



Original Article

The Safety and Analgesic Effect of *Datura Flos* Pharmacopuncture in Sprague-Dawley Rats



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ABSTRACT

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Background: The aim of this study was to investigate the safety and analgesic effects of *Datura Flos* pharmacopuncture (DFP).

Methods: The analgesic effects of DFP were assessed using mechanical (hot plate), chemical (formalin test), and thermal (von Frey filament test) pain tests. Forty male Sprague Dawley rats were assigned randomly into DFP (75 mg/kg, 150 mg/kg), lidocaine 0.5%, or normal saline group for treatment on Kl3. Gross pathology, histopathology, biochemistry and hematology were performed.

Results: In the hot plate test, DFP at a high dose (HDDFP; 150 mg/kg) produced a significant analgesic effect, at 10 and 20-minutes post injection ($p < 0.01$). Low dose DFP (LDDFP; 75 mg/kg) also showed an analgesic effect at 10 minutes post injection ($p < 0.01$). In the formalin test, HDDFP produced an analgesic effect, for 0-10 and 10-20 minutes ($p < 0.01$) post treatment, whereas LDDFP showed analgesic effects between 10-20 minutes ($p < 0.05$). In the von Frey filament test, DF-H produced an analgesic effect, 10 ($p < 0.01$) and 20 minutes post treatment ($p < 0.05$). LDDFP showed analgesic effect at 10 minutes ($p < 0.05$). In the acupuncture response test, HDDFP produced an analgesic effect at 10 minutes post treatment ($p < 0.05$). DF-H did not cause any anatomical changes to the liver or kidney and there were no abnormalities in biochemistry or hematology.

Conclusion: DF-H was not toxic and provided short term analgesia, suggesting it may be useful in the management of pain.

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Introduction

Pharmacopuncture was first introduced into Korea in the 1960s. It was a new acupuncture method where refined herbal medicines were injected into acupuncture points or tender sites [1,2]. Pharmacopuncture increases the effectiveness of treatment by stimulating the acupuncture points while simultaneously providing herbal effects. In addition, pharmacopuncture is useful when it is difficult to take medications by standard routes, or if a specific site is preferred for the injection of medication. Pharmacopuncture is used in various diseases, but especially in musculoskeletal diseases [3,4].

In Chinese medicine, DF (a dried flower of *Datura metal* L. *Datura innoxia* Mill. or *Datura stramonium* L.) has been used in the treatment of asthma, cough, spasms, rheumatism, anesthesia,

pain relief, sedation, antidiuretic use and in detoxification, and is widely used in chronic bronchitis, pain syndromes, and bruises [5]. DF is mainly composed of alkaloids, distributed through the entirety of the plant. These alkaloids produce analgesic effects by interacting with the opioid system. Opioids act on the grey matter neurons of the brainstem and the spinal cord to induce an analgesic effect, and its action is mainly inhibitory to neurons [6]. While the analgesic effects of DF seeds have been reported, the analgesic potential of the flower remains unexplored [7].

This study was performed based on the analgesic properties of DF observed in Chinese medicine. In order to confirm these analgesic effects, DF pharmacopuncture (DFP) was performed in rats and was assessed using a hot plate, rat paw formalin injection, von Frey test, and acupuncture stimulus test.

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Methods and Materials

Animals

The experimental animals used were male Sprague Dawley rats (SD rats), 10-weeks old, weighing approximately 250 g. The animals were housed at 23°C-24°C, 40%-60% humidity, with 12-h light/dark cycle, and access to sterile distilled water and food. Experimentation began 1 week after acclimatization. All the procedures in this study were conducted in accordance with the regulations and policies of the Animal Experiment Ethics Committee of Dong-Eui University (Approval No.: R2018-023).

Measuring dermal 50% lethal dose

SD rats and BALB/c mice were used to measure the 50% dermal lethal dose. Each group had 4 animals, and were carefully monitored for compound-related mortality following DF dermal injection into the dorsal region (100 mg/kg and 1,000 mg/kg).

On the day of the procedure (Day 0), the general condition (type of toxic symptom, time of onset, recovery period) and mortality were monitored at 30 minutes after the procedure, 1-hour, 2-, 3- and 6-hours post procedure. Between Day 1 and up to Day 14 after the procedure, the animals were monitored once a day for mortality.

Pharmacopuncture preparation

DF powder was obtained from dried Yangjinhua (Guangxi Province, China). First, 5 g of the prepared DF powder was alkalized by adding 100 mL of 25% ammonium hydroxide (NH₄OH-Daejungchem-EP, Korea) and stirring for 1 hour. The solids were recovered and an additional 5 g was added and stirred for 1 hour.

