

PREPARATION AND ANALGESIC ACTIVITY TEST OF CAPSICUM FRUTESCENS OLEORESIN[#]

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Abstract. Capsaicin is an alkaloid in capsaicinoids group known as a spicy flavour ingredient in chilies and some pungent foods. Recently, capsaicin has been used not only as an additive in food but also as a medicine like analgesic, anticancer, and antidiabetic drug due to its' pharmacological properties. In this study, capsicum oleoresin was extracted via heating chili powder, *Capsicum frutescens* (L.), with 80 % ethanol at 70 °C in 120 minutes, then the extract was solvent evaporated in low pressure. Capsaicin was determined by thin-layer chromatography (TLC) and qualified by high-performance liquid chromatography (HPLC) method. Finally, the analgesic activity of capsicum oleoresin was tested on mice. The results show that capsicum oleoresin extracted from 2 kilograms of capsicum powder was 368.84 grams with 2.84 % capsaicin. The central analgesic and peripheral analgesic effects of capsicum oleoresin were 90 minutes and 10 - 15 minutes after treatment, respectively.

Keywords: *Capsicum frutescens* (L.), capsaicin, capsaicinoids, extraction, chilies, analgesic activity.

Classification numbers: 1.2.1, 1.3.1.

1. INTRODUCTION

Capsaicin, an alkaloid belongs to capsaicinoids group, is derived from chili peppers of the genus *Capsicum*, which includes the Tabasco Pepper (*Capsicum frutescens* (L.)) commonly used as food additives [1]. It is presented mostly in the placental tissue (which holds the seeds) of Tabasco Pepper with the total content of around 0.05 - 2 % [2]. This compound was first studied

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by scientists in the early 19th century, but its pharmacological effects such as antioxidants, anticancer, promotion of energy metabolism, pain relief, suppression of fat accumulation, and anti-inflammation have not until recent been discovered [3, 4]. Among the effects, temporary analgesic activity is one of the most well-known effects of capsaicin. Capsaicin with concentration ranged from 0.025 % to 0.1 % has the potential to significantly relieve pains, which is utilized as topical ointments, sprays or cream to temporarily reduce the skeletal muscle and joints/bones pains. It achieves the pain-relieving effect by two ways: (1) reversibly depleting sensory nerve endings of substance P, and (2) reversibly reducing the density of epidermal nerve fibers [5]. Although capsaicin can be produced by alternative methods such as chemical synthesis, enzymatic synthesis, and cell or tissue culture, the utilize of capsaicin derived from chili pepper fruits has remained its mainstay role thanks to their commonly traditional applications in Eastern as well as the tendency to use natural compounds in Western.

In Viet Nam, chili peppers are widely cultivated in all regions of the country. The area and production of chili pepper have been constantly increasing from 56,339 ha and 83,903 tons in 2008 to 65,925 ha and 95,844 tons in 2017 [6]. However, chili peppers have been consumed or exported mostly as food materials for years, meanwhile applications in pharmaceuticals are still in limitation. Accompanied with the copious supplies of chilies, the research studies on modulating chilies extraction containing capsaicin and evaluating its biological activities in order to apply chilies extracts to medicinal products is requisite. That could not only add commercial value to chili peppers, but also contribute producing highly effectives and cost-effective medicaments in Viet Nam.

In this study, chili extracts were prepared by stirring and heating the chili powder in 80 % ethanol (food-grade), following by rotatory evaporation to remove the solvent. The content of capsaicin was then determined by HPLC (high pressure liquid chromatography). The pain relieve activity of chili extract was *in vivo* tested on mice.

