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Phytochemical profiling and anti-inflammatory activity of *Citrus hystrix* DC. Peel essential oil

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ABSTRACT

Chronic inflammatory diseases are a major global health problem. This study was conducted to evaluate the anti-inflammatory activity of kaffir lime peel essential oil (*Citrus hystrix* DC.) obtained using Microwave-Assisted Extraction with a network pharmacology approach and *in vivo* tests using Wistar rats. Identification of compounds in the essential oil was carried out by Gas Chromatography–Mass Spectrometry analysis, which obtained 60 identified compounds such as (−)-citronellal, β-pinene, D-limonene, and linalool. Network pharmacology analysis identified 265 inflammation-related targets, with TP53, IL6, and MAPK3 identified as hub genes. Functional enrichment analysis showed significant involvement in cytokine-cytokine receptor interactions and chemokine signaling pathways. *In vivo* experiments showed that oral kaffir lime peel essential oil administration (50–400 mg/kgBW) significantly reduced carrageenan-induced paw swelling in Wistar rats compared to negative controls ($p < 0.05$). Even higher doses (200 and 400 mg/kgBW) produced effects nearly comparable to those of diclofenac sodium (2.25 mg/kgBW). Neutrophil infiltration decreased, while immunohistochemical analysis confirmed decreased TNF -α and IL-6 expression, particularly at the higher doses. These findings suggest that kaffir lime peel essential oil has promising anti-inflammatory activity as a natural alternative to conventional anti-inflammatory drugs.

1. INTRODUCTION

Inflammation is a biological process that protects against threats such as infection, toxins, or injury and starts the healing of tissues [1,2]. Chronic inflammation is a factor in degenerative diseases such as cardiovascular disease, diabetes, cancer, and chronic kidney disease. These diseases together kill almost half of all people worldwide, and they keep getting worse as people get older and live modern lives [3,4]. Synthetic anti-inflammatory drugs, including both steroidal and nonsteroidal, effectively reduce inflammation. However, they can hurt the stomach, kidneys, and heart. Long-term use can also cause oxidative stress, disrupt electrolyte balance, induce fluid retention, decrease renal perfusion, and, in some cases, suppress bone marrow, leading to decreased blood cell counts. These combined effects increase the risk of stomach ulcers, kidney failure, arrhythmias, osteoporosis, and anemia [5–7]. Essential oils from medicinal plants are a safer option, and they are widely reported to have several pharmacological effects, including anti-inflammatory properties [8].

Kaffir lime (*Citrus hystrix* DC.), a member of the *Rutaceae* family, has long been used in traditional medicine to treat digestive problems, fever, and other ailments. Its antimicrobial and anti-inflammatory properties make this possible [9–12]. The fruit peel of kaffir lime is often considered as

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waste, even though it contains many bioactive compounds such as limonene, β-pinene, and terpinene-4-ol, which are reported to have anti-inflammatory activity [13–15]. Furanocoumarins contained in the fruit peel have also been shown to inhibit lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 macrophages and inhibit Cyclooxygenase-2 (COX-2) expression in colon cancer cells (HT-29 and HCT116) [10]. Previously, our study showed that essential oil from *C. hystrix* peel significantly reduced NO production *in vitro* using RAW 264.7 cells [16]. Many studies have used this cell line to study inflammatory mechanisms, particularly to activate the inhibition of NO and other pro-inflammatory mediators induced by LPS [17]. This makes it a good model for assessing anti-inflammatory potential. Due to its flavonoid content, *C. hystrix* exhibits antioxidant properties and anti-inflammatory effects [18]. These flavonoids neutralize free radicals in the DPPH assay. Evidence shows that aqueous and ethanolic extracts of leaves and fruit peels reduce reactive oxygen species levels and enhance cell migration [19–21].

Essential oil extraction methods are mainly carried out by conventional hydrodistillation methods, which are time-consuming, energy-intensive, and relatively inefficient, and often cause degradation of heat-sensitive bioactive compounds. So, to increase efficiency, shorten processing time, and reduce thermal degradation of bioactive compounds, in this study, we used the Microwave-Assisted Extraction (MAE) method [22,23]. Furthermore, we identified compounds in the extracted kaffir lime peel essential oil (KEO) using Gas Chromatography–Mass Spectrometry (GC–MS). Then, we investigated its potential as an anti-inflammatory agent with a computational approach (network pharmacology), and *in vivo* validation will provide a strong foundation as a promising candidate in the development of anti-inflammatory therapy.

2. MATERIALS AND METHODS

2.1. Extraction of essential oil from Kaffir Lime peel

Kaffir lime fruit was collected from Tapak Tuan, Trumon, South Aceh, Indonesia, and was identified at the Plant Systematics Laboratory, Herbarium Medanense, Universitas Sumatera Utara (Grant number 2222/MEDA/2024). Essential oil from kaffir lime peel KEO was extracted using the MAE method by weighing 50 g of dried fruit peel, then finely ground and put into a 1 L round-bottom flask containing 300 ml of distilled water. Extraction was performed by heating the mixture in a microwave oven at a temperature of 90°C ± 5°C for 30 minutes. To maximize yield, the distillation was repeated in three consecutive cycles. The oil layer was separated from the water layer using a separatory funnel, transferred into an amber glass vial, and then stored at 4°C until analysis.

2.2. Determination of the chemical composition of KEO

The chemical composition of the essential oil was analyzed using GC–MS with a TG-5MS capillary column (30 m × 0.25 mm internal diameter, 0.25 µm film thickness). The carrier gas utilized was helium, which flowed consistently at a rate of 1.0 ml/minute. The oven temperature protocol was initiated at 40°C and continued for one minute. The temperature subsequently increased to 280°C at a rate of 3°C per minute and sustained that level for five minutes. The sample was introduced in split injection mode at a 100:1 ratio. The system pressure was set to 50 kPa, the ion source to 200°C, and the electron impact ionization to 70 eV. We utilized retention indices and mass spectra to identify the chemicals by comparing them with entries in the NIST and Wiley spectral databases.

2.3. Target gene prediction

Two databases, SwissTargetPrediction (http://www.swisstargetprediction.ch/) and the Comparative Toxicogenomics Database (CTD; http://ctdbase.org/), were utilized to identify potential gene targets of chemicals in KEO based on GC-MS analysis. We converted the chemical structure of each molecule into the usual SMILES format and submitted it to SwissTargetPrediction, specifying "*Homo sapiens*" as the target organism. We utilized the GeneCards database (https://www.genecards.org/) and the Open Targets Platform (https://www.opentargets.org/) to identify genes associated with inflammation. We examined inflammation-related genes from both databases and selected those that were common to both as the principal targets in the inflammatory process.

