time Series with 3 intervals and 61 cycles to start the experiment; find the focus, set the exposure to live continuous.

Reagents

- 1st 2 μl AITC 100 mm stock was diluted 1/100 and injected into 98 μl of the Na-Hepes + Ca^2 buffer in one e-tube for a positive control.
- 2nd 2 μl AITC 100 mm stock was diluted 1/100 and injected into 96 μl of the Na-Hepes + Ca^2 buffer + 2 μl HC-030031 for a negative control.
- 3rd 80 μl of the Dicentra Formosa which is (45% ethanol, 20% glycerol, 20% H20 and 15% of the Dicentra Formosa tincture V/V / V/W = 15 g/100 μl= 0.015 g or 1 ml/100 = 1.5% of the extract is Dicentra Formosa) was injected with 2 μl AITC 100 mm stock diluted 1/100 into 18 μl of the Na-Hepes + Ca^2 buffer into 1 e-tube.
- 4th 2 μl AITC 100 mm stock diluted 1/100, was injected into 88 μl of the Na-Hepes + Ca^2 buffer into 1 e-tube.
- 5th 80 μl of Dicentra Formosa which is (45% ethanol, 20% glycerol, 20% H20 and 15% of the Dicentra Formosa tincture V/V / V/W = 15 g/100 μl= 0.015 g or 1 ml/100 = 1.5% of the extract is Dicentra Formosa) was injected with 2 μl AITC 100 mm stock diluted 1/100 and injected into 19 μl of the Na-Hepes + Ca^2 buffer into 1 e-tube.
- The dilutions from e-tubes 4-5 were used as a double injection in the 4th

well of the 8 well. The dilutions from e-tubes 1-3 were used for the first 3 wells of the 8 wells.

Behavioral experiments

Von Frey test - Mice were place in cages with a mesh grid floor. Mice were allowed to acclimate for a minimum of 30 minutes before testing. Plantar surface of the left hind foot was poked with von Frey filaments of different thickness. Withdrawal thresholds to von Frey filaments were determined when animals lifted hind paw and that evoked response was considered as a mechanical withdrawal threshold. All behavior experiments were performed on six week (C57Bl/6KO Mice). T-test statistics were derived using the Duncan's Multiple Range Test for RESPONSE.

Hargreaves test - This test was performed using a standard apparatus, Ugo Basile Biological research Apparatus. A mouse was placed in a transparent acrylic box and an infrared heat lamp was positioned underneath the targeted hind paw. A radiant stimulus was then applied to the plantar surface and paw withdrawal latency was measured. T-test statistics were derived using the Duncan's Multiple Range Test for RESPONSE.

Dorsal Root Ganglion culture

For isolation of DRG neurons, DRGs were dissected from mice, collected in cold culture medium (4 °C) containing a mixture of DMEM and F-12 solution, 10% (vol / vol) fetal bovine serum (Gibco BRL), 1 mM sodium pyruvate, 50–100 ng ml^{-1} nerve growth factor (Alomon) and 100 units per ml of

penicillin/streptomycin. Isolated DRGs were washed with DMEM/F-12 mixture and incubated for 30 min in a warm (37 °C) same medium containing 1 mg ml^{-1} collagenase (Type II, Worthington Biomedical), after washed three times and incubated with gentle shaking in Hank's solution containing 2.5 mg ml^{-1} of trypsin (Roche Diagnostics) for 30 min at 37 °C. The trypsin-containing solution was then centrifuged at 100 g for 10 min. The centrifuged pellets were washed gently two to three times with DMEM/F-12 mixture, and then gently triturated with a fire-polished Pasteur pipette and plated onto round glass coverslips (Fisher) treated with poly-L-lysine (0.5 mg ml^{-1}), in small Petri dishes (35 × 12 mm). Cells were then placed in a 37 °C incubator in a 95% air/5% CO_2 atmosphere. Cells were used 2–4 d after isolation.

Whole-cell current recording

Whole-cell current recordings were obtained using a voltage-clamp technique with an Axopatch 200B amplifier (Molecular Devices). Whole cells were formed after breaking the plasma membrane under the pipette tips. Resistance of glass pipettes was about 3 mΩ. Junctional potentials were adjusted to zero. Pipette solutions contained 140 mM KCl, 5 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA and 10 mM HEPES, adjusted to pH 7.2. Bath solutions contained 140 mM NaCl, 5 mM KCl, 2 mM MgCl2, and 10 mM HEPES, also to adjust pH 7.2. Whole-cell currents were amplified and stored in a personal computer after digitization using Digidata 1440 (Molecular Devices).