The extraction procedure was performed as described by Paula Sramka [8]. The solids were dried in a fume hood (ADC-3A1, ESCO, Singapore) for 1 hour to remove residual ammonia water. The dried solid was categorized as Sample-1, filtered with 25% ammonium hydroxide, and the remaining filtered solid was categorized as Sample-2. To each sample, 500 mL of ethyl acetate (Daejungchem-EP, Korea) was added, and the active ingredient was extracted by ultrasonic stimulation in an ultrasound bath, for 15 minutes. After extraction, Sample-1 was subjected to membrane filtration and concentrated under reduced pressure, at 40°C, on a rotary evaporator (Buchi, Switzerland). The ethyl acetate layer separated from the liquid-liquid separation was subjected to membrane filtration, and concentrated under reduced pressure, using a rotary evaporator (Buchi, Switzerland). Both concentrated Sample-1 and Sample-2 were dissolved in 15 mL of tertiary distilled water. Samples were acidified to pH 3 using sulfuric acid with a 0.2 µm syringe filter (Sartorius, Germany). Next, the aqueous layer was obtained from twice liquid-liquid separation with hexane (40 mL) and diethylether (40 mL) respectively. The aqueous layer was pH adjusted to pH 0 using 25% NH₄OH. The organic phase was obtained from twice liquid-liquid separation with CHCl₃ (20 mL). Each sample was concentrated under reduced pressure, on a rotary evaporator (Buchi, Switzerland), at 40°C and lyophilized (FD8508, Ilijin Biotech, Korea) to obtain 3.3 mg of Sample-1 and 7.2 mg of Sample-2. Electrolytes were adjusted by adding 100 mL of injection water, and 0.9 g of NaCl (KP) to 10.5 mg of the DF extract, and adjusted to pH 7.4 (OnoLab, pH 720 WTW, Germany) using 0.5 M NaOH. After the prepared solution was sterilized with a 0.2 µm bottle top filter (Corning, USA), the solution was transferred to a sterilized vial with a 0.2 µm syringe filter (Sartorius, Germany).

Classification of the study groups

The rats were randomly assigned into 4 groups; the low dose DF [DF-L; 75 mg/kg, 1/8 capacity of lethal dose (LD) 50] group administered with low dosage pharmacopuncture, the high dose DF (DF-H; 150 mg/kg, 1/4 capacity of LD50) group, the untreated control group which was administered with saline, and the positive control group administered with 0.5% Lidocaine. All groups had access to sterile distilled water and feed during the experimental period.

Pharmacopuncture injection

SD rats were injected with a DF-L or DF-H into the right leg at Kl3 using a 1 mL syringe at a volume of 0.2 mL and 0.4 mL, respectively. The untreated control group was administered 0.2 mL of physiological saline, whilst 0.2 mL of 0.5% lidocaine was injected into the positive control group.

Pain response to thermal stimulation

The hot plate test was used to determine the analgesic effect during thermal stimulation, using a modification of Zhang's method [9]. The RB-200 intelligent hot plate apparatus was used. The temperature of the hot plate was set to 55 ± 0.5°C. The reaction time was measured from the time that the animal was placed on the hot plate surface, to licking of feet, or jumping behavior was observed, to avoid heat-induced pain. The response test was recorded at 10-, 25-, 40- and 60-minutes post-treatment. In order to prevent damage to skin tissues in animals with heat-tolerance, the cut-off time on the plate was set at 60 seconds (Fig. 1).

Pain response to chemical stimulation

The analgesic effect of chemical stimulation was tested using the rat formalin test [10]. Ten minutes post treatment, animals were placed in an acrylic cage, and 50 µl of 0.5% formaldehyde (diluted in physiological saline) was injected into the subcutaneous tissue of the left rear footpad. Immediately after the formalin injection, behavioral observations were noted, and observations continued for 60 minutes. Flinching, licking, and biting the injected feet behavioral responses were recorded at 10, 20, 30, 40 and 60 minutes after the treatment (Fig. 1).

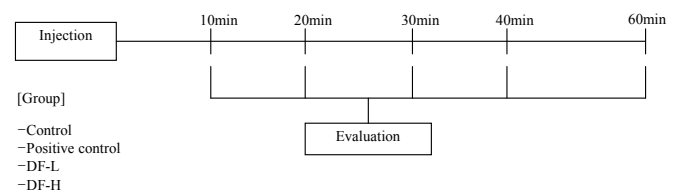


Fig. 1. Scheme showing experimental design.

Control : Sprague-Dawley rat group injected with normal saline 0.2 ml.

Positive control : Sprague-Dawley rat group injected with 0.5% lidocaine 0.2 ml.

DF-L : Sprague-Dawley rat group injected with low dose (0.2 ml, 75 mg/kg) of *Datura Flos* pharmacopuncture.

