Pharmacy Education (2023) 23(4) 242 - 246
https://doi.org/10.46542/pe.2023.234.242246

ICMHS 2022 SPECIAL EDITION

RESEARCH ARTICLE

Activities of ethanol-extract of red ginger (*Zingiber officinale* var. Rubrum) on Completed Freund's Adjuvant-induced arthritis in mice

Fifteen Aprila Fajrin[1,2], Putri Robiatul Khasanah[2], Yokta Esa Mikailla[2], Diana Holidah[1,2], Ika Puspita Dewi[1,2]

[1] Department of Clinical and Community Pharmacy, Faculty of Pharmacy, University of Jember, East Java Indonesia
[2] Preclinical Pharmacology Research Group, Faculty of Pharmacy, University of Jember, East Java Indonesia

Keywords
Arthritis
Hyperalgesia
Leukocyte
Oedema
Spinal cord

Correspondence
Fifteen Aprila Fajrin
Faculty of Pharmacy
University of Jember
Jember
East Java
Indonesia
fifteen.farmasi@unej.ac.id

Abstract

Background: Red ginger has been known to have anti-inflammatory and analgesic potency. **Objective:** This study aimed to analyse the ethanolic extract of red ginger (EERG) in mice's arthritis model induced by Completed Freund's Adjuvant (CFA). **Method:** Red ginger was extracted using ethanol 96%. Arthritis mice (n = 25) were injected with CFA i.p., and sham mice (n = 5) were injected with normal saline. On day seven, mice were divided into six groups: sham, CFA, gabapentin 100 mg/kg BW, and EERG (dose 200; 400; 600 mg/kg BW). Treatments were administered orally once a day for seven days. Latency time and plantar thickness were measured on days Zero, one, three, five, seven, eight, ten, twelve, and fourteen. On day 15, the mice were sacrificed, and the blood and spinal cords were collected. The haematology profiles were determined. **Result:** The EERG significantly prolonged the latency time towards thermal stimulus and decreased plantar thickness in arthritis mice, the same as gabapentin which served as the control. The EERG also reduced the number of leukocytes, lymphocytes, and neutrophils, and improved the morphology of the spinal cord's dorsal horn of arthritis mice. **Conclusion:** The EERG of 400 mg/kg BW significantly affects arthritis-induced hyperalgesia.

Introduction

Osteoarthritis is one of the most common diseases affecting joints, which attacks multiple joints including the hip, spine, knee, and foot (Tong *et al*., 2022). Arthritis is characterised by inflammation due to synovitis, articular cartilage, and subchondral bone damage (Kong *et al*., 2022). Inflammation in arthritis was also related to the changes in hematology profile especially leukocytes (Mehrani *et al*., 2023). Prolonged inflammation is usually followed by chronic pain which is shown as hyperalgesia (Kong *et al*., 2022).

The International Association for the Study of Pain (IASP) defines pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage stimulated by noxious stimuli (Raja *et al*., 2020). Some pain need time to cure (more than six months) and are called chronic pain (Dueñas *et al*., 2016).

Currently, adjuvant analgesics are often an option in chronic pain therapy, one of which is gabapentin (Wiffen *et al*., 2011). However, based on research, these synthetic analgesic drugs have undesirable long-term side effects, especially problems of physical dependence, misuse, or abuse (Carter *et al*., 2014; Paul *et al*., 2021).

One plant known to have a lot of activities is red ginger (*Zingiber officinale* var. Rubrum). Most people in Indonesia commonly use red ginger as a beverage, called "*jamu*". Based on previous studies, red ginger acted as an analgesic and anti-inflammatory for acute pain and inflammation (Zhang *et al*., 2022). This study aimed to analyse the ethanolic of red ginger extract (EERG) in mice's arthritis model using Completed Freund's Adjuvant (CFA) induction.

Methods

Materials

Zingiber officinale powder was collected and determined from Herbal Laboratorium "*Materia Medica*" Malang, East Java Indonesia, with number 074/227A/102.7/2020. CFA was purchased from Sigma. Gabapentin which served as the control was obtained from Indofarma, Indonesia. All solvents were purchased from Merck.

Extraction

The 96% ethanol (1:4) extracted Zingiber officinale var. Rubrum using the maceration method for 48 hours. Ethanol was evaporated using a rotary evaporator at a temperature of 50^{o}C to get the thick extract. Phytochemical analyses were determined qualitatively using thin-layer chromatography.