Results

D. Formosa inhibits Carrageenan-induced inflammatory pain.

Inflammatory pain is one of the most common forms of chronic pain in humans. Carrageenan was subcutaneously injected to induce inflammation. To test the analgesic effect of Dicentra Formosa on inflammatory chronic pain, 5 ml of Dicentra Formosa or Vehicle (45% Glycerol, 20% D.W, 20% Ethanol) was subcutaneously injected 1 hour after carrageenan-induced inflammation. A vehicle was created because the Dicentra Formosa extract also contained other properties that might have an analgesic effect. The vehicle contained all of the other properties in the Dicentra Formosa extract with the exception of Dicentra Formosa. Pain hypersensitivity was assessed by Hargreaves plantar assay and von Frey test, which were measured with the withdrawal latency or threshold on thermal and mechanical stimuli, respectively. Figure 1 showed that Dicentra Formosa-treated mice have increased thermal latency compared to vehicle-treated group suggesting that Dicentra inhibits thermal hyperalgesia induced by carrageenan-induced pain. When the mice were examined for von Frey assay, the mechanical threshold was higher in Dicentra-treated mice than vehicle treated mice (Figure 2). This result indicates that Dicentra reduced mechanical hypersensitivity in carrageenan-induced pain.

D. Formosa inhibits neuropathic pain.

Neuropathic pain like inflammatory pain is a common form of chronic pain. Neuropathic pain was induced to measure the analgesic effect of Dicentra Formosa in Spared nerve injury (SNI). Cutting tibial and common peroneal nerve branches among three branches of the sciatic nerve, also known as the L4 & L5 regions of the sciatic nerve. In the control experiments, the mouse had the same incisions on the right leg as the left but the sciatic nerve was not cut. Vehicle or Dicentra Formosa was injected intraperitoneally for pain relief 1 hour before behavioral experiments were performed. The thermal sensitivity in SNI model show higher withdrawal latency in Dicentra-treated mice rather than vehicle-treated groups, shown as Figure 4. And mechanical threshold was also increased in response to the application of Dicentra Formosa (Figure 5). These findings suggest that Dicentra has an inhibitory effect on thermal and mechanical hyperalgesia in neuropathic pain.

The Molecular target of D. Formosa; TRP channels

We determined through behavioral experiments that Dicentra Formosa exhibits significant thermal and mechanical analgesic effect in chronic pain mouse model. Now we needed to test the analgesic effect of Dicentra Formosa on a molecular level. TRPV1 and TRPA1 channels are known for their abilities to control pain in sensory neurons. Calcium imaging and Patch clamp experiments were performed to test the inhibition of Dicentra Formosa in TRPV1 and TRPA1 channels. When TRPV1 was overexpressed in HEK293 cells, capsaicin (100 μM) selectively activates Ca^{2+} influxes (Figure 6). This

CAP-evoked Ca^{2+} influx was completely blocked by the TRPV1 antagonist capsazepine, suggesting that this Ca^{2+} influx is mediated only by TRPV1. However the reagent of Dicentra Formosa rarely inhibited capsaicin-evoked Ca^{2+} influx, suggesting that Dicentra does not have an inhibitory effect on TRPV1 channel, if any. For further examination of the inhibition possibilities of Dicentra Formosa in the TRPV1 channel, a patch clamp recording was done to test the effect of Dicentra Formosa in HEK 293 cell transfected with TRPV1 gene. In whole cell configuration, inward current in heterologous expression of human TRPV1 was induced by capsaicin application, which was not inhibited by Dicentra Formosa (Figure 7). When capsaicin (1 μM) was recorded it caused an electrical current measured by 1 nano ampere over 1 min, later 1% of Dicentra Formosa was added and the electrical current showed no blockage or inhibition. When capsaicin (1 μM) was added again as a recovery another electrical current was measured, confirming that Dicentra Formosa does not inhibit the TRPV1 channel. These results suggest that the pain-relieving effect of Dicentra is not mediated by TRPV1. The calcium imaging experiment was performed for the effect of Dicentra Formosa in HEK293 cell transfected with TRPA1 gene. The TRPA1 channel is a promising therapeutic target for chronic inflammatory or neuropathic pain. In HEK293 cells transfected with TRPA1 gene, the extracellular application of AITC (Allyl isothiocyanate, 100 μM), a selective TRPA1 agonist, irreversibly increased Ca^{2+} influx. In contrast, the reagents of Dicentra Formosa + AITC inhibited AITC- induced Ca^{2+} influx of the TRPA1 channel (Figure 7). The data shows Dicentra Formosa as an antagonist comparable to the known antagonist of the TRPA1 channel, HC-030031

