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Research article

Kaempferia galanga L. alleviates the expression of COX-2 and NF-kappaB-p65 in the oral mucosa ulcer of Wistar rats and exhibits no irritation toward the vascular membrane of chicken eggs and the skin of albino rabbits

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ABSTRACT

The global incidence of recurrent aphthous stomatitis in 2018 reached 5–66 % of the population, while in Indonesia 8 %. Moreover, the prevalence of oral mucosal fibrosis and recurrent aphthous stomatitis among male doctors and nurses in China was 21.24 % and 24.27 %, respectively. Our previous study has shown that the ethanol extract of *Kaempferia galanga* L. rhizome (EKGR) revealed an accelerated wound-healing effect in the oral mucosa ulcer of Wistar rats. This study aims to explore the effects of EKGR on the expression of NF-kappaB-p65 and COX-2 in the tongue tissue of male Wistar rats by Western blot analysis and immunohistochemistry technique, its safety towards the vascular membrane of the egg chorioallantoic membrane, and its single-dose application on the skin of male rabbits. The rats were randomly assigned into 7 groups: the normal control; the negative control; the positive control (treated with triamcinolone acetonide); and 4 treatment groups of EKGR (0.5 %; 1 %; 2 %; 4 %). Western blot and immunohistochemistry methods were used to measure the expression of NF-kappaB-p65 and COX-2. The hen's egg test-chorioallantoic membrane assay was employed to predict the safety of EKGR towards the vascular membrane. Moreover, the effect of 200 mg/kg BW EKGR application on the dorsal skin of male albino rabbits was also evaluated. EKGR inhibits the expression of NF-kappaB-p65 and COX-2 as proven by WB and IHC results. In the HET-CAM assay, all concentrations of EKGR do not induce irritation responses, which elicits the safety of EKGR. The administration of EKGR causes mild irritation to the dorsal skin of male rabbits but does not induce erythema and edema, no significant changes in BW, no toxic effects on organ macroscopic examination or histopathology, and does not induce abnormalities in the hematological profile of male albino rabbits. EKGR has confirmed its anti-inflammatory activity by suppressing the expression of COX-2 and NF-kappaB-

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p65 in the oral mucosa ulcer of Wistar rats. EKGR is safe as it does not exhibit irritating potential and harmful effects.

1. Introduction

Stomatitis is an inflammatory oral mucosa disease often found in humans, with mild, moderate, and severe degrees. Regardless of its lack of potential to cause death, a recurrent or chronic condition of this ailment may interfere with the quality of life (QoL) of the patients [1,2]. It was reported that the global incidence of recurrent aphthous stomatitis in 2018 reached 5–66 % (average of 20 %) of the population [3], while in Indonesia 8 % [4]. Moreover, the prevalence of oral mucosal fibrosis and recurrent aphthous stomatitis among male doctors and nurses in China was 21.24 % and 24.27 %, respectively [5]. Stomatitis is often associated with the presence of systemic or autoimmune disease. Topical anti-inflammatory drugs for stomatitis therapy belong to the steroid group, such as triamcinolone acetonide, prednisone, or dexamethasone, however, these drugs were reported for several adverse effects. The condition of stomatitis sometimes gets worse, especially in patients with systemic diseases, or those with poor oral hygiene, due to the nature of the systemic disease and the use of steroid drugs itself affecting or reducing the local immunity of the oral mucosa, leading to the transformation of oral commensal microbes to pathogens, resulting in secondary infections. Steroid administration may exacerbate these existing conditions [6–8].

Steroid treatment is widely known to provoke problems such as host resistance that leads to a gradually increased dose required in therapy [8]. Moreover, these steroid topical anti-inflammatories for stomatitis currently prescribed are expensive, therefore, raising the need for the discovery of add-on or replacement therapy derived from natural resources. Plants have begun to draw the attention of many researchers and industries to be scientifically explored. Most medicinal plants for stomatitis and oral mucosal inflammation show good safety and mild adverse effects [9,10].

Our previous study has explored the potential of *Kaempferia galanga* L. rhizome as an anti-oral mucosal ulcer drug. The rhizome of *K. galanga* L. cold-extracted using 70 % ethanol was reported to contain secondary metabolites, e.g., polyphenols, flavonoids, alkaloids, triterpenoids, saponins, and tannins, that contribute to their pharmacological properties. One of the metabolites, namely ethyl p-methoxycinnamate (EPMC), is known to be the main component in the ethanol extract of *K. galanga* L. (EKGR) [11], which was also reported in other studies [12–14]. The *in vitro* study confirmed that EKGR at concentrations of 15.625 ppm and 31.25 ppm, revealed better anti-inflammatory effects ($p < 0.05$), by inhibiting the formation of PGG2 catalyzed by COX-2 compared to higher concentrations, with a relative IC_{50} of 39.85 ppm [15]. A previous *in vivo* study confirmed that the EKGR doses of 0.5–2% exhibited an anti-inflammatory effect and accelerated the healing of ulcers on the oral mucosa of Wistar rats [16]. Toxicity studies of *K. galanga* L. extract revealed its safety for animals. Administration of an oral single dose of up to 5000 mg/kg BW did not cause mortality in animal models. It was reported that a daily oral administration of 1000 mg/kg BW for 30 days and doses of 25, 50, or 100 mg/kg BW/day for 28 days resulted in no mortality in rats [17,18].

Considering this, our study aims to explore the effects of EKGR on the expression of NF-kappaB-p65 and COX-2 in the tongue tissue of male Wistar rats by Western blot analysis (the relative expression of the protein normalized to beta-actin) and immunohistochemistry technique (the % protein expressed in the tissue), its safety towards the vascular membrane of the egg chorioallantoic membrane (the irritation responses indicated by vasoconstriction, hemorrhagic, coagulation, and lysis), its acute dermal irritation test on the skin of male albino rabbits (indicated by erythema and edema score), and its single-dose application on the skin of male rabbits (body weight, rectal temperature, relative vital organ weight, hematology profile, erythema and edema on the dorsal skin, and histopathology of the dorsal skin tissue). All parameters were assessed to support the objective of studying the anti-ulcer activity and the safety of *Kaempferia galanga* rhizome extract (EKGR). NF-kappaB p65 and COX-2 are proteins involved in the inflammatory process. The safety of EKGR was confirmed using the HET-CAM assay, acute dermal irritation test, and single-dose application skin test. The hen's egg-chorioallantoic membranes (HET-CAM) are generally utilized to evaluate the anti-irritant properties of plant extracts [19]. The skin irritation test is carried out by applying the plant extract on the shaved dorsal area of albino rabbits and observing the signs of erythema and edema, followed by histopathological examination [20].

2. Material and methods

2.1. Plant materials

The fresh rhizomes were purchased from Subang, West Java, Indonesia. For the taxonomy identification process, 1–2 rhizomes were washed with 70 % ethanol, put in a plastic pouch, and sealed. The specimen was identified and stored at the Herbarium Bandungense, SITH, Bandung Institute of Technology (ITB), Indonesia, and was confirmed as *Kaempferia galanga* L. with descriptions matched those listed in https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:797160-1.

