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Research Article
Anti-Inflammatory and Cytoprotective Effect of *Kaempferia galanga* Extracts by Targeting NF-κB Activity

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Abstract

Background and Objective: Skin disease and inflammation are often treated with traditional medicine, which has been the case since ancient times. Controlling activation and gene expression of inflammatory signalling pathways, such as the Nuclear Factor-kappa B (NF-κB) pathway, is important for protecting the skin from adverse inflammation and preventing the acceleration of pathologic disease processes. Therefore, the present study aimed to identify natural anti-inflammatory products that could target NF-κB activity by screening Indonesian medicinal plants, with particular focus given to *Kaempferia galanga* extracts as promising candidates. **Material and Methods:** We investigated the inhibition of NF-κB activation in 35 natural medicinal plants (1–35) cultivated in Indonesia traditionally used to treat skin disease symptoms. Specifically, a 4T1 cell line, a breast cancer cell line and RAW 264.7 macrophages expressing the firefly luciferase gene under the control of an NF-κB response element were used for this purpose. Inhibition of NF-κB activity and cell viability were determined by reporter assays and WST-8 methods. **Result:** Based on the screening of 35 natural medicinal plants, we identified two *K. galanga* extracts (29 and 35) from different rhizome types that strongly suppressed NF-κB activity without affecting cell viability. In further investigation of the anti-inflammatory effects of these extracts, Tumour Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) was found to cause cellular damage to human HaCaT keratinocytes: however, both extracts 29 and 35 from *K. galanga* had protective effects against TRAIL. **Conclusion:** Collectively, these results indicate that the identified *K. galanga* extracts represent potential candidates for the development of novel anti-inflammatory natural medicines.

Key words: Anti-inflammation, *Kaempferia galanga*, Indonesian herbal medicine, NF-κB, dyspepsia, dermopathy, tumour necrosis factor

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In traditional medicine, plants are used as natural resources to produce treatments that have traditionally been applied in human healthcare since ancient times. Generally, single plants or plant formulas are used to treat or prevent disease[1]. Indonesia is endowed with immense biodiversity in its flora and fauna, which presents remarkable opportunities for drug discovery[2]. In the past, drug discovery screening largely occurred by chance and drug development was based on clinical practice. In many plants, secondary metabolites possess structural diversity that makes them promising candidates in terms of their medicinal properties. Indeed, medicinal plants have played an important role in the development of the pharmaceutical industry[2,3].

The members of the Zingiberaceae family, which is distributed throughout Southeast Asia, are widely used as medicinal plants[3]. These plants are known for their use as ethnomedicines and are important in Indonesian traditional medicine. Specific Zingiberaceae family species, such as *Kaempferia rotunda*, *Zingiber aromaticum* and *Curcuma domestica*, have shown promising results in terms of their efficacy as traditional medicines[4,5].

Kaempferia galanga is a plant native to Southeast Asia that is believed to have originated in Burma[6]. In Indonesia, it is commonly known as "kencur" and is used in local cuisines and traditional medicine. The rhizome of the plant has long been used in Indonesian traditional medicine as "jamu," a plant material for "Jamu gendong" (a traditional medicine in liquid or other forms that are freshly prepared without preservation and sold without a label)[7]. Many Southeast Asian countries use *K. galanga* as traditional medicine or in other products. In Chinese traditional medicine, it is used to treat cholera, contusion, constipation and stomach ache: in Thailand, it is used as a treatment for menstrual disorder and dyspepsia: in Bangladesh, it is used for scenting vinegar, hair shampoo, cosmetics, flavourings and beverages and in Indian Ayurveda, it is used to treat muscular swelling and rheumatism[8-10].

Analysis of the macro-and micro-components of *K. galanga* has shown that it contains protein, fibre and high amounts of essential minerals (e.g., potassium, phosphorous and magnesium), along with considerable amounts of iron, manganese, zinc, cobalt and nickel[6]. In addition, compounds such as isopimarane, abietane, labdane and clerodane diterpenoids, along with flavonoids, phenolic acids, phenyl-heptanoids, curcuminoids, tetrahydropyran-phenolics and steroids have been isolated from Kaempferia species[11]. Previous studies have also reported that the biological activities of the plant include larvicidal, insect repellant, anticancer, antimicrobial, antioxidant, anti-obesity–induced dermatopathy, anticholinesterase, wound healing, antinociceptive, neuroprotective, antiallergenic and anti-inflammatory activities[11,12].