supporting the behavioral data. For further confirmation, a patch clamp recording testing the effect of Dicentra Formosa in HEK 293 cell transfected with TRPA1 gene was done. Patch clamp recording show the reagent Dicentra Formosa antagonist effect when 0.1% is applied in TRPA1 expressing cells, but no inhibition in TRPV1 genes (Figure 8). This data indicates the analgesic effect of Dicentra Formosa presumably through inhibition of TRPA1. To identify the molecular mechanism at which Dicentra Formosa exhibits alleviation of pain to use in vivo, patch clamp recordings were performed from mouse DRG. At -60 mV whole cell recording, AITC-induced inward currents were diminished in a dose- dependent manner by co-treatment both Dicentra Formosa and AITC but not capsaicin, as shown in Figure 9.

In conclusion, Dicentra Formosa showed significant reduction in thermal and mechanical hyperalgesia in inflammatory and neuropathic pain model. Dicentra Formosa fails to block TRPV1 activity in heterologous expression system and DRG neuron. Dicentra Formosa prominently reduced TRPA1-induced current in heterologous expression system and DRG neuron. Therefore, the Dicentra Formosa shows analgesic effect through TRPA1 inhibition, which provides some potential of Dicentra Formosa as an alternative in herbal pain relief.

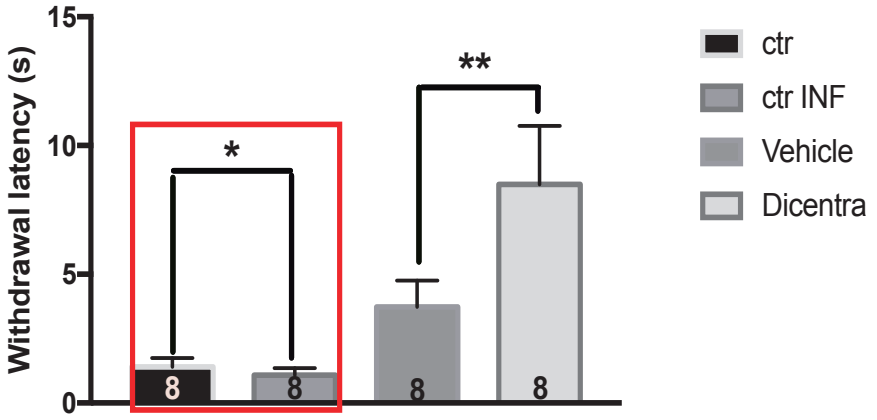


Figure 1. The effect of Dicentra Formosa in carrageenan induced inflammation

Carrageenan was subcutaneously injected to induced inflammation. To test the analgesic effect of Dicentra Formosa on inflammatory chronic pain, 5 ml of Dicentra Formosa or Vehicle (45% Glycerol, 20% D.W, 20% Ethanol) was subcutaneously injected 1 hour after carrageenan-induced inflammation. A vehicle was created because the Dicentra Formosa extract also contained other properties that might have an analgesic effect. The vehicle contained all of the other properties in the Dicentra Formosa extract with the exception of Dicentra Formosa. Pain hypersensitivity assessed by Hargreaves plantar assay measured the withdrawal latency on thermal nociception. Withdrawal latency measurement, show a higher withdrawal latency in six week C57BL/6 mice treated with Dicentra Formosa. Results are presented as means ± SEM of 8 mice per group, per experiment, and are representative of 4 separate experiments; One-way ANOVA followed by Duncan analyses gave a *P value < 0.05, **p<0.01. Red box indicates the significance between the control and inflamed hind paw.