2.4. Protein–protein interaction (PPI) network analysis

The overlap between the anticipated targets of KEO drugs and inflammation-associated genes was analyzed using a Venn diagram created using Venny 2.1.0. The overlapping genes were subsequently submitted to the STRING database (https://string-db.org/) with a minimum interaction confidence score of 0.9. We utilized Cytoscape software version 3.10.2 to visualize the resulting PPI network. Hub genes were identified utilizing the CytoHubba plugin by analyzing topological measures such as degree centrality, betweenness centrality, and closeness centrality (CC). For additional analysis, we concentrated on the maximum values for these parameters.

2.5. Enrichment and pathway analysis

To evaluate the biological relevance of the predicted target genes, functional enrichment analysis was performed using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations via the DAVID platform (https://david.ncifcrf.gov/). GO analysis encompassed three main categories: biological process (BP), molecular function (MF), and CC. The enrichment results were visually presented using the SRPlot platform (https://www.bioinformatics.com.cn/) to facilitate interpretation.

2.6. Animal design and preparation

A total of 30 male Wistar rats (*Rattus norvegicus* L.), approximately two months old and weighing 200 g, were used. Male rats were used to minimize biological variability associated with hormonal fluctuations in females during the estrous cycle, which can affect inflammatory responses. This approach is consistent with standard practices in preclinical research to maintain data consistency [24,25]. The rats were sourced from the Pharmacology Laboratory, Faculty of Pharmacy, Universitas Sumatera Utara, and acclimatized for 7

days to reduce stress that can affect changes in physiological responses, including stress hormones that can affect the immune and inflammatory systems. The experiment utilized six groups: one negative control, one positive control, and four treatment groups administered KEO at dosages of 400, 200, 100, and 50 mg/kgBW. The sample size was determined based on Federer's criterion for six treatment groups ($r \geq 4$). A total of five animals per group ($n = 5$) was selected as a compromise between statistical power and ethical considerations (3R principle), consistent with sample sizes commonly used in carrageenan-induced paw edema models. The repeated-measures design enhanced statistical sensitivity, while the final decision also considered resource limitations and standard practices in similar preclinical studies. A double geometric series was used to construct a KEO dose range of 50–400 mg/kgBW. This facilitates the visualization of the dose-response relationship. This range is similar to that used in preclinical studies of essential oils, which are often active at tens to hundreds of mg/ kgBW and are still safe for short-term use. Oral administration was selected, with the suspension volume adjusted to not exceed 1 ml per animal, hence preventing stomach distension and variability in absorption. The dosages were 1.00, 0.50, 0.25, and 0.125 ml at a suspension concentration of 8% (80 mg/ml). The KEO suspension (8%) was formulated by dissolving 0.964 g of KEO in 1 ml of 5% Tween-80, thereafter diluting with distilled water to get a final volume of 10 ml. 5% Tween-80 served as the negative control, whereas diclofenac sodium at a dosage of 2.25 mg/kg body weight was utilized as the positive control. Diclofenac sodium is a nonsteroidal anti-inflammatory drug commonly used as a positive control in anti-inflammatory activity studies, both *in vitro* and *in vivo*. The selection of diclofenac as a positive control is based on its precise pharmacological mechanism of action, stability of effects, and widespread clinical use as a standard anti-inflammatory agent. Because of its widely proven effectiveness, diclofenac sodium is often used as a comparator (positive control) to validate the anti-inflammatory activity of new test compounds, such as plant extracts, secondary metabolites, and synthetic compounds [26,27].

Inflammation induction with 0.1 ml of carrageenan solution in the left hind paw was performed after 30 minutes of oral administration. Inflammation volume measurements were performed using a plethysmometer before induction and at 30, 60, 90, 120, 150, 180, 240, 300, and 360 minutes after injection. The anti-inflammatory effect was evaluated by calculating both the percentage of inflammation and the percentage of inhibition of inflammation according to the following formulas [28]:

$$\text{Percentage of inflammation} = \frac{V_t - V_0}{V_0} \times 100$$

where: V_t= volume (or weight) of the rat paw after inflammation induction
V_0 = volume (or weight) of the rat paw before inflammation induction

$$\text{Percentage of inhibition} = \frac{C - T}{C} \times 100$$

where: C= mean volume (or weight) of the rat paws of the negative control group
T= mean volume (or weight) of the rat paws of the treated group

2.7. Histology and analysis of Interleukin-6 (IL-6) and Tumor Necrosis Factor alpha (TNF-α) expression

After completing the inflammatory volume measurements, the animals were euthanized using ketamine, and the inflamed paw tissue was collected for histological analysis. Hematoxylin–eosin (HE) staining was performed to evaluate neutrophil infiltration in the histologically inflamed paw tissues. HE-stained sections were analyzed using a light microscope at 400x magnification, and the number of neutrophils was measured using ImageJ software in ten fields of view for each specimen.

Immunohistochemical (IHC) analysis assessed the expression of the pro-inflammatory cytokines IL-6 and TNF-α. The tissue was fixed in 10% buffered formalin, then subjected to a stepwise dehydration process in alcohol, clarification with xylol, and paraffin infiltration at 60°C. Tissue sections of 3–4 µm thickness were mounted on poly-L-lysine-coated glass slides and incubated overnight at 40°C. After deparaffinization and rehydration, endogenous peroxidase blocking was performed using 0.3% H_2O_2 and nonspecific blocking with 10% normal serum. The samples were then incubated with primary antibodies against IL-6 and TNF-α at 4°C for 18–22 hours, followed by a universal secondary antibody for 30 minutes. Staining was performed using 3,3-diaminobenzidine chromogen and Mayer's hematoxylin counterstain. Expression was observed under a light microscope at 400 × magnification, with each specimen analyzed across 10 fields of view. The percentage of protein expression was calculated by dividing the number of expressed proteins by the total number of proteins, with the assistance of ImageJ [29].

2.8. Statistical analysis

The research data were analyzed using SPSS version 26. All data are presented as mean ± standard deviation (SD). Differences between groups were assessed using one-way ANOVA followed by Tukey's post hoc test. A p-value < 0.05 was considered statistically significant. Graphs were generated using GraphPad Prism 9.