2. MATERIALS AND METHODS

2.1. Materials and equipment

Fresh chilies (*Capsicum frutescens* (L.)) was originated during February 2020 in Dong Thap, Viet Nam. Absolute ethanol and food-grade ethanol were purchased from Chemsol (Viet Nam). Acetic acid (glacial) was supplied from Guangdong Guanghua (China). Normal phase silica thin layer for thin layer chromatography was purchased from Merck (USA). Acetonitrile (HPLC grade) and phosphoric acid (98 %) was purchased from Fisher (USA). Capsaicin reference standard (capsaicin 100 mg, 98 %) was purchased form Sigma-Aldrich (USA). Efferalgan Codein[®] (Paracetamol 500 mg, Codein 30 mg) was purchased from UPSA (France). Voltaren Emulgel[®] (Diclofenac diethylamine 1 %) was purchased from Novartis (Switzerland). All other agents were of analytical grade and used directly without further processing.

Adult Swiss albino mice of both sexes in healthy condition and no observable deformities, weighed from 18 - 25 g were supplied by Institute of Vaccines and Biological Medical – IVAC, from Nha Trang, Viet Nam.

The product's samples were evaluated on its capsaicin content by HPLC Perkin Elmer, L11 reversed-phase silica column, Supelco.

2.2. Extraction processes

Chilies were harvested at red-ripe appearances, with pedicle removed, followed by cleaned with water and air dried in airy, cool condition. After all water content was removed, the chilies were cut to 1 - 2 mm pieces, cold dried at 50 - 55 °C in 24 hours and grinded to powder.

Capsicum extraction was prepared on small scale with 10 g chili powder in various concentrations of ethanol. The optimal ethanol's concentration is 80 % and the ratio of chili powder and ethanol is 1:7 (w/v). Following that step, the process of extraction was scaled up to 2 kg chili powder. First, the powder was placed into extraction flask and moisturized by solvent. Then 14 liters of food-grade ethanol were added and stirred at a constant speed, temperature of 70 °C in 120 minutes. After the extracted solution was filtered, the residue was once again gone through the extraction process in the similar condition. The extracted solution of the two processes were mixed, placed in rotary evaporator to remove solvent at 60 °C to obtain the final capsicum oleoresin (Figure 1).

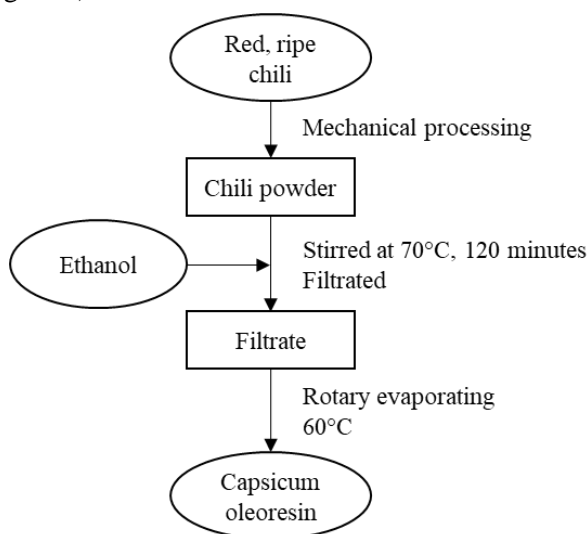


Figure 1. Extraction process of capsicum oleoresin.

2.3. Analytical methods

The capsicum oleoresin was qualified of its alkaloid properties by Bouchadat reagent and Dragendorff reagent.

Capsaicin in the extract was determined by normal phase thin layer chromatography. The capsaicin standard sample and capsicum extract were applied as dots to the silica plate, which was putted into a saturated chamber using the mixture of chloroform and methanol (95:5, v/v) as developing solvent system. Develop the chromatograms over a distance of 8 cm. Remove the plate from the chamber, dry, and derivatize with H₂SO₄ 10 %, and heat until bands develop.

The quantity of capsaicin was determined by HPLC based on the pharmacopeia USP 43. The provided condition for the chromatography run included: L11 stationary column, mixture of acetonitrile and phosphoric acid 0.1 % (4:6, v/v) as mobile phase, flow rate of 1 mL/minute, injecting volume of 20 µL, column temperature at 30 °C and the UV detector was set at 281 nm. Before injection, the standard capsaicin and capsicum oleoresin were dissolved in methanol and filtrated by 0.45 µm filter.