Animals

The thirty male mice (Balb-C strain) aged two to three months and weighed 20-30 grams were obtained from the Pharmacology Laboratory of the University of Jember. The mice were maintained at 25^{o}C ± 2 with a 12 h light/dark cycle and free access to food and water. The mice were adapted for one week before the experiment. The *in vivo* procedures were approved by The Animal Ethical Committee and Committee from Jember University (No. 976/UN25.8/KEPK/DL/2020).

Experimental design

Thirty male mice were divided into sham (n=5) and CFA (n=25). Before induction, all mice were determined latency time and plantar thickness as a baseline. Immadiately, mice in the CFA group were induced arthritis using 40 µL of CFA intraplantar, and sham mice were injected using normal saline with equal volume as CFA.

In-vivo study

After considering arthritis, which was characterized by prolonged hyperalgesia and oedema, CFA groups were randomly divided into five subgroups: arthritis CMC Na (1%), drug control (Gabapentin 100 mg/kg BW), ethanolic extract of red ginger (EERG) with dose 200, 400, and 600 mg/kg BW. The treatment was given orally once a day for seven days.

Arthritis assessment

Plantar thickness analysis

Plantar thickness was evaluated using callipers. The plantar thickness was measured on days zero, one, three, five, seven, eight, ten, twelve, and fourteen (Fajrin *et al*., 2019).

Hyperalgesia analysis

The latency time test of the heat stimulus was carried out using a hot/cold plate. Mice were placed individually on a hot/cold plate set at a temperature of 50^{o}C ± 0.5. The pain behaviour of each mouse was observed individually. This latency time was tested on days zero, one, three, five, seven, eight, ten, twelve, and fourteen (Fajrin *et al.,* 2019).

Hematology analysis

The total leukocyte was calculated using a Neubauer counting room under a microscope with a magnification of 100X. The counting of neutrophils and lymphocytes was carried out by staining with 15% Giemsa. The sample was examined under a light microscope at 400X magnification. Based on these 100 nucleated cells, the numbers of lymphocytes and neutrophils were calculated (Navarro-Alvarez *et al*., 2018).

Histology analysis

On day 15, mice were sacrificed by cervical dislocation. The spinal cord was isolated and fixated in neutral buffer formalin 10%. The spinal cord was stained using hematoxylin-eosin (HE), and the morphology of the dorsal horn was observed using a light microscope with 1000X magnification.

Data analysis

All data were expressed in mean ± SEM. Data were analysed using an independent t-test and one-way ANOVA with a 95% confidence level. The significant difference of each group was determined if $p < 0.05$.

Results

Extraction and phytochemical screening

The extraction from 500.16 g red ginger powder using ethanol 96% resulted in a thick extract weighing 100.67 g. The yield of this process was 20.13%. The phytochemical test results showed that red ginger extract contained alkaloids, flavonoids and tannins.

The effect of CFA injection on the plantar thickness of mice

Before CFA injection, there was no significant difference in the plantar thickness between the sham (2.35 ± 0.09 mm) and arthritis groups (2.32 ± 0.03 mm;

$p > 0.05$). One day after the CFA injection, there was an increase in plantar thickness in the arthritis group (4.38 ± 0.09 mm) which is significantly different from the sham group (2.60 ± 0.08 mm; $p < 0.05$). This condition occurred continuously until the seventh day (CFA group 3.57 ± 0.05 mm; sham group 2.56 ± 0.20; $p < 0.05$) which indicated that the test animals had arthritis.

The effect of CFA injection in hyperalgesia of mice

The increase in plantar thickness was followed by a decrease in the latency time to heat stimuli. The day after administering CFA (d-1), the latency-time-to-heat stimuli in the arthritis group (6.0 ± 0.11 s) decreased significantly compared to the sham group (8.8 ± 0.24 s; $p < 0.05$). The decrease in latency- time-to-heat stimuli in the arthritis group occurred continuously until the seventh day (4.2 ± 0.19 s). It was shown that the arthritis group was already in a state of hyperalgesia.

The effect of the EERG administration in reducing plantar thickness and hyperalgesia after CFA induction in mice

The EERG administration could significantly reduce oedema compared to the arthritis group ($p < 0.05$). However, the plantar thickness of the highest dose is still bigger than that of the sham group ($p < 0.05$). The administration of the EERG also prolongs the mean latency time to heat stimuli, better than that of the sham group ($p < 0.05$). After seven days of treatment (d-14), the EERG dose of 600 mg/kg BW had the best antihyperalgesic activity, as shown in Table I.