3. RESULTS AND DISCUSSION

3.1. Characterization and chemical profiling of KEO via GC–MS

The characterization results indicated that KEO possessed a specific gravity of 0.83 ± 0.01 g/ml at 25°C. This finding aligns with prior reports indicating that KEO generally displays a specific gravity between 0.85 and 0.89 g/ml [30–32]. MAE is acknowledged as a contemporary extraction technique that surpasses traditional procedures like hydrodistillation, as it facilitates reduced extraction durations, necessitates

less solvent, and diminishes the degradation of volatile chemicals [33,34]. This is especially significant as the primary components of KEO, including (−)-citronellal, β-pinene, and D-limonene, are extremely volatile and susceptible to degradation at elevated temperatures. Consequently, the application of MAE facilitates the manufacture of essential oils that meet quality and physical characteristic standards. GC–MS analysis of KEO identified 60 volatile compounds, as shown in Table 1 and Figure 1 (chromatogram), with (−)-citronellal as the predominant component, followed by β-pinene, sabinene, and D-limonene. Ten major components were selected based on their relative abundance, and most of them have been reported to exhibit important biological activities, particularly anti-inflammatory effects. β-Pinene, for instance, has been shown to reduce edema and leukocyte migration [35]. α-Pinene provides neuroprotection against neonatal brain inflammation [36]. α-Terpineol suppresses IL-4, IL-13, and β-hexosaminidase release [37]. D-limonene and linalool significantly diminish the generation of TNF-α, IL-6, IL-1β, and reactive oxygen species [38]. Citronellol demonstrates anti-inflammatory characteristics and gastroprotective advantages [39]. The principal component, (−)-citronellal, has been shown to inhibit leukocyte infiltration, reduce edema, and provide redox protection [40]. Citronellal, or 3,7-dimethyl-6-octenal, is a simple monoterpene aldehyde distinguished by an extended carbon chain, associated with many biological activities including immunosuppression, antibacterial effects, anticancer properties, and anti-inflammatory actions [41,42].

3.2. Network pharmacology analysis of KEO compounds and inflammation-related genes

The network pharmacology analysis focused on the 10 principal constituents of KEO discovered using GC–MS. The target prediction for the chosen KEO constituents identified 523 distinct genes linked to these chemicals. Concurrently, inflammation-associated genes were extracted from two principal databases: GeneCards (19,480 genes) and Open Targets (2,388 genes). The convergence of both datasets identified 2,259 shared genes (11.5%) (Fig. 2a), illustrating the complementary characteristics of the two platforms. GeneCards consolidates various genomic and proteomic information, while Open Targets focuses on clinically and experimentally substantiated medicinal targets. The 2,259 overlapped genes were designated as the inflammation-related gene collection for subsequent study. A comparison with the 523 anticipated KEO targets revealed 265 shared genes (10.5%) (Fig. 2b). Despite the seemingly tiny percentage, the absolute count of overlapping genes is substantial, offering a robust basis for subsequent network development and functional enrichment analysis. These findings underscore that the bioactive constituents of KEO may affect critical molecular pathways associated with inflammation. The substantial percentage of KEO targets identified within the inflammation-related gene set highlights the pharmacological significance of KEO components in this setting.

The overlapping target profiles of key KEO ingredients, including D-limonene, α-pinene, and linalool, indicate that the anti-inflammatory activity is probably attributable to the synergistic action of many bioactive chemicals. While direct evidence of additive or synergistic interactions remains scarce, prior research has indicated that terpenoid combinations may amplify anti-inflammatory effects by inhibiting MAPK and NF-κB pathways and downregulating pro-inflammatory mediators such as IL-6, TNF-α, and NO in activated macrophages. This suggests that the anti-inflammatory properties of KEO may arise from interactions among multiple components rather than from a singular element, a theory that necessitates additional exploration in future investigations on compound combinations [43,44].

3.3. Protein–protein interaction (PPI) network and hub gene analysis

The analysis of the PPI network, utilizing 265 overlapping genes, identified numerous pivotal hub genes linked to KEO's anti-inflammatory properties. TP53, IL6, and MAPK3 had the highest degree centrality, indicating robust connections with other genes (Fig. 3a). The betweenness centrality metric identified TP53, PLA2G4A, and GNAS as significant bridging nodes (Fig. 3b), whereas closeness centrality indicated TP53, Jun oncogene (JUN), and ESR1 as the most central nodes in the network (Fig. 3c). TP53 consistently rated as the foremost gene in all three analyses, highlighting its significant function in tumor suppression and the regulation of inflammatory responses [45,46]. Nonetheless, due to the generic role of TP53, JUN, and MAPK3 (ERK1) in inflammation, these genes are maintained as predictive but have not yet been established as validated functional indicators. In contrast, IL6 and TNF-α were identified as better-suited indicators due to their recognized functions as principal pro-inflammatory cytokines in the inflammatory cascade [47,48]. The depiction of the PPI network (Fig. 3) illustrates node influence by color intensity, with red signifying elevated centrality scores. The ten principal hub genes identified for each centrality metric are also emphasized in the respective networks.

3.4. Functional enrichment and pathway analysis of hub genes in KEO

Functional enrichment analysis indicated that the hub genes identified from the PPI network are significantly linked to transcriptional control and inflammatory signaling. Within the BP category, the most prominent phrases encompassed reaction to lipopolysaccharide and positive control of transcription by RNA polymerase II. In the CC category, abundant phrases such as cytosol, plasma membrane, and cytoplasm suggest that the anticipated targets are predominantly situated in areas essential for signal transduction. In the MF category, identical protein binding and enzyme binding were the most prevalent (Fig. 4a). Pathway enrichment analysis further underscored significant correlations with inflammation-related signaling pathways. The most enriched KEGG pathways were cytokine–cytokine receptor interaction, cytokine receptor binding, and the chemokine signaling pathway, all of which are pivotal in the onset and advancement of inflammation (Fig. 4b). These findings align with the GO results, validating the idea that KEO influences cytokine activity.

Table 1. GC–MS analysis results of KEO components.

