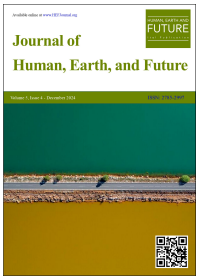
Available online at www.HEFJournal.org

ISSN: 2785-2997

Journal of
Human, Earth, and Future

Vol. 5, No. 4, December, 2024



The Potential of Ethanol Extract of *Aleurites Moluccanus* Leaves as TNF-α Inhibitor in Oral Incision Wound Care Model

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Received 16 July 2024; Revised 21 November 2024; Accepted 29 November 2024; Published 01 December 2024

Abstract

Candlenut (*Aleurites moluccanus*) is a plant that has active components and is believed to have medical benefits in every part of this plant. This study aims Identified the active compounds in the ethanol extract of candlenut leaves from the Seulawah Mountains, Aceh, Indonesia, and evaluated their effectiveness in reducing inflammation through the inhibition of TNF-α. The extraction of candlenut leaves was conducted using ethanol as a solvent. The active compounds in the candlenut leaves extract were identified using phytochemical screening, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, gas chromatography-mass spectrometry (GC-MS), and their biological activity was assessed using molecular docking. This study further examined the effects of candlenut leaves extract on wound healing in male white rats in vivo and the inhibition of TNF-α levels using the ELISA method through candlenut leaves extract mouthwash formulations at concentrations of 12.5%, 25%, and 50%. Phytochemical screening results revealed that the ethanol extract of candlenut leaves contains abundant secondary metabolites, including alkaloids, flavonoids, steroids/terpenoids, tannins, phenols, and saponins. Antioxidant analysis demonstrated that the ethanol extract exhibited strong antioxidant activity with an IC_{50} value of 89.221 ppm. GC-MS analysis identified 54 individual compounds in the extract, with five major compounds: n-hexadecanoic acid, isophytol, 9,12-octadecanoate-1-ol, octadecanoic acid, and squalene. These major compounds have significant bioactivities, including antioxidant, antibacterial, and antimicrobial properties. Molecular docking tests identified stigmasta-5,22-dien-3-ol and cycloheptadecanol as showing strong docking activity against TNF-α. Rats treated with the extract showed significant wound size reduction over 14 days, along with an increase in body weight. The extract also demonstrated an inhibitory effect on TNF-α concentration based on the dosage used. The anti-inflammatory effect of certain active compounds can reduce the regulation of specific cytokines, thereby inhibiting inflammation. These findings suggest that the active compounds in the ethanol extract of candlenut leaves have the potential to inhibit TNF-α, a key of pro-inflammatory cytokine, through significant antioxidant and anti-inflammatory activities.

Keywords: Aleurites Moluccanus; Anti-Inflammatory Activities; Antioxidant; Candlenut Leaves; TNF-α.

1. Introduction

Oral health is a critical component of overall health and can significantly influence other health conditions, thereby affecting the overall quality of life [1]. Dental and oral health issues rank first among the top 10 most common diseases suffered by the Indonesian population and have been increasing year by year [2]. These health issues are caused by

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http://dx.doi.org/10. 28991/HEF-2024-05-04-010

pathogenic and mutualistic bacteria that evolve within the oral cavity. One of the pathogenic microbial flora responsible for dental caries is *Streptococcus mutans* [3]. In addition to dental caries, injuries to the oral mucosa are also a serious concern. Over the past few centuries, numerous studies have employed various methods to treat wounds in the oral mucosa [4]. Effective wound healing is essential because injuries in the oral cavity can have significant consequences due to the susceptibility of these wounds to bacterial exposure and pathogenic infections [5]. In the field of healthcare, wound management has undergone rapid development, supported by advancements in technology that greatly facilitate the wound healing process [6].

Excessive use of chemicals, whether topically or through other means, has adverse side effects that can delay wound healing. The use of mouthwash is believed to clean the surfaces of teeth and the mouth, including areas between teeth that are not reachable by a toothbrush [7], as well as aid in wound healing and tissue regeneration [8, 9]. Many commercially available mouthwashes contain the chemical Chlorhexidine. Based on previous studies, chlorhexidine has demonstrated antibacterial properties that function by modifying cell membrane permeability [10]. The use of chlorhexidine shows potential in managing inflammation in the oral cavity; however, daily use at high concentrations has been shown to result in clinical deterioration in various studies [11]. Other studies have also revealed that long-term use may cause adverse side effects for users [12, 13].

An alternative to Chlorhexidine in mouthwash is the use of natural herbal ingredients with antibacterial properties to reduce side effects; candlenut leaves are one of them. Candlenut tree (*Aleurites moluccanus*). It is widely found in tropical rainforest regions of Indonesia and has a relatively fast growth rate. Every part of the tree, including the trunk, seeds, bark, sap, and leaves, has multiple uses [14]. The tree has long been used to produce herbal or traditional medicine. Several studies have reported the analgesic, anti-inflammatory [15], antipyretic, and antihyperlipidemic (fat-reducing mechanism) effects of candlenut leaves [16, 17].

Phytochemical screening of candlenut leaves extract, as reported in previous studies, has shown that the ethanol extract of candlenut leaves contains various active secondary metabolite compounds such as alkaloids, flavonoids [18], saponins, and tannins [16]. Screening results for the hexane fraction were positive for alkaloids. The ethyl acetate fraction was positive for alkaloids and tannins, while the residue fraction was found to contain alkaloids, flavonoids, saponins, and tannins [16].

To date, although several studies on herbal mouthwash have been conducted, research on the potential of Candlenut leaves for mouthwash formulation remains limited. Therefore, and based on the above-mentioned findings, this study is conducted to identify the key active compounds of Candlenut leaves collected from the valley of Mount Seulawah Aceh, Indonesia, and to evaluate their effectiveness in reducing inflammation through the inhibition of TNF-α as a model for oral incision wound care.

2. Research Methodology

2.1. Sample Preparation

An amount of 10 kg candlenut leaves was collected from the valley of Mount Seulawah, Saree District, Aceh Besar Regency. The finest leaves were picked and washed thoroughly before being laid to dry (no direct exposure of sunlight) for 7 days. The dried candlenut leaves were then grounded to powder and stored for future research.