2.2. Preparation of the ethanol extract of K. galanga (EKGR)

The preparation of the rhizome extract was carried out by adapting a previous procedure [10,15]. The rhizomes were cleaned to remove dirt, soil, and rotten parts, sliced, and dried under sunlight for several days. At room temperature, the dried rhizomes were soaked in 70 % ethanol for 3 × 24 h. The extract was filtered and the excess solvent was removed using a rotary evaporator (Buchi®,

R-220) at 40–45 °C to a viscous consistency. The cold maceration method for the extract preparation is the most suitable for obtaining optimal secondary metabolites because of its thermolabile nature.

2.3. Analysis of EPMC in EKGR

Analysis of the ethanol extract of *K. galanga* rhizome, abbreviated to EKGR, was performed by operating a reversed-phase high-performance liquid chromatography (RP-HPLC) (Waters Alliance, 2695) adapting a procedure described elsewhere [21] with modifications as follows: 10 μL of the standard EPMC at a concentration of 20 μg/mL (Tokyo Chemical Industry Co., Ltd., 24393-56-4) and/or the EKGR extract (concentration of 1000 μg/mL), were separately inserted into the octadecylsilane (ODS) column (Merck Millipore LiChroCART; 250 mm × 4.6 mm) and were eluted using double-distilled water (API IPHA®, 7732-18-5) and acetonitrile (Merck®, 75-05-8), or H_2O–CH_3CN (40:60) in isocratic elution, with a flow rate of 1.0 mL/min, and a run time of 10 min. Detection (UV–vis detector Waters 2489) was adjusted at 308 nm, which is the maximum absorption wavelength of EPMC. The chromatogram of EKGR was collocated to that of the standard EPMC.

2.4. Determination of total phenols in EKGR

The determination of the total phenols was carried out by following a previous method [22,23]. Briefly, a solution of EKGR was added with 2.5 mL of 10 % Folin-Ciocalteu reagent (Sigma-Aldrich, F9252), left at room temperature for 1 min, then added with 2 mL of 7.5 % sodium carbonate (Na_2CO_3; Sigma-Aldrich, 497-19-8), and incubated for 15 min in a water bath at 45°C and the absorbance was measured at 765 nm. The standard curve was created by plotting the concentration versus the absorbance of gallic acid ($C_7H_6O_5$; Merck Millipore, 149-91-7) and the total phenols are expressed in gallic acid equivalents (GAE)/g dry extract weight.

2.5. The effect of EKGR on the expression of NF-kappaB-p65 and COX-2 animals and ethical considerations

Thirty-five healthy male Wistar albino rats (*Rattus norvegicus*), aged 10–11 weeks, weighing 150–200 g, were purchased from the Iratco® Animal Experiment Laboratory, Bogor, Indonesia. The rats were randomly assigned to 7 cages (n = 5 rats/cage) with the dimensions of the cage of 55 cm length x 45 cm width x 45 cm height. There were 7 groups of rats consisting of normal control, negative control, TRA, 0.5 % EKGR, 1 % EKGR, 2 % EKGR, and 4 % EKGR groups. The number of rats in each group was 5 based on calculations using the Federer formula. During the acclimatization and research period, the 3R and 5F principles were implemented following the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments). The rats were acclimatized for 7 days with a relative humidity of 70 %, a dark-light cycle of 12 h, and a room temperature of 18–24 °C. The rats were given standard pellet feed (75 g/cage/day, containing 5 % fibers, 20 % protein, and 5–10 % fat) and water (350 mL/day).

This study was approved by the Ethical Committee of Health Research of Universitas Padjadjaran (document number 523/UN6.KEP/EC/2020 and the amendment document number 031/UN6.KEP/EC/2021).

2.6. Experimental design

The male Wistar rats were randomly assigned into 7 groups (n = 5 rats/cage): Group 1 (the normal control) was not treated nor induced; Group 2 (the negative control) was treated with a 2 % CMC Na suspension and 100 μL of 70 % acetic acid was applied to the ventral of the rat's tongue for 2 min, to induce oral mucosal stomatitis; Group 3 (the positive control or TRA) was treated with triamcinolone acetonide 0.1 % in oral-base (Kenalog in oral-base, Taisho®) for 6 days, and a 100 μL of 70 % acetic acid was applied to the ventral of the rat's tongue for 2 min, to induce oral mucosal stomatitis on day 1; Groups 4–7 were treated with EKGR doses of 0.5 % (4); 1.0 % (5); 2.0 % (6): and 4.0 % (7), respectively, once daily for 6 days, and a 100 μL of 70 % acetic acid was applied to the ventral of the rat's tongue for 2 min, to induce oral mucosal stomatitis on day 1.

A mouthwash solution containing 0.2 % chlorhexidine gluconate in a sterile solution was used as the antiseptic before the induction process was carried out. The procedure followed previous works with a few modifications [24–26]. The induction technique was standardized for all animals and performed by the same person.

At the end of the study, the rats were euthanized using intraperitoneal sodium pentobarbital. This drug is commonly used in euthanasia because of its narrow safety margin, only needs small volumes, and has a rapid onset [27]. The tongue of the rats was taken as the specimen, put in a 5 mL tube containing 10 % formaldehyde (Indokimia®, 50-00-0), and kept at −20 °C for further use. The remains of the rats were put in a freezing box and were sent to the incinerator.

2.7. Western blot analysis

The Western blot analysis was carried out by following a previous method [28]. The protein in the tongue tissue of the rats was extracted by several steps: (1) the first step is done by adding a homogenizer buffer (RIPA buffer; Sigma-Aldrich, R0278), containing 10 mM Tris-HCl pH 7.8, 150 mM sodium chloride (NaCl), 1 mM ethylene diamine tetra acetic acid (EDTA), 1 % Nonidet P-40, and protease inhibitors, then the homogenates were centrifuged for 5 min at 5200 rpm to separate the supernatant; (2) the supernatants were collected and used as protein extracts (lysate) to measure the quantity of protein using bicinchoninic acid assay (BCA) reagents (Thermo Sci, 23228).

The tissue lysate was then incubated at 4 °C and separated by adding sodium dodecyl sulfate (SDS/$NaC_{12}H_{25}SO_4$; Merck Millipore)

and polyacrylamide gel (Merck Millipore, MP10W1), transferred to the nitrocellulose membrane (Thermo Sci, 77010). The nitrocellulose membrane was blocked with a phosphate buffer saline (Indokimia®, 7778-77-0) solution for 1 h and further incubated for 1 night at 4°C with specific primary antibodies for protein COX-2 antibody (D5H5, XP® Rabbit mAb; Cellsignal, 1:1000), NF-kappaB-p65 (Mouse mAb; Santacruz Biotechnology, 1:1000), and internal β-actin antibodies (G-9; Santacruz Biotechnology, 365062, 1:1000). After 24 h the membrane was washed with PBS three times each for 5 min, then incubated again with a secondary antibody (horseradish peroxidase anti-mouse antibody and substrate/C-2; Sigma-Aldrich, sc-8432, 1: 3000) for 1 h, and washed again with PBS three times each for 5 min. Finally, the membrane was placed on a blotting scanner and the bands were observed visually using a computer connected to the scanner (Oddysey Clx, Li-Cor® C Digit tool). The relative intensity of the bands was measured using ImageJ® computer software (https://imagej.nih.gov/ij/). The COX-2 bands appeared at 74 kDa, NF-kappaB-p65 at 65 kDa, and β-actin at 42 kDa.