Identifying new anti-inflammatory drug candidates is important for improving the treatment of inflammation-related diseases such as cancer, dermatitis and rheumatoid arthritis. One documented regulator protein associated with such diseases is Nuclear Factor-kappa B (NF-κB). NF-κB pathways are essential for regulating host defence responses to stress, injury and infection[13]. Inflammation is a normal biological response to injury and infection: it involves the recruitment of the immune system to neutralize invading pathogens, repair injured tissues and promote wound healing. Increasing the levels of proteins involved in inflammation, such as inducible nitric oxide synthase, cyclooxygenase 2, TNF α, interleukin-1, interleukin-6 and type I interferon, accelerates the production of key inflammatory mediators[14,15]. This complex generates an intracellular signalling cascade that sequentially phosphorylates and activates NF-κB, which translocates to the nucleus and plays a crucial role in pro-inflammatory gene transcription and expression. The excessive production of inflammatory mediators, especially nitric oxide, is associated with chronic inflammation and is thought to cause inflammatory diseases such as rheumatoid arthritis, atherosclerosis, inflammatory bowel disease, inflammatory airway disease and even cancer[16-18].

Inhibition of proinflammatory gene expression regulated by NF-κB is likely to reduce the production of inflammatory mediators and control excessive inflammation, which would help prevent inflammatory disease[19]. Therefore, the present study aimed to identify natural anti-inflammatory products that could target NF-κB activity by screening Indonesian medicinal plants, with particular focus given to *K. galanga* extracts as promising candidates.

MATERIALS AND METHODS

Study area: This work was carried out All the experiments were performed from January-December, 2020 in Biofarmaka Laboratory, Faculty of Pharmacy, Hasanuddin University, Indonesia and Section of Host Defences of Institute of Natural Medicine, University of Toyama, Japan.

Table 1: List of the collected medicinal plants

Local name	Plant part	Latin name	Family
Klika Lelak	Bark	*Uvaria rufa* blume	Annonaceae
Akar wangi	Root	*Chrysopogon zizanioides* (L.) Roberty	Poaceae
Kersen	Leaf	*Muntingia calabura*	Muntingiaceae
Afrika	Leaf	*Vernonia amygdalina* Delile	Compositae
Rambai Laut	Leaf	*Sonneratia caseolaris* (L.) Engl	Lythraceae
Lakka-lakka	Rhizome	*Curculigo orchioides* Gaertn	Hypoxidaceae
Temu Kunci	Rhizome	*Boesenbergia rotunda* (L.) Mansf	Zingiberaceae
Kapuk Randu	Leaf	*Ceiba pentandra* (L.) Gaertn	Malvaceae
Faloak	Stem	*Sterculia abbreviata* E.L. Taylor ex Mondragón	Malvaceae
Degan	Fruit	*Dillenia serrata* Thunb	Dilleniaceae
Degan	Leaf	*Dillenia serrata* Thunb	Dilleniaceae
Kapuk Randu	Root	*Ceiba pentandra* (L.) Gaertn	Malvaceae
Kesambi	Leaf	*Schleichera oleosa* (Lour.) Merr	Sapindaceae
Faloak	Bark	*Sterculia abbreviata* E.L. Taylor ex Mondragón	Malvaceae
Leileum	Leaf	*Sterculia abbreviata* E.L. Taylor ex Mondragón	Malvaceae
Kersen	Leaf	*Muntingia calabura* L.	Muntingiaceae
Akar Laka	Daun	*Dalbergia parviflora* Roxb	Leguminosae
Temu Ireng	Rhizome	*Curcuma aeruginosa* Roxb	Zingiberaceae
Temu Putih	Rhizome	*Curcuma zedoaria* (Christm.) Roscoe	Zingiberaceae
Curcuma Mangga	Rhizome	*Curcuma mangga* Valeton and Zijp	Zingiberaceae
Brotowali	Leaf	*Tinospora crispa* (L.) Hook. f. and Thomson	Menispermaceae
Bangle	Rhizome	*Zingiber montanum* (J. Koenig) Link ex A. Dietr.	Zingiberaceae
Lengkuas	Rhizome	*Alpinia galanga* (L.) Willd	Zingiberaceae
Jahe Merah	Rhizome	*Zingiber officinale* Roscoe	Zingiberaceae
Jahe Segar	Rhizome (fresh)	*Zingiber officinale* Roscoe	Zingiberaceae
Kunyit Putih	Rhizome	*Curcuma zedoaria* (Christm.) Roscoe	Zingiberaceae
Kunyit	Rhizome	*Curcuma longa* L.	Zingiberaceae
Pangkal Kunyit Putih	Stem	*Curcuma zedoaria* (Christm.) Roscoe	Zingiberaceae
Kencur Besar	Rhizome (large)	*Kaempferia galanga* L.	Zingiberaceae
Temulawak	Rhizome	*Curcuma zanthorrhiza* Roxb	Zingiberaceae
Jahe kering	Rhizome (dry)	*Zingiber officinale* Roscoe	Zingiberaceae
Bawang Putih Tunggal	Tuber (single)	*Allium sativum* L.	Amaryllidaceae
Lempuyang	Rhizome	*Zingiber zerumbet* (L.) Roscoe ex Sm	Zingiberaceae
Temu Giring	Rhizome	*Curcuma heyneana* Valeton and Zijp	Zingiberaceae
Kencur Kecil	Rhizome (small)	*Kaempferia galanga* L.	Zingiberaceae