No	Compound name	Retention time (minutes)	Area (%)
1	(-)-Citronellal	7.449	16.34
2	β-Pinene	4.150	12.51
3	Sabinene	4.106	11.94
4	D-Limonene	4.973	11.72
5	(-)-Terpinen-4-ol	8.041	6.07
6	Linalool	6.218	5.17
7	α-Terpineol	8.323	4.56
8	Citronellol	9.095	3.86
9	α-Pinene	3.514	2.92
10	Cadina-1[10],4-diene	16.347	2.37
11	Copaene	12.813	2.34
12	γ-Terpinene	5.439	2.26
13	Caryophyllene	13.905	1.75
14	1H-Cyclopenta[1,3]cyclopropa[1,2]benzene	13.139	1.51
15	6-Octen-1-ol, 3,7-dimethyl-, acetate	12.099	1.45
16	1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	4.694	1.40
17	Cyclohexene, 3-methyl-6-(1-methylethylidene)-	6.011	1.16
18	8-Isopropyl-1-methyl-3-methylenetricyclo[4.4.0.0²,⁷]decane (rel-)	15.367	1.09
19	2-Furanmethanol, 5-ethenyltetrahydro-α,α,5-trimethyl-, cis-	5.687	0.94
20	3,7,11,11-Tetramethylbicyclo[8.1.0]undeca-2,6-diene	15.731	0.74
21	o-Cymene	4.827	0.56
22	1,4,7-Cycloundecatriene, 1,5,9,9-tetramethyl-, (Z,Z,Z)-	14.707	0.50
23	4-Isopropyl-1-methylcyclohex-2-enol	6.711	0.41
24	Bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)-	3.392	0.37
25	3,7-Cyclodecadiene-1-methanol, α,α,4,8-tetramethyl-, [s-(Z,Z)]	16.915	0.33
26	Geraniol	9.684	0.33
27	α-Cadinol	19.340	0.32
28	β-Ocimene	5.167	0.32
29	endo-Borneol	7.745	0.31
30	Camphene	3.725	0.28
31	(1R,2R,5S)-5-Methyl-2-(prop-1-en-2-yl)cyclohexanol	7.562	0.27
32	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-, cis-	7.119	0.27
33	5-Isopropyl-2-methylbicyclo[3.1.0]hexan-2-ol	5.596	0.26
34	τ-Muurolol	19.061	0.26
35	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-	15.938	0.20
36	1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-, (E)-	17.170	0.20
37	Bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)-	4.497	0.20
38	2-Cyclohexen-1-ol, 3-methyl-6-(1-methylethyl)-, trans-	8.402	0.19
39	1-Naphthalenepropanol, α-ethenyldecahydro	26.509	0.19
40	3,7-Dimethyloctahydro-1H-cyclopenta[1,3]cyclopropa[1,2]-	16.153	0.17
41	2-Cyclohexen-1-ol, 3-methyl-6-(1-methylethyl)-, trans-	8.646	0.15
42	Octanal	4.381	0.14
43	6-Octen-1-ol, 3,7-dimethyl-, (R)-	9.568	0.14
44	2-Cyclohexen-1-one, 3-methyl-6-(1-methylethyl)-	9.779	0.13
45	1H-Cyclopenta[1,3]cyclopropa[1,2]benzen-3-ol	15.663	0.13
46	2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (Z)-	12.367	0.12
47	Cubenol	18.755	0.12

Continued

No	Compound name	Retention time (minutes)	Area (%)
48	(-)-Globulol	17.768	0.12
49	(1S,2R,5R)-2-(2-Hydroxypropan-2-yl)-5-methylcyclohexanol	11.670	0.12
50	Ethanone, 1-(1,2,2,3-tetramethylcyclopentyl)-, (1R-cis)-	8.742	0.11
51	(-)-Spathulenol	17.615	0.11
52	(2S,4R)-4-Methyl-2-(2-methylprop-1-en-1-yl)tetrahydro-2H-pyran	6.432	0.11
53	Unidentified component	18.836	0.11
54	Decanal	8.510	0.11
55	Cyclohexanol, 3-ethenyl-3-methyl-2-(1-methylethenyl)-	19.601	0.10
56	2H-Pyran, tetrahydro-4-methyl-2-(2-methyl-1-propenyl)-	6.800	0.04
57	Cyclopropanemethanol, 2-methyl-2-(4-methyl-3-pentenyl)-	7.687	0.03
58	1-Naphthalenol, 1,2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl-	19.142	0.03
59	Alloaromadendrene oxide-[2]	18.955	0.02
60	2-Naphthalenemethanol, decahydro-α,α,4a-trimethyl-8-methylene	19.265	0.02
	Total		100.00

Note: The chemical components were arranged according to their relative peak area percentages obtained from the GC–MS analysis. The ten major compounds with the highest percentages were selected for subsequent *network pharmacology* analysis.

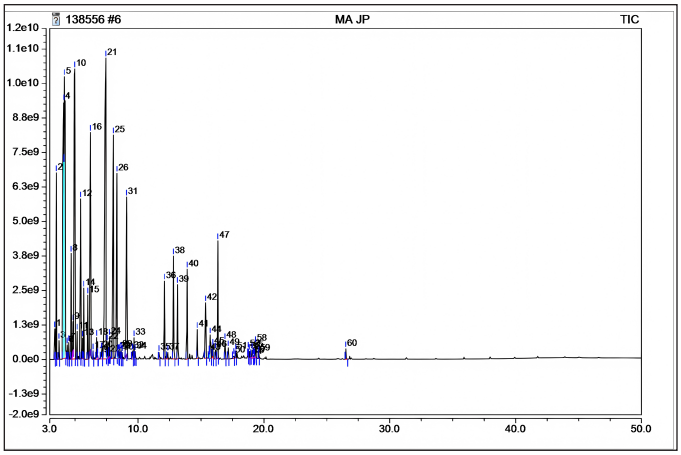


Figure 1. GC–MS chromatogram of KEO.

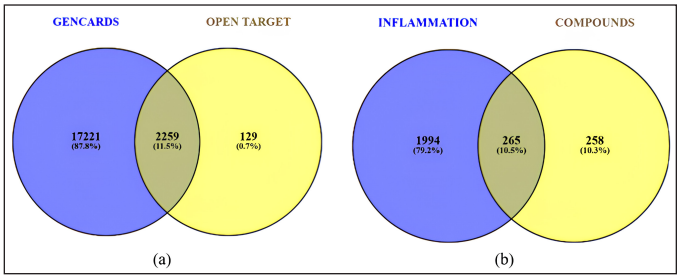


Figure 2. Overlapping inflammation-related genes identified from GeneCards and Open Targets (a). Venn diagram showing the intersection between 523 predicted KEO target genes and 2,259 inflammation-related genes, identifying 265 shared targets (b).