2.8. Immunohistochemistry method

The immunohistochemistry step was performed by following a previous procedure with modification [29]. The oral mucosal tissues were cut using a microtome with a thickness of 3 μm, then processed using Mouse and Rabbit Specific HRP/DAB (Abcam, Detection IHC kit ab64264), and incubated with 100 μL primary antibody anti-COX-2 protein (D5H5; XP® Rabbit mAb, Cellsignal, 1:800) and NF-kappaB-p65 (Mouse mAb, Santacruz Biotechnology, 1:300), overnight at room temperature. After washing, the tissue pieces were dripped with 100 μL biotinylated goat anti-polyvalent anti-rabbit/anti-mouse (Abcam, ab64264), and incubated for 30 min at room temperature, then washed again with PBS (3 × 5 min) and incubated with 100 μL streptavidin HRP secondary antibody (Abcam, ab64264), for 30 min. The tissues were washed again with PBS (3 × 5 min) and dripped with 100 μL of DAB substrate (Abcam, ab64264), incubated for 15 s in the dark, rinsed, counterstained using hematoxylin (Merck®, 115938) for 10 s, washed again using distilled water (Indokimia®) for 5 min, dehydrated, cleared, and examined under a compound microscope (Zeiss ®, Primo Star 3) connected to a computer and a Lumenera scientific microscope camera (Infinity1-microscope camera) at 40 × 10 magnification. The data was documented as digital files. Immune response was noted as a brown stain on the epithelial cytoplasm for COX-2 and in the nucleus for NF-kappaB p65. The extent of expression was noted from lamina propria to basal and superficial areas. Cytoplasms with granular to diffuse brown staining, irrespective of staining intensity, were regarded as positive. The percentage of brown-stained cells was measured using ImageJ® computer software (https://imagej.nih.gov/ij/).

2.9. The effect of EKGR on the egg chorioallantoic membrane

The hen's egg test-chorioallantoic membrane (HET-CAM) has been widely used in various studies to measure the irritant potential of a chemical substance. This method is an alternative procedure to the Draize's test [24,25]. Fresh fertile hen eggs 7 days old were collected from the Iratco® farm and animal experiment Laboratory, Bogor, Indonesia, with weight ranges from 50 to 60 g/egg, and were used in this study. Eggs were cleaned before use and examined against a candlelight and nonviable or defective eggs or cracked were discarded. The eggs were acclimatized for 3 days in an incubator at a temperature of 37–38 °C with a relative humidity of 62 ± 7.5 %. A surface disinfection on the eggs was carried out, and the outer shell layer was opened carefully using a special electric cutter until the inner membrane (the chorioallantoic membrane) was visible.

300 μL of the control and EKGR in suspension (0.25 %, 0.5 %, and 1.0 % EKGR dissolved in 2 % Na-CMC) was applied directly using a micropipette to the chorioallantoic membrane (CAM) of the Hen's egg, which is rich in vascular networks. The normal control or non-irritant control was 0.9 % physiological NaCl and the irritant control was 1 % SDS. Each treatment was carried out in triplicates on

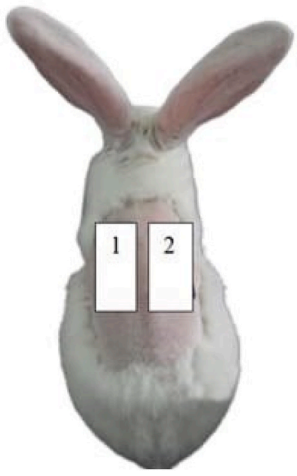

Fig. 1. The application areas on the dorsal skin of the rabbits for acute dermal irritation assay: (1) the area where EKGR is applied for 4 h; (2) the control area.

three different egg embryos.

The observation was carried out under a stereomicroscope (Olympus SD30®), in the form of the presence or absence of signs of irritation, such as vasoconstriction, hemorrhage, lysis, and coagulation, and the structure of the blood vessels, by an expert veterinarian. Data were recorded at 0, 15, 30, 60, 100, and 300 s.

2.10. The acute dermal irritation test of EKGR on the skin of male albino rabbits

2.10.1. Animals and ethical considerations

Nine healthy male albino rabbits (*Oryctolagus cuniculus*), aged 3–6 months, with a body weight of 1.5–2.5 kg, were obtained from Bio Farma's Animal Breeding Facility, Cisarua, West Java, Indonesia. The rabbits were placed in 9 cages (n = 1 rabbit/cage) with the dimensions of the cage at 42 x 61 × 57 cm^3 and were acclimatized for 5 days with a relative humidity of 49–52 %, a dark-light cycle of 12 h, and a room temperature of 16–22 °C. The rabbits were given standard feed containing protein 15 %, fat 5 %, raw fiber 16 %, calcium 1.35 %, and phosphor 0.7 %, and drank freely. This study was approved by the Ethical Committee of Health Research of Universitas Padjadjaran (document number 1122/UN6.KEP/EC/2022).

2.10.2. Experimental design

The rabbits were assigned into 3 groups (n = 3), and the hairs on the dorsal skin were shaved (10 × 15 cm^2) each for different concentrations of EKGR (0.25 %, 0.5 %, and 1 %). 0.5 g of the extract was applied to the dorsal area of the rabbits (Fig. 1), covered with a sterile cotton gauze, for 4 h. The extract was only applied once on day 1 with a predetermined concentration (0.25 %, 0.5 %, and 1 %). Area 1 was the control area which was not being applied with extract treatment, meanwhile, area 2 was the test area, applied with extract. Irritation reactions in terms of erythema and edema were observed and calculated using the Draize skin irritation score (Table 1). The observation was conducted at 1 h, 24 h, 48 h, and 72 h post-application, and the intensity of the redness was measured using ImageJ (https://imagej.net/ij/).

The primary irritation index was calculated by employing the following equation:

$$\text{The primary irritation index} = \frac{A - B}{C}$$

Where:

A: total erythema and edema scores on area 1 (EKGR) at 24 h, 48 h, and 72 h.

B: total erythema and edema scores on area 2 (control) at 24 h, 48 h, and 72 h.

C: the number of animals.

2.11. The effects of single-dose EKGR on the hematology, macro-pathology, and histopathology of the skin of male albino rabbits

2.11.1. Animals and ethical considerations

The skin irritation test was performed by following previous methods with modifications [20,30]. Four healthy male albino rabbits (*Oryctolagus cuniculus*), aged 3–6 months, weighing 1.5–2.5 kg, were obtained from Bio Farma's Animal Breeding Facility, Cisarua, West Java, Indonesia. The rabbits were placed in 4 cages (n = 1 rabbit/cage) with the dimensions of the cage at 42 x 61 × 57 cm^3 and were acclimatized for 5 days with a relative humidity of 49–52 %, a dark-light cycle of 12 h, and a room temperature of 16–22 °C. The rabbits were given standard feed containing protein 15 %, fat 5 %, raw fiber 16 %, calcium 1.35 %, and phosphor 0.7 %, and drank freely. This study was approved by the Ethical Committee of Health Research of Universitas Padjadjaran (document number 1122/UN6.KEP/EC/2022).