2.2. Candlenut (*Aleurites moluceanus*) Leaf Extraction

The extraction process of candlenut leaves is carried out using the maceration method with 96% ethanol solvent, the extraction process is carried out for 3×24 hours filtered with occasional stirring to maximize the extraction process. After 3×24 hours, filtration process is then being carried out, followed by separating the residue and filtrate. The collected filtrate is then being concentrated using a rotary evaporator instrument to obtain candlenut leaves ethanol extract [19].

2.3. Phytochemical Screening of Candlenut Leaves (*Aleurites moluccanus*)

The analysis of candlenut leaves compounds aims to determine the presence of secondary metabolites. These secondary metabolites can be grouped based on basic frameworks, including alkaloids, terpenoids, steroids, saponins, and flavonoids [20].

2.4. Analysis of Antioxidant Activity of Candlenut Leaves (*Aleurites moluccanus*) using 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Assay

An ethanol extract of candlenut leaves, weighing 0.01 g, was dissolved in absolute ethanol up to 100 mL and then diluted to 20 ppm, 40 ppm, 60 ppm, 80 ppm, and 100 ppm. The absorbance of the test solution was then measured at a wavelength of 515 nm. The calculation of % inhibition data was performed using the formula:

$$\text{inhibitory concentration (\%)} = \frac{\text{blank absorbance} - \text{sample absorbance}}{\text{blank absorbance}} \times 100 \quad (1)$$

To calculate IC_{50}, the formula from the regression equation between concentration and % inhibition, $y = ax + b$, was used.

2.5. Characterization of Candlenut (*Aleurites moluceanus*) Leaves

Identification of compounds contained in candlenut leaves was carried out using GC-MS QP2010 Plus. Identification of phytochemicals in the sample was carried out using QP2010 gas chromatography with Thermal Desorption System, TD 20 coupled with Mass Spectroscopy. The ionization voltage was 70 eV, Gas Chromatography was performed in temperature programming mode with Restek Column (0.25 mm, 60 m, XTI-5). The initial column temperature was 80°C for 1 min and then increased linearly at 70°C min to 220°C, held for 3 min followed by a linear temperature increase of 10°C min to 290°C for 10 min. The temperature of the injection port was 290°C and the GC-MS surface temperature was maintained at 290°C. The sample was introduced through an all-glass injector working in split mode, with a helium carrier gas rate of 1.2 ml min'. Identification of compounds was carried out by comparison of retention times and fragmentation patterns, as well as by GC-MS mass spectra (Lai and Fiehn, 2018). Characterization of chemical compounds of candlenut (Aleurites moluccanus) leaves was carried out using GC-MS QP2010 Plus [21].

2.6. Molecular Docking

The receptors used for molecular docking was *Tumor Necrosis Factor-α* (TNF-α) (PDB ID: 2AZ5) [22]. The molecular docking process was conducted using Autodock Vina, then the results of molecular docking results was visualized using BIOVIA Discovery Visualizer [23].

2.7. Preparation of Mouthwash Formulation

The ethanol extract used in the mouthwash formulation process consisted of three different formulas, with varying concentrations of 12.5%, 25%, and 50%. The preparation of the mouthwash formulation began with creating the aqueous phase, which involved dissolving xylitol, calcium lactate, and potassium thiocyanate separately in water. These solutions were then combined and mixed until homogeneous. Additionally, ingredients with low water solubility, such as benzoic acid and butylated hydroxyanisole, were dissolved in oleum menthe. Subsequently, the candlenut leaves extract was emulsified with PEG-40 hydrogenated castor oil. Propylene glycol was then gradually added and mixed until homogeneous. Next, 70% sorbitol was gradually incorporated into the mixture, stirred to homogeneous. Sodium benzoate was dissolved in water until a homogeneous solution was obtained, which was then added to the oleum menthe mixture, adjusting the pH to 6-7.

2.8. Wound Measurement in Rats

The anti-inflammatory test was conducted on 30 male white rats, each weighing between 250-300 grams. The rats were obtained from the Faculty of Veterinary Medicine, Syiah Kuala University, Banda Aceh, Indonesia. All rats underwent a surgical procedure under local anaesthesia using procaine cream to create a 0.5 cm incision wound in the oral area, extending to the subcutaneous layer at the vertebral region. The wounded rats were divided into 5 groups: Group 1 (T1) served as the negative control, where the wound surface was covered with aquadest. Group 2 (T2) was the positive control, with the wound surface treated with 0.2% chlorhexidine. Treatment Groups (T3, T4, and T5) received mouthwash treatments with ethanol extract of candlenut leaves at concentrations of 12.5%, 25%, and 50%, respectively. The wounds were treated twice daily at 08:00 and 18:00 for a duration of 14 days

2.9. Body Weight Measurement

The body weight of male white rats was measured before and after the treatment was administered.

2.10. Evaluation of Tumor Necrosis Factor- α (TNF-α) Inhibition via Enzyme Linked Immunoassay (ELISA) Method

The inhibition of TNF-α was evaluated using immunohistochemical expression methods. The staining procedure was conducted using monoclonal anti-rat vascular endothelial growth factor (VEGF) antibodies on tissue samples. The process began with deparaffinization using xylene followed by decreasing concentrations of ethanol. Subsequently, a 3% peroxide solution was applied to remove endogenous peroxidase, and the samples were washed with PBS. Next, the sections were treated with 0.025% trypsin in PBS for 6 minutes at 37°C, followed by incubation with monoclonal VEGF primary antibodies for 30 minutes and washing with PBS. This was followed by incubation with a secondary antibody for 30 minutes, washing with PBS, and then incubation with streptavidin-HRP for 30 minutes. The samples were washed again with PBS, then incubated with a chromogen substrate for 5 minutes, washed with PBS and distilled water, and finally stained with Mayer's Hematoxylin for 6 minutes before being rinsed with running water. The staining results were observed, and macrophages expressing VEGF were counted in 5 different fields of view at a magnification of 400x.