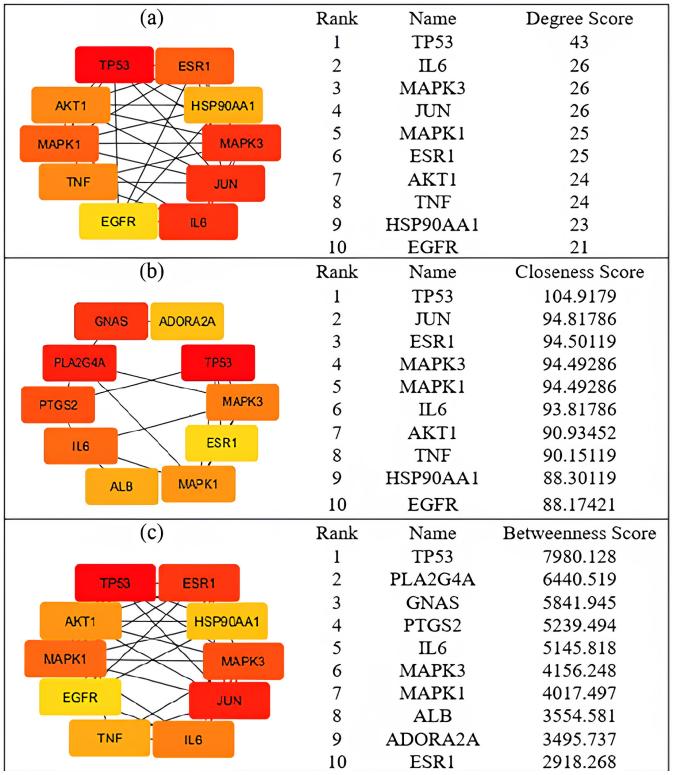


Figure 3. Hub gene analysis of the *Citrus hystrix* essential oil–inflammation PPI network using Cytoscape (CytoHubba). Degree centrality (a), Betweenness centrality (b), and Closeness centrality (c). Nodes are colored according to centrality scores, with red indicating higher influence within the network.

Cytokine receptor interactions and chemokine signaling have emerged as pivotal pathways in inflammatory development, highlighting their potential as therapeutic targets [49]. The enhancement of transcriptional control facilitated by RNA polymerase II indicates that KEO components may affect gene expression patterns induced by pro-inflammatory stimuli like lipopolysaccharides. The primary cellular location in the cytosol, plasma membrane, and cytoplasm indicates their role in both membrane-associated and intracellular signaling

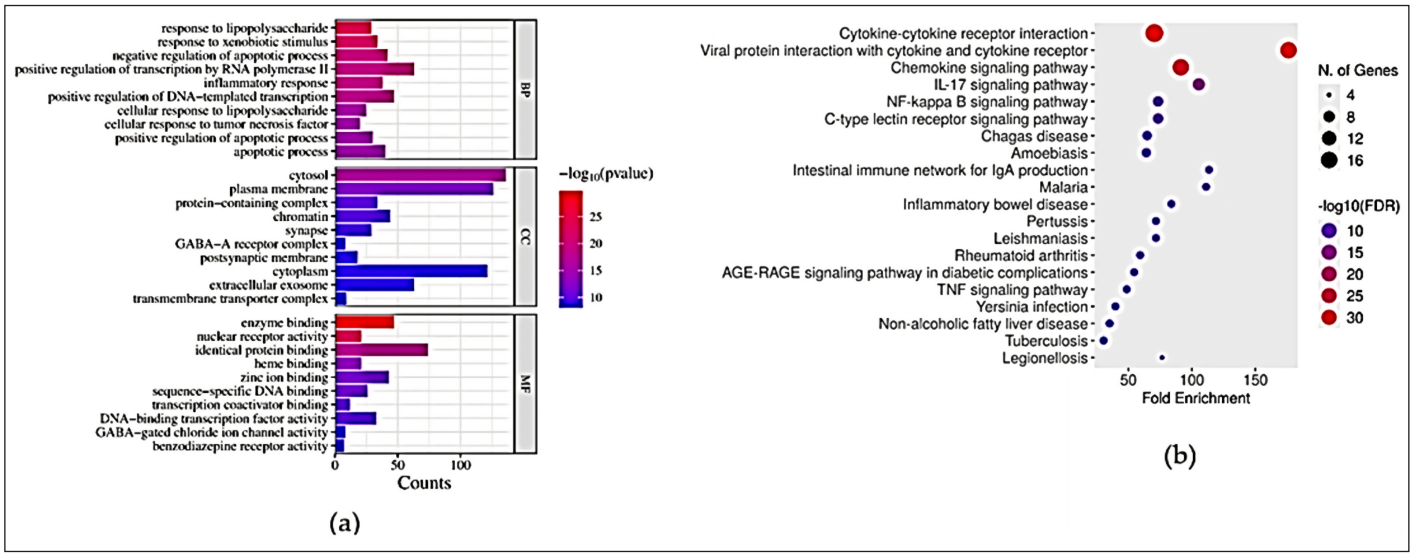


Figure 4. Functional enrichment and pathway analysis of KEO hub genes. Gene Ontology (GO) enrichment across three domains: biological process (BP), cellular component (CC), and molecular function (MF) (a). KEGG pathway enrichment of hub genes, highlighting key signaling pathways involved in inflammation (b).

processes [50]. These results collectively demonstrate that hub genes controlled by KEO are fundamentally implicated in immune responses and inflammatory signaling. Notable bioactive substances, including (−)-citronellal, α-pinene, β-pinene, and D-limonene, have been documented to diminish pro-inflammatory mediators such as TNF-α and IL-1β, while also offering protective benefits against oxidative stress-induced tissue damage [35–40,51–53]. The integrated evidence from GO and KEGG enrichment analyses provides significant biological insights and establishes a strong foundation for further validation of the anti-inflammatory activity of KEO through *in vitro* and *in vivo* studies using inflammation models. Although *in vitro* assays were not conducted in the present study, our previous findings demonstrated that KEO reduced nitric oxide production in LPS-induced RAW 264.7 macrophages [16], supporting the rationale for subsequent *in vivo* evaluation.

3.5. Anti-inflammatory activity test results

The anti-inflammatory activity of KEO was evaluated based on the percentage of inflammation and the percentage of inflammation inhibition, as shown in Figure 5. At 30 minutes, no significant differences ($p > 0.05$) were observed between the KEO-treated groups, the diclofenac sodium group, and the negative control. However, from 60 to 360 minutes, significant differences ($p < 0.05$) were found between the treatment groups and the negative control. The lowest percentage of inflammation was observed in the diclofenac sodium group, followed by KEO at doses of 400, 200, 100, and 50 mg/kg BW. The groups treated with 50 and 100 mg/kg BW of KEO showed lower inflammation inhibition, whereas doses of 200 and 400 mg/kg BW demonstrated the highest inhibitory effects. Between 60 and 360 minutes, low doses (50 and 100 mg/kg BW) differed significantly ($p < 0.05$) compared to diclofenac sodium, whereas high doses (200 and 400 mg/kg BW) showed no significant difference ($p > 0.05$), suggesting that higher doses have greater anti-inflammatory potential.

