

Research Article

Anti-inflammatory activity of *Zingiber montanum* (J.König) Link ex Dietr. extracts prepared by deep frying in coconut oil

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Phlai; *Zingiber montanum*; Rat ear edema; Anti-inflammatory activity; Coconut oil

ABSTRACT

The *Zingiber montanum* (J.König) Link ex Dietr. (Phlai) rhizome has been used for the treatment of muscular and joint pain. An essential oil from this has been developed into various dosage forms, but there are no studies on the traditional Phlai hot oil extract, which is widely used among Thai traditional practitioners. Commonly a high temperature has been suggested for extraction but some traditional doctors claimed the efficacy of the low temperature extract (LTE, 70-90°C). The objectives of this study were to determine the effect of temperature on the anti-inflammatory activity and investigate the minimum concentration of the most potent extract. The anti-inflammatory activity was determined using the ear-edema model. Phlai oil extracts were prepared traditionally by deep frying the sliced rhizome in hot vegetable oil using three different temperatures. The density and viscosity of all the extracts were recorded. The extract using a high temperature (HTE, 240-260°C) showed the significantly highest anti-inflammation activity ($P < 0.05$). The minimum concentration that presented a reduction in ear edema was 25% of HTE in coconut oil ($P < 0.05$). A thin layer chromatogram of the extracts demonstrated that some compounds in the volatile oil remained in the extracts that were exposed to a low temperature, but those were not found in the medium temperature extract (MTE, 170-190°C) and HTE. All extracts contained (E)-4-(3',4'-dimethoxyphenyl)but-3-en-2-ol (compound-D). Other phenylbutanoids and cassumunoids in the HTE may be responsible for anti-inflammation and should be revealed, while quality control should be studied further.

1. INTRODUCTION

The medicinal plant, *Zingiber montanum* (J.König) Link ex Dietr. called Phlai^{1,2} is commonly used externally for the treatment of muscular and joint pain³. In Thai traditional medicine Phlai extracts are prepared by deep-frying in coconut oil. It has been recorded in the list of herbs recommended for primary health care and the national list of herbal medicinal products since 2011⁴. The rhizome of Phlai contains essential oils, phenylbutanoids and cassumunoids which were reported

to be responsible for anti-inflammatory activity^{5,6}. Many reports of the anti-inflammatory activity of the extracts of the rhizome of Phlai were revealed including the essential oil, which has been developed into various dosage forms⁷. Although traditional Phlai hot oil extracts have been widely used among Thai traditional practitioners and a lot of products are available on the market, no study on its effect has been published. Commonly, a high temperature has been suggested for this hot oil extraction, but some traditional doctors simmered the rhizome in oil with a water bath or using a low temperature to extract instead. Theoretically, the volatile oil, which may play a role in anti-inflammation is vaporized during extraction if using a high temperature, so it is interesting that the use of low temperature may sustain the volatile oil in the extract and providing more efficacy than the high temperature exposing extraction. Furthermore, different concentrations of the hot oil extract in the dosage forms available on the market varies from 5 to 90%, but no study revealed the effective concentration. According to traditional Thai textbooks⁸, coconut oil, which has long been used as food and in traditional medicine, was selected to use in the extraction⁹. The aims of this study were to investigate the effect of temperature on anti-inflammatory activity and examine the minimum effective concentration of the most potent extract. The TLC pattern and the physical properties of the extract would be revealed.

2. MATERIALS AND METHODS

2.1. Materials

The rhizome of *Zingiber montanum* (J.König) Link ex Dietr., Zingiberaceae was purchased from Chao Krom Po herbal drug store, Chakkrawat Road, Samphanthawong, Bangkok. Coconut milk (*Cocos nucifera* L.) was purchased from Thewet fresh-food market, Samsen Road, Dusit, Bangkok. Ethyl phenylpropionate (EPP) and indomethacin were purchase from Sigma-Aldich Co., Germany. Phlai essential oil and Compound D were received from Dr. Natthinee Anantachoke.