Plant extracts: Plants were collected from the South Sulawesi Province, Indonesia. The leaves or rhizomes of plants were extracted with 70% ethanol using the maceration method[20]. Liquid extracts were then evaporated and lyophilized to obtain an EtOH extract. In total, extracts were collected from 35 natural medicinal plants, which were collected from a local traditional healer (Table 1).

Cells and reagents: Murine 4T1 cell lines were obtained from the American Type Culture Collection and maintained at 37°C in Eagle's minimal essential medium or RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd.) containing 10% fetal bovine serum (FBS: Nichirei Biosciences, Inc.). Human HaCaT keratinocytes (provided by Dr. Takeda, Juntendo University, Tokyo, Japan) were maintained in a culture medium consisting of Dulbecco's modified Eagle's medium (DMEM: Nissui Pharmaceutical Co., Ltd.) supplemented with 10% FBS, 100 U L^{-1} penicillin G and 100 mg L^{-1} streptomycins at 37°C with 5% CO_2. Human recombinant Tumour Necrosis Factor-related Apoptosis-inducing Ligand (rTRAIL) was purchased from PeproTech, Inc. Mouse RAW 264.7-NF-κB-luc cells were cultured in DMEM containing 10% FBS, 100 U L^{-1} penicillin G and 100 mg L^{-1} streptomycins in a 5% CO_2 atmosphere at 37°C. In the experiments, cells were seeded in 35 mm dishes (Corning Inc., Corning, NY, USA) and confluent cells were incubated in DMEM for 24 hrs. To establish RAW 264.7 cells expressing the NF-κB-mediated luciferase gene (RAW 264.7-NFκB-luc2), RAW 264.7 cells (RCB0535) (2×10^5 $well^{-1}$) were seeded in 6-well plates and transfected with the pGL4.32 vector using Lipofectamine 2000. The cells were selected with hygromycin B (100 μg mL^{-1}) and cloned by limiting dilution. To evaluate the cellular response to NF-κB *in vitro*, RAW 264.7-NF-κB transfectants or RAW CMV control cells (2×10^5 $well^{-1}$) were cultured in 96-well plates and treated with LPS (100 ng mL^{-1}). After 6 hrs of incubation, the luciferase activity of cells was measured with a microplate reader (Sunrise™: Tecan Group Ltd., Männedorf, Switzerland).