The fundamental process is probably linked to citronellal content, which might inhibit inflammatory mediators, thereby diminishing cell migration and paw edema induced by carrageenan. Carrageenan provokes inflammation by interaction with the TLR-4 receptor, leading to the production of inflammatory mediators such as prostaglandins, histamine, serotonin, bradykinin, nitric oxide, TNF-α, IL-1β, and IL-6 [54,55]. Furthermore, an elevation in prostaglandin E2 levels three hours after stimulation further facilitates cell migration and edema [56]. Citronellal in KEO is believed to impede these mediators, resulting in reduced cell migration and edema [40,41].

3.6. Histological observation

The findings indicated a substantial decrease ($p < 0.05$) in neutrophil infiltration in muscle and connective tissues in the treatment groups relative to the negative control. Both diclofenac sodium and KEO reduced neutrophil counts, with the 200 and 400 mg/kg BW KEO dosages exhibiting no significant difference ($p > 0.05$) in comparison to diclofenac sodium (Fig. 6).

Neutrophils constitute the primary defense mechanism of the immune system, directly addressing infections and participating in phagocytosis and the annihilation of alien entities. They can secrete pro-inflammatory cytokines that may harm tissues and initiate an inflammatory response [57]. Recent studies have emphasized the variability and adaptability of neutrophils, indicating that diminished recruitment is frequently associated with a reduction in the severity of acute inflammation [58]. In carrageenan-induced paw edema models, neutrophil infiltration is a characteristic histological feature of the initial inflammatory phase. The decrease in neutrophil counts post-treatment indicates anti-inflammatory effectiveness, aligning with data that pharmacological drugs and phytochemicals can inhibit neutrophil recruitment and enhance histological results [59,60]. The proposed mechanisms include the inhibition of NF-κB/MAPK signaling, suppression of chemotactic mediators

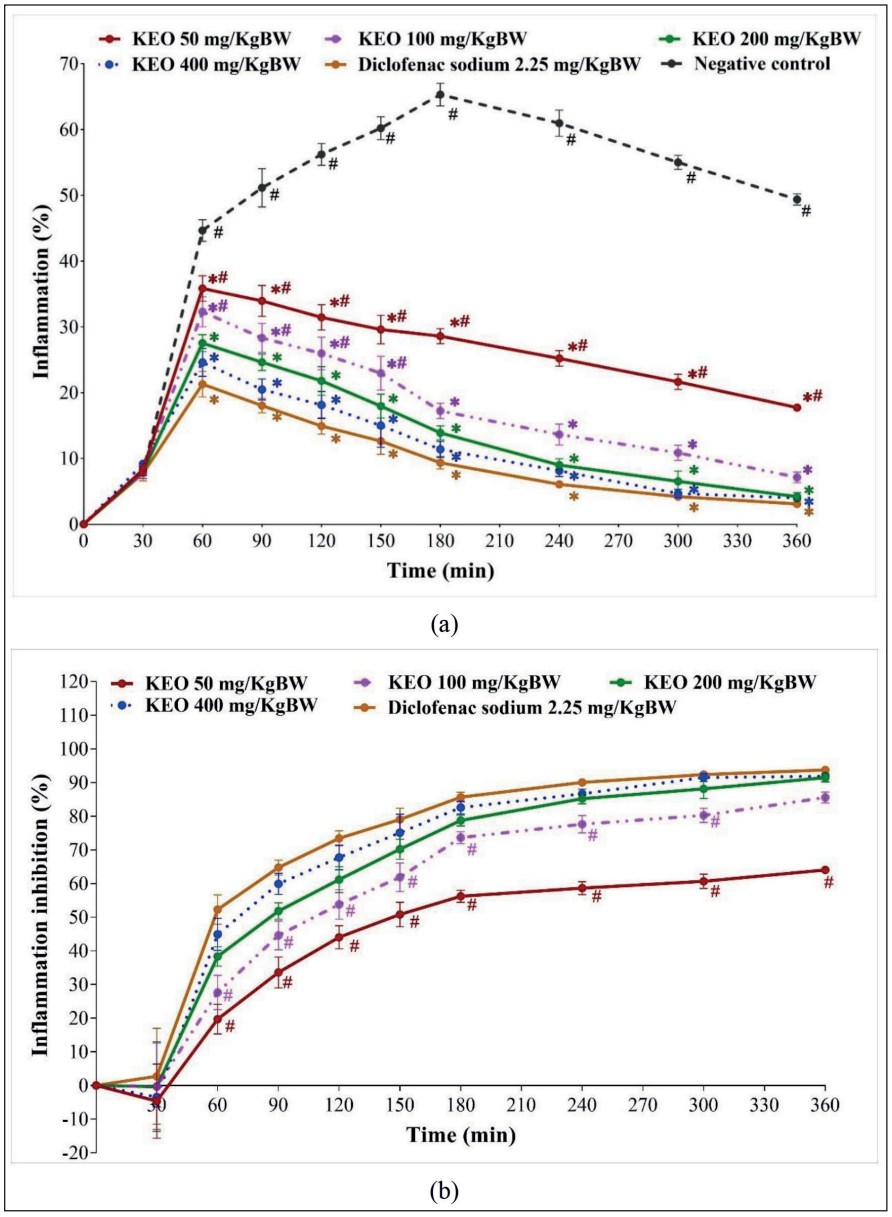


Figure 5. Anti-inflammatory effects of KEO on carrageenan-induced paw edema in rats. Percentage of inflammation (a), and percentage of inflammation inhibition. Data are presented as mean ± SD ($n = 5$). *: indicates a significant difference from negative control ($p < 0.05$); #: indicates a significant difference from diclofenac sodium 2.25 mg/KgBW ($p < 0.05$).

(chemokines, prostaglandins), and a decrease in oxidative stress [61,62].

These histological findings strengthen the functional data (inflammation and inhibition percentage), demonstrating that KEO—particularly at doses of 200–400 mg/kg BW—exerts significant anti-inflammatory activity comparable to diclofenac sodium. This effect is likely due to active terpenes and terpene alcohols in KEO, such as citronellal and caryophyllene, which have been shown to inhibit pro-inflammatory cytokine production and arachidonic acid metabolism, thus diminishing neutrophil migration and tissue damage in both acute and chronic inflammation [63–65].