2.2. Materials

Density of all extracts was carried out using Hake™ RotoVisco™ (USA), TLC chromatogram were developed by Camag Linomat 5 (Switzerland).

2.3. Coconut oil preparation

Coconut oil was prepared by heating 5 kg of coconut milk in the pot and heated at temperatures between 80-100°C until the water was evaporated. The coconut oil was filtered and the filtrate was kept in a closed container.

2.4. Preparation of extracts

The fresh rhizomes were cleaned and divided into three portions. Six hundred grams of the rhizomes for each portion were cut into thin pieces. Three hundred grams was deep fried in 300 ml of coconut oil at a controlled temperature until the pieces became dry and crispy. The residues were removed and then the other half of the thin pieces of the rhizome were put into the same coconut oil. The same procedure was followed. The hot oil extract was filtered. After recording the yield, the hot oil extract was kept in closed containers in a refrigerator until use. According to the temperature used, the extracts were categorized as follows: high temperature extract (HTE): 240-260°C, medium temperature extract (MTE): 170-190°C and low temperature extract (LTE): 70-90°C.

2.5. Determination of relative densities and viscosities of oil extracts

The relative densities of the extracts were calculated by the equation:

$$d_r = \frac{d_{(\text{sample})}}{d_{(\text{water})}}$$

where d_r is the relative density, $d_{(\text{sample})}$ is the density of each extract and $d_{(\text{water})}$ is the density of water. The densities of the samples and water were measured using the method presented in the British Pharmacopoeia Volume III, 2016¹⁰. A sample of 100 ml of each extract was added into a cylinder and weighed at room temperature. The density of each extract was calculated using the following equation:

$$d = \frac{m}{V}$$

where d is the density, m is the weight and V is the volume.

The viscosities of the extracts were measured using a rheometer (HAAKE™ RotoVisco™) by putting each 40 ml Phlai oil extract and coconut oil into the cup, where the cylinder rotor was used and the temperature was set at 40 °C. Shear rate was increased from 200 to 1400 /s in 2 min¹¹.

2.6. Thin layer chromatography determination

Samples were prepared by dissolving each Phlai oil extract so it was at 60%*v/v* concentration with dichloromethane. Then, 1 mg of (E)-4-(3',4'-dimethoxyphenyl)but-3-en-2-ol (compound D) was dissolved in 200 ml of dichloromethane to be a standard marker, 0.1 mg of curcuminoids was dissolved in 200 ml of dichloromethane and 1 ml of Phlai essential oil was dissolved in 900 ml of dichloromethane.

One microliter of each Phlai oil extracts and standard marker were streaked on a Silica gel 60 GF₂₅₄ pre-coated TLC plate by Camag Linomat 5. Two mobile phases were used, which were dichloromethane and a mixture of toluene and ethyl acetate (9:1). All developed plates were examined under a 254 nm wavelength.

2.7. Determination of anti-inflammatory activity of Phlai oil extracts

2.7.1. Animals

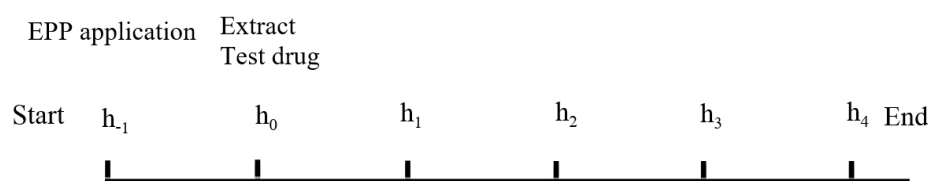
Male Sprague-Dawley rats (100-120g) from the National Laboratory Animal Center, Mahidol University were used. The animals were housed in groups of three per cage. They were kept in a temperature controlled room (25±1°C) under a 12 hours light/dark cycle for one week before the experimental study. The experiment protocol was approved by the Institutional Animal Care and Use Committee, Faculty of Pharmacy, Mahidol University. The experiments were undertaken according to the ethics of the care of laboratory animals guidelines.