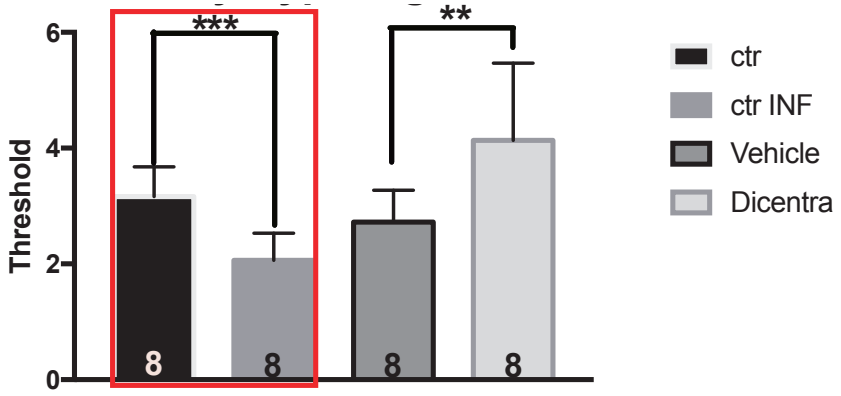


Figure 2. The effect of Dicentra Formosa in Carrageenan induced inflammation

Carrageenan was subcutaneously injected to induced inflammation. To test the analgesic effect of Dicentra Formosa on inflammatory chronic pain, 5 ml of Dicentra Formosa or Vehicle (45% Glycerol, 20% D.W, 20% Ethanol) was subcutaneously injected 1 hour after carrageenan-induced inflammation. A vehicle was created because the Dicentra Formosa extract also contained other properties that might have an analgesic effect. The vehicle contained all of the other properties in the Dicentra Formosa extract with the exception of Dicentra Formosa. Pain hypersensitivity assessed by von Frey filaments measured the withdrawal threshold or mechanical nociception. Withdrawal threshold measurement, show a higher withdrawal threshold in six week C57BL/6 mice treated with Dicentra Formosa. Results are presented as means ± SEM of 8 mice per group, per experiment, and are representative of 4 separate experiments; One-way ANOVA followed by Duncan analyses gave a ** P value < 0.01, ***p<0.001. Red box indicates the significance between the control and inflamed hind paw.

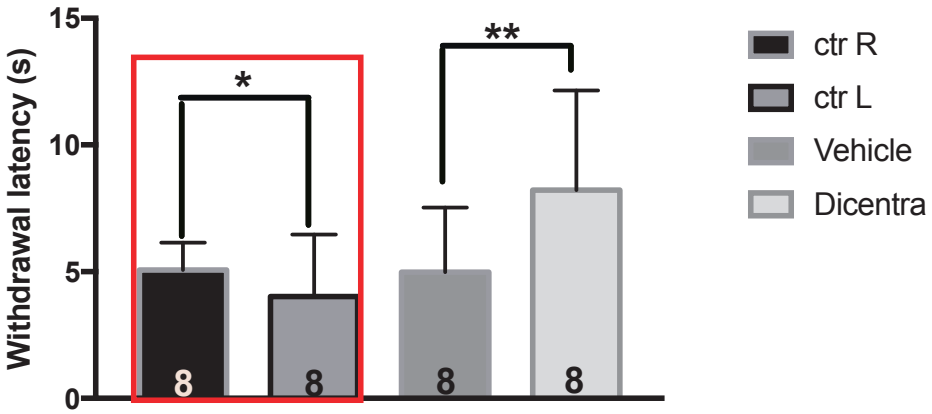


Figure 3. The effect of Dicentra Formosa on SNI

Spared nerve injury was induced in mice through cutting tibial and common peroneal nerve branches among three branches of the sciatic nerve, also known as the L4 & L5 regions of the sciatic nerve. In the control experiments, the mouse had the same incisions on the right leg as the left but the sciatic nerve was not cut. Vehicle or Dicentra Formosa was injected intraperitoneally for pain relief 1 hour before behavioral experiments were performed. SNI test show higher withdrawal latency on thermal nociception in response to the application of Dicentra Formosa. The graph shows Dicentra Formosa alleviates neuropathic behavior 1 week after spared nerve injury. Results are presented as means ± SEM of 8 mice per group, per experiment and are representative of 4 separate experiments; One-way ANOVA followed by Duncan analyses gave a ** P value < 0.01. Red box indicates the significance between control group and SNI model.

