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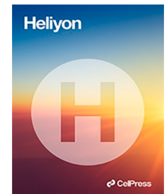

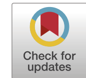

Piper retrofractum ameliorates imiquimod-induced skin inflammation via modulation of TLR4 axis and suppression of NF-κB activity

Subehan Lallo[a,*], Besse Hardianti[b,c], Yulia Yusrini Djabir[a], Ismail Ismail[a], Maulita Indrisari[b], Muhammad Aswad[a], Ai Hertati[d], Habibie Habibie[a], Yoshihiro Hayakawa[c,**]

[a] *Faculty of Pharmacy, Hasanuddin University, Makassar 90245, Indonesia*
[b] *Sekolah Tinggi Ilmu Farmasi Makassar, Makassar 90242, Indonesia*
[c] *Institute of Natural Medicine, University of Toyama, Toyama 930-0194, Japan*
[d] *Pusat Riset Rekayasa Genetika Jl. Raya Jakarta-Bogor No.KM 46, Pakansari, Kec. Cibinong, Kabupaten Bogor, Jawa Barat 16911, Indonesia*

A R T I C L E I N F O

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A B S T R A C T

Chronic inflammation is a significant concern due to its association with various pathological conditions. As a result, extensive research has been conducted to identify new natural products that can effectively treat acute inflammation, which has the potential to inhibit the chronic inflammation. In our study, we aimed to identify Indonesian medicinal plants with the ability to inhibit proinflammatory agents, specifically targeting NF-κB, a crucial regulator of gene transcription involved in the production of proinflammatory proteins/cytokines. Through a series of identification processes, we found that *Piper retrofractum* (Javanese chili) extract demonstrated promising inhibitory effects on NF-κB and proinflammatory molecules.

Further investigation was conducted using a variety of assays, including reporter assay, viability test, ELISA, and Western blotting. The results revealed that the extract significantly reduced LPS, NO, COX-2, IL-6, IL-1, and NF-κB through the TLR4 axis. Notably, *Piper retrofractum* extract was found to enhance the survival of human keratinocytes by protecting them from cell death induced by TRAIL, a member of the TNF superfamily. Moreover, immunohistochemistry analysis in an Imiquimod-induced skin inflammation mice model showed downregulation of COX-2 and IL-1β expression upon treatment with the extract.

In conclusion, our findings suggest that *Piper retrofractum* extract possesses anti-inflammatory properties by reducing proinflammatory cytokine production through inhibition of NF-κB signaling pathway. These promising results highlight the potential of *Piper retrofractum* extract as a candidate for future drug development in the clinical treatment of inflammation-related conditions, offering hope for the advancement of therapeutic interventions.

* Corresponding author.
** Corresponding author.
E-mail addresses: subehan@unhas.ac.id (S. Lallo), haya@inm.u-toyama.ac.jp (Y. Hayakawa).

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1. Introduction

Research on novel drugs with the potential to exert pharmacological effects on pathological conditions is encouraged. Plant-based raw materials continue to play a significant role. The exploration for biologically active compounds from medicinal plants is encouraged due to the potential discovery of numerous compounds that could serve as pharmaceutical drugs. Indonesia is one of the 17 countries with a megadiverse biosphere of floral acuity [1]. Indonesia ranks fifth in plant diversity with over 38,000 species, and approximately 18,700 are endemic, being the highest (>6%) of total global flora. This tropical territory is renowned for its abundance of fascinating traditional medicinal plants [2], including natural herbs belonging to the genus Piper, containing approximately 1000 species. It has been reported that there are three distinct stem types in this genus: creeping, climbing, and branching [3]. Additionally, each plant has a unique leaf color and shape. Apart from pharmacological sectors, indigenous cultures use *Piper* trees as a support material for decoration, cultural celebration, groceries, and beverages. Many reports have been documented that the compounds isolated from *Piper retrofractum* are amides, the secondary metabolite main components of this species, with the majority being unsaturated amides and piperine [3–5]. Fruit extracts containing fibre (28.8%), sugar (63.4%), whole fat (2.97%), ash (4.29%) and crude protein (11.4%), were reported by Ref. [3]. *Piper retrofractum* fruit are also rich in copper, calcium, iron, phosphorus, magnesium, sodium, potassium, and zinc. n-Hexane and ethyl acetate extracts were also reported to contain quinone, sterol, glycosides, alkaloid, and tannin. The fruit methanol extract contains alkaloids and <1% of phenolics [6]. In traditional medicine, *P. retrofractum* fruits are used for their antiflatulent, antioxidant, cough suppressant, antitussive, antimicrobial, and appetizing properties [7,8]. Piperine is the most effective adjuvant for optimizing the effectiveness of TRAIL-induced apoptosis in triple negative breast cancer cells via suppression of cells survival as well as p65 phosphorylation [9]. There has been numerous reports on the extract's benefits; however, researchers believe it is critical to ascertain the significance of the *Piper retrofractum* cultivated in Indonesia and investigate the possibility of suppressing inflammation via downregulation of the NF-κB signaling pathways. NF-κB obtains information flowing from the microenvironment signaling configurations, which downstream effectors then read to regulate cellular behavior [10]. This gene's product specifically and rapidly regulates many proteins and has a critical role in immune responses. Therefore, NF-κB regulates inflammatory progression and many pathological conditions, including cancer [11]. It has been reported that the NF-κB family has a crucial role in expressing proinflammatory genes like inducible nitric oxide synthase (iNOS), interleukin-6 (IL-6), cyclooxygenase-2 (COX-2) and interleukin-1β (IL-1β) [12–14]. Additionally, toll-like receptor (TLR) has become one of the promising targets for treating inflammatory diseases [15,16]. Extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 (mitogen-activated protein kinase [MAPK] signaling pathways) are important for the transcriptional regulation or translocation of p65 NF-κB subunit [17,18]. Thus, the nuclear translocation of NF-κB is a sequence of inflammatory reaction that may be manipulated to develop anti-inflammatory drugs. Consequently, this study was aimed to identify plants that can inhibit the production of proinflammatory factors. Various plants were selected for investigation based on traditional medicine used by healers.

2. Materials and methods

2.1. Extraction of the plants

The plants were harvested in the province of South Sulawesi, Indonesia. All plants were identified and voucher specimens were deposited in Laboratory of Pharmacognosy, Faculty of Pharmacy, Hasanuddin University. Ethanol (70%) was used to macerated the dried leaves or rhizomes. After evaporating and lyophilizing the liquid extracts, an EtOH extract was obtained (Table 1). Piperine content in *Piper retrofractum* extract (PRE) was determined using a TLC densitometer (Camag TLC Scanner 3, Muttenz, Switzerland) with a TLC plate using EtoAc:Hexane: (2:8) as a mobile phase and silica gel GF_{254} as a stationary phase. GC–MS was used with a GC-MS

Table 1
List of medicinal plants.