3.7. Observation of TNF-α and IL-6 protein expression

Protein expression was indicated by a brown color, whereas non-expressing proteins were marked by a purple color. This principle is consistent with the IHC method, which is based on the use of specific antibodies to detect and label target proteins in tissues. The primary antibody binds to the target protein, in this case, TNF-α and IL-6. Subsequently, the secondary antibody binds to the primary antibody. The enzyme then reacts with a specific substrate to produce a brown color that marks the site of protein expression within the cell or tissue. Areas without protein expression retain the background color, such as purple from hematoxylin staining [66,67]. IHC enables visualization of the spatial distribution and intensity

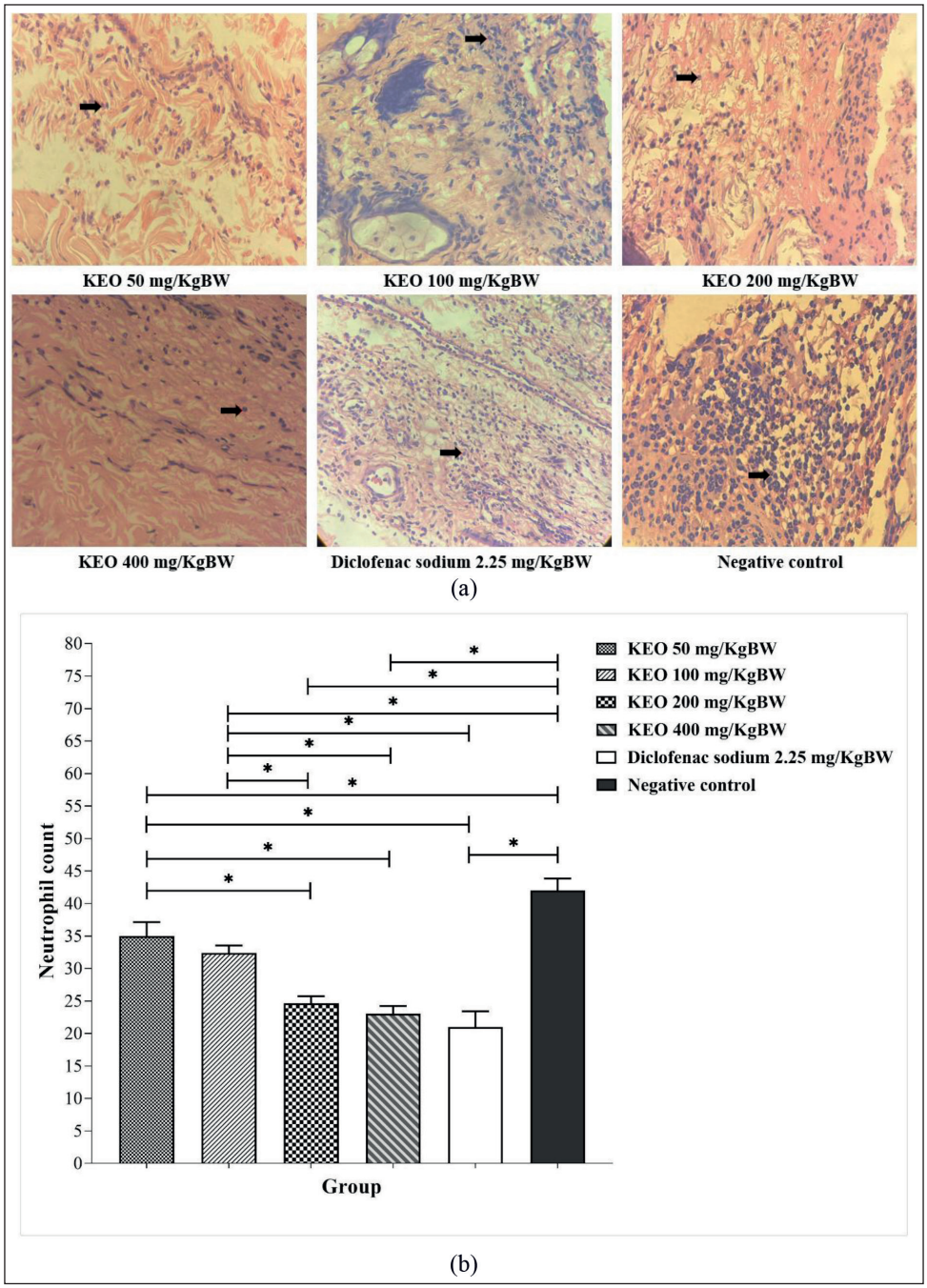


Figure 6. Histological observation of rat paw tissue stained with hematoxylin–eosin (a). Percentage of neutrophils in the inflamed area (b). Data are presented as mean ± SD (n = 5). *: indicates a significant difference ($p < 0.05$); ↑: indicates neutrophils.

of target proteins within the tissue context, making it useful for evaluating inflammatory responses or specific molecular activities in animal research models [29].

IHC analysis in Figure 7 indicated that the treatment of KEO and sodium diclofenac markedly decreased the production of the proinflammatory proteins TNF-α and IL-6 in comparison to the negative control ($p < 0.05$). The TNF-α expression % in the KEO 400 mg/kgBW group and IL-6 expression in the KEO 200 and 400 mg/kgBW groups did not exhibit significant differences when compared to sodium diclofenac ($p > 0.05$), suggesting that KEO possesses anti-inflammatory capability akin to sodium diclofenac. TNF-α and IL-6 are principal proinflammatory cytokines that regulate acute and chronic inflammatory responses, encompassing inflammatory cell recruitment, endothelial activation, and enhanced vascular permeability. They are synthesized by diverse immune cells, including macrophages, T lymphocytes, and endothelial cells, in addition to non-immune cells such as adipocytes