DF-H : Sprague-Dawley rat group injected with high dose (0.4 ml, 150 mg/kg) of *Datura Flos* pharmacopuncture.

Pain response test for physical stimulation

The Seltzer method was used to assess physical stimulation. The rats were placed in a plastic cage with a wire mesh floor at 10, 20, 30, 40, and 60 minutes post treatment. The response values were calculated by using the von Frey filaments. The stimulation was applied to the skin at the center of the hind paw for about 5 seconds, and a quick paw flick was measured. The range of stimulation was applied stepwise from 10 g to 100 g. The paw withdrawal mechanical threshold was determined by recording the minimum bending force that causes active foot avoidance (at least 3 times), when stimulated with each von Frey filaments (5 times). The maximum usable bending force was set at 100 g of the von Frey filament. The avoidance response was considered to be positive when lifting, licking, shuffling of soles or running away during stimulation, were observed (Fig. 1).

Pain response test for acupuncture stimulation

Experimental animals were placed in a plastic cage with a wire netting at 10, 20, 30, 40, and 60 minutes post injection. The rapid avoidance of feet stimulation was measured when the acupuncture (stress free needle from Dongbang medical, 0.25 mm × 40 mm) was vertically applied to the skin, at the center of the hind paw. In each group, the number of attempts to avoid foot stimulation was measured when the subjects were tested 5 times.

Hematological examination

The experimental animals were injected with 0.2 mL, and 0.4 mL of DFP (DF-L and DF-H) respectively, and the hematological changes were observed. The next day, animals were anesthetized with CO₂, and a sample of the blood obtained from the abdominal vein was treated with EDTA, an anticoagulant. The total leukocyte count, total erythrocyte count, hemoglobin, hematocrit, mean cell volume, hemoglobin, and hemoglobin concentration, and platelet count were measured with an automatic hemocytometer (Hemavet, Drew Scientific Co, Oxford, CT, USA).

Blood biochemistry test

Whole blood was collected and centrifuged (3,000 rpm, Eppendorf, Hamburg, Germany) for 15 minutes, and the collected serum analyzed using an automatic analyzer (Hitachi 7060, Hitachi, Tokyo, Japan). Total protein, albumin, total bilirubin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, creatinine, blood urea nitrogen, total cholesterol,

glucose, and triglycerides were measured.

Visual examination, weighing of major organs and histopathology examination

The SD rats were treated with DF-L and DF-H. The following day the animals were autopsied and the heart, liver, lung, kidney, brain, spleen, thymus, testis, ovary and gastrocnemius were weighed and observed for gross abnormalities. Fixed liver and renal tissues were sectioned and stained with hematoxylin-eosin to observe histopathological changes.

Statistical process

The results of the experiment were verified using the student's t-test for all measured data. DF-L and DF-H groups were compared with the control groups. The levels of significance for test groups and controls were $p < 0.05$ or $p < 0.01$. The DF-L and DF-H groups were compared and level of significance was set as $p < 0.05$. Statistical analysis was performed using SAS program (version 9.1.3, SAS Institute Inc., USA).

Results

Lethal Dose (LD50)

At concentrations of 100, 200, 300, and 400 mg/kg DF, the SD rats were 100% viable, but only 75% survived at 500 mg/kg, 50% survived at 600 mg/kg and all were deceased at 700 mg/kg. Hence, the LD50 of the SD rats was confirmed to be 600 mg/kg (Table 1).

The BALB/c mice were 100% viable at 100 mg/kg DF, however, only 75% survived at 200 and 300 mg/kg, 50% survived at 400 mg/kg and all were deceased at 600 mg/kg. The LD50 for BALB/c mice was 400 mg/kg (Table 1).

Pain response to thermal stimulation (hot plate)

In the positive control group, the reaction time was delayed to 15.6 ± 0.8 seconds after 10 minutes but gradually shortened with time. The DF-L group showed a significant improvement in their reaction time (8.3 ± 0.4 seconds) to thermal stimulation 10 minutes after treatment injection compared with the untreated control ($p < 0.01$). The DF-H group showed a significant difference in reaction time at 10 and 20 minutes (8.7 ± 0.5 seconds, and 6.8 ± 0.6 seconds, respectively), after the treatment injection compared with the untreated control ($p < 0.01$; Fig. 2).

Table 1. LD50 Test of *Datura Flos* Pharmacopuncture in Sprague-Dawley Rats and Balb/c Mice.