No.	Latin Name	Plant Part	Family	Local Name
1.	*Alpinia galanga* L.	Rhizome	Zingiberaceae	Lengkuas
2.	*Curcuma zanthorrhiza* Roxb	Rhizome	Zingiberaceae	Temulawak
3.	*Zingiber officinale* Roscoe	Rhizome	Zingiberaceae	Lengkuas
4.	*Andrograpis paniculata* (Burm.f) Nees	Stem	Acantaceae	Sambiloto
5.	*Piper retrofractum* Vahl.	Fruit	Piperaceae	Cabe Jawa
6.	*Zingiber zerumbet* (L.) Roscoe ex Sm.	Rhizome	Zingiberaceae	Lempuyang
7.	*Smallanthus sonchifolius* (Poepp.) H.Rob	Leaf	Compositae	Insulin
8.	*Myristica fragrans* Houtt	Seed	Myristicaceae	Pala
9.	*Pimpinella anisum* L.	Seed	Apiaceae	Adas Manis
10.	*Physalis angulata* L.	Stem	Solanaceae	Leppo-leppo
11.	*Lannea coromandelica* (Houtt.) Merr.	Leaf	Anacardiaceae	Aju Jawa
12.	*Tinospora sinensis* (Lour.) Merr.	Stem	Menispermaceae	Brotowali
13.	*Spatholobus littoralis* Hassk.	Stem	Leguminosae	Aju Lampeng
14.	*Terminalia catappa*	Leaf	Combretaceae	Katapang
15.	*Zingiber montanum* (J.Koenig) Link ex A.Dietr.	Rhizome	Zingiberaceae	Panini
16.	*Murraya paniculata* (L.) Jack	Stem	Rutaceae	Aju Wellang
17.	*Murraya paniculata* (L.) Jack	Leaf	Rutaceae	Aju Wellang
18.	*Lansium parasiticum* (Osbeck) K.C. Sahni and Bennet	Bark	Meliaceae	Tebba lasse

QP-2010 plus Autosampler AOC-20i (Shimadzu, Japan). We used an SH-Rxi-5Sil MS capillary column (30 cm in length and 0.25 mm in diameter). The temperature of the column was set as follow: 2 min at 70 °C, then 280 °C at 10 °C/min and then temperature was hold for 9 min at 280 °C. Split injector was ran at 250 °C with the split ratio at 1:10. As the carrier gas, high purity Helium was used with a flow rate of 14 mL/min. The electron-impact mode of the ion source was used with ionization energy at 70 eV.

2.2. *Cells and reagents*

RAW264.7 cells (ATCC, USA) and RAW264.7-NFκB-Luc2 cells [19,20] were maintained in Eagle's Minimal Essential Medium (EMEM, Nissui Seiyaku, Tokyo, Japan) supplemented with 10% FBS (Nichirei Biosciences, Tokyo, Japan), 100 mg/mL of streptomycin, 100 units/mL of penicillin, 1 mM/L-glutamine (Life Technologies, MD, USA), and 0.2% $NaHCO_3$. Cells were incubated at 37 °C with CO_2 (5%).

2.3. *Cells viability*

The viability of the cells was determined according to Ref. [21]. Briefly, 2×10^4 cells/well of RAW264.7 cells or RAW264.7--NFκB-Luc2 cells were plated in a 96 well plate and incubated for 24 h. The cells were treated with 50 μM of compound or 50 μg/mL of extract followed by 24 h incubation. WST-8 reagent (10 μl/well, Wako Pure Chemical Corporation, Osaka, Japan) was pipetted to each well followed by 1 h incubation with CO_2 (5%) at 37 °C. The absorbance was determined using a microplate reader (Sunrise™; Männedorf, Switzerland) at 450/620 nm wavelengths. The viability of the cells was showed as a percentage of the viability of control cell [20].

2.4. *Analyses of nitrite*

RAW264.7 cells (1×10^5 cells per well) were plated in a 24 well plate and cultured overnight with CO_2 (5%) at 37 °C. PRE (50 μg/mL) and isolated piperine from *Piper retrofractum* (50 μM) was administered to the cells for 1 h prior to stimulation with TLR ligands LPS (100 ng/mL). To measure the concentration of NO_2^-, supernatants from RAW264.7 macrophage cells cultures were collected 3 h after stimulation of lipopolysaccharide (LPS). A total of 50 μl of each were pipetted in duplicate in a 96-well microplate. Additionally, we used a standard solution based on the protocol described in Technical Bulletin #TB229 Kit Promega Griess Reagent System cat. no. G2930. Additionally, 5 μl/well was added to both the standards and the samples on the microplate, followed by mixing for approximately 10 s before it was measured using a microplate-reader (Sunrise™; Männedorf, Switzerland). The NO_2^- concentration was determined using the standards' calibration curve.

2.5. *NF-κB reporter gene assay*

RAW264.7-NFκB-Luc2 cells (2×10^4 cells per well) were cultured for 24 h at 37 °C with CO_2 (5%) in a 96-well plate. Subsequently, the extracts or vehicle were added to the cells and cultured for another 24 h. After 24 h, 900 μg/mL of D-luciferin was pipetted to the well followed by 30 min incubation. Luminescence of firefly luciferase was used to determine the luciferase activity. IVIS LUMINA II and Living Image 4.2 software (Caliper Life Science) were used to measure the luciferase activity via determination of the light that the cells released. Luciferase activity was demonstrated as photons/seconds, and the formula (extracts total flux divided by vehicle total flux) was used to quantified the fold of NFκB activity [20,21].

2.6. *Measurement of cytokines*

RAW264.7 cells (10^5 cells) were cultured in a 24 well plate followed by overnight incubation at 37 °C with CO_2 (5%). One hour before stimulation with 100 ng/mL LPS (TLR ligands), PRE (50 μg/mL) and 50 μM piperine compound were administered to the cells. After 24 h, culture media were harvested and stored at −80 °C until further analysis. The concentration of IL-6 was measured using ELISA reagent (BioLegend) based on the manufacturer's instructions [22].

2.7. *Real-time PCR*

Total RNA of RAW264.7 cells was isolated by employing the Qiagen RNeasy Plus Mini Kit (Hilden, Germany). ABI Prism 7300 (Life Technologies, Carlsbad, USA) was used to determine the expression of IL-1β mRNA and normalized to the mRNA of GAPDH.

The primers are as follow:
IL-1β mRNA:
(forward) 5′- CTGGAGCCCA CCAAGAACGA-3′
(reverse) 5′- GCCTCCGACT TGTGAAGTGGT-3.
GAPDH mRNA:
(forward) 5′- CAC CTC ACA CGA GGC ACA AG-3′;
(reverse) 5′-GCA GCA ACA GCA TCA GAG ACA-3′ [23].