2.4. Evaluating analgesic activity of capsicum oleoresin

2.4.1. Tail-immersion testing model

The tail-flick test for determining loss of pain sensation following the method described by Ramabadran *et al.* [7]. Briefly, the lab mice were fixed in a falcon with tail hanged freely outside. Tail section was immersed 5 cm deep from the tip into heated water maintained at 55 ± 0.5 °C with magnetic hot plate stirrer. The tail-flick reaction time of mice was recorded and counted from the immersion to the reflexive flick of its tail clearly out of the heated water. Lab mice to be chosen were the ones with reaction time less than 5 seconds.

Low concentrations of capsaicin were included in over-the-counter analgesic creams. Capsaicin was robustly absorbed from the skin upon topical administration. Most trials were used with 0.025 % - 1 % of capsaicin gel applied for pain relief [8]. Thus, in our study, the obtained capsicum oleoresin was diluted in 70 % ethanol into a solution containing 0.05 % capsaicin. The selected lab mice were shaved at dorsal region with area $2 \text{ cm} \times 2 \text{ cm}$ one day prior to the experiments.

The experimental mice were divided randomly into the three following groups:

- Control group (n = 8): received no treatment.
- Positive control group (n = 8): received Efferalgan Codein effervescent in aqueous solution with Paracetamol/Codein dosage ratio of 123/7.38 mg/kg.
- Capsicum oleoresin group (n = 8): received topical treatment by applying the processed capsicum oleoresin on the shaved dorsal area twice, repeated twice and each time was 5 minutes separated from the other.

The reaction time of mice's tail flick were recorded before the drug treatment, and then at 30, 60, 90, 120 minutes after drug treatment. At each time interval, the reaction time was recorded twice and the longer value was used as the recorded data. Experimented mice's tails were wipe-dried by clean cotton, and if no tail flick was seen after 10 seconds, tail can be removed to avoid heat damage.

Recorded times of tail flick (tail-flick latency) out of heated water bath were compared between 3 groups, and the extended time of the latency in experimental groups when compared with that of control group will indicate that central analgesic effect has been achieved.

2.4.2. Acetic acid- induced writhing test

Chili pepper/capsaicinoid compounds can be used by various routes, in which the topical administration is well absorbed and there are also transdermal preparations containing low concentrations of capsaicin for pain relief and inflammation. Other researches just reported acetic acid-induced writhing test for oral administration, but commercial preparations of capsaicin were used on skin, so in this research, acetic acid-induced writhing test was applied for skin application [9]. Briefly, the experiment was processed as mice were shaved along the spinal area one day prior to the experiment, with $2 \text{ cm} \times 2 \text{ cm}$ areas. Capsicum oleoresin was diluted in 70 % ethanol to obtain solution of 0.075 % capsaicin.

The experimental mice were divided randomly into the following groups:

- Control group (n = 10): received no treatment.
- Positive control group (n = 9): received topical treatment by applying Voltaren cream on shaved dorsal area.

- Capsicum oleoresin group (n = 8): received a topical treatment by applying the processed capsicum oleoresin (0.075 % capsaicin) on the shaved dorsal area.

At time interval of 30 minutes after the topical treatment, all experimental mice were injected (peritoneal injection) with acetic acid 0.7 % solution at dosage 0.1 mL/10 g body weight. Each mouse was placed in separated bocal, and the writhing phenomena were recorded as numbers per 5 minutes, 10 minutes and 40 minutes after acetic acid injection. The number of abdominal writhing by mice of different experimenting groups were compared at the same time interval. The decreased amounts of cramping from treatment groups in comparison with blank control group will indicate for the successful peripheral analgesic effect caused by the tested substance.