Animals	Dose (mg/kg)	100	200	300	400	500	600	700	800	1,000
Sprague-Dawley rats	Mortality	4/4	4/4	4/4	4/4	3/4	2/4	1/4	0/4	0/4
	%	100	100	100	100	75	50	25	0	0
Balb/c mice	Mortality	4/4	3/4	3/4	2/4	0/4	0/4	0/4	0/4	0/4
	%	100	75	75	50	0	0	0	0	0

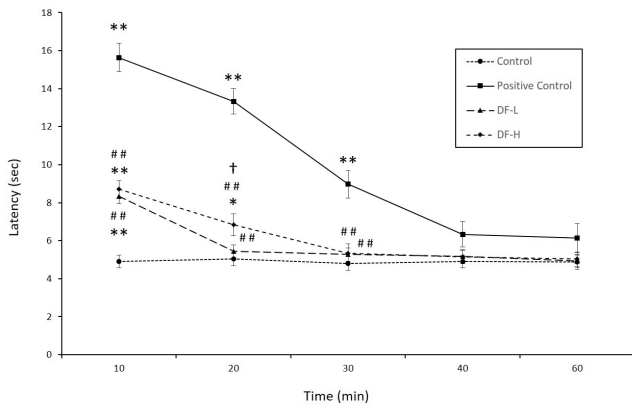


Fig. 2. Hot-plate test on different times after treatment.

Data are presented as mean \pm SE.

* $p < 0.05$, ** $p < 0.01$ compared with control group by student's t-test.

$p < 0.01$ compared with positive control group by student's t-test.

† $p < 0.05$ compared with DF-L group by student's t-test.

Control: Sprague-Dawley rat group injected with normal saline 0.2 ml.

Positive control: Sprague-Dawley rat group injected with 0.5% lidocaine 0.2 ml.

DF-L: Sprague-Dawley rat group injected with low dose (0.2 ml, 75 mg/kg) of *Datura Flos* pharmacopuncture.

DF-H: Sprague-Dawley rat group injected with high dose (0.4 ml, 150 mg/kg) of *Datura Flos* pharmacopuncture.

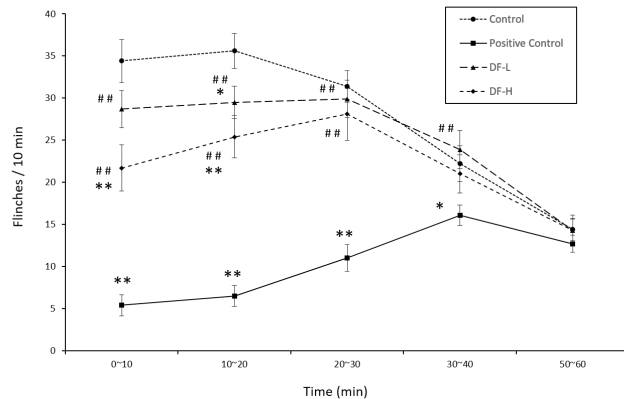


Fig. 3. The rat formalin test on different times after treatment.

Data are presented as mean \pm SE.

* $p < 0.05$, ** $p < 0.01$ compared with control group by student's t-test.

$p < 0.01$ compared with positive control group by student's t-test.

† $p < 0.05$ compared with DF-L group by student's t-test.

Control: Sprague-Dawley rat group injected with normal saline 0.2 ml.

Positive control: Sprague-Dawley rat group injected with 0.5% lidocaine 0.2 ml.

DF-L: Sprague-Dawley rat group injected with low dose (0.2 ml, 75 mg/kg) of *Datura Flos* pharmacopuncture.

DF-H: Sprague-Dawley rat group injected with high dose (0.4 ml, 150 mg/kg) of *Datura Flos* pharmacopuncture.

Pain response to chemical stimulation (formalin test)

The rat formalin test was performed and in the control group, the number of times flinching, licking, biting, or spontaneous shaking in the injected foot occurred, after the injection of formalin, was recorded 34.4 ± 2.6 times in the first 10 minutes, gradually decreasing over time. In the positive control group injected with lidocaine, the response was markedly decreased to 5.4 ± 1.3 times

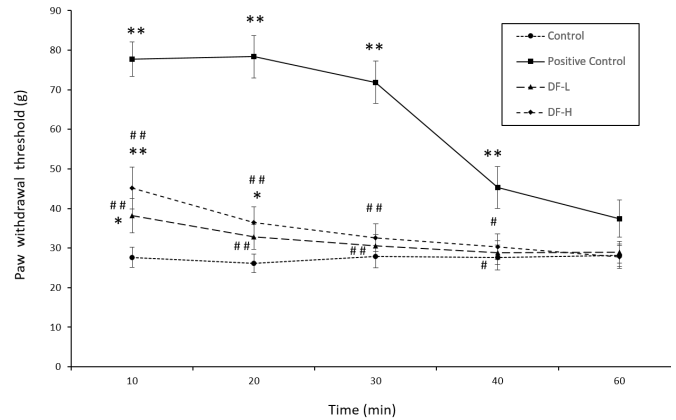


Fig. 4. Paw withdrawal threshold in response to von Frey filaments at several time points after treatment.