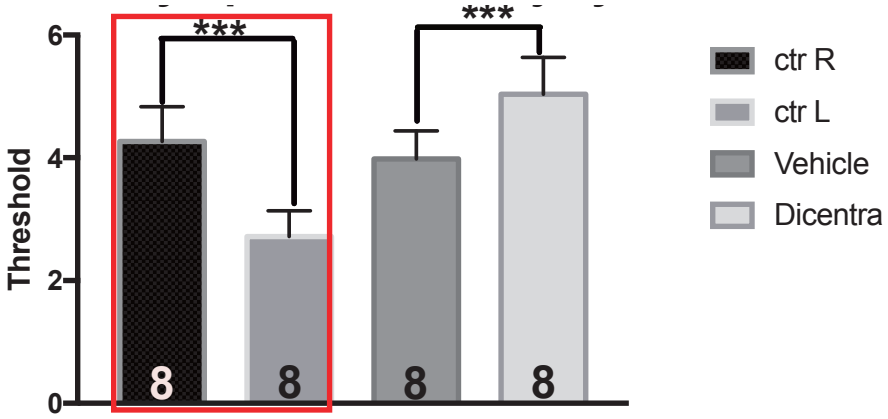


Figure 4. The effect of Dicentra Formosa on SNI

Spared nerve injury was induced in mice through cutting tibial and common peroneal nerve branches among three branches of the sciatic nerve, also known as the L4 & L5 regions of the sciatic nerve. In the control experiments, the mouse had the same incisions on the right leg as the left but the sciatic nerve was not cut. Vehicle or Dicentra Formosa was injected intraperitoneally for pain relief 1 hour before behavioral experiments were performed. SNI test show higher withdrawal threshold or mechanical nociception in response to the application of Dicentra Formosa. The graph shows Dicentra Formosa alleviates neuropathic behavior 1 week after spared nerve injury. Results are presented as means ± SEM of 8 mice per group, per experiment, and are representative of 4 separate experiments; One-way ANOVA followed by Duncan analyses gave a *** P value < 0.001. Red box indicates the significance between control group and SNI model.

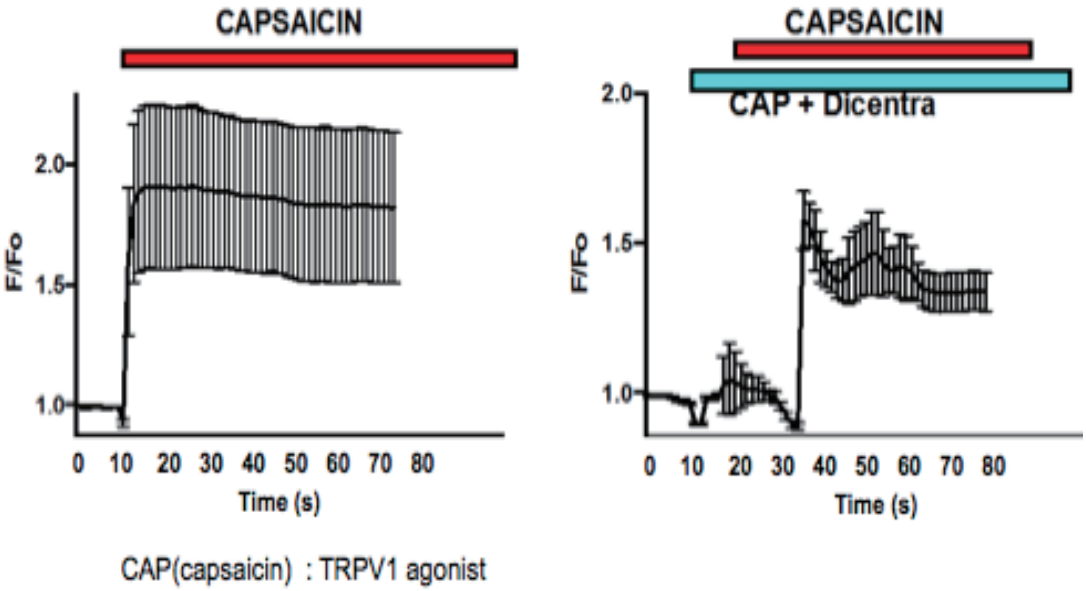
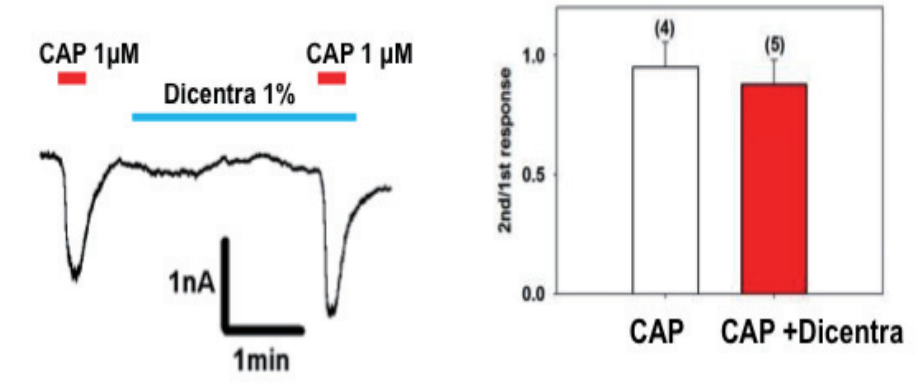


Fig 5. Calcium imaging effect of Dicentra Formosa in HEK 293 cells transfected with TRPV1 gene.