2.7.2. Determination of anti-inflammatory activity of Phlai oil extracts

The male Sprague-Dawley rats were randomly divided into six groups. Ear edemas were induced by applying 1 mg/20µl/ear of ethyl phenylpropionate (EPP) on the inner and outer surface of a rat's ear. One hour after EPP application, 20 µl of each extract (LTE, MTE, HTE), 1 mg/µl indomethacin, 20 µl of 0.9%N/S or 20 µl of coconut oil was topically applied on each ear at the same area as the EPP application. Ear thickness was measured using a micrometer before (h_0) and after (1, 2, 3 and 4 h - h_1 , h_2 , h_3 and h_4) application of the controls or samples. The increases in ear thicknesses of the treated groups were compared with that of the control group. The most active Phlai oil extract was selected for further study.

2.7.3. Determination of minimum concentrations of active Phlai oil extract

Three dilutions of the effective Phlai oil extract in coconut oil, 25%, 50% and 75% (*v/v*%), were prepared. All dilutions and the effective Phlai oil extract were investigated using the EPP induced ear-edema model. Indomethacin, 0.9%N/S and coconut oil (20µl/ear) were used as controls. Ear thickness was measured before (h_0) and then at 1, 2, 3 and 4 hours (h_1 , h_2 , h_3 and h_4) after inflammation using a micrometer. The increase in ear thickness of treated group was compared with those of control group. The percentage of activity and inhibition were calculated by the following equation.



$$\text{Ear thickness} = h_a - h_0$$

$$\% \text{Swelling} = \frac{100 \times (h_a - h_0)}{h_0}$$

$$\% \text{Inhibition} = \frac{100 \times (ET_c - ET_t)}{ET_c}$$

Where; h_0 = ear thickness before treatment

h_a = ear thickness after treatment (a = 1, 2, 3, 4 and 5 hours)

ET_c = ear thickness of the control group

ET_t = ear thickness of the each treatment group

2.7.4. Statistical analysis

The results of the study were expressed as means \pm S.E.M. Statistical comparisons between groups were undertaken using an analysis of variance (ANOVA) and Tukey's multiple range test, which were performed with the SPSS computer program.

3. RESULTS AND DISCUSSION

3.1. Density and viscosity of extracts

As the temperature and heating time affected the relative density and viscosity of the oil extract, these properties should be determined

every batch^{12,13}. LTE showed a relative density and viscosity significantly higher than MTE ($P < 0.05$). The viscosity of the LTE was also higher than the viscosity of the HTE, as shown in Table 1. Since the temperature used in the LTE was under the boiling point of water, and some water remained in the extract that showed the turbid solution was different from the other clear extracts. The water content might affect the relative density and viscosity. The density and viscosity of the vegetable oil changed after being exposed to the heat, so it should be recorded every batch to monitor the process.

Table 1. Density and viscosity of Phlai extracts at three different temperatures (LTE, MTE, HTE)

Sample	Relative density of extracts (d_4) (g/cm ³)	Viscosity (cP)
Coconut oil	0.755 \pm 0.003	26.67 \pm 0.053
LTE	0.878 \pm 0.002 ^a	27.14 \pm 0.068 ^a
MTE	0.868 \pm 0.004 ^{a,b}	26.91 \pm 0.030 ^{a,b}
HTE	0.878 \pm 0.004 ^{a,c}	26.99 \pm 0.053 ^{a,b}

^a significantly different from coconut oil ($P < 0.05$), ^b significantly different from LTE ($P < 0.05$), ^c significantly different from MTE ($P < 0.05$); express as mean \pm SD (n=3)

Viscosity by rheometer (HAAKETM RotoViscoTM) with cylinder rotor at 40 °C. Shear rate was increased from 200 to 1400 /s in 2 min.