Data are presented as mean \pm SE.

* $p < 0.05$, ** $p < 0.01$ compared with control group by student's t-test.

$p < 0.05$, ## $p < 0.01$ compared with positive control group by student's t-test.

Control: Sprague-Dawley rat group injected with normal saline 0.2 ml.

Positive control: Sprague-Dawley rat group injected with 0.5% lidocaine 0.2 ml.

DF-L: Sprague-Dawley rat group injected with low dose (0.2 ml, 75 mg/kg) of *Datura Flos* pharmacopuncture.

DF-H: Sprague-Dawley rat group injected with high dose (0.4 ml, 150 mg/kg) of *Datura Flos* pharmacopuncture.

for the first 10 minutes after the formalin injection. However, the number of reactions gradually increased over time and decreased during the last 10 minutes. In both the DF-L and DF-H groups, the number of reactions was reduced compared with the untreated control group post-treatment injection, but only statistically significantly lower for the DF-H group at 0-10 minutes post-injection ($p < 0.01$).

At 10-20 minutes, there was a statistically significant reduction in the number of reactions for both DF-L ($p < 0.05$) and DF-H groups compared with the untreated control ($p < 0.01$; Fig. 3).

Pain response to physical stimulation (von Frey filaments test)

In the control group, the average paw withdrawal mechanical threshold of 27.6 ± 2.6 g was measured 10 minutes after injection. Thereafter, similar values were observed. On the other hand, the positive control group injected with lidocaine showed paw withdrawal mechanical threshold values of 77.7 ± 4.4 g after 10 minutes of injection. In both the DF-L (38.2 ± 4.3 g) and DF-H (45.2 ± 5.3 g) groups after the injection, the threshold value before the paw was withdrawn, increased compared with the untreated control group. In the DF-L group, there was a statistically significant difference at 10 minutes after injection ($p < 0.05$). The HDDFP showed a statistically significant difference at 10 minutes ($p < 0.01$) and 20 minutes after injection ($p < 0.05$). The DF-L and DF-H groups showed significant differences at 10, 20, 30 and 40 minutes after lidocaine administration, suggesting this was not as effective as lidocaine (Fig. 4).

Pain response test to acupuncture (mechanical) stimulation

In the untreated control group, 5 escape reactions were seen in all measured time points. On the other hand, lidocaine injected positive group showed 2.4 ± 0.3 escape reactions at 10 minutes after the procedure, showing a marked decrease. The escape reaction decreased to 2.1 ± 0.2 after 20 minutes, 3.0 ± 0.4 after 30

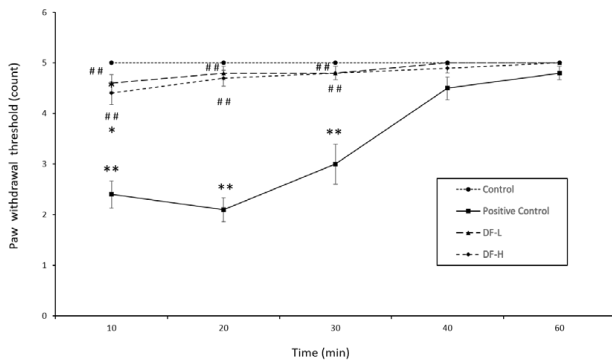


Fig. 5. Paw withdrawal threshold in response to acupuncture at several time points after treatment.

Data are presented as mean \pm SE.

* $p < 0.05$, ** $p < 0.01$ compared with control group by student's t-test.

$p < 0.01$ compared with positive control group by student's t-test.

† $p < 0.05$ compared with DF-L group by student's t-test.

Control: Sprague-Dawley rat group injected with normal saline 0.2 ml.

Positive control: Sprague-Dawley rat group injected with 0.5% lidocaine 0.2 ml.

DF-L: Sprague-Dawley rat group injected with low dose (0.2 ml, 75 mg/kg) of *Datura Flos* pharmacopuncture.

DF-H: Sprague-Dawley rat group injected with high dose (0.4 ml, 150 mg/kg) of *Datura Flos* pharmacopuncture.

Table 2. Hematological Values of Rats in the Single Toxicity Study of *Datura Flos* Pharmacopuncture.