3. RESULTS AND DISCUSSION

3.1. Capsicum oleoresin

The capsicum oleoresin extracted from 2 kg chili powder was triplicated to achieve stable data resulted with average oleoresin weight of 368.84 g, capsaicin percentage in capsicum oleoresin of 2.84 % and the average extracting efficiency reached 0.52 % (Table 1).

Table 1. The capsicum oleoresin weight and capsaicin percentage in 2kg-scale extraction (n=3).

Extraction number	Capsicum oleoresin weight (g)	Capsaicin percentage in oleoresin (%)	Capsaicin percentage in chili powder (%)
1	375.25	2.87	0.54
2	361.73	2.93	0.53
3	369.55	2.71	0.50
Average	368.84	2.84	0.52



Figure 2. Capsicum oleoresin.

3.2. Analytical results

Alkaloid properties qualification in the extraction samples given the positive result with Bouchadat reagent (formation of reddish-brown precipitate) and Dragendorff reagent (formation of orange-red precipitate).

Collected data from TLC analytical assay shown that the capsicum oleoresin created various stripes with R_f ranged from 0 to 1, in the which include the stripes like R_f of standard capsaicin ($R_f = 0.75$) (Figure 3).

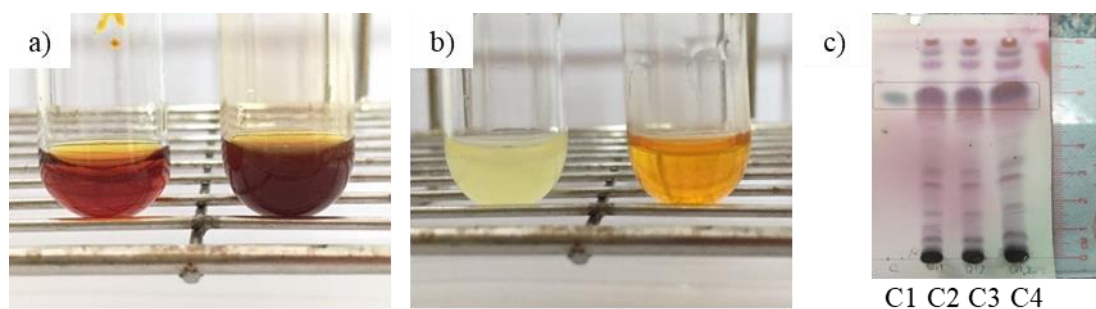


Figure 3. Alkaloid properties qualification of extract with a) Bouchadat reagent and b) Dragendorff reagent. The left test tube is blank, the right one is samples. c) Thin layer chromatography results of standard capsaicin (C1) and capsicum oleoresin (C2, C3, C4).

The resulted HPLC data indicated that the capsicum oleoresin extraction has the peak at the same retention time with standard capsaicin peak (Figure 4). With combined results from the qualitative assays conducted above, it can be proved that capsaicin was present in the extracted capsicum oleoresin. Capsaicin percentages in the oleoresin's samples can be determined by HPLC and the standard curve plotted by standard capsaicin. The linear regression equation given as: $y = 12140x - 22454$, $R^2 = 0.9997$.

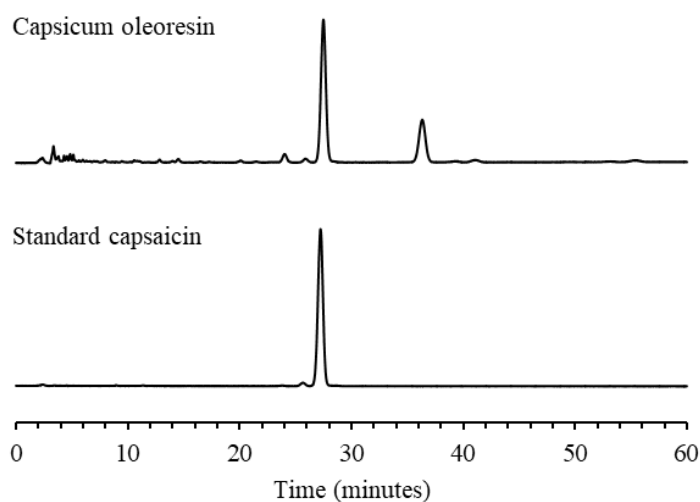


Figure 4. HPLC results of standard capsaicin and capsicum oleoresin.