2.8. Western blotting

RAW264.7 and RAW264.7 NFκB-Luc Cells (1×10^6 cells/well) were cultured for 12 h with tested samples and PRE (50 μg/mL) after 1 h challenged with or without LPS (100 ng/mL) as well as with or without 20 μM Imiquimod (IMQ). Cells were harvested in PBS followed by centrifugation for 10 min at 14.000 rpm in 4 °C. Cell lysates were collected in lysis buffer (0.3 M NaCl, 25 mM HEPES pH 7.7, 10 mg/mL of leupeptin, 1.5 mM $MgCl_2$, 1 mM phenyl-methylsulfonyl fluoride, 0.2 mM EDTA, 20 mM β-glycerophosphate, 0.1% TritonX-100, 1 mM sodium orthovanadate, 1 mM dithiothreitol and 10 mg/mL of aprotinin). Cell lysates were loaded for proteins separation onto 10% SDS-PAGE. Furthermore, proteins were shifted to Immobilon-P nylon membranes (Millipore, USA). Block Ace was used to blocked the membranes (Dainippon Pharmaceutical, Japan) for 2 h before incubated with primary antibodies against p65 (#6956, Cell Signaling, USA), COX-2 antibody #4842 (Cell Signaling, USA), ERK1/2 sc-514302 -C-9, (Santa Cruz, USA), TLR4 sc-293072 (Santa Cruz, USA), and β-actin (C4, sc-47778, Santa Cruz, USA) overnight at 4 °C, followed by HRP-conjugated secondary antibodies incubation (1:1000) at room temperature. The bands were visualized using ECL reagents (Amersham Biosciences, USA) [21].

2.9. Animal model of psoriasis-like skin inflammation induced with IMQ

Balb/c mice (7–8 weeks old, Male, 18–19 g) were bought from the Agriculture, Food, Marine and Fisheries Department of Bantul, Indonesia. Use and Care of Laboratory Animals guidelines no. 11766/UN.14.1/TP.February 01, 2021 were followed for all experiments.

A modified version of previously published psoriasis model [24] was used in this study. For 7 days, mice received a topical application of PRE (10 g in 100 mL of dimethyl sulfoxide, DMSO), Daivobet® gel (LEO Laboratories Ltd, Dublin 12, Ireland) or vehicle on the right part of the ear 3 h prior to the administration of 20 mg of 5% IMQ cream (Aldara; iNova Pharmaceuticals Pte Ltd (Singapore), Selangor, Malaysia). Three hours after IMQ application, the thickness of the ear was determined. Daivobet® gel contains

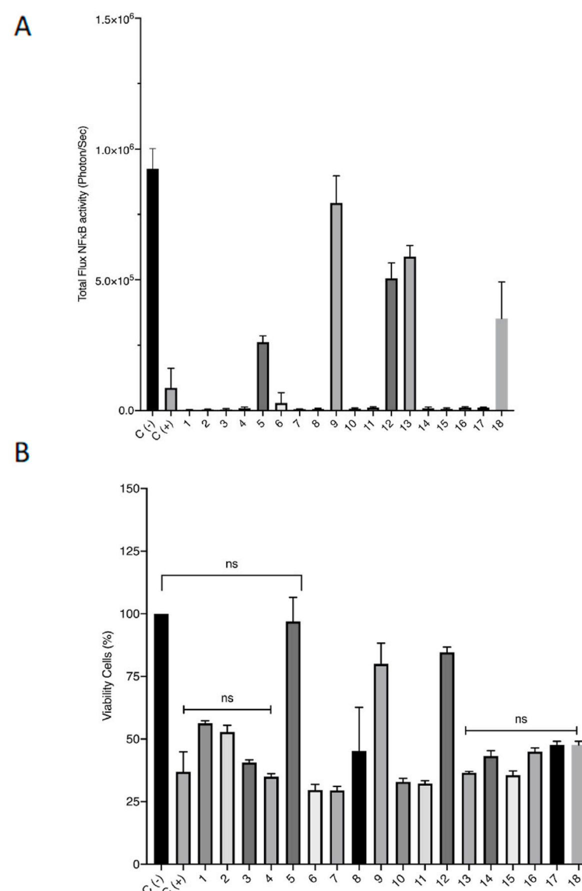

Fig. 1. Effect of Indonesian plants extracts on NF-κB activity (A) and cell viability of RAW264.7- NFκB luc (B) cells. RAW264.7-NFκB luc cells were cultured for 24 h with plant extracts (50 μg/mL). Cell viability and NF-κB activity were determined using WST-8 and Luciferin Reporter Assay. Cell viability and NF-κB activity are shown as the percentage of viability observed in untreated cells (Bonferroni post hoc test; ns: nonsignificant differences).

20 mg of calcipotriol/betamethasone and used as a positive control.

2.10. Immunohistochemistry (IHC)

Microtome was used to obtain thin sections of ear tissue from Balb/c mice. Each tissue section was transferred to gelatin-coated slides, then deparaffinized and rehydrated. As previously stated, the preparate was first rehydrated with 100%, 95% and 70% of ethanol respectively for 2 min before rehydrated with water for 1 min. Furthermore, it was left to soak in peroxidase blocking solution for 10 min at 25 °C. The preparate were later blocked in a prediluted blocking serum for 10 min at 25 °C. Hereafter, primary antibody against COX-2 or IL-1β (1:400, Santa Cruz, Sao Paulo, Brazil) were applied overnight at 4 °C. Afterward, the preparate were washed with phosphate-buffered saline (PBS) for 5 min and further incubated with a streptavidin-HRP-conjugated secondary antibody (Biocare Medical, USA) for 10 min at 25 °C, followed by a 5 min wash with PBS to remove any remaining secondary antibodies. The next step involved incubating the preparate with the chromogen diaminobenzidine (DAB) at 25 °C for 10 min, then incubating the preparate with hematoxylin and eosin for 3 min before being washed with running water. The preparate were cleaned, dripped with mounting media, and covered with a cover glass to complete the process. The positive staining of COX-2 and interleukin-1β was calculated using a light microscope Olympus CX 31 with 400× magnification and five fields of view; the results were reported afterward.

2.11. Statistical analysis

Data were calculated using a one-way analysis of variance continued by a Bonferroni correction test. SPSS version 25 software (IBM Corp, USA) was employed to analyze the data. $P < 0.05$ are considered as significantly differences.

3. Results

3.1. NF-κB inhibition and viability cell against RAW264.7-NFκB luc cell line by medicinal plants extract

Medicinal plants were selected based on information provided by traditional healers from several tribes (Table 1). Several collected plant extracts have shown to suppress the NF-κB activity and the viability of RAW264.7-NFκB luc cells (Fig. 1A and B).