Index	Control	DF-L	DF-H
WBC (103/ μ l)	3.20 \pm 0.16	3.17 \pm 0.14	3.348 \pm 0.13
RBC (106/ μ l)	8.28 \pm 0.19	8.42 \pm 0.21	8.33 \pm 0.26
Hemoglobin(g/dL)	10.65 \pm 0.37	10.56 \pm 0.46	11.26 \pm 0.25
HCT (%)	32.59 \pm 0.71	31.59 \pm 0.75	31.72 \pm 0.69
Platelet (103/ μ l)	939.47 \pm 19.88	947.53 \pm 14.13	931.37 \pm 26.48
MCV (fl)	43.73 \pm 1.25	41.38 \pm 2.07	42.75 \pm 2.27
MCH (pg)	13.17 \pm 0.43	12.90 \pm 0.55	12.96 \pm 0.42
MCHC (g/dL)	32.77 \pm 1.19	33.27 \pm 0.98	31.09 \pm 0.90

Data are presented as mean \pm SE.

Control: Sprague-Dawley rat group injected with normal saline.

DF-L: Sprague-Dawley rat group injected with low dose (0.2ml, 75mg/kg) of *Datura Flos* pharmacopuncture.

DF-H: Sprague-Dawley rat group injected with high dose (0.4ml, 150mg/kg) of *Datura Flos* pharmacopuncture.

WBC: Total leucocyte count.

RBC: Total erythrocyte count.

HCT, hematocrit; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration.

minutes, but increased after 40 minutes. On the other hand, in the LDDF and DF-H groups the threshold value for paw withdrawal reduced at 10 minutes after the injection but only in the DF-H group, and only at that time point ($p < 0.05$). In addition, DF-L and DF-H groups showed significant differences at 10, 20 and 30 minutes after lidocaine administration, indicating that it was not as effective as treatment with lidocaine (Fig. 5).

Table 3. Serum Biochemical Values of Rats in the Single Toxicity Study of *Datura Flos* Pharmacopuncture.

Index	Control	DF-L	DF-H
Total protein (g/dL)	4.93 \pm 0.15	5.02 \pm 0.13	5.06 \pm 0.17
Albumin (g/dL)	1.47 \pm 0.06	1.50 \pm 0.07	1.49 \pm 0.08
Total Bilirubin (mg/dL)	0.15 \pm 0.01	0.12 \pm 0.01	0.13 \pm 0.01
ALP (IU/L)	40.63 \pm 1.35	41.70 \pm 0.84	41.42 \pm 0.86
AST (IU/L)	48.64 \pm 1.37	51.04 \pm 1.50	50.15 \pm 1.77
ALT (IU/L)	20.82 \pm 0.52	19.94 \pm 0.60	19.45 \pm 0.60
Creatinine (mg/dL)	0.52 \pm 0.03	0.49 \pm 0.02	0.51 \pm 0.02
BUN (mg/dL)	17.54 \pm 0.65	17.63 \pm 0.79	18.76 \pm 0.84
Cholesterol (mg/dL)	150.99 \pm 6.19	152.25 \pm 7.16	154.87 \pm 6.67
Triglyceride (mg/dL)	67.06 \pm 2.10	69.45 \pm 3.89	70.82 \pm 3.71
Glucose (mg/L)	167.59 \pm 8.17	164.29 \pm 6.82	165.23 \pm 7.45

Data are presented as mean \pm SE.

Control: Sprague-Dawley rat group injected with normal saline 0.2 ml.

DF-L: Sprague-Dawley rat group injected with low dose (0.2ml, 75mg/kg) of *Datura Flos* pharmacopuncture.

DF-H: Sprague-Dawley rat group injected with high dose (0.4ml, 150mg/kg) of *Datura Flos* pharmacopuncture.

ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; TG, triglyceride.

Hematological examination

The animals were anesthetized with ether, and blood was collected from the abdominal aorta. Hematologic markers (total leukocyte count, total erythrocyte count, hemoglobin, hematocrit, platelet, mean cell volume, mean cell hemoglobin, and mean cell hemoglobin concentration) were tested with an automatic hematology analyzer. The hematological indices showed no statistically significant differences between control and experimental groups (Table 2).

Blood biochemistry test

The day after injecting DFP, total protein, albumin, total bilirubin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, creatinine, blood urea nitrogen, total cholesterol, triglycerides and glucose were tested. There were no statistically significant differences in the blood biochemical parameters between the experimental groups and the control groups (Table 3).

Weight change of major organs

The day after the DFP injection, the organs of each of the experimental group were isolated and weighed. No gross pathological findings were observed in the organs of the experimental groups treated with DF-L and DF-H, and the untreated control (Table 4).

Anatomical changes of liver and kidney

After the liver extraction, hepatic tissues were observed follow-

Table 4. Relative Organ Weight Values of Rats in the Single Toxicity Study of *Datura Flos* Pharmacopuncture.