3.3. Analgesic activity results

3.3.1. Central analgesic effect on mice's tail flick test

The resulted tail-flick latency of experimental mice in different batches are described in Table 2 and Figure 5. At initial time, latency of the tail-flick has no statistical significance. For the positive control group (paracetamol/codein), tail-flick latency increased and has statistical significance, compared to the blank control batches, from time interval 30 minutes to time interval 120 minutes ($p < 0.05$); this proved that the product has central analgesic effect with combination treatment using paracetamol/codein on mice tail-flick testing model.

Table 2. Tail-flick latency (seconds) of lab mice by time intervals.

Group	Before topical/oral treatment	After 30 minutes	After 60 minutes	After 90 minutes	After 120 minutes
Blank control	2.04 ± 0.25	2.43 ± 0.30	2.17 ± 0.26	2.31 ± 0.24	2.14 ± 0.11
Positive control	2.49 ± 0.30	4.19 ± 0.66*	5.33 ± 0.97*	7.33 ± 0.66**	9.04 ± 0.56**
Capsicum oleoresin	2.04 ± 0.11	2.37 ± 0.24	2.72 ± 0.21	3.94 ± 0.37**	7.88 ± 0.86**

(*) $p < 0.05$, (**) $p < 0.01$ when compared to blank control batch at the same time interval.

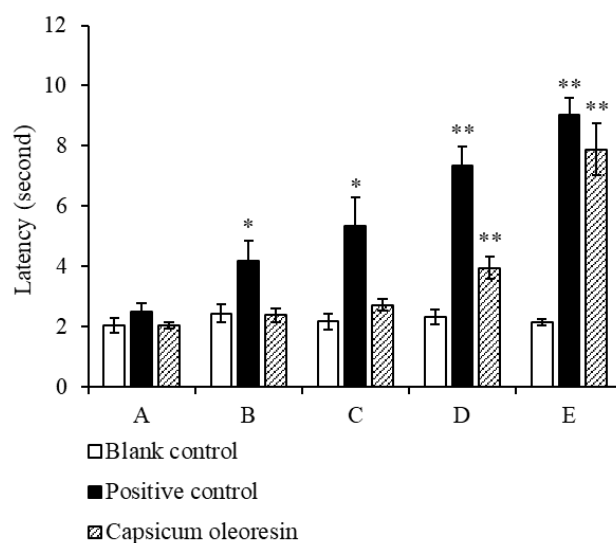


Figure 5. Graphical comparison of tail-lick latency (second) of experimental mice's group by time interval. A) Before topical/oral treatment, B-E) 30, 60, 90, 120 minutes after treatment, respectively. (*) $p < 0.05$, (**) $p < 0.01$ when compared to blank control batch at the same time interval.

For treatment group using capsicum oleoresin, tail-flick latency, although increased with increased time, but there were no differences between batches at 30 and 60 minutes. At time interval of 90 minutes, the groups's tail-flick latency with the oleoresin treatment increased significantly compared to the blank control group ($p < 0.01$). At time interval of 120 minutes, latent phase rapid tail movement also increased and has statistical significant ($p < 0.01$), which were close to the latent phases of the controlled group treated by drugs.

In summary, capsicum oleoresin has shown the ability to exert analgesic effect on tail-flick testing model with slow affecting time, at 90 minutes after the topical application. At time interval of 120 minutes, the analgesic effect was expressed clearer and close to that of the group treated with paracetamol/codein.