The flowchart of the research methodology that was used to achieve the study's aims is shown in Figure 1.

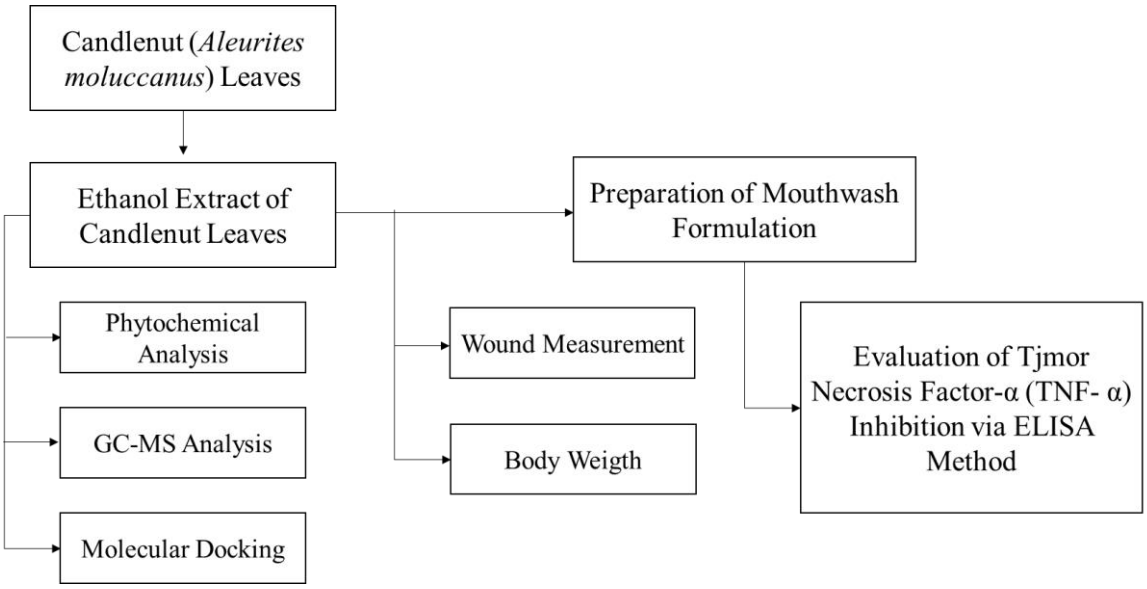

Figure 1. Flowchart of the research methodology

3. Results

3.1. Phytochemical Screening of Candlenut Leaves (*Aleurites moluccanus*)

The characterization of candlenut leaves extracts began with phytochemical screening. This study used ethanol as the solvent in the extraction process of candlenut leaves. The results of the phytochemical screening of the ethanol extract of candlenut leaves are presented in Table 1. Based on Table 1, the ethanol extract of candlenut leaves exhibited positive results in all types of phytochemical tests conducted, including tannins, flavonoids, saponins, alkaloids, and steroids.

Table 1. Phytochemical screening results of candlenut (*Aleurites moluccanus*) leaves extract

Phytochemical Screening	Result
Tannin	+
Flavonoid	+
Saponin	+
Alkaloid	+
Steroid	+

3.2. Characterization of Candlenut Leaves (*Aleurites moluccanus*)

The results of the antioxidant analysis of the candlenut leaves extract are shown in Table 2. Based on the analysis, the ethanol extract of candlenut leaves exhibits strong antioxidant activity, demonstrated by an IC_{50} value of 89.221 ppm.

Table 2. Antioxidants activity test results of candlenut (Aleurites moluccanus) leaves extract

Concentration	Absorbance	Control Absorbance	% inhibition	IC_{50}
20 ppm	0.451		8.146	
40 ppm	0.410		16.496	
60 ppm	0.354	0.491	27.902	89.221
80 ppm	0.315		35.845	
100 ppm	0.173		64.765	

GC-MS analysis was conducted to detect, identify, and quantify the number of compounds extracted from a plant [24]. Based on the results of this study, there were 54 individual compounds detected in the ethanol extract produced by candlenut leaves through GC-MS analysis as shown in Figure 2.