Organ	Control	DF-L	DF-H
Heart	0.14 ± 0.04	0.13 ± 0.02	0.14 ± 0.03
Liver	1.33 ± 0.18	1.30 ± 0.16	1.31 ± 0.20
Kidney(R)	0.25 ± 0.03	0.24 ± 0.04	0.23 ± 0.04
Kidney(L)	0.23 ± 0.03	0.25 ± 0.04	0.24 ± 0.03
Brain	0.43 ± 0.05	0.43 ± 0.05	0.44 ± 0.07
Lung	0.24 ± 0.04	0.25 ± 0.04	0.26 ± 0.06
Spleen	0.13 ± 0.03	0.13 ± 0.04	0.14 ± 0.04
Thymus	0.09 ± 0.02	0.10 ± 0.03	0.10 ± 0.03
Testis	0.08 ± 0.02	0.08 ± 0.02	0.08 ± 0.02
Gastrocnemius m.	0.10 ± 0.03	0.10 ± 0.03	0.11 ± 0.04

Data are presented as mean ± SE.

Control: Sprague-Dawley rat group injected with normal saline 0.2 ml.

DF-L: Sprague-Dawley rat group injected with low dose (0.2ml, 75mg/kg) of *Datura Flos* pharmacopuncture

DF-H: Sprague-Dawley rat group injected with high dose (0.4ml, 150mg/kg) of *Datura Flos* pharmacopuncture.

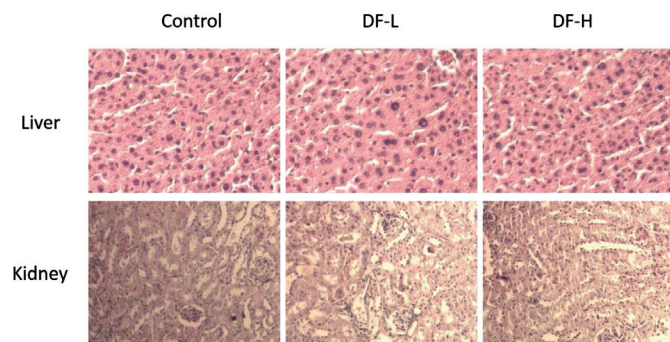


Fig. 6. Hematoxylin & Eosin stained liver and kidney tissues from rats injected once with *Datura Flos* pharmacopuncture.

Magnification, x100.

Control: Sprague-Dawley rat group injected with normal saline 0.2 ml.

DF-L: Sprague-Dawley rat group injected with low dose (0.2 ml, 75 mg/kg) of *Datura Flos* pharmacopuncture.

DF-H: Sprague-Dawley rat group injected with high dose (0.4 ml, 150 mg/kg) of *Datura Flos* pharmacopuncture.

ing hematoxylin and eosin staining. The hepatic veins and parenchyma of the untreated control group maintained their normal structure. In the DF-L group and DF-H group, no hepatic cell necrosis, inflammatory cell infiltration, or fatty degeneration was found, and in the kidney tissue, dense cytoplasm, normal glomerulus, convoluted tubule or epithelial cells, and no tubular expanding or epidermal detachment was observed. There were no significant differences between treated and untreated control groups (Fig. 6).

Discussion

In order to establish the safety and efficacy (analgesic) of DFP, several tests were performed including, hot plate, formalin,

von Frey test, acupuncture stimulation, hematology, blood biochemistry, weight and anatomical change of organs were conducted. LD50, was used as the primary toxicity indicator for the study [11]. The LD50 of the SD rats and BALB/c mice was 600 mg/kg and 400 mg/kg, respectively. Based on these results, a dose of 150 mg/kg, which is 1/4 of LD50, was used as the HDDFP and 75 mg/kg, which is 1/8 of LD50 was set as the LDDFP.

The hot plate test is similar to the tail flick test and is associated with the upper spinal pathway [12]. The von Frey test, developed by Maximilian von Frey, and is a mechanical nociceptive threshold test for the analgesic effect on physical harmless pain [13]. The von Frey test is where physical stimulation is applied to uninhibited rats (which can prevent other stress factors from changing the outcome). The pain response test as observed by acupuncture stimulation was measured by counting the number of times the foot avoidance reaction occurred when the skin at the center of the rats' foot was tested with a 0.25 × 40 mm acupuncture needle, 5 times.

In the hot plate test and the von Frey test, the DF-L and DF-H groups showed an increase in threshold value compared with the untreated control group after the acupuncture injection. In both the DF-L and the DF-H groups, 10 minutes after treatment, the number of escape reactions decreased significantly. However, only the DF-H group for both the hot plate test and the von Frey test showed a significant improvement, compared with the untreated control for 20 minutes after the treatment. The mechanical pain test stimulated by acupuncture showed significant results after 10 minutes for HDDFP. The analgesic effect of pharmacopuncture was better at the higher concentration, but a long-term analgesic effect was not seen.