Cell viability: 4T1-NF-κB-Luc2 cells were plated at a final concentration of 2×10^4 cells well^{-1} in a 96-well plate. After 24 hrs of incubation, the cells were pretreated with 50 g mL^{-1} of 35 extracts and again incubated for 24 hrs. Subsequently, 10 µL of WST-8 (FUJIFILM Wako Pure Chemical Corporation) solution was added to the cells, which were incubated for an additional 1 hr in a humidified atmosphere (37°C and 5% CO_2) to allow the formation of formazan dye and to increase sensitivity. Then, the absorbance was measured with a microplate reader (Sunrise™: Tecan Group Ltd., Männedorf, Switzerland) at wavelengths of 450/620 nm. Cell viability was determined based on the absorbance of the soluble formazan dye generated by the living cells. The same method was followed to determine the viability of RAW 264.7-NF-κB-luc2 cells treated with selected extracts in a concentration-dependent manner. HaCaT cells were seeded on a 96-well plate at a density of 104 cells/well and then pretreated with 20 ng mL^{-1} rTRAIL for 1 hr. The cells were then cultured with or without selected extracts (4, 8, 16 and 32 µg mL^{-1}) for 24 hrs. After incubation, the WST-8 solution was added and used according to the manufacturer's instructions: Then, absorbance was measured at 450 nm using a microplate reader. The cell viability of the treated cells was calculated as a percentage of control cell viability.

NF-κB reporter gene assay: 4T1-NF-κB-Luc2 cells and RAW 264.7-NF-κB-luc2 cells [stable 4T1 (ATCC) and RAW (ATCC) cell lines with NF-κB-driven luciferase reporters] were maintained in DMEM supplemented with 10% FBS, 1-mM treated with the extracts and an equal concentration of the solvent vehicle was included as control after 24 hrs. At the end of the assay, 900 µg mL^{-1} D-luciferin was added and the L-glutamine and antibiotics (100 U L^{-1} penicillin and 100 mg L^{-1} streptomycins) at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. The cells were seeded in black 96-well plates at a density of 2×10^4 cells per well and then incubated for 24 hrs. Subsequently, they were plates were incubated for another 30 min. Luciferase activity was measured according to the luminescence of firefly luciferase, which was quantified using IVIS LUMINA II and Living Image 4.2 software (Caliper Life Science) by determining the light emitted from cells. Data were expressed as photons/s and the fold NF-κB activity was calculated as a total flux of extracts (photons/s) is divided by a total flux of vehicle as control (photons/s).

Statistical analysis: All data are presented as the mean±standard error of the mean of three independent experiments. SPSS version 23 software (IBM Corp.) was used to analyze data. Data were analyzed using a one-way analysis of variance followed by a Bonferroni correction. p-values of <0.05 were considered to indicate a statistically significant difference.

RESULTS

Identification of extracts of Indonesian natural medicines that inhibit NF-κB activation: We investigated the inhibition of NF-κB activation in 35 natural medicinal plants (1-35) traditionally used to treat skin disease symptoms: these were screened using a reporter assay method. Inhibitory activity against NF-κB was determined by co-culturing 4T1-NF-κB-Luc2 cells with extracts at 50 µg mL^{-1} for 24 hrs (Fig. 1). Twelve extracts showed active inhibition of NF-κB in transfected cells (Fig. 2). NF-κB inhibition results were compared with those

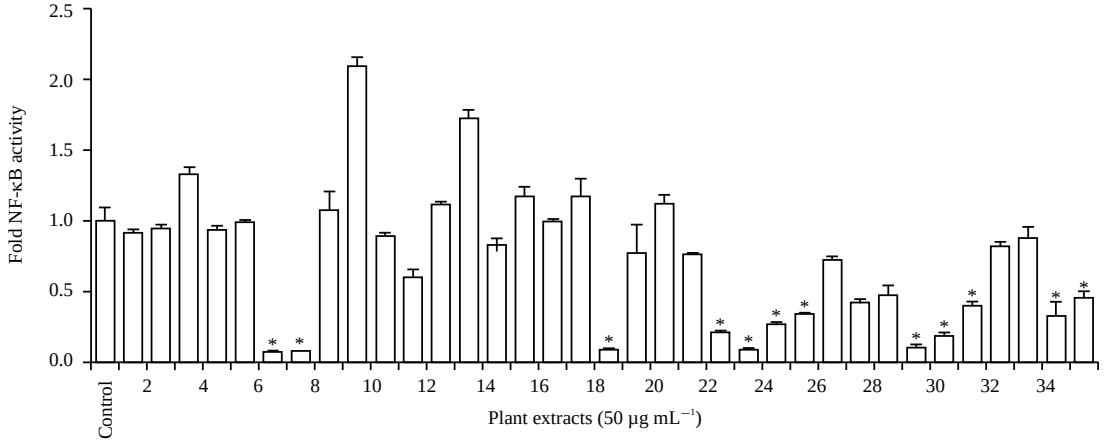

Fig. 1: 4T1-NF-κB-Luc2 cells were co-cultured with 35 plant extracts at 50 µg mL^{-1} for 24 hrs