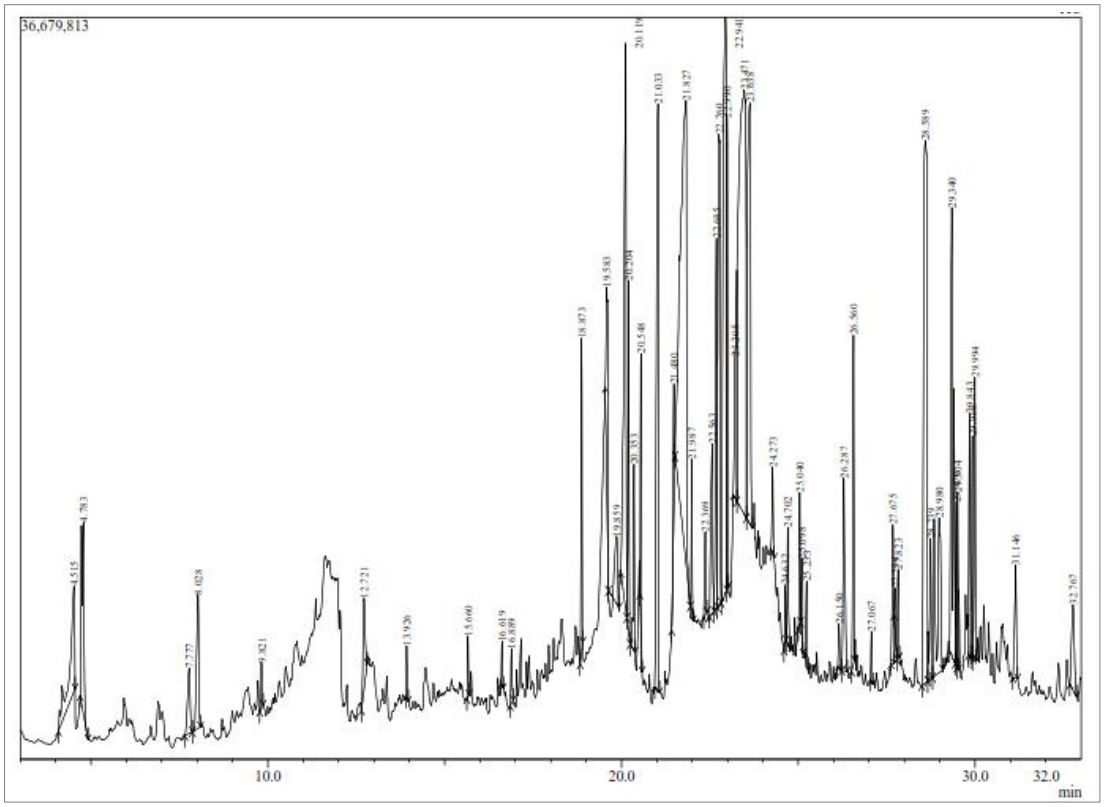

Figure 2. GC-MS Chromatogram of Ethanol Extract of Candlenut (*Aleurites moluceanus*) leaf

The active principles with retention time (RT), molecule formula and molecular weight (MW) and peak area as percentage are presented in Table 3. The results of this analysis showed the presence of 5 main compounds at retention times 21.83 (10.86%), 22, 94 (7.94%), 23.47 (12.02%), 23.64 (5.45%) and 28.59 (7.37%). The main biological compounds have been tabulated in Table 4. The five main compounds identified in this GC-MS analysis consist of n-hexadecanoic acid, 9,12-Octadecadien-1-ol (CAS: Octadec), squalene, isophytol, and Octadecanoic acid (CAS: Stearic acid).

Table 3. Bioactive Compounds from GC-MS Analysis of Ethanol Extract of Candlenut Leaves (*Aleurites moluceanus*)

No.	Retention Time (min)	Compounds	Molecular Formula	Molecular Weight (g/mol)	Peak Area (%)
1.	4.52	Acetic Acid	CH_3COOH	60.05	1.94
2.	4.78	2-Propanone, 1-hydroxy-	C_3H_6O	74.08	1.80
3.	7.78	Butyrolactone	$C_4H_6O_2$	86.09	0.68
4.	8.03	2-Cyclopenten-1-one, 2-hydroxy-	$C_5H_6O_2$	98.10	1.13
5.	9.82	1,5-Heptadiene, 2,3,6-trimethyl-	$C_{10}H_{18}$	138.25	0.20
6.	12.72	Benzofuran, 2,3-dihydro-	C_8H_8O	120.15	0.81
7.	13.93	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150.17	0.22
8.	15.66	5,9-Undecadien-2-one, 6,10-dimethyl-, (Z)-	$C_{13}H_{22}O$	194.31	0.20
9.	16.62	(.+/-.)-Lavandulol, chlorodifluoroacetate	$C_{12}H_{17}ClF_2O_2$	226.71	0.15
10.	16.89	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-	$C_8H_{10}O_2$	138.16	0.24
11.	18.87	Tetradecanoic acid, methyl ester (CAS) Me	$C_{15}H_{30}O_2$	242.40	1.15
12.	19.58	9-Octadecenoic acid (Z)- (CAS) Oleic acid	$C_{18}H_{34}O_2$	282.50	1.40
13.	19.86	(-)-Loliolide	$C_{11}H_{16}O_3$	196.24	0.56
14.	20.12	NEOPHYTADIENE	$C_{20}H_{38}$	278.50	4.80
15	20.20	2-Pentadecanone, 6,10,14-trimethyl-	$C_{18}H_{36}O$	268.50	1.42
16.	20.53	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [	$C_{20}H_{40}O$	296.50	0.75