3.3.2. Peripheral analgesic effect on acetic acid-induced writhing in mice model

Table 3 describes the number of abdominal writhes after peritoneal injection of acetic acid in experiment mice's groups that were recorded in 5 minutes, with 10 minutes time separated between each time, and the total time recording of 40 minutes.

The control group treated with topical application by diclofenac cream has less number of abdominal writhes and possessed statistical significance compared to blank control batches in all 3 time intervals of examination ($p < 0.01$), which proved that the analgesic effect of diclofenac by induced muscle cramping on mice by acetic acid model has been achieved. Similarly, capsicum oleoresin also reduced the number of writhes on experimented mice in all 3 time interval and have statistical significance compared to blank control group with p-value < 0.01 . Besides, the number of cramps in oleoresin treatment group had no difference with drug-treated control group in all time intervals (Figure 6).

Therefore, the peripheral analgesic effect of capsicum oleoresin were expressed early, in about 5 - 10 minutes up to 30 - 45 minutes after topical treatment.

The results also indicated that acetic acid-induced writhing test can be applied for skin administration.

Table 3. Number of average abdominal writhes in experimental mice by time interval.

Group	5-10 minutes	20-25 minutes	30-45 minutes
Blank control	19 ± 1.04	10.2 ± 0.95	6.5 ± 0.79
Positive control	6.56 ± 2.22**	3.56 ± 1.07**	1.33 ± 0.55**
Capsicum oleoresin	4.00 ± 1.54**	2.88 ± 0.93**	1.63 ± 0.56**

(*) $p < 0.05$, (**) $p < 0.01$ when compared to blank control batch at the same time interval.

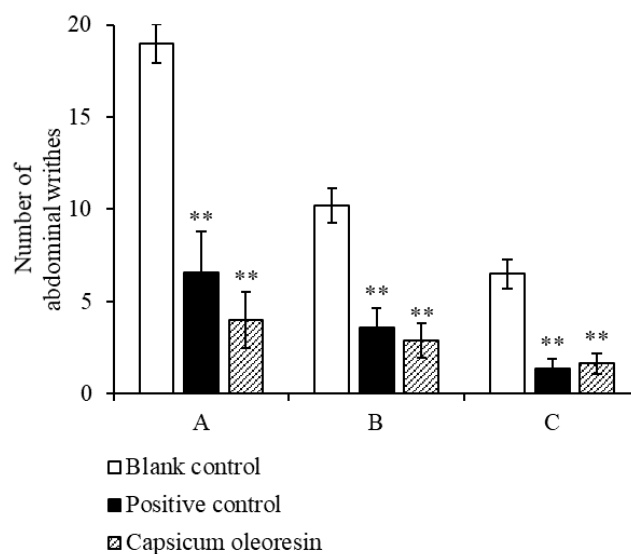


Figure 6. Graphical comparison of number of average abdominal writhes in experimental mice by time interval. A) 5-10 minutes, B) 20-25 minutes and C) 30-45 minutes after treatment. (*) $p < 0.05$, (**) $p < 0.01$ when compared to blank control batch at the same time interval.

4. CONCLUSIONS

In this study, capsicum oleoresin was extracted from chili powder by heat extraction in ethanol solvent and has been proved to present in the product at the content of 2.84 %, confirmed by HPLC. The results of analgesic effect test on mice's tail-immersion testing model and acetic acid-induced writhing model showed that capsicum oleoresin exerted its ability to establish central analgesic effect in slow fashion, at 90 minutes after topical application; and peripheral analgesic effect in faster fashion, at 10 - 15 minutes after topical treatment and lasted as long as 30 - 45 minutes. The collected results indicated that capsicum oleoresin extracted from local chili possesses substantial potential to be applied as analgesic preparation, which not only utilizes local chili sources but also increases the value of domestic chili.

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Declaration of competing interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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