Data are normalized to the untreated controls and presented as Mean±Standard error of the mean (n = 3, *p<0.05 vs. untreated control)

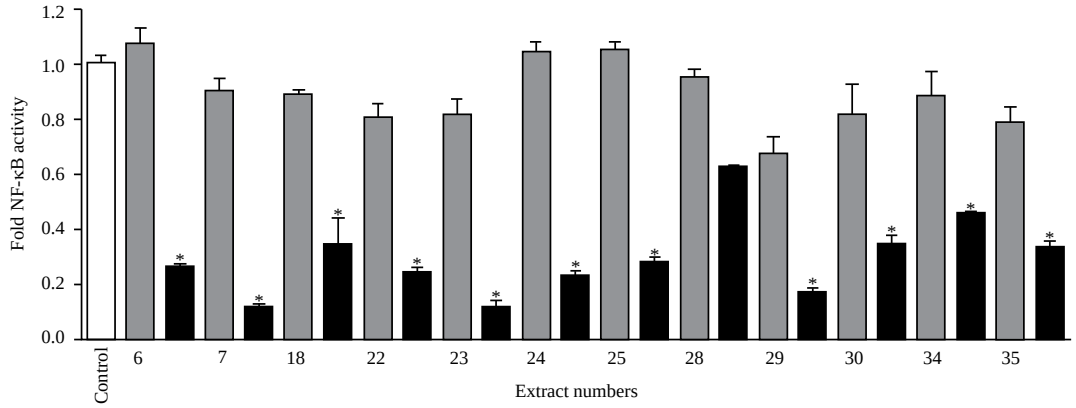

Fig. 2: 4T1-NF-κB-Luc2 cells were co-cultured with 12 selected plant extracts at 1 (grey bar) and 50 (black bar) μg mL^{-1} for 24 hrs
Data are normalized to the untreated controls and presented as Mean±Standard error of the mean (n = 3: *p<0.05 vs. untreated control)

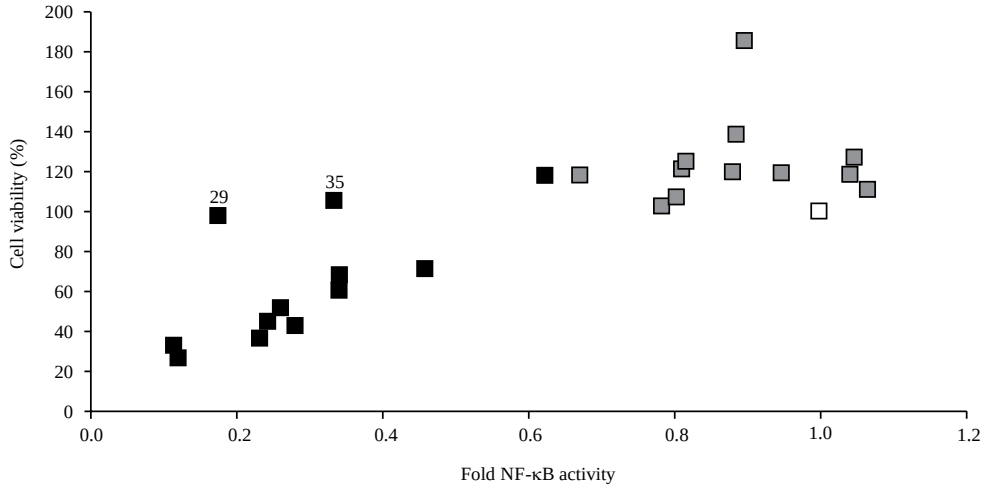

Fig. 3: Inhibitory effects of 12 active plant extracts at 1 (grey) and 50 (black) μg mL^{-1} (24 hrs exposure)
Inhibitory activity of the extract on NF-κB activation in 4T1-NF-κB-Luc2 cells (relative to untreated controls) versus cell viability (as a percentage of untreated control cell viability)