3.2. Identification of piperine, NF-κB inhibition and viability cell against RAW264.7-NFκB luc cells by *Piper retrofractum* extract (PRE)

The used *Piper retrofractum* extract (PRE) was characterized by identifying piperine content (the main compound commonly reported from this plant). Analysis with the densitometry method revealed 13% of piperine and GC-MS spectra analysis identified the presence of 5.2% of piperine (supplemental information 1) among the other compounds. The RAW264.7-NFκB luc cells were cultured

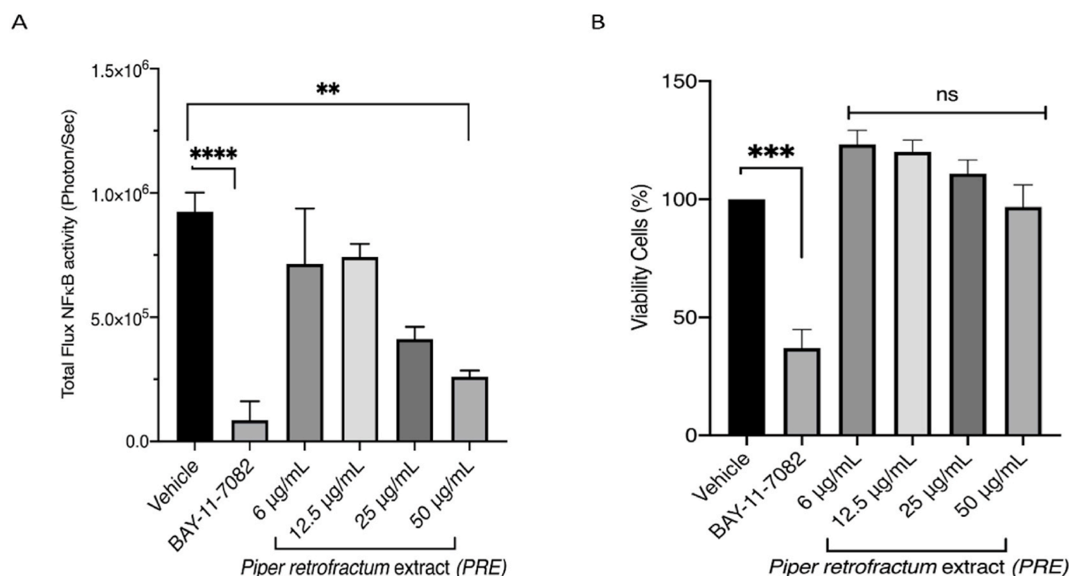

Fig. 2. Effect of NF-κB *Piper retrofractum* extract (PRE) on NF-κB activity (A) and cell viability (B) of RAW264.7-NFκB luc cells. RAW264.7-NFκB luc cells were cultured for 24 h with or without treating with PRE at a concentration of 6, 12.5, 25, and 50 μg/mL, and cell viability was determined using WST-8 and Luciferin Reporter Assay. NF-κB activity and cell viability are expressed as a percentage of viability observed in untreated cells (Bonferroni post hoc test; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$). ns: nonsignificant differences.

with different concentrations of PRE. NF-κB activity decreased significantly in transfected cells treated with extracts, as well as in the cells that survived the test; cell viability decreases in a concentration dependent manner by PRE (Fig. 2A and B). in the other hand, the IκB kinase (IKK) inhibitor (BAY-11-7082) with the 10 μM concentration is quite toxic to the cell. Furthermore, a strong reduction to NF-κB activity is comparable to piperine, the main compound of *Piper retrofractum*.

3.3. NF-κB inhibition and NO production on RAW264.7-NFκB luc cell line

The PRE has the same potent inhibitory effect on lipopolysaccharides-induced NF-κB activity as the piperine inhibitory effect (Fig. 3A). Numerous studies have shown that increased nitric oxide (NO) causes cell damage and inflammation. In this study, we used the Griess Reagent system to assess the role of lipopolysaccharides to the production of NO in RAW264.7 cells and whether PRE can reduce lipopolysaccharides-induced NO production. Our results showed that the extract reduces NO production (Fig. 3B).

3.4. IL-6 and IL-1β inhibition RAW264.7 cell line

Our study demonstrated that the proinflammatory cytokines IL-6 and IL-1β has a crucial role for immune cells activation. The ELISA method was performed to measure IL-6 production, and IL-1β was measured by RT-PCR.

Our data demonstrated that LPS administration induced IL-6 in RAW 264.7 cells, whereas extract treatment decreased. LPS was responsible for an increase in IL-6 production, which was strongly reduced by the administration of PRE (Fig. 4A). The generation of IL-1β also decreased in IL-1β mRNA before and after LPS induction using the RT-PCR technique (Fig. 4B).

3.5. Molecular mechanism of PRE on RAW264.7 cell line

To investigate the mechanism of PRE and its inhibition of the COX-2 and TLR4 expressions, RAW 264.7 cells were cultured with LPS and imiquimod (IMQ) and investigated the influence of PRE to COX-2 and TLR4 by Western blot analysis. We assumed that the PRE activity was related to the NF-κB protein level and the effects of PRE on ERK1/2, an upstream protein of NF-κB. Our results indicated that PRE reduced ERK1/2 overexpression, whereas LPS and IMQ did not affect ERK1/2 overexpression. Moreover, treatment of LPS or IMQ reduced NF-κB protein expression. We hypothesized that PRE inhibits LPS binding to TLR4, thus inhibiting NF-κB activation via ERK1/2 inhibition (Fig. 5).

3.6. Viability cell human keratinocytes (HaCaT) cell Line against TRAIL and PRE

The viability of human keratinocytes (HaCaT cells) was investigated and showed that PRE could protect cells against rTRAIL, a member of the TNF superfamily of proinflammatory cytokines. Similarly, the protective effect on normal skin cells was observed morphologically (Fig. 6).