17.	20.55	NEOPHYTADIENE	$C_{20}H_{38}$	278.50	0.88
18.	21.03	Hexadecanoic acid, methyl ester (CAS) Me	$C_{17}H_{34}O_2$	279.50	4.66
19.	21.48	Dibutyl phthalate	$C_{16}H_{22}O_4$	278.34	0.57
20.	**21.83**	**n-Hexadecanoic acid**	**$C_{16}H_{32}O_2$**	**256.42**	**10.86**
21.	21.99	Heptadecanoic acid, methyl ester	$C_{18}H_{36}O_2$	284.50	0.52
22.	22.37	cis-9-Hexadecenal	$C_{16}H_{30}O$	238.41	0.41
23.	22.56	Heptadecanoic acid (CAS) Margaric acid	$C_{17}H_{34}O_2$	270.50	1.28
24.	22.68	9,12-Octadecadienoic acid (Z,Z)-, methyl e	$C_{19}H_{34}O_2$	294.50	2.64
25.	22.76	11,14,17-Eicosatrienoic acid, methyl ester	$C_{21}H_{36}O_2$	320.50	3.91
26.	**22.94**	**Isophytol**	**$C_{20}H_{40}O$**	**296.50**	**7.94**
27.	22.99	Octadecanoic acid, methyl ester (CAS) Met	$C_{19}H_{38}O_3$	314.50	1.20
28.	23.20	Phytol, acetate	$C_{22}H_{42}O_2$	338.60	1.68
29.	**23.47**	**9,12-Octadecadien-1-ol (CAS) OCTADEC**	**$C_{18}H_{34}O$**	**226.50**	**12.02**
30.	**23.64**	**Octadecanoic acid (CAS) Stearic acid**	**$C_{18}H_{36}O_2$**	**284.50**	**5.46**
31.	24.27	9-Octadecenoic acid (Z)- (CAS) Oleic acid	$C_{18}H_{34}O_2$	282.50	0.39
32.	24.63	Tetradecanoic acid, 2,3-dihydroxypropyl es	$C_{17}H_{34}O_4$	302.40	0.19
33.	24.70	Eicosanoic acid, methyl Arachidate	$C_{21}H_{42}O_2$	326.6	0.53
34.	25.04	4,8,12,16-Tetramethylheptadecan-4-olide	$C_{21}H_{40}O_2$	324.50	0.50
35.	25.10	Eicosanoic acid	$C_{20}H_{40}O_2$	312.50	0.27
36.	25.23	FARNESYL ACETONE B	$C_{18}H_{30}O$	262.40	0.35
37.	26.15	Cycloheptadecanol	$C_{17}H_{34}O$	254.50	0.21
38.	26.28	Hexadecanoic acid, 2-hydroxy-1-(hydroxy	$C_{34}H_{67}NO_3$	537.90	1.40
39.	26.56	1,2-Benzenedicarboxylic acid, bis(2-ethylh	$C_8H_6O_4$	166.13	1.42
40.	27.06	Tricosanoic acid, methyl ester	$C_{24}H_{48}O_2$	368.60	0.18
41.	27.67	9-Octadecenoic acid, 1,2,3-propanetriyl est	$C_{57}H_{104}O_6$	885.40	0.85
42.	27.73	ETHYL LINOLEOLATE	$C_{20}H_{36}O_2$	308.50	0.11
43.	27.82	Heptacosanoic acid, methyl ester	$C_{28}H_{56}O_2$	424.70	0.40
44.	**28.59**	**Squalene**	**$C_{30}H_{50}$**	**410.70**	**7.37**
45.	28.74	(E,E,E)-3,7,11,15-Tetramethylhexadeca-1,3	$C_{20}H_{32}$	272.50	0.97
46.	29.98	2H-1-Benzopyran-6-ol, 3,4-dihydro-2,8-di	$C_{27}H_{46}O_2$	402.70	2.50
47.	29.34	GERANYL LINALOOL ISOMER B	$C_{20}H_{34}O$	290.50	4.13
48.	29.47	Squalene	$C_{30}H_{50}$	410.70	0.74
49.	29.50	Hexadeca-2,6,10,14-tetraen-1-ol, 3,7,11,16-	$C_{20}H_{34}O$	290.50	0.63
50.	29.84	1,6,10,14,18,22-Tetracosahexaen-3-ol, 2,6,	$C_{30}H_{50}O$	426.7	1.19
51.	29.92	2,6,10,14,18-Pentamethyl-2,6,10,14,18-eico	$C_{25}H_{42}$	342.6	1.17
52.	29.99	NERYL LINALOOL ISOMER	$C_{20}H_{34}O$	290.5	1.29
53.	31.14	dl-.alpha.-Tocopherol	$C_{29}H_{50}O_2$	430.7	0.78
54.	32.77	Stigmasta-5,22-dien-3-ol, (3.beta.,22E)- (C	$C_{29}H_{48}O$	412.7	0.94

The results of the molecular docking tests indicated the potential biological activity of the active compounds obtained from candlenut leaves ethanol extract. This potential biological activity was demonstrated by the inhibitory ability or binding affinity values (Kcal/mol). The target receptor is TNF-α (PDB ID: 2AZ5). The results of the study indicate that the compounds stigmasta-5,22-dien-3-ol and cycloheptadecanol exhibit strong docking activity. The biological activity results of the active compounds from candlenut leaves can be seen in Table 4.

Table 4. The binding affinity value between receptor TNF-α and bioactive compounds of Ethanol Extract of Candlenut Leaves (*Aleurites moluceanus*)

Compounds	Nilai Binding Affinity (Kkal/mol)
Chlorhexidine (Control positive)	-8.1
Acetic Acid	-3.7
2-Propanone, 1-hydroxy-	-3.7
Butyrolactone	-3.9
2-Cyclopenten-1-one, 2-hydroxy-	-4.4
1,5-Heptadiene, 2,3,6-trimethyl-	-5.2
Benzofuran, 2,3-dihydro-	-5.1
2-Methoxy-4-vinylphenol	-5.6
5,9-Undecadien-2-one, 6,10-dimethyl-, (Z)-	-6.0
(.+/-.)-Lavandulol, chlorodifluoroacetate	-6.2
2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-	-5.3
Tetradecanoic acid, methyl ester (CAS) Me	-5.1
9-Octadecenoic acid (Z)- (CAS) Oleic acid	-5.7
(-)-Loliolide	-6.8
NEOPHYTADIENE	-5.7
2-Pentadecanone, 6,10,14-trimethyl-	-6.0
2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, (2E,7R,11R)-	-6.0
Hexadecanoic acid, methyl ester (CAS) Me	-5.5
Dibutyl phthalate	-6.6
n-Hexadecanoic acid	-4.8
cis-9-Hexadecenal	-5.2
Heptadecanoic acid (CAS) Margaric acid	-5.3
9,12-Octadecadienoic acid (Z,Z)-, methyl ester	-5.9
Isophytol	-5.8
Phytol, acetate	-6.1
9,12-Octadecadien-1-ol (CAS) OCTADEC	-5.2
4,8,12,16-Tetramethylheptadecan-4-olide	-6.3
FARNESYLACETONE	-6.3
Cycloheptadecanol	-7.8
Squalene	-7.5
(E,E,E)-3,7,11,15-Tetramethylhexadeca-1,3,6,10,14-pentaene	-6.0
2H-1-Benzopyran-6-ol, 3,4-dihydro-2,8-dimethyl-2-((4R,8R)-4,8,12-trimethyltridecyl)-, (2R)-	-7.7
Geranyllinalool	-6.2
Hexadeca-2,6,10,14-tetraen-1-ol, 3,7,11,16-tetramethyl-	-6.5
2,6,10,14,18-Pentamethyl-2,6,10,14,18-eicosapentaene	-6.4
e-Neryl linalool	-6.0
dl-.alpha.-Tocopherol	-7.6
Stigmasta-5,22-dien-3-ol, (3beta,22E)-	-9.3

3.3. Anti-inflammation Activity of Candlenut Leaves (Aleurites moluccanus)

Based on the research results, the body weight of white male rats increased during the treatment period, as determined by ANOVA and t-test, which aimed to clarify the significant differences between treatment groups. The measurements of the rats' body weight are shown in Table 5. The body weight in both the treatment group and the control group showed a significant difference on days 3 and 7 ($p<0.05$), while no significant difference was observed on day 14 ($p>0.05$) (Figure 3).