2.11.2. Experimental design

The rabbits were assigned into 2 groups (n = 2): the control group and the EKGR 200 mg/kg BW group (single-dose application skin test group) and the hairs on the dorsal skin were shaved (10 × 15 cm^2). 0.5 g of the extract was applied to the dorsal area of the rabbits,

Table 1
The Draize skin irritation score.

Erythema and eschar evaluation	Score
No erythema	0
Very slightly erythema	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet to crimson red) to slight eschar formation (injuries in depth)	4
Edema evaluation	**Score**
No edema	0
Very slightly edema	1
Well defined edema	2
Moderate to severe edema (edges of area well-defined with definite raising)	3
Severe edema (raised approximately 1 mm)	4

covered with a sterile cotton gauze, for 24 h. The rabbits' BW and rectal temperature (measured using an animal thermometer; Onemed®, ThermoOne Alpha 3) were observed on day 1, day 7, and day 14. Hematology parameters by blood sampling from the ear marginal vein were measured on day 1 and day 14 using a Hematology Analyzer VetScan® HM5 v2.31. On day 14, the rabbits were euthanized using intraperitoneal sodium pentobarbital. Moreover, their organs were separated and weighed, and the skin of the rabbits was histopathology examined at the Faculty of Medicine, Universitas Padjadjaran, West Java, Indonesia.

2.11.3. Statistical analysis

Excel app software was used for statistical analyses (Microsoft® Excel® 2021 MSO; Version 2304 Build 16.0.16327.20200; 64-bit). The significant differentiation between two categorical variables was analyzed using one-way ANOVA and Wilcoxon Signed Rank Test. $P < 0.05$ was considered significant.

3. Results

3.1. Analysis of EPMC and total phenols in EKGR

The RP-HPLC chromatogram of standard EPMC (20 μg/mL) (Fig. 2a) reveals a major peak eluted at 5.890 min with a % AUC of 99.29, whereas EKGR (1000 μg/mL) (Fig. 2b) shows several minor peaks with a major eluted at 5.880 min with a % AUC of 89.45. Moreover, the determination of the total phenols demonstrated that EKGR contains 398.52 mg GAE/g extract.

3.2. EKGR inhibits the expression of COX-2 and NF-kappaB-p65 (western blot technique)

The expression of inflammatory proteins (COX-2 and NF-kappaB-p65) in the oral mucosa of Wistar rats was analyzed using the

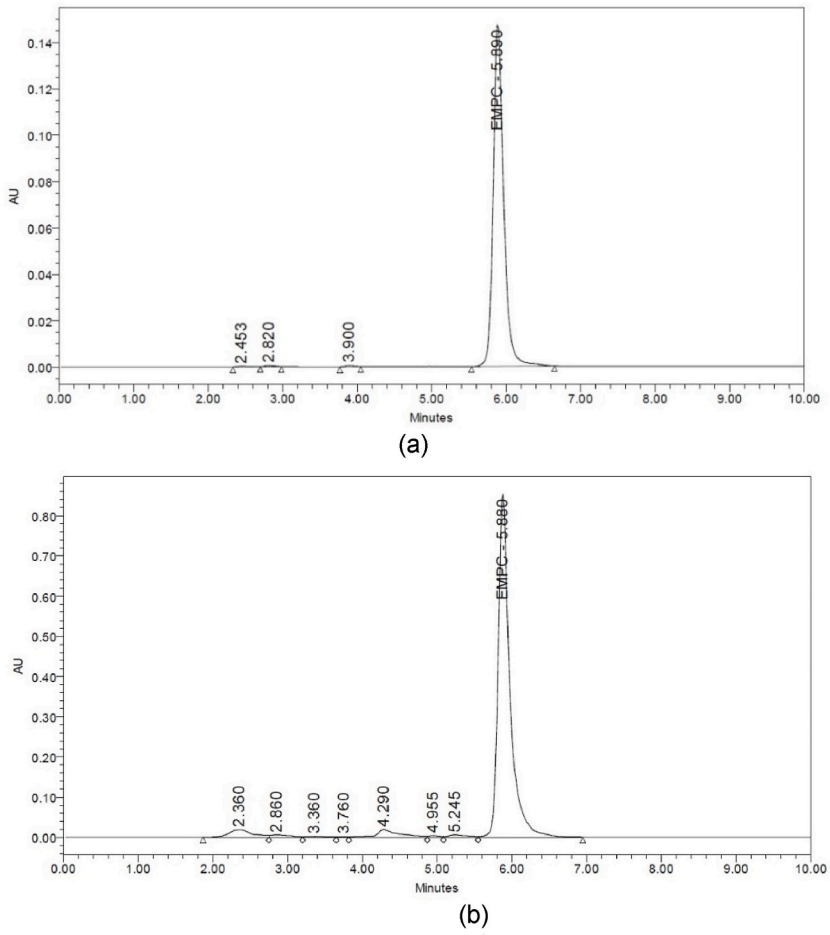

Fig. 2. RP-HPLC chromatogram of (a) standard EPMC 20 μg/mL and (b) EKGR, using an ODS column, H_2O–CH_3CN (40:60) as the mobile phase, flow rate of 1.0 mL/min, and detection at 308 nm. The HPLC chromatogram of the EKGR extract reveals numerous peaks that represent the constituents contained in the extract. EPMC is eluted as a major peak at 5.880 min.

Western blot technique and depicted in Fig. 3 (supplementary material for the "Western blot images of COX-2 and NF-kappaB-p65 expression" is available at click here). The Western blot bands of the inflammatory protein COX-2 (74 kDa), NF-kappaB-p65 (65 kDa), and beta-actin (42 kDa) as the internal control were shown in Fig. 3a. The lowest average relative expression of COX-2 (Fig. 3b) was affected by TRA (0.59 ± 0.06) and the highest was affected by 0.5 % EKGR 1.27 ± 0.33. In general, EKGR showed inhibitory activity on COX-2 expression but was weaker than that of TRA. TRA and EKGR doses of 2 % and 4 % showed significant differences compared to the negative control.

The lowest average relative expression of NF-kappaB p65 (Fig. 3c) was affected by 4 % EKGR (0.59 ± 0.13) and the highest was affected by 2 % EKGR (0.98 ± 0.43). TRA and EKGR indicated an inhibitory activity on NF-kappaB-p65 expression, but only TRA and 4 % of EKGR showed significant differences when compared to the negative control. Several standard errors in the results of this study exhibit quite large variations in values, this is expected because each rat as an experimental animal can reveal different immunological responses. Differences in the immunological response can be seen both from the response to ulcer induction using acetic acid, as well as the response to the same therapy in each group.

3.3. EKGR inhibits the expression of COX-2 and NF-kappaB-p65 (immunohistochemistry)

Immunohistochemistry results reveal the expression of COX-2 (depicted in Fig. 4) and NF-kappaB-p65 (depicted in Fig. 5), both are inflammatory cytokines.