3.2. TLC chromatogram

Compound D and the essential oil are the chemical compounds in the rhizome of Phlai that were reported to show anti-inflammatory activity^{5,6}. The traditional preparation of Phlai oil extracts in this study presented compound D, which exhibited the quenching bands at 0.16 and 0.10 (dichloromethane and a mixture of toluene and ethyl acetate (9:1), respective) for the Rf value when observed under UV 254 nm. The fingerprint of the essential oil was demonstrated. Some compounds in the essential oil remained in the extracts, especially in the LTE, but most compounds with a low molecular weight might evaporated when exposed to the medium or high temperature heats, as seen in Figure 1.

3.3. Anti-inflammatory activity of Phlai oil extracts

A topical application of EPP on the rat's ear demonstrated an inflammatory response and produced ear swelling that was observed one hour

after its application^{14,15}. Indomethacin, distilled water, and coconut oil were used as positive, negative and vehicle control, respectively. Phlai oil extracts significantly reduced ear edema ($P < 0.05$) throughout the period of study when compared to the control group, as seen in Figure 2. The coconut oil also showed a significant reduction of edema compared to distilled water. The low temperature extract (LTE) exhibited low potency on the reduction of ear edema, which was the same as coconut oil for the first two hours after its application. However, its activity was better than the coconut oil from the third hour until the end of the experiment. The MTE and HTE showed inhibition of inflammation at 2, 3 and 4 h after the application of the extracts and the activity was significantly greater than the coconut oil ($P < 0.05$). Moreover, the HTE showed the most potent anti-inflammatory activity from the first hour and the thickness of the ears almost returned to normal at the fourth hour. Therefore, HTE was chosen to study the minimum effective concentration.

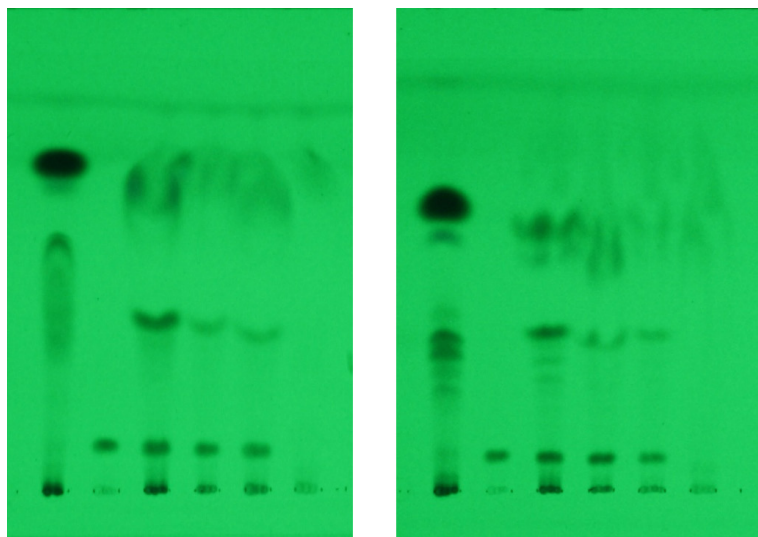


Figure 1. TLC fingerprint of Phlai extracts prepared by deep-frying in coconut oil under three different temperatures (LTE, MTE, HTE), 50% Phlai essential oil, coconut oil and compound D; 1 = essential oil, 2 = compound D, 3 = LTE, 4 = MTE, 5 = HTE, 6 = Coconut oil. Mobile phase I (left), dichloromethane; Mobile phase II (right), toluene: ethyl acetate (9:1). Detectors: UV 254 nm.