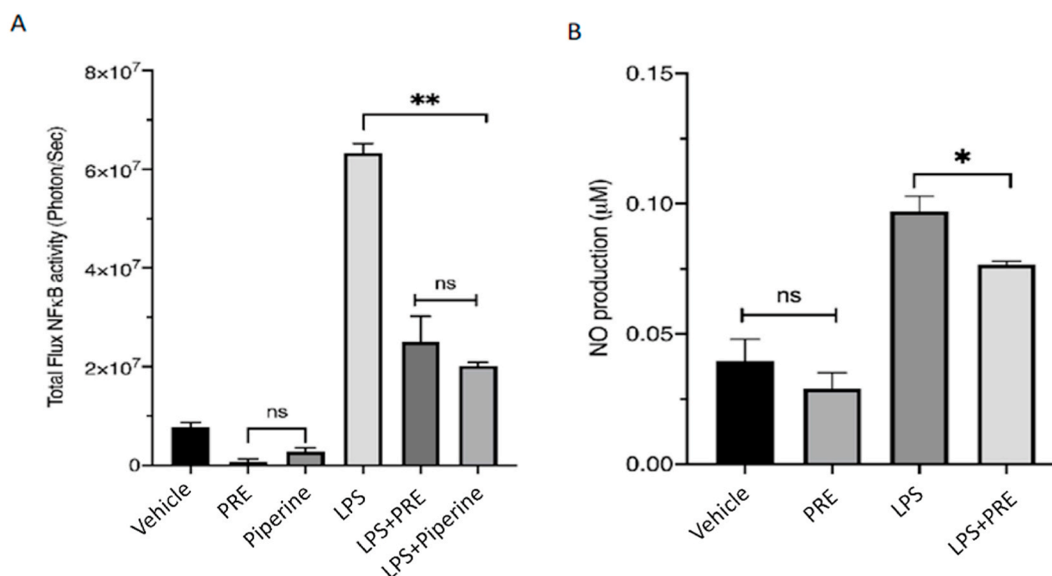

Fig. 3. Inhibitory activity of NF-κB on RAW264.7-NFκB Luc cell line (A) and NO production of RAW 264.7 (B) were cultured for 24 h, induced with and without LPS (100 ng/mL) and pre-treatment 30 min with PRE. NF-κB activity and NO production were expressed as normalized by untreated cells (Bonferroni post hoc test; *$p < 0.05$, **$p < 0.01$). ns: nonsignificant differences.

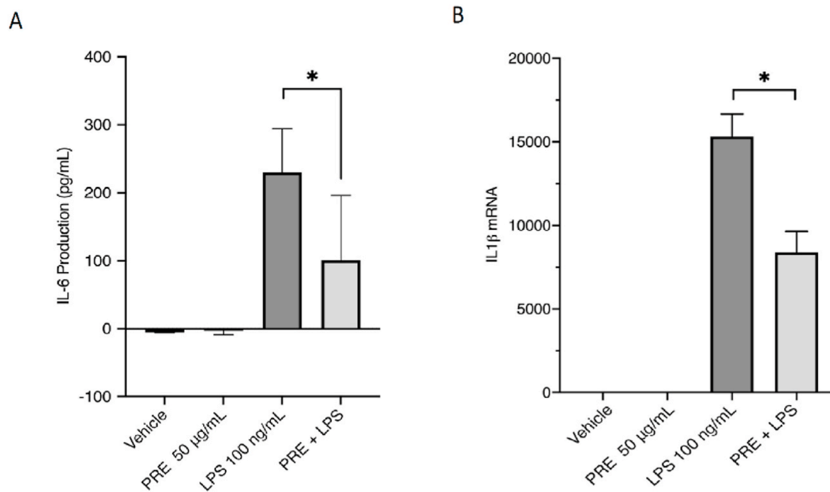

Fig. 4. (A) RAW264.7 were cultured for 24 h, induced with and without 100 ng/mL LPS pre-treatment 30 min with PRE. IL-6 production was measured from the supernatant of cells with an ELISA assay. (B). IL-1β mRNA was measured by RT-PCR. The results are means SEM. IL-6 and IL-1β mRNA, standardized to the untreated controls and displayed as the mean standard error of the mean; n = 3. (Bonferroni post hoc test; $^*p < 0.05$). ns: nonsignificant differences.

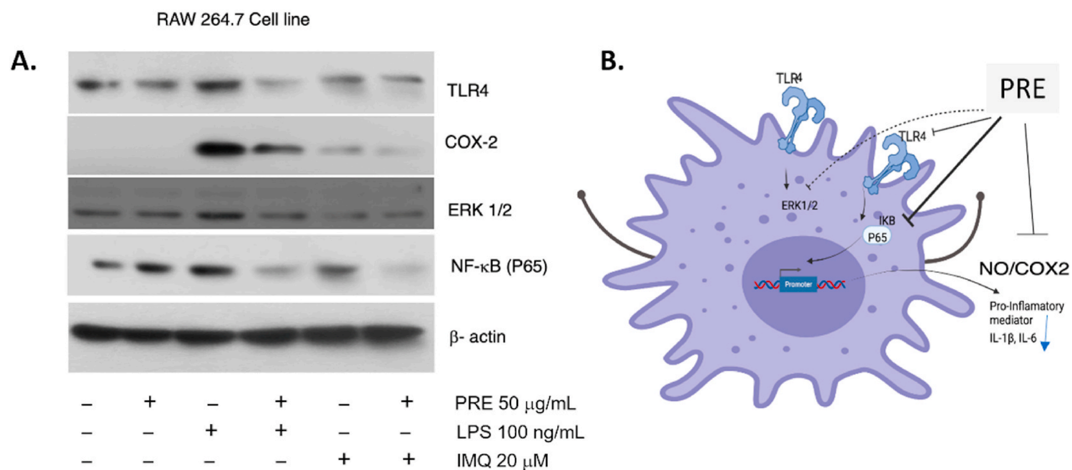

Fig. 5. (A) RAW264.7 were cultured for 24 h, induced with and without 100 ng/mL LPS or imiquimod (IMQ) 20 μM prior to pre-treatment 30 min with PRE. Protein level of cell lysates was identified by Western blot analysis on TLR4, COX-2, ERK1/2, and p65 expression. (B) The role of PRE on macrophage RAW264.7 cell line.

3.7. *In vivo assay of the ear edema model*

Further investigation was confirmed by an in vivo assay. Imiquimod (IMQ) induced psoriasis-like skin by stimulating innate and acquired immune responses, resulting in the infiltration of inflammatory cells into the drug application area, followed by the death of the diseased tissue. Balb/C mice were treated by daivobet® as a positive control compared to PRE at a concentration of 10%. Edema of ear thickness was measured (Fig. 7A), and the expression of COX-2 (Fig. 7B), as well as IL-1β (Fig. 7C), were identified by immunohistochemistry testing.

4. Discussion

Inflammation is an important event in the immune responses to tissue damage or an invading pathogen. This response aids in tissue repair, turnover, and adaptability. However, aging may impair acute inflammatory action to pathogen invasion and increase the risk of infection [25]. Because of that, acute inflammation must be resolved before chronic inflammation begins.