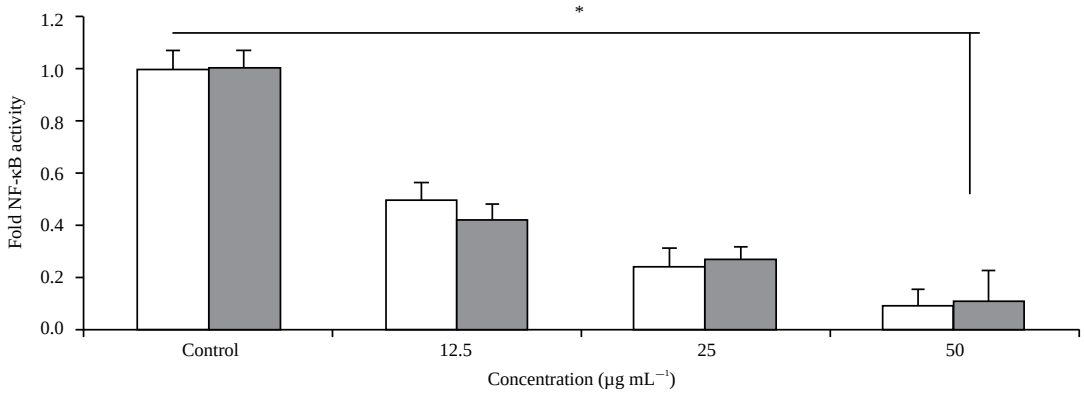

Fig. 4: 4T1-NF-κB-Luc2 cells were co-cultured with extract 29 (white bars) and extract 35 (gray bars) at 12.5, 25.0 and 50.0 μg mL^{-1} for 24 hrs
Inhibitory effects of each plant extract on NF-κB activation relative to the untreated controls are shown, Data are normalized to the untreated controls and presented as Mean±standard error of the mean (n = 3, *p<0.05 vs. untreated control)

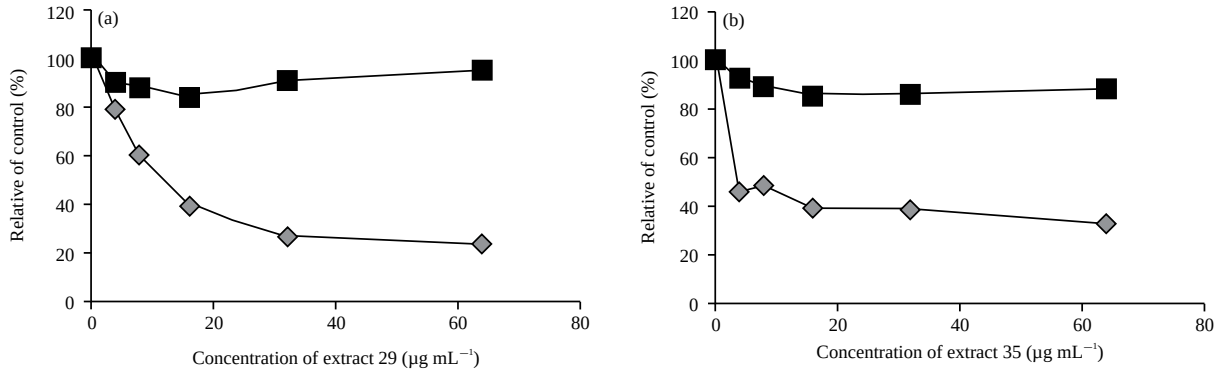

Fig. 5(a-b): RAW 264.7-NF-κB-Luc cells were co-cultured with (a) Extracts 29 and (b) 35 at various concentrations for 24 hrs

The inhibitory effect (grey) of each plant extract on NF-κB activation relative to untreated controls is shown. Cell viability (black) was determined using a WST-8 assay and is shown as a percentage of untreated control cell viability