The reagents were added at the time points indicated horizontal bars. Capsaicin (CAP) (100 μM) selectively activates TRPV1 Dependent Ca^{2+} influxes. Dicentra Formosa partially inhibited Capsaicin, but when Capsaicin was applied as a recovery; Dicentra Formosa showed no inhibition making it not an antagonist in HEK 293 cells transfected with TRPV1 gene comparable to capsazepine.



CAP: TRPV1 agonist

Figure 6. Patch clamp recording effect of Dicentra Formosa in HEK 293 cells transfected with TRPV1 gene.

A whole cell heterologous overexpression of human TRPV1 wasn't inhibited by Dicentra Formosa. When Capsaicin (CAP) (1 µM) was recorded it caused an electrical current measured by 1 nano ampere over 1 min, later 1% of Dicentra Formosa was added and the electrical current showed no blockage or inhibition. When Capsaicin (CAP) (1 µM) was added again as a recovery another electrical current was measured, confirming that Dicentra Formosa doesn't inhibit the TRPV1 channel.

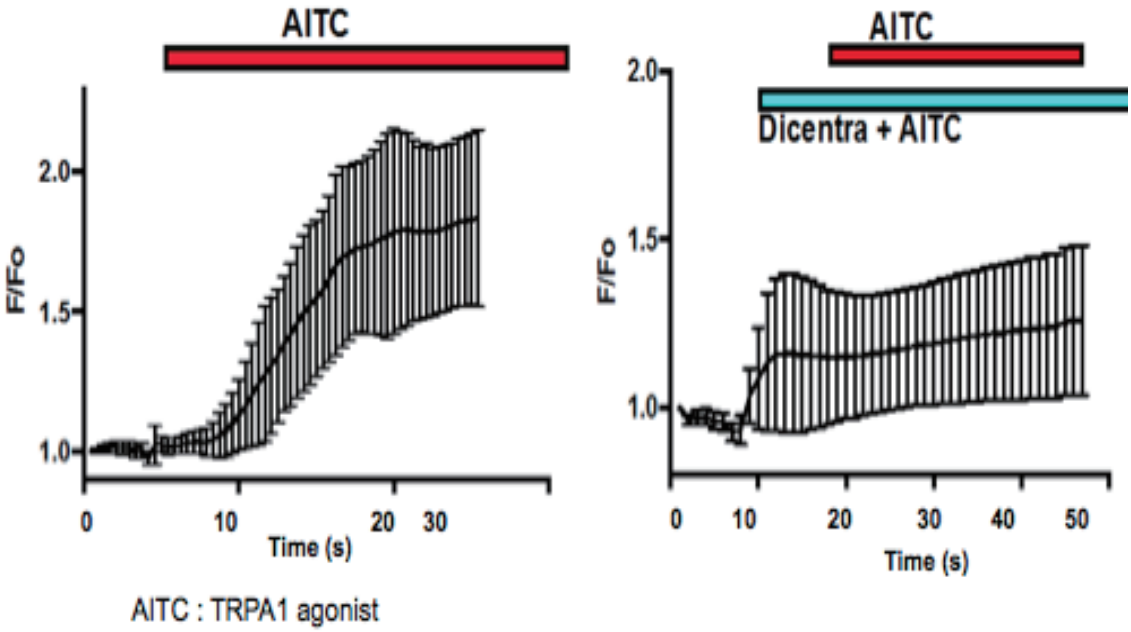


Figure 7. Calcium imaging effect of Dicentra Formosa in HEK 293 cells transfected with TRPA1 gene.