The results of the study on the oral mucosal tissues of Wistar rats treated with TRA and 0.5 %–4 % EKGR showed lower expression of COX-2 (Fig. 6) and NF-kappaB-p65 (Fig. 7) compared to the negative control. The lowest expression of COX-2 was affected by 1 % EKGR (2.13 ± 1.60) and the highest was affected by TRA (8.82 ± 1.79). Meanwhile, the lowest expression of NF-kappaB-p65 was affected by 0.5 % EKGR (1.37 ± 0.43), and the highest was affected by 4 % EKGR (4.87 ± 2.58). Taken together, all doses of EKGR significantly reduced the expression of COX-2 in the tongue tissue of acetic acid-induced male Wistar rats (Fig. 6), however, only EKGR 0.5 % and 1 % significantly lowered the expression of NF-kappaB (Fig. 7).

3.4. EKGR does not induce an irritation response on the chorioallantoic membrane

Fig. 8 depicts the results of the HET-CAM test which reveals the safety of EKGR (0.5 %–2 %) as it does not induce irritation responses on the chorioallantoic membrane (CAM) during the observation period (0–300 s). Irritation responses are indicated by vasoconstriction (black arrow), hemorrhagic (white arrow), coagulation, and lysis as shown in CAM treated with SDS, particularly at 60–300 s. Conversely, treatment with NaCl 0.9 % reveals normal blood vessels.

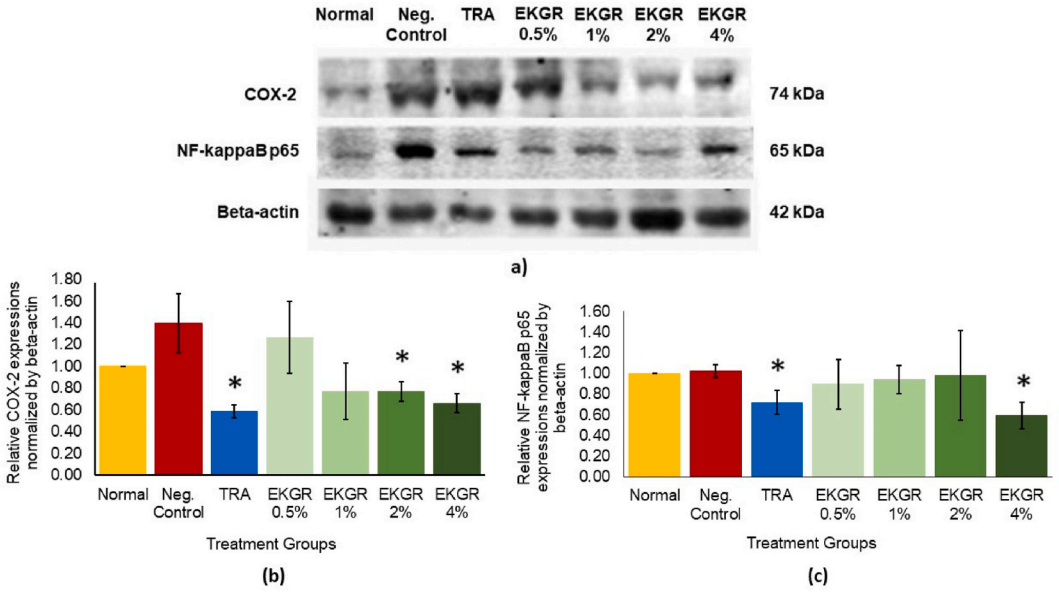

Fig. 3. a) The bands represent COX-2 (74 kDa), NF-kappaB p65 (65 kDa), and beta-actin (42 kDa) expression in the oral mucosa of Wistar rats by Western blot analysis. The asterisk symbol (*) in the **b)** and **c)** figures indicated $p < 0.05$ (significantly different compared with the negative control, calculated by One-way ANOVA followed by post hoc Bonferroni test). TRA = triamcinolone acetonide; EKGR = ethanol extract of *K. galanga* L. rhizome.

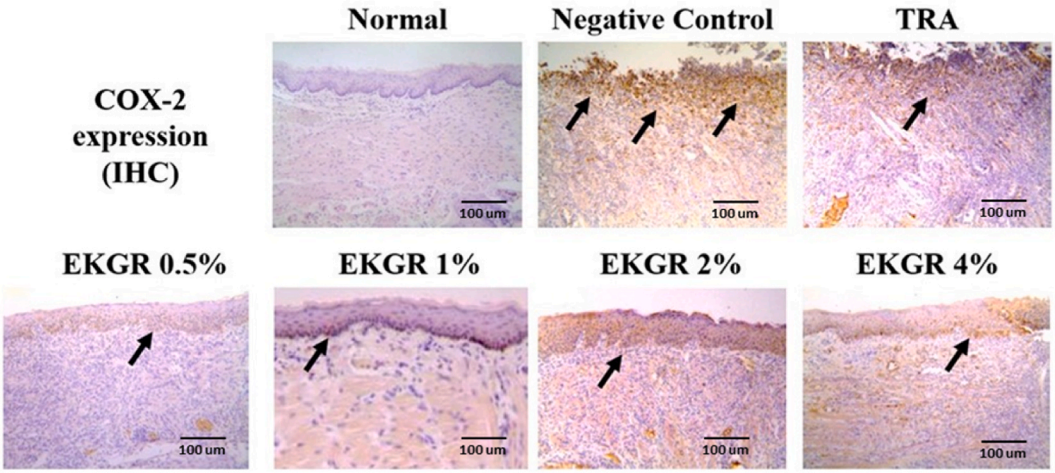

Fig. 4. COX-2 expression between treatment groups using IHC. TRA = triamcinolone acetonide; EKGR = ethanol extract of *K. galanga* L. rhizome; IHC = immunohistochemistry. The expression of COX-2 is demonstrated by a brown color in the cell cytoplasm (black arrow). Magnification = 400×. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

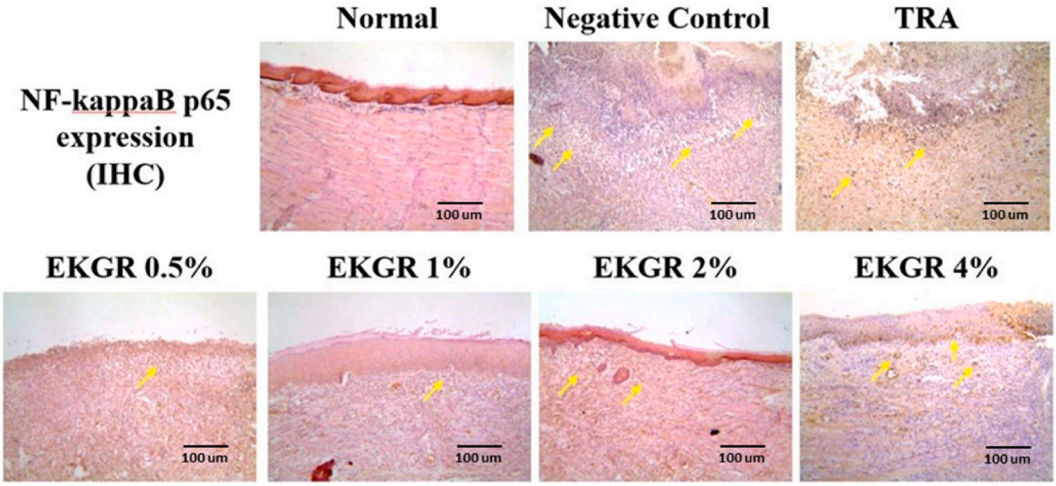

Fig. 5. NF-kappaB-p65 expression between treatment groups using IHC. TRA = triamcinolone acetonide; EKGR = ethanol extract of *K. galanga* L. rhizome; IHC = immunohistochemistry. The expression of NF-kappaB-p65 is demonstrated by a brown color occurs in the cell nucleus (yellow arrow). Magnification = 400×. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.5. EKGR does not induce erythema and edema on the dorsal skin of male albino rabbits

EKGR was proven safe as its application on the dorsal skin of male albino rabbits for 72 h does not induce erythema and edema (Draize skin irritation total scores at each point = 0 and the primary irritation index = 0, which are similar to those of the control). EKGR shows no difference in the intensity of redness compared to control, measured using the ImageJ® software (Table 2). Fig. 9 explains the comparison of rabbit skin redness as a response to acute dermal irritation from the administration of the 3 concentrations of the extract being tested, observed at scheduled times.