Table I: Plantar thickness and latency time of each group after treatments for seven days

Groups	Plantar thickness (mm ± SEM) and Latency time (sec ± SEM) day-							
	PT	LT	PT	LT	PT	LT	PT	LT
	8		10		12		14	
Sham	3.22 ± 0.08^{a}	8.9 ± 0.26^{a}	2.85 ± 0.05^{a}	7.9 ± 0.16^{a}	2.68 ± 0.03^{a}	7.8 ± 0.17^{a}	2.50 ± 0.01^{a}	8.3 ± 0.20^{a}
Arthritis	3.80 ± 0.40^{b}	4.7 ± 0.26^{b}	3.65 ± 0.35^{b}	4.9 ± 0.24^{b}	3.25 ± 0.05^{b}	3.7 ± 0.25^{b}	3.25 ± 0.10^{b}	4.3 ± 0.19^{b}
Gabapentin 100 mg/kg BW	3.41 ± 0.03^{b}	7.3 ± 0.13^{c}	3.15 ± 0.09^{b}	7.8 ± 0.34^{a}	3.15 ± 0.20^{b}	8.6 ± 0.33^{c}	3.00 ± 0.15^{c}	9.3 ± 0.50^{c}
EERG 200 mg/kg BW	3.61 ± 0.11^{b}	7.3 ± 0.22^{c}	3.35 ± 0.15^{b}	8.0 ± 1.16^{a}	3.38 ± 0.08^{bc}	8.7 ± 0.24^{c}	3.00 ± 0.09^{c}	9.7 ± 0.30^{c}
EERG 400 mg/kg BW	3.46 ± 0.11^{b}	7.8 ± 0.40^{c}	3.06 ± 0.05^{bc}	7.9 ± 0.25^{a}	3.02 ± 0.60^{c}	10.1 ± 0.60^{d}	2.92 ± 0.06^{c}	9.7 ± 0.33^{c}
EERG 600 mg/kg BW	3.42 ± 0.13^{b}	8.3 ± 0.20^{a}	3.15 ± 0.10^{bc}	9.2 ± 0.28^{c}	2.95 ± 0.10^{c}	12.0 ± 0.25^{d}	2.80 ± 0.05^{c}	14.9 ± 0.26^{d}

PT = plantar thickness; LT = latency time; EERG = ethanolic extract of red ginger. Data were presented as the mean ± standard of error (SEM). The analysis used one-way ANOVA continued with Tukey. The confidence interval was 95%. The different superscript letters indicated significant differences between groups ($p < 0.05$)

The effect of red ginger extract administration on total leukocyte number, percentage of lymphocytes and neutrophils

Table II showed that the number of leukocytes, neutrophils, and lymphocytes in the negative group is significantly different ($p < 0.05$) from the sham group. CFA-induced arthritis caused a significant increase in leukocytes, neutrophils, and lymphocytes. The administration of the EERG dose of 400 mg/kg BW appeared to reduce the number of leukocytes, neutrophils, and lymphocytes in mice induced by CFA.

Table II: Total number of leukocytes, neutrophils, and lymphocytes of each group after treatments

Groups	Treatments	Leukocyte (x10^{3}/mm^{3} ± SEM)	Neutrophyl (% ± SEM)	Lymphocyte (% ± SEM)
Sham	CMC Na 1%	6.81 ± 0.44^{a}	17.50 ± 9.04^{a}	78.75 ± 8.69^{a}
Arthritis	CMC Na 1%	11.2 ± 1.14^{b}	48.75 ± 4.43^{b}	48.50 ± 5.80^{b}
Gabapentin	100 mg/kg BW	8.07 ± 0.83^{c}	28.75 ± 7.93^{a}	66.25 ± 4.57^{a}
EERG	200 mg/kg BW	7.55 ± 1.05^{c}	22.75 ± 2.75^{a}	75.25 ± 3.77^{a}
EERG	400 mg/kg BW	7.14 ± 0.50^{c}	25.75 ±10.30^{a}	68.75 ± 9.54^{a}
EERG	600 mg/kg BW	7.85 ± 0.39^{c}	26.25 ± 8.46^{a}	70.50 ± 8.19^{a}

EERG = ethanolic extract of red ginger. Data were presented as the mean ± standard of error (SEM). The analysis was done using one-way ANOVA and Tukey. The confidence interval was 95%. The different superscript letters indicated significant differences between groups ($p < 0.05$).