TRPA1 channel is a promising therapeutic target for chronic inflammatory or neuropathic pain. Dicentra Formosa, AITC, HC-030031 reagents were carried out at the time points indicated by horizontal bars. In HEK 293 cells transfected with TRPA1 gene, extracellular application of AITC (100 μM) irreversibly increased [Ca^{2+}] influx. In contrast, the reagents of Dicentra Formosa + AITC inhibited AITC known influx of the TRPA1 channel. The data shows Dicentra Formosa as an antagonist, comparable to the known antagonist HC-030031 supporting the behavioral data.

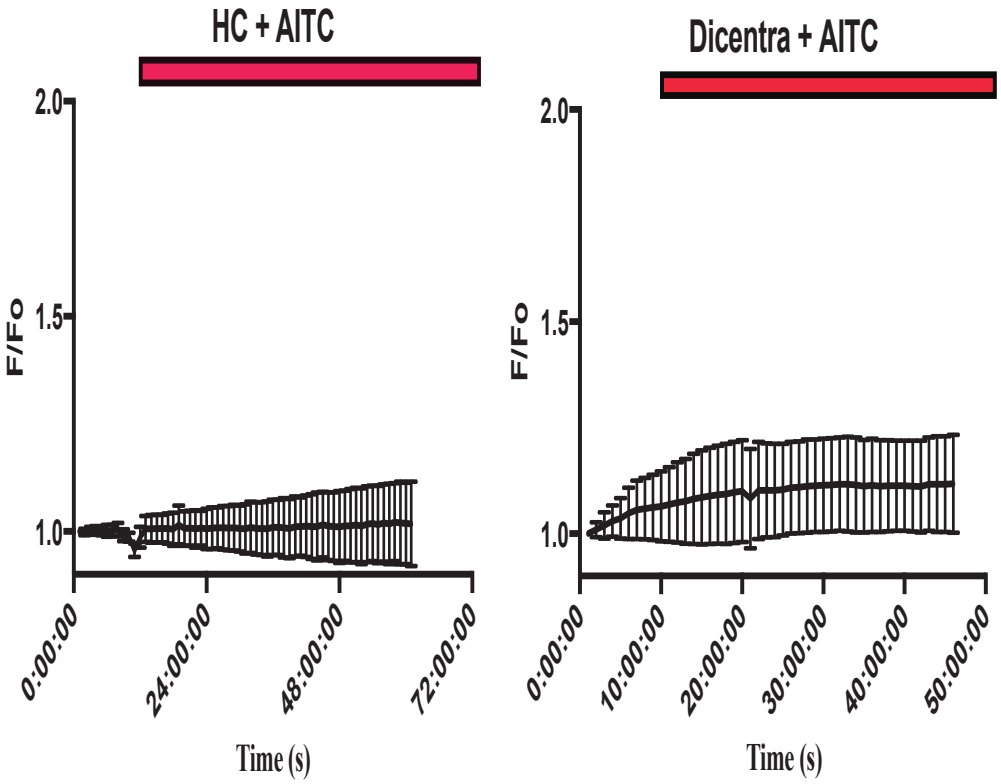


Figure. 8. Calcium Imaging effect of Dicentra Formosa on [Ca^{2+}] in HEK 293 cells transfected with TRPA1 genes.

Indicated by horizontal bars, the reagents HC-030031 + AITC, Dicentra Formosa + AITC inhibited AITC know influx of HEK 293 cells transfected with human TRPA1 gene. The data shows Dicentra Formosa as an antagonist similar to the known antagonist HC-030031 supporting the behavioral data.