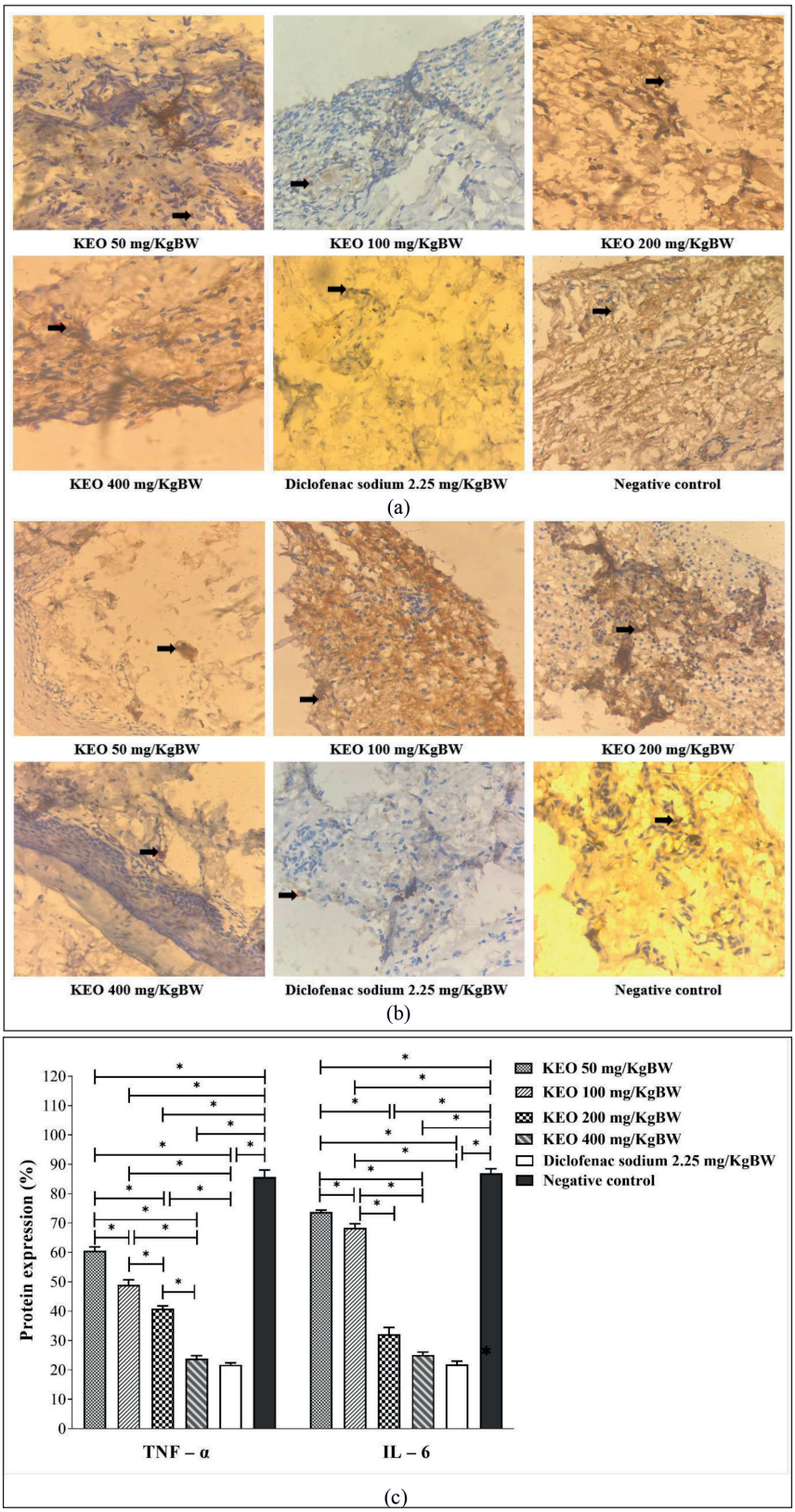


Figure 7. Analysis of protein expression by immunohistochemistry: TNF-α (a); IL-6 (b); and percentage of TNF-α and IL-6 protein expression in the inflamed area (c). Data are presented as mean ± SD ($n = 5$). *: indicates a significant difference ($p < 0.05$); ↑ indicates TNF-α or IL-6 protein expression.

[68]. TNF-α serves as a crucial mediator in the inflammatory response, initiating immune cell activation, enhancing vascular permeability, and stimulating the synthesis of other cytokines; high levels of TNF-α can lead to tissue damage and exacerbate chronic inflammatory disorders [69]. IL-6 functions both as an inflammatory mediator and as a regulator of immunological responses, with increased levels frequently detected in chronic inflammatory diseases, so serving as a valuable prognostic marker. Both cytokines serve as biomarkers for evaluating inflammation levels and monitoring therapeutic responses [70]. The immunohistochemistry findings corroborate additional histological and functional evidence, strengthening the notion that KEO efficiently mitigates acute inflammatory responses in a rat paw edema model. However, this IHC analysis is semi-quantitative and based on visual assessment, so it may not fully reflect the actual level of protein expression.

Although this study demonstrates the potential anti-inflammatory effects of KEO, several limitations should be acknowledged. First, the mechanistic interpretation of these effects remains preliminary, as the analysis was limited to the immunoreactivity of TNF-α and IL-6, while key molecular pathway markers such as NF-κB, COX-2, and MAPK3, as well as serum cytokine levels, were not evaluated. Second, the network pharmacology analysis provided predictive insights into potential target genes and pathways, but has not yet been experimentally validated. Third, the *in vivo* experiment employed only an acute inflammation model without evaluation in a chronic inflammation model. In addition, the immunohistochemical analysis was semi-quantitative and based on visual assessment, which may not fully represent the actual level of protein expression. Nevertheless, this study can serve as a foundation for future research integrating serum cytokine analysis, quantitative gene and protein expression assays, and chronic inflammation models to confirm and expand upon the current findings.

4. CONCLUSION

The essential oil of Kaffir lime (*Citrus hystrix* DC.) peel KEO was successfully extracted using the MAE method. GC–MS analysis identified major active constituents, including (−)-citronellal, β-pinene, sabinene, D-limonene, (−)-terpinen-4-ol, linalool, α-terpineol, citronellol, α-pinene, and cadina-1[10],4-diene. A network pharmacology approach revealed that these compounds interact with target genes involved in inflammatory pathways, with key hub genes such as TP53, IL6, MAPK3, and JUN. However, only the modulation of IL6 was experimentally validated *in vivo*, while the involvement of MAPK3 (ERK1) and other hub genes remains predictive and requires further validation. Additional studies, such as gene and protein expression analyses, including phosphorylation assays for MAPK3 (p-ERK) or AP-1 pathway analyses related to JUN, are warranted to confirm the mechanistic roles of these genes. GO and KEGG enrichment analyses demonstrated the involvement of these hub genes in transcriptional regulation, lipopolysaccharide response, and cytokine–chemokine signaling pathways. *In vivo* evaluation using a carrageenan-induced paw edema rat model showed that KEO significantly reduced the percentage of inflammation and increased inhibition of swelling compared to the negative control ($p < 0.05$). Notably, higher doses (200–400 mg/kg BW) produced anti-inflammatory effects comparable to sodium diclofenac at 2.25 mg/kg BW ($p > 0.05$). Histological analysis confirmed a reduction in neutrophil infiltration within inflamed tissues, while immunohistochemistry demonstrated decreased expression of the pro-inflammatory proteins TNF-α and IL-6. Collectively, these findings highlight that KEO possesses significant anti-inflammatory potential, mediated through the suppression of inflammatory mediators, inhibition of neutrophil recruitment, and modulation of key cytokines TNF-α and IL-6, supporting its potential as a natural alternative to conventional anti-inflammatory drugs.

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6. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

7. CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

The study protocol was approved by the Animal Research Ethics Committee, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Indonesia [Approval No.: 0657, KEPH-FMIPA/2024, dated July 25, 2024].

9. DATA AVAILABILITY

All data generated and analyzed are included in this research article.

10. PUBLISHER'S NOTE

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11. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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