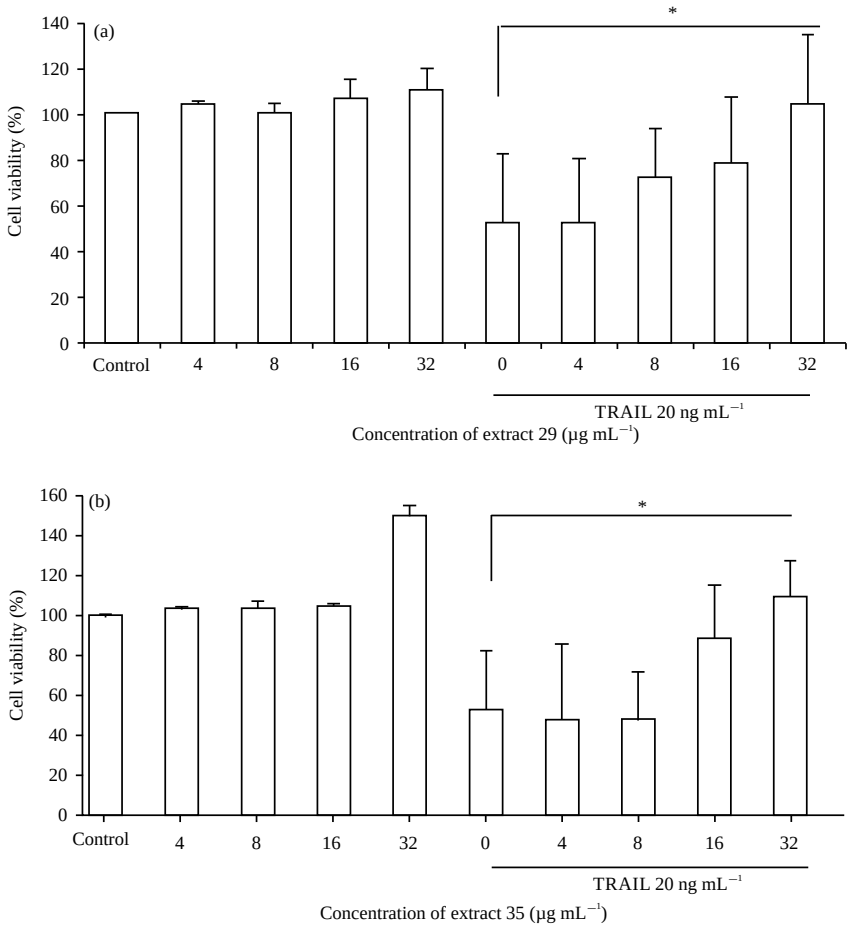

Fig. 6(a-b): Cytoprotective effects of extracts 29 (a) and 35 (b) against rTRAIL-induced cellular damage in humans keratinocytes

Data are normalized to the untreated controls and presented as Mean±standard error of the mean (n = 3: *p<0.05 vs. cells without extract treatments)

from a viability assay in which WST-8 was used. Results indicated that both assays were appropriate tools for identifying extracts that potentially act against inflammation-associated NF-κB modulation factors. Among the 12 selected extracts, extracts 29 and 35 were from the same species, namely *K. galanga* nd both showed properties suggesting they could act as anti-inflammatory agents (Fig. 3). These findings were validated by

performing reporter assays on 4T1-NF-κB-Luc2 cells co-cultured with extracts 29 and 35 for 24 hrs: Results showed that the extracts produced clear inhibitory effects at concentrations of 12.5, 25.0 and 50.0 μg mL^{-1} (Fig. 4). Importantly, these two active extracts inhibited NF-κB activity without affecting the cell viability of 4T1-NF-κB-Luc2 cells (Fig. 5a-b). The result of Fig. 5a illustrated the RAW 264.7-NF-κB-Luc cells were co-cultured with extracts 29, this extract effectively inhibited NF-κB activity without affecting viability cells. The result of Fig. 5b illustrated the RAW 264.7-NF-κB-Luc cells were co-cultured with extracts 35, this extract effectively inhibited NF-κB activity without affecting viability cells.

Cytoprotective effects of *K. galanga* extract on TRAIL-induced cytotoxicity in HaCaT cells: The biological activity of *K. galanga* in extracts 29 and 35 was evaluated by incubating HaCaT cells with rTRAIL as a mediator of inflammatory stimuli that activate NF-κB activity and cytotoxicity. Pretreatment with extracts 29 and 35 did not show toxic effects on HaCaT cells, whereas rTRAIL induced a significant reduction in the viability of cells. Furthermore, pretreatment with extract 29 and 35 at concentrations of 16 and 32 g mL^{-1} protected cell growth against rTRAIL-induced cellular damage (Fig. 6a-b). Cytoprotective effects of extracts 29 (Fig. 6a) and 35 (Fig. 6b) against tumour necrosis factor-related apoptosis-inducing ligand (rTRAIL)-induced cellular damage in human keratinocytes.