3.6. Single-dose EKGR does not alter the body weight and rectal temperature of male albino rabbits

EKGR was proven safe as its application on the dorsal skin of male albino rabbits does not alter the body weight and rectal temperature of male albino rabbits for 14 days (Table 3).

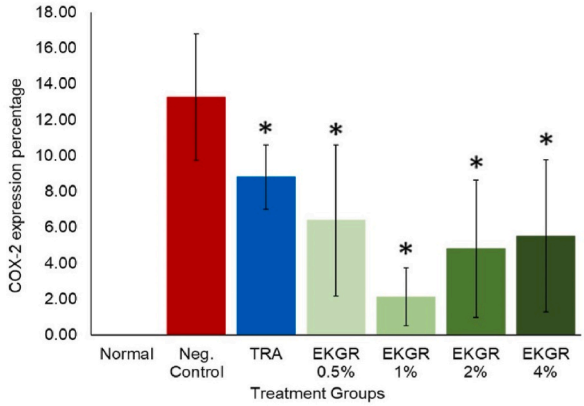

Fig. 6. COX-2 expression percentage using immunohistochemistry. The asterisk symbol (*) indicated $p < 0.05$ (significantly different compared with the negative control, calculated by One-way ANOVA followed by the post hoc Bonferroni test).

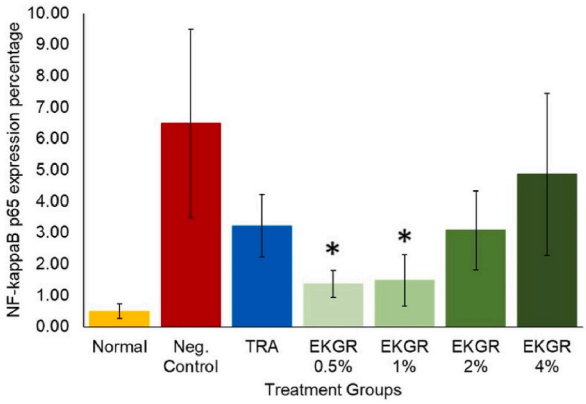

Fig. 7. NF-kappaB-p65 expression percentage using immunohistochemistry. The asterisk symbol (*) indicated $p < 0.05$ (significantly different compared with the negative control, calculated by One-way ANOVA followed by the post hoc Bonferroni test).

3.7. Single-dose EKGR does not alter the relative organ weight of male albino rabbits

EKGR was proven safe as its application on the dorsal skin of male albino rabbits does not alter the relative organ weight of male albino rabbits for 14 days (Table 4).

3.8. Single-dose EKGR does not alter the hematology parameters of male albino rabbits

EKGR was proven safe as its application on the dorsal skin of male albino rabbits does not alter the hematology parameters of male albino rabbits after 14 days (Table 5).

3.9. Single-dose application of EKGR causes mild irritation in the skin of the male albino rabbit

EKGR was proven safe as its application on the dorsal skin of male albino rabbits does not induce erythema or edema after 14 days, however, a mild irritation as indicated by the infiltration of inflammatory cells, is observed. Fig. 10a–b showed the histopathology of dorsal skin tissue of male albino rabbits in the normal control group, meanwhile, Fig. 10c–d showed a mild expression of inflammatory cells in the group of rabbits that received single-dose EKGR (black arrows indicating the inflammatory cells).

4. Discussion

In this study, the ethanol extract of the rhizome of *K. galanga* (Zingiberaceae) has been proven to consist of ethyl p-methoxy cinnamate (EPMC) as a major constituent in the RP-HPLC chromatogram. Likewise, the existence of EPMC was described by former studies [10,13,14,28,31–35]. The main secondary metabolites in the extract are EPMC (30 %), ethyl cinnamate (25 %), p-methoxy cinnamic acid, monoterpenes ketone, and 3-carene-5-one [36].

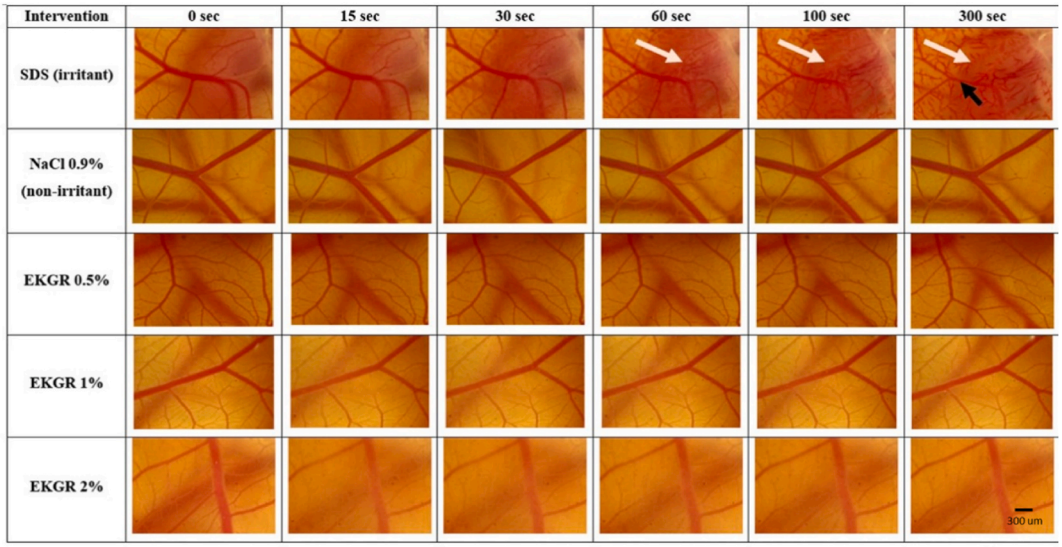

Fig. 8. HET-CAM test results illustrate the sequence effect of SDS (irritant), 0.9 % NaCl (saline) solution, and EKGR 0.5–2% on the chorioallantoic membrane over 300 s. Irritation responses are observed in the CAM exposed to SDS irritants, as shown by the occurrence of hemorrhagic (white arrow starting at 60 s) and vasoconstriction (black arrow starting at 300 s). EKGR = ethanol extract of *K. galanga* L. rhizome; SDS = sodium dodecyl sulfate.