Formalin testing is a method used to observe the behavior of rats following chemically induced pain. It can be divided into the early phase and late phase. Non-steroidal anti-inflammatory drugs do not work in the first phase (3-5 minutes after the injection of formalin) of formalin testing, but are effective in the late phase (15-20 minutes after injection of formalin). In the early phase, transient and intense pain is experienced when C-fibers are activated, and substance P and bradykinin are released through stimulating the peripheral nerves caused by direct chemical stimulation. Thereafter, the pain-induced behavior is reduced, and late phase occurs after about 15-20 minutes of formalin infusion, followed by persistent and intense pain. The late phase is maintained for about 20-40 minutes. It has been shown that functional changes occurring in the central nervous system affect the peripheral nervous system. This is due to the combined action of the inflammatory reaction (substances such as histamine, serotonin, and prostaglandin) induced by the formalin in the peripheral tissues, and functional changes in the spinal cord [14-17]. The formalin test shows the mechanism of 2 different pains. In the present study, both HDDFP and LDDFP showed significant analgesic effect between 10-20 minutes after treatment, and HDDFP also showed significant analgesic effects between 0-10 minutes after formalin treatment. In the case of HDDFP, both early and late phase showed significant results. However, LDDFP showed significant decrease only in late phase. Therefore, though the DFP treatment was more effective for a central analgesic effect, if used in a sufficient dose, it may have efficacy during inflammatory pain. A previous study reported a significant effect in both the early and late phase of formalin testing using *Datura stramonium* seed [7]. Therefore, it is considered that the use of a sufficient dosage may be effective not only for chronic pain but also for the treatment of acute inflammatory pain.

The positive control, lidocaine (5 mg/mL) showed a significant effect for 30-40 minutes after the injection in all behavioral tests such as thermal, physical and chemical stimulation. On the other

hand, the DFP showed a short-term analgesic effect of 10-20 minutes in general, which is a shorter effect compared with that of lidocaine.

DF contains about 60 kinds of alkaloids in the roots, stems, leaves, flowers and seeds [18,19]. The major alkaloids are atropine and scopolamine, which are anticholinergic drugs. They act as an antagonist to the muscarine cholinergic receptor, and inhibit the parasympathetic nerves such as vagus nerve. It is used as central nervous system sedatives [20]. However, higher doses cause side effects such as mouth dryness, pharyngeal disturbance, paralysis of the oculomotor nerve, delayed response to the light and delayed or lost corneal reflex. When heart nerve paralysis occurs, the pulse is accelerated, the smooth muscles of the bronchi and the stomach are relaxed, and the skin blood vessels expand so that the skin appears red. It can also affect breathing and body temperature control, and can lead to death if serious [21-23]. Scopolamine is also used as a central nervous system anesthetic as it is toxic to the central nervous system.

Studies on the analgesic efficacy of atropine and scopolamine have been performed in various ways, but the precise mechanism is unclear [7, 24]. Historically, atropine was used for relieving pain in ancient Rome. Additionally, the *Historia Naturalis*, the world's first encyclopedia, recorded the use of *Mandragora officinarum* or *Hyoscyamus niger* juice which contains atropine for preoperative analgesic effects. Ghelardini et al [25] reported that low doses of atropine increased cholinergic function in rodents, and caused analgesic effects without side effects. In a previous study, scopolamine had no effect on the stimulation of non-opioid stress analgesia, but showed an effective difference in the endogenous opioid pain inhibitory system. As a result of this study, it is possible to demonstrate the existence of a synapse that mediates opioid-induced stress in the central nervous system pathway, and it can be demonstrated that the action of scopolamine has an analgesic effect. The analgesic effect of scopolamine is different from analgesia of opioid drugs such as morphine. According to the study by Pert in 1975 [26], morphine analgesia weakens the affective-motivational response, whereas scopolamine analgesia is caused by attention defects such as an increased threshold for pain, and slow response. Several studies have shown that atropine and scopolamine are analgesic, but the mechanism has not been determined.

DFPs showed significant analgesic effect in this current study. However, further studies are needed to determine the mechanism of pain suppression of atropine and scopolamine, which are the major components of pharmacopuncture. In this study, it was concluded that the medicinal herbs used had low and short-term analgesic effects when compared with lidocaine. Therefore, it is necessary to study the method of extracting pharmacopuncture drugs which have a stronger analgesic effect. This study was performed to observe the analgesic action of DFP and safety in SD rats. Therefore, further studies are essential to evaluate clinical applications for DFP.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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