Chronic inflammation shares many characteristics with acute inflammation but is typically milder and more persistent, resulting in degenerative tissue changes. Chronic inflammation can be caused by different mechanisms [22], such as infiltrating leukocytes

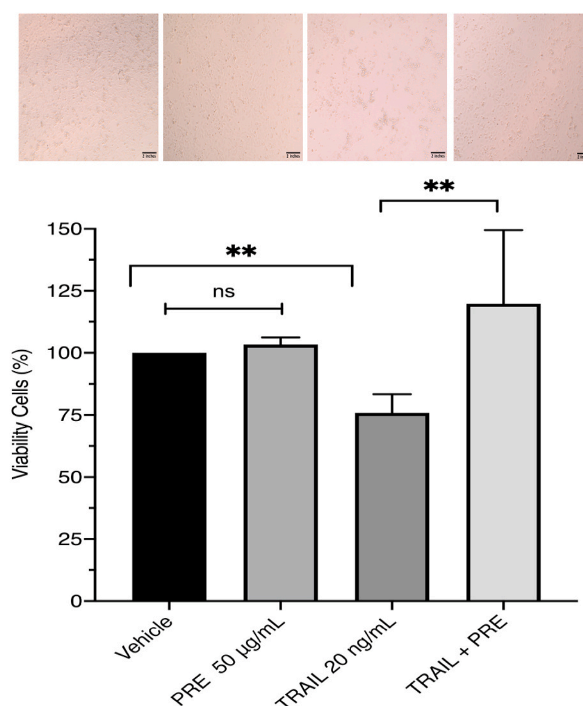

Fig. 6. Anti-apoptotic activity of *Piper retrofractum* (PRE) on human keratinocytes (HaCaT) cells. HaCaT cells were co-cultured with rTRAIL (20 ng/mL) with or without extract at the indicated concentration. After 24 h incubation, viability cell was measured by WST-8. Viability cell are standardized to the untreated controls and displayed as the mean standard error of the mean; n = 3. (Bonferronni post hoc test; *$p < 0.05$; **$p < 0.01$). ns: non significant differences.

producing reactive molecules including NO_2-, IL-6, or TNFα, which are necessary for pathogen elimination but eventually damage the structural and cellular features of tissues. It also can damage non-immune cells and modify the phenotypes of neighboring cells [26].

Genes involved in inflammation, such as proinflammatory interleukins, chemokines, cytokines, and adhesion molecules, are controlled by NF-κB, which acts as a master transcriptional regulator. Proinflammatory, stress, infection, and cell senescence transmission are activation factors [27,28]. Among the plant extract, *Piper retrofractum* (Javanese chili) showed potential as an anti-inflammatory drug via suppression of the well-characterized NF-κB pathway (Fig. 1A and B). Further characterization of the extract was performed to ensure its reproducibility. The main compound from the Piperaceae family is the piperamide-type of compound. Among the piperamides, piperine is mainly collected from *P. retrofractum, P. nigrum*, and *P. tuberculatum.* Piperine is used as a standard to measure solvent extraction efficiency [29]. GC-MS analysis showed that the PRE contained 5.2% of piperine, and further analysis with densitometry indicated that the piperine content was 13% in the 70% ethanolic extract. Other studies revealed the percentage of piperine from *P. retrofractum* as 6–8% from the fruit ethanol extract [30]. Piperine has also been reported from *P. retrofractum* as decreasing with the increasing maturity of the fruits [8].

Further investigation into the inhibitory effects of *Piper retrofractum* extract (PRE) using immune cells RAW264.7-NFκB-Luc and parental RAW264.7 cells as an in vitro model showed that this extract decreased NF-κB, but its immune cells were saved from death. Furthermore, we found that the extracts significantly decreased the activity of NF-κB, but secured cell survival. On the other hand, the positive control BAY-11-7082 (an IKK inhibitor) was quite toxic at 10 μM (Fig. 2A and B). LPS has been widely recognized as a TLR4 receptor ligand (antigen from *E. coli*) and has been shown to activate NF-κB. Thus, in this experiment, we attempted to induce RAW 264.7 NFκB-Luc cells using Lipopolysaccharides in order to observe the effect when treated with and without PRE, 1 h prior to LPS administration. A significant decrease in NF-κB activity was observed, comparable to the piperine, a well-known compound found in this plant. This finding demonstrates that the extract has the same potent inhibitory effect on NF-κB activity induced by LPS as the piperine itself (Fig. 3A). Numerous reports have indicated that increased NO_2^- has a detrimental effect on cell damage and the development of inflammatory conditions. Therefore, we conducted as a test using the Griess Reagent system to evaluate the effects of LPS on NO_2^- secretion in RAW264.7 cells to determine whether treatment with the extract could reduce NO_2^- production induced by LPS. We discovered that the extract could reduce NO_2^- production (Fig. 3B). IL-6 and IL-1β have a crucial role in activating immune cells and triggering persistent inflammation. Both of these cytokines are involved in tissue inflammation. Further investigation was conducted on the effects of LPS administration on IL-6 using the ELISA method to determine IL-6 secretion in RAW 264.7 cells. Our results revealed that LPS was responsible for an increase in IL-6 secretion but it was significantly downregulated by PRE administration (Fig. 4A). We found the same pattern on IL-1β gene expression, where we observed that PRE significantly suppressed the effect of LPS to induce IL-1β mRNA expression (Fig. 4B).

Modulation in the expression of cyclooxygenase-2 (COX-2) followed by an increase production of prostaglandin E 2 (PGE 2) are