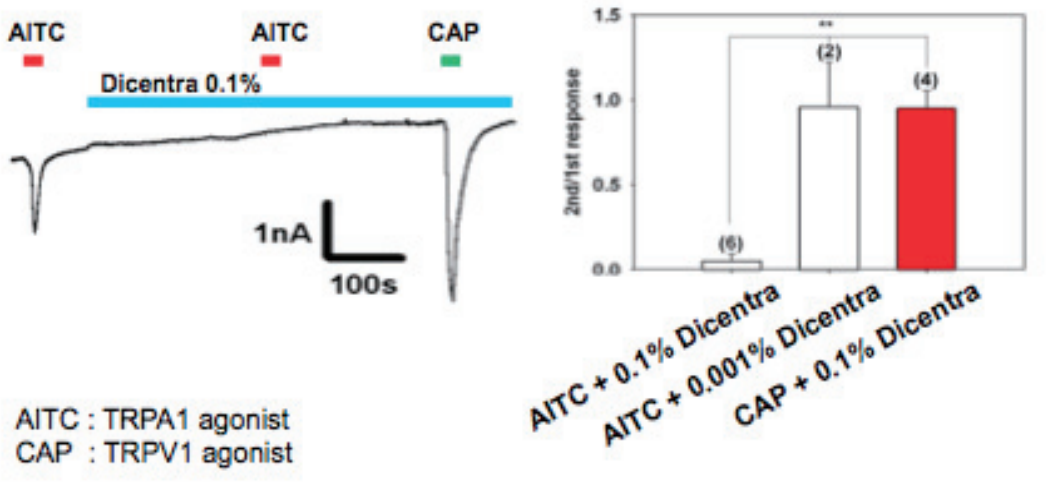


Figure 9. Patch clamp recording effect of Dicentra Formosa in Mouse DRG neurons

Patch clamp recording show reagent application 0.01% of Dicentra Formosa antagonist effect in TRPA1 genes but no inhibition in TRPV1 genes. This data confirms the analgesic effect of Dicentra Formosa in behavioral and path clamp experiments. 2nd and 1st whole cell recordings are quantified in the bar graph with a comparison of reagent Dicentra Formosa + Capsaicin treated in TRPV1 genes. To identify the molecular mechanism where Dicentra Formosa exhibits alleviation of pain to use in vivo, Patch clamp recordings performed from mouse DRG. At -60 mV whole cell recording, AITC (Allyl isothiocyanate), TRPA1 channel which is widely known as major target channel protein for mechanical allodynia Agonist, response is inhibited in concentration dependent by co-treatment both Dicentra Formosa and AITC but not Capsaicin. TRPV1 channel was also targeted because it is widely known as major target protein for thermo hyperalgesia Agonist. Whole cell recording advantage is that the larger opening at the tip of the patch clamp electrode provides lower resistance and better electrical access to the inside of the cell.

Discussion

In this study, we determined the anti-nociceptive effect of Dicentra Formosa; showing reduced thermal and mechanical hyperalgesia in inflammatory and neuropathic pain models. We then examined the molecular target for alleviating pain. In HEK 293 cells overexpressing TRPA1, the application of AITC irreversibly increased Ca influx. In contrast, Dicentra Formosa and AITC inhibited AITC-induced Ca influx through the TRPA1 channel, comparable to the known antagonist HC-030031. Patch clamp recordings of mouse DRG showed AITC response was inhibited in a concentration-dependent manner by co-treatment both Dicentra Formosa and AITC but not capsaicin, confirming Dicentra Formosa antagonist abilities on TRPA1.

Neuropathic and inflammatory behavioral experiments provided supporting data for Dicentra Formosa as an alternative in herbal pain relief. Dicentra Formosa was injected intraperitoneally or subcutaneously for pain relief 1 hour before behavioral experiments were performed. SNI and Hargreaves test show higher withdrawal threshold, latency or mechanical, thermal nociception in response to the application of Dicentra Formosa.

With conclusive data showing that Dicentra Formosa alleviates Inflammatory, neuropathic pain and inhibits TRPA1 channel; several challenges for proving the role of Dicentra Formosa analgesic effect could be developed. One is the toxicity of Dicentra Formosa if administered orally, injected or in the

form of a patch. Although the rhizomes, roots and leaves of Dicentra Formosa have been used medicinally for decades they also contain Alkaloids that are toxic. If the LD50 and LC50 (Lethal dosage and Concentration) were measured to discover its acute toxicity, Dicentra Formosa is a potential candidate to provide neuropathic and inflammatory pain relief in many forms. Whether administered by capsule, patch, or as an elixir, Dicentra Formosa healing properties could help millions suffering from pain. Therefore further research should be done to determine the LD50 and LC50 in Dicentra Formosa followed by clinical trial studies.

Another is to finding out the active compounds contained in Dicentra Formosa targeting TRPA1. Dicentra Formosa contains alkaloid toxins such as protopine, a well-known voltage and receptor gated Ca^{2+} channel blocker. Even though TRPA1 is not just voltage gated, TRPA1 is known to have voltage dependent inactivation. Using various techniques such as MALDI-TOF mass spectroscopy, the active compounds in Dicentra Formosa can be identified and synthesized, which may allow bulk production. The development of the active ligands may be of utility in boosting herbal pain relief in the future.

These findings provide a mechanistic rationale of the analgesic actions and motivate further analysis of the effect of Dicentra Formosa on pain perception.

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Thesis topic: Molecular Mechanisms of Dicentra Formosa Analgesic effect

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