The effect of red ginger extract administration on the histology of the spinal cord of mice

CFA-induced inflammation changed the morphology of the dorsal horn's spinal cord of mice (Figure 1B). Chronic inflammation causes vasodilatation, nucleus protrusion, and cell necrosis. After treatment using EERG (Figure 1D-F), the improvement of nerve cells was shown as diminished vasodilatation, nucleus protrusion, and cell necrosis. The higher doses were seen quite similar to gabapentin used as the control (Figure 1C).

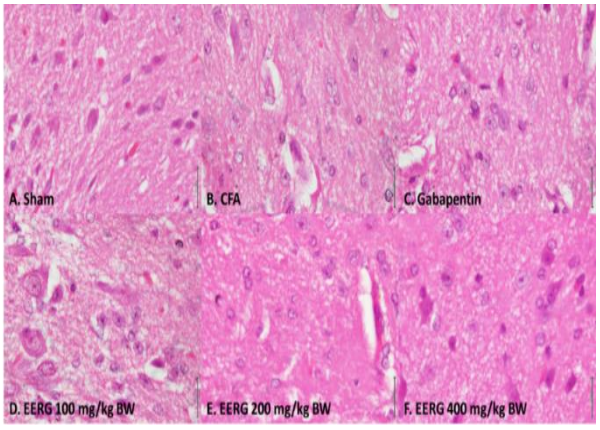

Figure 1: Histology of dorsal horn's spinal cord from the sham group (A), CFA group (B), gabapentin (C), EERG 100 mg/kg BW (D), EERG 200 mg/kg BW, and EERG 400 mg/kg BW (F). Stained using H & E with 1000x magnification. EERG = ethanolic extract of red ginger

Discussion

CFA which contains a mycobacteria suspension that has been heat-killed is effective in causing arthritis by releasing various pro-inflammatory mediators such as leukotrienes, prostaglandin E2, histamine, bradykinin, and serotonin (Patil *et al*., 2019; Jang *et al*., 2020).CFA causes the release of glutamate and activates the NMDAR2B subunit, then increases pain sensitisation. This study showed that CFA can induce long-term inflammation with strong thermal hyperalgesia (Zhang & Ren, 2011; Zhang *et al*., 2021).

When inflammation occurs, the number of leukocytes in the blood will increase (Abdulkhaleq *et al*., 2018). In chronic inflammation, the increasing number of chemokines, and cytokines also caused the re-activation of neutrophils followed by lymphocyte activity (Sugimoto *et al*., 2016).

After the treatment of the EERG, there was a decrease in plantar thickness and an increase in latency-time-to-heat stimuli in arthritis mice. Even so, the plantar thickness of all mice in the treatment groups did not return to baseline. This condition is possible because of the complex mechanism of chronic pain involving the immune system (Vijayalaxmi *et al*., 2015).

The EERG has been reported to have anti-inflammatory effects due to its secondary metabolites such as flavonoids and alkaloids. Shogaol and gingerol were the important phenolic compounds in red ginger which were reported to have strong anti-inflammatory activity by inhibiting cyclooxygenase activity (COX) and lipoxygenase (LOX) and reducing prostaglandin release, an important inflammatory mediator in pain (Zhang *et al*., 2022). Flavonoids as well, could inhibit leukocyte accumulation, neutrophil degranulation, and the release of inflammatory mediators such as histamine and prostaglandins (Mao *et al*., 2019). Flavonoids are also reported to stimulate lymphocyte proliferation and reduce lymphocyte mortality, causing the number of lymphocytes in the blood to increase (Maheswari *et al*., 2022).

Limitations

As a limitation, our study did not measure other pain responses such as screaming or pain responses to mechanical stimuli. It could be analysed further to ensure the effect of the EERG in treating arthritis in humans

Conclusion

The EERG doses of 400 mg/kg BW had the optimum effect as anti-hyperalgesic activity in mice as well as reduced leukocytes and neutrophils and the number of lymphocytes in response to system repair due to a CFA-induced arthritis. Based on the results of this study, the EERG can be developed as an anti-hyperalgesia in arthritis.

Acknowledgement

The authors appreciate the University of Jember through "Hibah *Reworking Skripsi/Tesis 2021*" for the kind funding support to finalise this manuscript.

Source of funding

This study was funded through the Hibah Reworking Skripsi/Tesis 2021 Universitas Jember. No. 3021/UN25.3.1/LT/2021

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