Table 5. The body weight of the rats during the treatment period

Group	Body weight on day-3	Body weight on day-7	Body weight on day-14
T1	234.00±20.30	293.00±10.15	296.67±37.00
T2	269.67±5.51	267.00±13.00	302.33±21.59
T3	228.00±11.79	278.00±11.36	299.67±2.52
T4	249.33±15.95	275.67±9.87	317.00±21.00
T5	235.33±4.51	286.33±6.81	307.00±20.95

Description: T1 = negative control; T2 = positive control (chlorhexidine 0.2%); T3, T4 and T5 = treatment groups with the administration of candlenut leaves ethanol extract mouthwash with concentrations of 12.5%, 25% and 50%.

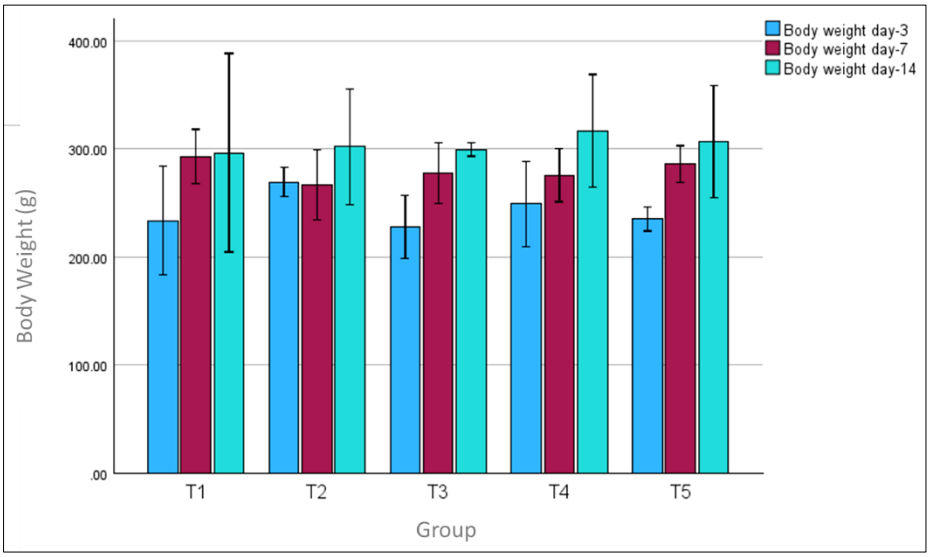

Figure 3. Comparison of body weight of the rats during the treatment period. T1 = negative control; T2 = positive control (chlorhexidine 0.2%); T3, T4 and T5 = treatment groups with the administration of candlenut leaves ethanol extract mouthwash with concentrations of 12.5%, 25% and 50%.

The anti-inflammatory test was conducted by creating a 0.5 cm wound on the mouth area of white male rats, followed by analyzing the reduction in wound size over 14 days. Based on the test results, a significant difference in wound size was observed after 14 days ($p<0.05$). The differences in wound size can be seen in Figure 4.

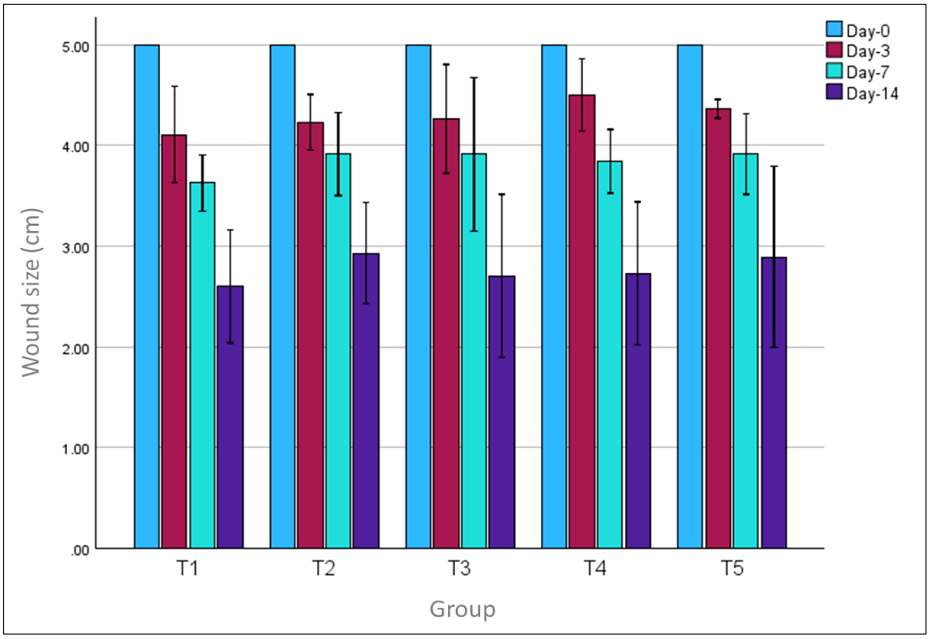

Figure 4. Comparison of wound size during the treatment period. T1 = negative control; T2 = positive control (chlorhexidine 0.2%); T3, T4 and T5 = treatment groups with the administration of candlenut leaves ethanol extract mouthwash with concentrations of 12.5%, 25% and 50%.

The measurement of wound size reduction over the 14-day treatment period can be seen in Table 6. Based on the results obtained, the wound size reduction during the first 3 days showed a significant difference in scar reduction, whereas the average wound reduction calculated over 7 and 14 days did not show a significant reduction.

Table 6. The wound size of the rats during the treatment period

Group	Treatment		
	Day-3*	Day-7	Day-14
T1	0.25±0.25	0.22±0.09	0.29±0.09
T2	0.35±0.33	0.25±0.83	0.33±0.08
T3	0.15±0.22	0.23±0.11	0.30±0.06
T4	0.45±0.11	0.32±0.37	0.38±0.00
T5	0.50±0.00	0.30±0.46	0.31±0.07

* Superscript indicated significant difference ($p<0.05$).