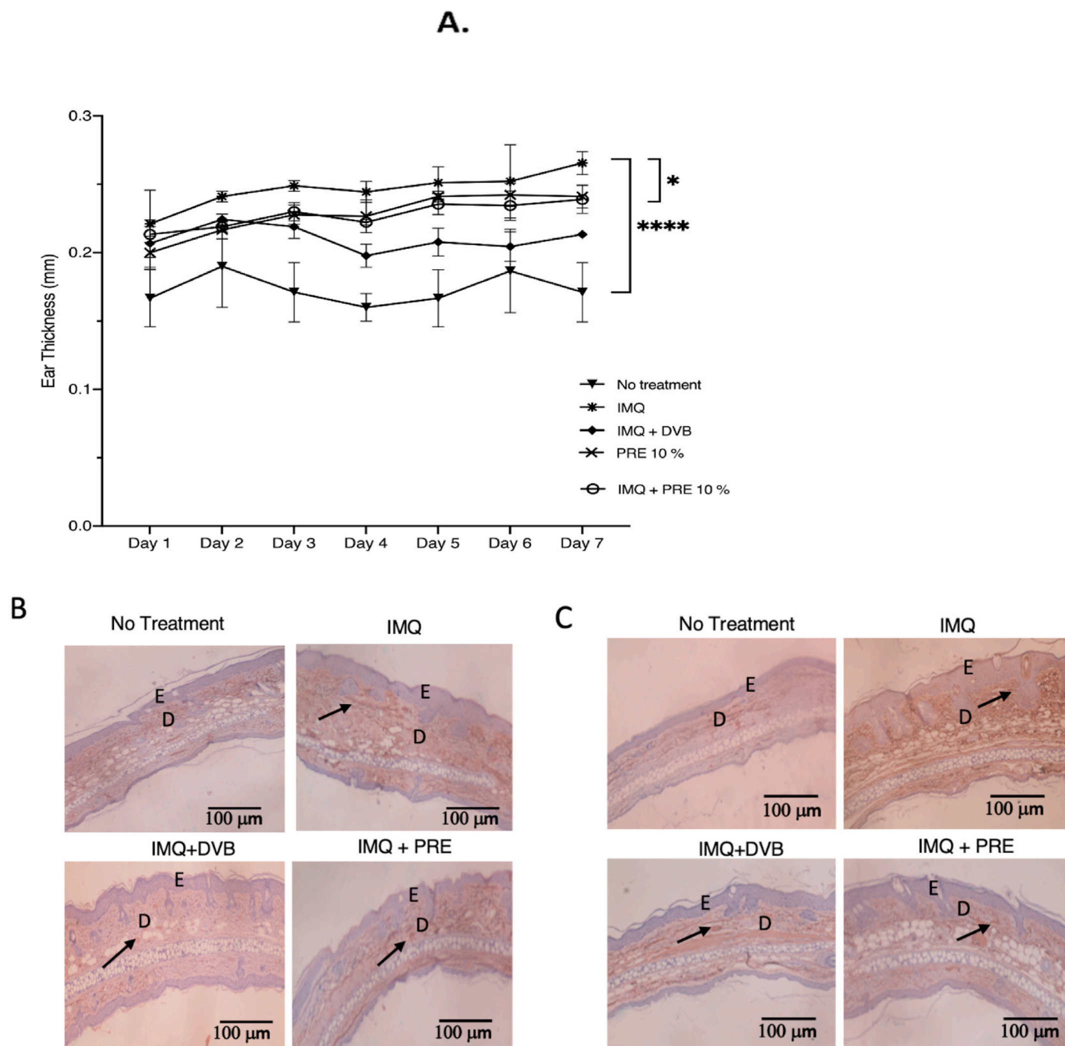

Fig. 7. (A) Ear thickness of mice induced by IMQ, treated mice with daivobet® (DVB) and PRE. (B) Immunohistochemical COX-2, and (C) IL-1β reactivity image. Arrows point to COX-2 or IL-1β-reactive cells for inflammatory polymorphonuclear infiltration (dermis) or epidermis thickening (epidermis). n = 5 mice. Abbreviations: E = Epidermis, D = Dermis.

widely accepted as a major factors to the progression of chronic inflammation [31]. Based on this knowledge, we sought to demonstrate the effects of administering LPS or IMQ (both known to act via TLR receptors). According to our findings, the extract could inhibit COX-2 and be mediated by the TLR4 level. Additionally, to better understand the effect of LPS and IMQ administration on NF-κB, their effect on ERK1/2, an upstream protein of NF-κB was evaluated and discovered that administration of PRE was able to suppress ERK1/2 overexpression. Moreover, in response to NF-κB, we identified that by administering pretreated PRE against LPS or IMQ, the protein expression of NF-κB decreased. Based on these results, we hypothesized that PRE acts by inhibiting LPS binding to TLR4, thereby suppressing NF-κB activation via inhibition of ERK1/2 activation (Fig. 5A and B). Our research also demonstrated that PRE's has an anti-inflammatory role by suppressing the secretion of proinflammatory IL-6, IL-1β and COX-2 through TLR4/ERK1/2/NF-κB axis. In order to supplement the data, the protective properties of this extract against human skin cell lines were determined by inducing damage to human keratinocytes (HaCaT cells) with rTRAIL. HaCaT cells were cultured with the extract 30 min after being induced with rTRAIL. Based on the viability test, PRE could protect cells from death even after being induced with the superfamily of proinflammatory cytokine TNFα, rTRAIL (Fig. 6) [32]. Similarly, a protective effect on normal skin cells was observed morphologically. Furthermore, in vivo analysis was conducted by applying PRE to the ears of mice and treated with IMQ (Fig. 7A). Imiquimod induce psoriasis-like skin inflammation by stimulating innate and acquired immune responses, leading to inflammatory cell infiltration and apoptosis of diseased tissue. Daivobet® was used as a positive control, and PRE was used at a concentration of 10% against IMQ. Consequently, ear thickness decreased compared with IMQ alone, and the expression of COX-2 and IL-1β positive staining were reduced as compared to IMQ (Fig. 7B and C). Collectively, we concluded that PRE possess sufficient pharmacological activity as an anti-inflammatory and protective agent against skin disorders and shed light on further investigations into this Indonesian plant for

clinical use.

5. Conclusions

Indonesia is endowed with an abundance of natural ingredients, and it is worthwhile to continue researching and screening their pharmacological benefits and applications. Among the numerous plant extracts screened, we discovered that the ethanolic extract of *Piper retrofractum* (PRE) inhibited NF-κB. This transcription factor regulates the production of various proinflammatory compounds via suppression of the TLR4/ERK1/2/NF-κB to decrease the production of NO, IL-1β, COX-2 and IL-6 in vitro and in vivo. Additionally, this extract can protect skin keratinocytes from damage caused by the cytokine rTRAIL, a member of the TNFα superfamily.

Ethics statement: The protocol of animal experiment was accepted by the Ethics Committee of Hasanuddin University (no. 11766/UN.14.1/TP.February 01, 2021 and date of approval at December 30, 2021) for studies involving animals.

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Author contribution statement

Subehan Lallo; Yoshihiro Hayakawa: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Besse Hardianti: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Yulia Yusrini Djabir; Ai Hertati; Habibie Habibie: Analyzed and interpreted the data; Wrote the paper.

Ismail Ismail; Muhammad Aswad: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Maulita Indrisari: Performed the experiments; Wrote the paper.

Data availability statement

Data included in article/supplementary material/referenced in article.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e20151.