Table 2
The effect of EKGR on the intensity of redness on the dorsal skin of male albino rabbits.

Concentration of EKGR	Replication	The intensity of rabbit skin redness (pixel)				
		Control	1 h	24 h	48 h	72 h
0.25 %	1	145.152	145.147	145.144	145.150	145.151
	2	148.552	148.550	148.552	148.548	148.547
	3	147.671	147.663	147.668	147.664	147.668
	Mean ± SD	147.125 ± 1.76	147.12 ± 1.77	147.121 ± 1.77	147.121 ± 1.76	147.122 ± 1.76
0.5 %	1	148.596	148.589	148.585	148.584	148.590
	2	148.725	148.720	148.725	148.721	148.722
	3	145.741	145.739	145.738	145.741	145.740
	Mean ± SD	147.687 ± 1.69	147.683 ± 1.68	147.683 ± 1.69	147.682 ± 1.68	147.684 ± 1.68
1.0 %	1	150.890	150.881	150.886	150.887	150.881
	2	147.546	147.546	147.542	147.540	147.537
	3	145.343	145.340	145.334	145.330	145.337
	Mean ± SD	147.926 ± 2.79	147.922 ± 2.79	147.921 ± 2.80	147.919 ± 2.80	147.918 ± 2.79

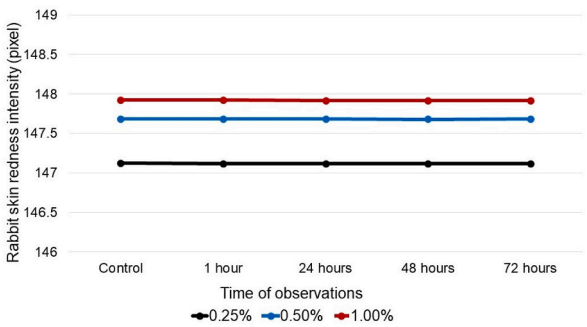

Fig. 9. This graph shows that the administration of each EKGR concentration did not cause an increase in the intensity of rabbit skin redness in line with changes in time, but it appears to be in line with an increase in the EKGR concentration tested.

Table 3
The effect of EKGR on the body weight and rectal temperature of male albino rabbits.

	Repetition	Day 1		Day 7		Day 14	
		BW (g)	RT (°C)	BW (g)	RT (°C)	BW (g)	RT (°C)
Control	1	2704	39.5	2730	38	2694	38.6
	2	2738	39	2797	38.7	2780	38.8
	Mean	2721	39.25	2763.5	38.35	2737	38.7
	± SD	± 24.04	± 0.35	± 47.38	± 0.49	± 60.81	± 0.14
EKGR 200 mg/kg BW	1	2931	38.4	2992	38.8	3046	38.1
	2	2992	39	3010	39.4	3074	39
	Mean	2961.5 ± 43.13	38.7	3001	39.1	3060	38.55
	± SD		± 0.42	± 12.73	± 0.42	± 19.80	± 0.64

Table 4
The effect of EKGR on the relative organ weight of male albino rabbits.

Organ	% relative organ weight ±SD			
	Control		EKGR 200 mg/kg BW	
	Day 1	Day 14	Day 1	Day 14
Liver	3.2615 ± 0.147 p = 0.655	3.2384 ± 0.108	2.9435 ± 0.456 p = 0.180	2.8597 ± 0.451
Spleen	0.0579 ± 0.008 p = 0.655	0.0575 ± 0.008	0.0459 ± 0.024 p = 0.180	0.0445 ± 0.023
Heart	0.3085 ± 0.065 p = 0,655	0.3068 ± 0.068	0.2212 ± 0.036 p = 0.180	0.2148 ± 0.034
Kidney	0.6209 ± 0.003 p = 0.655	0.6167 ± 0.004	0.5935 ± 0.047 p = 0.180	0.5764 ± 0.044
Lung	0.5161 ± 0.023 p = 0.655	0.5124 ± 0.017	0.4616 ± 0.008 p = 0.180	0.4484 ± 0.006

Statistical analysis was carried out using the Wilcoxon Signed Rank Test. Significance was indicated by $p < 0.05$.

Table 5
The effect of EKGR on the hematology parameters of male albino rabbits.

Hematology Parameter	Result		Unit	Normal range
	Control	EKGR		
Leucocyte	10.46	6.83	10^9/l	3.0–11.5
Lymphocyte	5.43	6.08	10^9/l	2.0–9.1
Monocyte	0.26	0.29	10^9/l	0.0–0.5
Erythrocyte	7.61	7.22	10^{12}/l	5.0–9.0
Hemoglobin	14.1	13.45	g/dl	12.7–16.3
MCV	70	67	fl	57.0–70.0
MCH	18.5	18.65	pg	17.5–23.5

There are three main findings of this work, (1) the anti-ulcer activity of EKGR is by inhibiting the expression of COX-2 and NF-kappaB-p65 in the oral mucosa tissue; (2) the best doses of EKGR in inhibiting COX-2 expression are 2 % and 4 %, while the best dose in inhibiting NF-kappaB p65 is 4 %; and (3) EKGR is safe for the vascular network, the skin, the body weight, the relative weight of the organs, the hematology parameters; and the histopathology of animal models. These results coincide with previous studies [35, 37–39].

In our study, EKGR showed inhibitory activity on COX-2 expression but was weaker than that of TRA. TRA, 2 %, and 4 % EKGR showed significant differences compared to the negative control. Moreover, TRA and EKGR indicated an inhibitory activity on NF-kappaB-p65 expression, but only TRA and 4 % of EKGR showed significant differences when compared to the negative control. Based on the data from this study, it can be concluded that the mechanism of action of EKGR as an anti-inflammatory is by inhibiting the expression of COX-2 and NF-kappaB-p65. COX-2 is a protein that works to catalyze prostaglandin synthesis [15], inflammatory mediators that cause pain, therefore if COX-2 can be inhibited then pain as one of the signs of inflammation can be suppressed. Meanwhile, NF-kappaB-p65 is a protein that is responsible for the synthesis of several inflammatory mediators, including the synthesis of COX-2, IL-6, and TNF-alpha [40]. Natural or herbal materials that can inhibit NF-kappaB-p65 expression have the potential to be developed into anti-inflammatory drugs.