The determination of the bioactive compounds in candlenut leaves and their effects on pro-inflammatory mediators in vivo was conducted in this study, where the inhibitory effect of the active compounds on TNF-α was evaluated using immunohistochemical analysis, as shown in Figure 5. The results indicated that the ethanol extract of candlenut leaves could reduce TNF-α concentration as a pro-inflammatory mediator in a dose-dependent manner. The higher the dose of the ethanol extract used, the greater the reduction in TNF-α concentration.

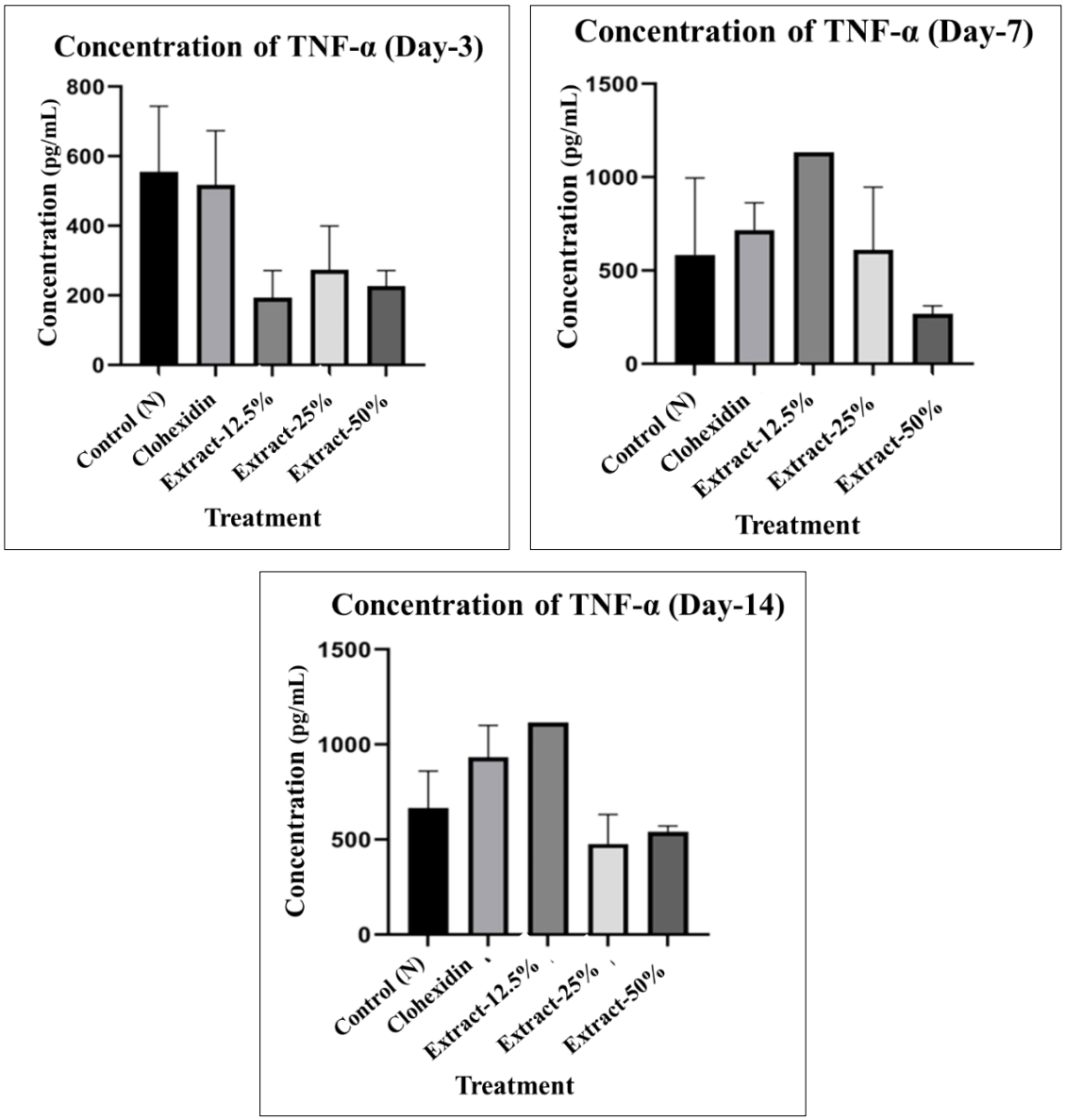

Figure 5. Comparison of wound size during the treatment period

4. Discussion

The antioxidant analysis conducted on the ethanol extract of candlenut leaves demonstrated strong antioxidant activity, as tests performed at various concentrations yielded a relatively low IC50 value (89.221 ppm). Gulcin (2020) stated that antioxidant activity is categorized as very strong if the IC50 value is <50 ppm; strong (50–100 ppm); moderate (100–150 ppm); weak (150–200 ppm); and very weak >200 ppm [25]. These findings were further supported by the results of phytochemical screening, which indicated that the candlenut leaf extract is rich in secondary metabolites. Previous studies revealed that candlenut leaves contain secondary metabolites such as alkaloids, flavonoids, phenols [18], steroids/terpenoids, tannins, and saponins [26]. Earlier researchers reported that candlenut leaves contain flavonoid and phenolic compounds, which are the primary contributors to antioxidant activity due to their ability to scavenge free radicals [27, 28]. Other studies have also highlighted the significant influence of flavonoid and phenolic compound content on lipid peroxidation inhibition and antioxidant capacity [27].

The GC-MS analysis data showed that the ethanol extract of candlenut leaves contains five major compounds: n-hexadecanoic acid, isophytol, 9,12-octadecadien-1-ol (CAS) Octadec, octadecanoic acid (CAS) stearic acid, and squalene. According to the GC-MS analysis results of this study, the main compounds obtained from the ethanol extract of candlenut leaves are terpenoids and fatty acids, which act as phytochemical agents. This is consistent with a study by Hakim et al. (2022), which stated that most active compounds detected in candlenut leaves are terpenoids.

Isophytol (7.94%) is a terpenoid molecule with antibacterial and antimicrobial activities [29]. According to prior study, isophytol compounds have a considerable effect on *Salmonella enterica* [30]. Isophytol is commonly utilized as an antibacterial component in essential oils [31]. In addition, squalene (7.37%) is a triterpenoid compound present in many plants. Previous research has shown that squalene molecules have antioxidant, anti-inflammatory, and anti-atherosclerotic properties [32]. Even in some studies determined that squalene molecules are the primary chemicals involved in antioxidant activity in the plants tested [33, 34].