References

[1] K. Von Rintelen, E. Arida, C. Häuser, A review of biodiversity-related issues and challenges in megadiverse Indonesia and other Southeast Asian countries, Research Ideas and Outcomes 3 (2017), e20860.
[2] N. Myers, R.A. Mittermeier, C.G. Mittermeier, et al., Biodiversity hotspots for conservation priorities, Nature 403 (6772) (2000) 853–858.
[3] W.M.N.H.W. Salleh, F. Ahmad, Phytopharmacological investigations of *Piper retrofractum* vahl.–A review, Agric. Conspectus Sci. 85 (3) (2020) 193–202.
[4] A. Banerji, M. Sarkar, R. Datta, et al., Amides from *Piper brachystachyum* and *Piper retrofractum*, Phytochemistry 59 (8) (2002) 897–901.
[5] A.A. Rajopadhye, A.S. Upadhye, A.M. Mujumdar, HPTLC method for analysis of piperine in fruits of Piper species, JPC–Journal of Planar Chromatography–Modern TLC 24 (1) (2011) 57–59.
[6] N. Jadid, B.A. Arraniry, D. Hidayati, et al., Proximate composition, nutritional values and phytochemical screening of *Piper retrofractum* vahl. fruits, Asian Pac. J. Trop. Biomed. 8 (1) (2018), 37.
[7] K.J. Kim, M.-S. Lee, K. Jo, et al., Piperidine alkaloids from *Piper retrofractum* Vahl. protect against high-fat diet-induced obesity by regulating lipid metabolism and activating AMP-activated protein kinase, Biochemical and biophysical research communications 411 (1) (2011) 219–225.
[8] M. Takahashi, N. Hirose, S. Ohno, et al., Flavor characteristics and antioxidant capacities of hihatsumodoki (*Piper retrofractum* Vahl) fresh fruit at three edible maturity stages, J. Food Sci. Technol. 55 (4) (2018) 1295–1305.
[9] S. Abdelhamed, S. Yokoyama, A. Refaat, et al., Piperine enhances the efficacy of TRAIL-based therapy for triple-negative breast cancer cells, Anticancer Res. 34 (4) (2014) 1893–1899.
[10] A. Maity, R. Wollman, Information transmission from NFκB signaling dynamics to gene expression, PLoS Comput. Biol. 16 (8) (2020), e1008011.
[11] K. Gaptulbarova, M. Tsyganov, A. Pevzner, et al., NF-κB as a potential prognostic marker and a candidate for targeted therapy of cancer, Exp. Oncol. 42 (2020) 263–269.
[12] S.-J. Suh, U.-H. Jin, K.-W. Kim, et al., Triterpenoid saponin, oleanolic acid 3-O-β-d-glucopyranosyl (1→ 3)-α-l-rhamnopyranosyl (1→ 2)-α-l-arabinopyranoside (OA) from Aralia elata inhibits LPS-induced nitric oxide production by down-regulated NF-κB in RAW 264.7 cells, Arch. Biochem. Biophys. 467 (2) (2007) 227–233.

[13] S.-J. Suh, C.-H. Kwak, K.-H. Song, et al., Triple inhibitory activity of *Cliona celata* against TNF-α-induced matrix metalloproteinase-9 production via downregulated NF-κB and AP-1, enzyme activity, and migration potential, Inflammation 35 (2) (2012) 736–745.
[14] S.-J. Kim, T.-W. Chung, H.-J. Choi, et al., Monosialic ganglioside GM3 specifically suppresses the monocyte adhesion to endothelial cells for inflammation, Int. J. Biochem. Cell Biol. 46 (2014) 32–38.
[15] K. Takeda, S. Akira, TLR signaling pathways, in: Seminars in Immunology, Elsevier, 2004.
[16] A. Rahman, F. Fazal, Blocking NF-κB: an inflammatory issue, Proc. Am. Thorac. Soc. 8 (6) (2011) 497–503.
[17] T.W. Chung, S.K. Moon, Y.C. Chang, et al., Novel and therapeutic effect of caffeic acid and caffeic acid phenyl ester on hepatocarcinoma cells: complete regression of hepatoma growth and metastasis by dual mechanism, Faseb. J. 18 (14) (2004) 1670–1681.
[18] J.-Y. Park, T.-W. Chung, Y.-J. Jeong, et al., Ascofuranone inhibits lipopolysaccharide–induced inflammatory response via NF-kappaB and AP-1, p-ERK, TNF-α, IL-6 and IL-1β in RAW 264.7 macrophages, PLoS One 12 (2) (2017), e0171322.
[19] N. Nwet Win, B. Hardianti, S. Kasahara, et al., Anti-inflammatory activities of isopimara-8(14),-15-diene diterpenoids and mode of action of kaempulchraols P and Q from *Kaempferia pulchra*rhizomes, Bioorg. Med. Chem. Lett 30 (2) (2020), 126841.
[20] N.N. Win, B. Hardianti, H. Ngwe, et al., Anti-inflammatory activities of isopimara-8 (9), 15-diene diterpenoids and mode of action of kaempulchraols B–D from *Kaempferia pulchra* rhizomes, J. Nat. Med. 74 (2) (2020) 487–494.
[21] S. Lallo, B. Hardianti, S. Sartini, et al., Ethyl P-methoxycinnamate: an active anti-metastasis agent and chemosensitizer targeting NFκB from *Kaempferia galanga*for melanoma cells, Life 12 (3) (2022), 337.
[22] C. Franceschi, M. Bonafè, S. Valensin, et al., Inflamm-aging: an evolutionary perspective on immunosenescence, Ann. N. Y. Acad. Sci. 908 (1) (2000) 244–254.
[23] L. Umeyama, B. Hardianti, S. Kasahara, et al., Anti-inflammatory effects of *Morus alba* Linne bark on the activation of toll-like receptors and imiquimod-induced ear edema in mice, BMC complementary medicine and therapies 21 (1) (2021) 1–10.
[24] L. Van Der Fits, S. Mourits, J.S. Voerman, et al., Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis, J. Immunol. 182 (9) (2009) 5836–5845.
[25] C. Franceschi, J. Campisi, Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases, Journals of Gerontology Series A: Biomedical Sciences and Medical Sciences 69 (Suppl_1) (2014) S4–S9.
[26] F. Rodier, J. Campisi, Four faces of cellular senescence, JCB (J. Cell Biol.) 192 (4) (2011) 547–556.
[27] A. Salminen, J. Huuskonen, J. Ojala, et al., Activation of innate immunity system during aging: NF-κB signaling is the molecular culprit of inflamm-aging, Ageing Res. Rev. 7 (2) (2008) 83–105.
[28] D. Jurk, C. Wilson, J. Passos, et al., Mann DA, von Zglinicki T. Chronic inflammation induces telomere dysfunction and accelerates ageing in mice, in: C. Fox, C. Lawless, R. Anderson, G. Hewitt, S.L.F. Pender, N. Fullard, G. Nelson, J. Mann, B. van de Sluis (Eds.), Nature Communications, vol. 2, 2014, p. 4172, 2014, 2, 4172. Nature Communications.
[29] I.M. Scott, H.R. Jensen, B.J. Philogène, et al., A review of *Piper* spp. (Piperaceae) phytochemistry, insecticidal activity and mode of action, Phytochemistry Rev. 7 (1) (2008) 65–75.
[30] B. Cahyono, E.F. Hasanah, M. Suzery, Analysis of piperine content in cabe jawa extracts (*Piper retrofractum* Vahl) using UV spectrophotometry and HPLC, in: IOP Conference Series: Materials Science and Engineering, IOP Publishing, 2019.
[31] A. Greenhough, H.J. Smartt, A.E. Moore, et al., The COX-2/PGE 2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment, Carcinogenesis 30 (3) (2009) 377–386.
[32] B. Hardianti, L. Umeyama, F. Li, et al., Anti-inflammatory compounds moracin O and P from *Morus alba* Linn. (Sohakuhi) target the NF-κB pathway, Mol. Med. Rep. 22 (6) (2020) 5385–5391.