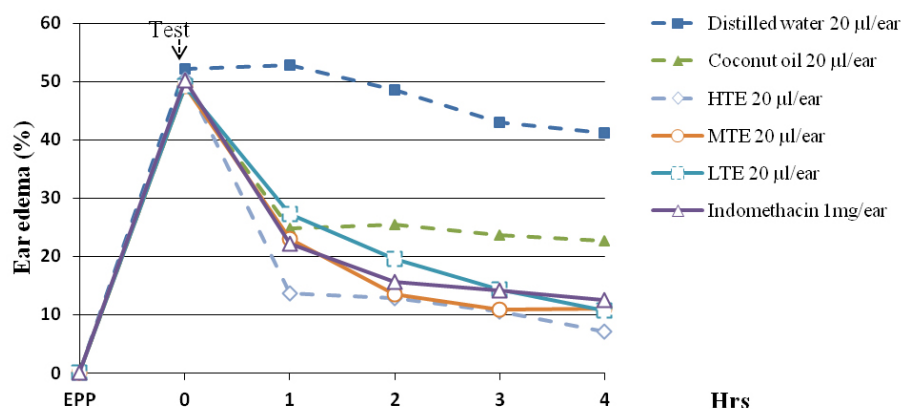


Figure 2. Effect of three Phlai extracts (LTE, MTE, HTE) on ear edema reduction. Data shown as mean±S.E.M. *Significantly different from control group, $P < 0.05$, #significantly different from coconut oil, $P < 0.05$.

3.4. Minimum effective concentrations of HTE

The three concentrations of HTE diluted with coconut oil (25%, 50%, 75%, v/v) and the HTE were investigated. All concentrations demonstrated a reduction in the ear edema when compared to the control group ($P < 0.05$) (Figure 3). The HTE showed a significant reduction ($P < 0.05$) in the ear edema from the first hour, whereas all dilutions of HTE started showing inhibition at the second hour. The 25% HTE demonstrated a significant

difference ($P < 0.05$) from coconut oil at the second hour and reduced the ear edema throughout the period of study when compared to the control group. Therefore, the 25% extract was considered to be the minimum effective concentration.

4. CONCLUSIONS

This study has revealed that Phlai oil extracts prepared by deep frying in oil using three different heating temperatures i.e., LTE (70-90°C), MTE

(170-190°C), HTE (240-260°C), demonstrated anti-inflammatory activity. The extract using a high temperature for the heating process showed the most effective activity. The concentration of the oil extract showing activity should not be less than 25%. The ratio of rhizome and oil in this study was 2:1, following the national list of essential medicines in Thailand⁴. Coconut oil showed a weak anti-inflammatory activity when compared to the negative control group, so it is suggested to use coconut oil

rather than other oils to enhance the activity rather than the rhizome itself. Moreover, the relative density and viscosity, which changed after exposure to the heat, were recorded. The TLC fingerprint showed that compound D was found in all three extracts, but some compounds in the volatile oil remained in the extract exposed to the low temperature heat. A study of other chemical content responsible for the activity as well as quality control should be further investigated.

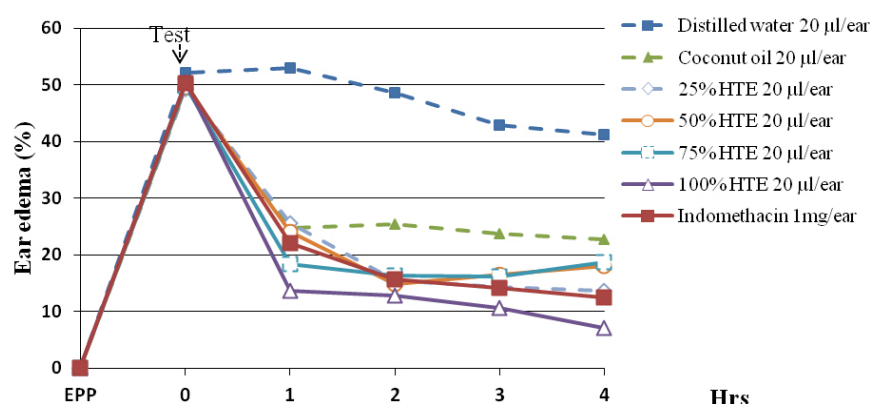


Figure 3. Effect of three dilutions and 100% of HTE on ear edema reduction.

Data shown as mean+S.E.M. *Significantly different from control group, $p < 0.05$, #significantly different from coconut oil, $p < 0.05$.

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Conflict of interest

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