Among the fatty acid compounds that dominate candlenut leaf extract, n-hexadecanoic acid ($C_6H_{32}O_2$), also known as palmitic acid (10.86%), is a saturated fatty acid compound that has antibacterial activity by damaging the structure of cell walls and membranes [35, 36]. The main compound obtained in this work was 9,12-octadecadien-1-ol, also known as linoleic acid (12.02%), which exhibits antibacterial and antifungal activities [37, 38]. The fifth dominant compound in the ethanol extract of candlenut leaves was octadecanoic acid, also known as stearic acid (5.45%). This chemical may be found in a variety of plant and animal fats. According to prior study, this chemical exhibit a variety of bioactivities including antioxidant, antibacterial, anti-analgesic, anti-malaria, anticonvulsant, anti-obesity, anti-cancer, and hypocholesterolemia [39, 40].

Based on the insilico analysis conducted, molecular docking was performed on 38 secondary metabolite compounds present in the ethanol extract of candlenut leaves, with TNF-α (PDB ID:) used as the target receptor. The results showed that the compounds stigmasta-5,22-dien-3-ol and cycloheptadecanol exhibited strong docking activity. This was evidenced by their high docking scores [41]. Stigmasta-5,22-dien-3-ol had the highest docking score, even when it was compared to the positive control, namely chlorhexidine, suggesting that this compound may have greater potential as a TNF-α inhibitor. This was consistent with previous studies, which stated that the compound stigmasterol 5,22-dien-3-ol exhibited anti-inflammatory activity. Furthermore, this compound was reported to be a potential therapeutic agent for inhibiting the expression of pro-inflammatory mediators, such as TNF-α [42, 43]. Similarly, prior research also reported comparable findings for the compound cycloheptadecanol [44]. Chlorhexidine is an active ingredient in commercial mouthwashes that can cause side effects in the mouth with long-term use [12, 13].

Inflammation resulting from wounds or injuries stimulates the formation of polymorphonuclear leukocytes (PMNL), which produce free radicals that can impair cellular function [45]. Free radicals cause oxidative damage to cell membranes, such as lipid peroxidation, as well as to proteins and nucleic acids, making antioxidant properties essential to inhibit free radical production [46]. Based on the antioxidant analysis conducted, the high antioxidant activity of candlenut leaf extract is believed to influence the progression of inflammation and reduce wound expansion. The bioactive compounds found in candlenut leaves suggest that they have potential in preventing inflammation. This finding is supported by previous studies [46, 47], which reported that the bioactive terpenoid compounds exhibit anti-inflammatory effectiveness.

The active compounds in candlenut leaves have previously been proven to exhibit antibacterial effects against Vibrio cholerae and Salmonella typhi, with the largest average inhibition zone observed at a concentration of 1.75%, measuring 12.46 mm [48]. According to Othman (2010), candlenut leaves can also inhibit the growth of Streptococcus pyogenes, Pseudomonas aeruginosa, and Klebsiella pneumoniae at concentrations of 6.25% and 12.5% [49]. Furthermore, lethal dose testing in other studies has indicated that the dosage range used remains safe [50]. However, to confirm the toxicity levels of the doses applied, further testing should be conducted in future research.

TNF-α is a critical cytokine in the inflammatory response. The immune system is greatly influenced by the levels of TNF-α, with a weakened immune system leading to increased TNF-α production, thereby reducing the body's ability to

fight infections. Various studies have demonstrated that the anti-inflammatory effects of certain active compounds can downregulate specific cytokines, thereby inhibiting inflammation [51, 52]. This is consistent with the findings of this study, where the active compounds in the ethanol extract of candlenut leaves were shown to reduce TNF-α concentration. Additionally, other researchers have found that candlenut leaves can lower TNF-α levels in the serum of type 2 diabetes mellitus rat models [53].

5. Conclusion

Candlenut leaves (Aleurites moluccanus) possess phytochemical agents with significant bioactive potential. Specifically, the antioxidant activity of the ethanol extract from candlenut leaves demonstrated a low IC50 value, confirming its strong antioxidant activity. Furthermore, the bioactive compounds in the mouthwash formulation of candlenut leaf extract exhibited significant anti-inflammatory potential. Molecular docking results revealed that compounds such as stigmasta-5,22-dien-3-ol and cycloheptadecanol showed strong binding affinity to TNF-α, indicating their potential as TNF-α inhibitors. Further in vivo experiments supported these findings, showing that the extract effectively reduced wound size and decreased TNF-α levels in a dose-dependent manner.

6. Declarations

6.1. Author Contributions

Conceptualization, E.Z., D.D., and C.S.; methodology, E.Z. and C.S.; software, D.D.; validation, U.B. and D.D.; formal analysis, C.S. and D.D; investigation, E.Z. and U.B.; resources, E.Z.; data curation, E.Z., C.S., and U.B.; writing—original draft preparation, E.Z., D.D., U.B., and C.S.; writing—review and editing, C.S. and D.D; visualization, U.B.; supervision, D.D., U.B., and C.S.; project administration, E.Z. and C.S.; funding acquisition, E.Z. All authors have read and agreed to the published version of the manuscript.

6.2. Data Availability Statement

The data presented in this study are available in the article.

6.3. Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

6.4. Acknowledgments

The author thanks to the promoters from Universitas Syiah Kuala for all their knowledge and support in this process.

6.5. Institutional Review Board Statement

The study was approved by Veterinary Ethics Committee, Faculty of veterinary Medicine, Syiah Kuala University, Banda Aceh, Indonesia (Approval Number: 246/KEPH/VIII/2023).

6.6. Informed Consent Statement

Not Applicable

6.7. Declaration of Competing Interest

The authors declare that there are no conflicts of interest concerning the publication of this manuscript. Furthermore, all ethical considerations, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancies have been completely observed by the authors.

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