It was described that the ethanol extract of *K. galanga* doses of 600 mg/kg and 1200 mg/kg revealed significant anti-inflammatory activity in the carrageenan-induced edema and cotton pellet granuloma model compared to that of the control group [32]. The powder of *K. galanga* rhizome dissolved in dimethylsulfoxide (DMSO), exhibited a strong anti-IL-6 activity with the IC_{50} value of 0.04 ± 0.01

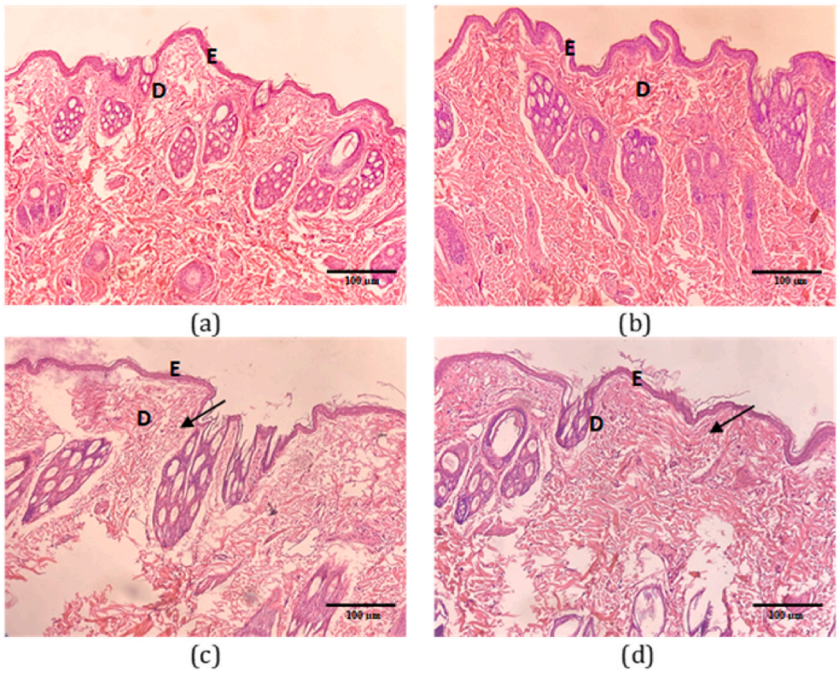

Fig. 10. Histopathology analysis of the dorsal skin tissue of male albino rabbits: (a–b) control; (c–d) single-dose EKGR. There were no inflammatory cells found on the histopathological picture of the dorsal skin tissue of control rabbits, whereas there was a mild expression of inflammatory cells in the group of rabbits that received single-dose EKGR. D = dermis; E = epidermis; black arrows = inflammatory cells. Magnification = 400×.

μg/mL [37]. Moreover, *K. galanga* influenced the expression of COX-2, TNF-α, IL-6, and IL-1 in the arthritic rat model [38]. The extract at doses of 50, 100, and 200 mg/kg administered orally could reduce pain in acetic acid-induced writhing, formalin, hot plate, and tail-flick tests in mice and rats [39]. *K. galanga* extracts from different rhizome types strongly blocked NF-kappaB activity in the 4T1 cell line, a breast cancer cell line, and RAW 264.7 macrophages, without affecting the viability of the cells [40]. However, the anti-inflammatory activity of *K. galanga* cannot be separated from the main contribution of its active constituent, EPMC.

It was proven that EPMC isolated from *K. galanga* could suppress inflammation by significantly inhibiting IL-1 and TNF-α in both *in vivo* and *in vitro* models [21]. In addition, EPMC dose-dependently blocked carrageenan-induced edema rats and non-selectively inhibited the activities of COX-1 and COX-2, with IC_{50} values of 1.12 μM and 0.83 μM, respectively [41]. COX-1 is expressed constitutively in many tissues, particularly in the gastric mucosa, and prostaglandins produced by COX-1 protect the gastric mucosa from the corrosive hydrochloric acid. Prostaglandins also regulate renal blood flow and platelet aggregation. Conversely, COX-2 is rapidly induced by stimuli, e.g., IL-1 and TNF-α, lipopolysaccharides, and growth factors, resulting in the excess production of prostaglandins in inflamed and neoplastic tissues. In summary, COX-2 is implicated in the processes of inflammation [42]. Inflammatory reactions involve the activation of numerous intracellular pathways that connect to the activation of genes that encode the expression of IL-1, TNF-α, COX-2, and other proinflammatory cytokines [43]. By inhibiting proinflammatory gene expression regulated by NF-kappaB, the production of inflammatory mediators and control of excessive inflammation, can be prevented [44].

In our study, the ethanol extract of *K. galanga* does not alter the vascular network, the skin, the body weight, the relative weight of the organs, and the hematology parameters of animal models, which confirms its safety. The safety of *K. galanga* extract was also described by previous studies [17,18]. Both the extract and EPMC exhibited moderate cytotoxic activity against the human CL-6 cell line and were well tolerated up to the maximum single oral dose of 5000 mg/kg and daily dose of 1000 mg/kg for 30 days [18]. The maximum tolerated dose (MTD) of *K. galanga* extract was up to 5000 mg/kg and no death was observed. Hematological analysis showed no difference in any parameter tested between the control and the test groups. No abnormality in histopathology and no irritation in the skin were observed [17].

Furthermore, the administration of EKGR did not irritate the blood vessels of the fertilized egg membrane, in observations up to 300 s. No signs of irritation such as bleeding, lysis, and coagulation of blood vessels were detected. In our study, blood vessel damages were observed based on the width of the primary, secondary, and tertiary blood vessels. The HET-CAM test is used as a test for potential irritation in the development of vaginal or ophthalmic drugs [45,46], while the oral mucosa also has a similar tissue structure and superficial blood vessels under the thin epithelial layer. The membranes of hen's egg-chorioallantoic are described as effectively employed for evaluating the anti-irritant properties of plant extracts [19].

To our knowledge, this is the first study to report the molecular mechanism by which the anti-ulcer activity of *Kaempferia galanga* extract in the oral mucosa of Wistar rats occurs. The underlying anti-ulcer mechanism of EKGR is predicted by disrupting the NF-kappaB signaling pathway, thus resulting in an inhibition of translocation of cytosolic NF-kappaB p65 to the nucleus, hence reducing the expression of NF-kappaB-dependent COX-2. EKGR is also indicated as safe for the vascular network, the skin, the body

weight, the relative weight of the vital organs, and the hematology and histopathology parameters of animal models. However, the different concentrations used in this study are the limitations of the methodologies. We used a lower range of concentrations in the HET-CAM test (0.5 %, 1.0 %, and 2 % EKGR, for the vascular irritation test) and in the rabbits (0.25 %, 0.5 %, and 1.0 %, for acute dermal irritation test), as well as a higher range of doses in rats (0.5 %, 1.0 %, 2.0 %, and 4.0 %) and single-dose in rabbits (pure EKGR 200 mg/kg BW). Despite the limitations, the ethanol extract of *Kaempferia galanga* rhizome could be considered a potential candidate for an anti-oral mucosal ulcer drug, but further clinical studies are needed to verify its efficacy and safety in humans.

5. Conclusion

Kaempferia galanga (Zingiberaceae) extract (EKGR) has confirmed its anti-inflammatory activity by alleviating the expression of proinflammatory cytokines COX-2 and NF-kappaB-p65 in the tongue tissue ulcer of Wistar rats caused by 70 % acetic acid-inducing oral mucosal stomatitis. EKGR is safe as it does not exhibit irritating potential and harmful effects.

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Availability of data and materials

The data generated in the present study may be requested from the first author upon reasonable request.

CRediT authorship contribution statement

Indah Suasani Wahyuni: Writing – original draft, Validation, Supervision, Methodology, Investigation. **Tresnafuty Rasyiida Diina:** Formal analysis. **Annisa Siti Salsabila:** Formal analysis. **Miski Aghnia Khairinisa:** Supervision, Investigation. **Irna Sufiawati:** Supervision, Project administration. **Jutti Levita:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Data curation, Conceptualization. **Aziiz Mardanarian Rosdianto:** Supervision, Methodology. **Wipawee Nittayananta:** Supervision, Methodology.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e35827.

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