DISCUSSION

In this study, we screened 35 natural medicinal plants that are used like traditional natural medicines (extracts 1-35) for their anti-inflammatory properties in the treatment of skin diseases symptoms. Specifically, we targeted NF-κB activity intending to discover drugs that could reduce inflammation and chronic inflammation leading to pathological disease[21]. Activation The activation of NF-κB is mainly initiated by bacterial endotoxins such as lipopolysaccharide and pro-inflammatory cytokines such as tumour necrosis factor and IL1[22]. The NF-κB family contains several inducible transcription factors that regulate many genes involved in the mechanisms of immune and inflammatory reactions[23].

To identify inhibitory effects against NF-κB, we used culture cells showing stable expression of NF-κB: we used a 4T1 (ATCC) breast cancer cell line, in which we constitutively expressed the firefly luciferase gene under the control of an NF-κB reporter. Unlikely most tumour models, 4T1 cell lines have several characteristics that make it a suitable experimental animal model for human mammary cancer[24]. From the screening of 35 natural medicinal plants, 12 extracts were found to decrease NF-κB activity. These effects were verified by examining NF-κB activity in conjunction with cell viability with extract concentrations up to 50 μg mL^{-1}. Interestingly, extracts 29 and 35 from *K. galanga* significantly suppressed NF-κB activity without affecting the viability of 4T1 or RAW cells: thus, these extracts may regulate NF-κB activity while maintaining the proliferation of cells. These results were further supported by our finding that extracts 29 and 35 significantly reduced NF-κB activity in a concentration-dependent manner (at 12.5, 25.0 and 50.0 g mL^{-1}) in 4T1-NF-κB-Luc2 cells. Many cancer cells using the NF-κB pathway to achieve resistance to death by radiation or drugs. Therefore it is interesting to understand NF-κB activity to evaluate cancer and normal cell responses[25]. Several natural products were reported for anti-cancer activity but toxic on normal cell line[26]. However, in tests of murine RAW 264.7, stable cells transfected with NF-κB extracts 29 and 35 were nontoxic to macrophage cells while NF-κB activity decreased significantly. Collectively, these data indicate that extracts 29 and 35 are promising candidates for targeting NF-κB in cancer cells, particularly because they do not affect normal cell viability. Furthermore, these extracts might be useful candidates for advancing proinflammatory research.

In additional tests, we evaluated the efficacy of extracts 29 and 35 in the HaCaT cell line, which has been widely used in the studies of skin biology and differentiation[27]. This spontaneously immortalized human keratinocyte cell line was used with and without pretreatment of TRAIL (20 ng mL^{-1}): TRAIL/Apo1 L is a death ligand, i.e., a cytokine that activates apoptosis through cell surface death receptors[28,29]. At concentrations of 4, 8, 16 and 32 μg mL^{-1} extract 29 and 35 did not affect the viability of HaCaT cells, whereas, at concentrations of 16 and 32 μg mL^{-1}, the extracts showed protective effects in cells pretreated with TRAIL. Resistance of TRAIL can occur at different points in the TRAIL-induced apoptosis signalling pathway[30]. Thus, these findings provide further evidence that the identified Kaempferia species extracts may be beneficial for the treatment of inflammatory disease. Although their exact mechanism of action remains to be determined, the extracts may act as novel anti-inflammatory or cytoprotective agents through the suppression of the NF-κB pathway.

CONCLUSION

Identification on natural anti-inflammatory by targeting NF-κB activity on 35 Indonesian medicinal plants showed *Kaempferia galanga* species extracts represent potential candidates for the development of novel anti-inflammatory natural medicines to protect the pathological condition to human skin in vitro and shed a light to further investigation on human dermatology diseases *in vivo* and clinically.

SIGNIFICANCE STATEMENT

This study discovered that two separate *Kaempferia galanga* extracts strongly suppressed NF-κB activity without affecting cell viability in various cell types. In addition, we found that these two *K. galanga* extracts had protective effects against damage induced by tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) in human HaCaT keratinocytes. We have identified two plant extracts that represent potential candidates for the development of novel anti-inflammatory natural medicines.

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