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Anti-Inflammatory Activity of Qutsh Al Hindi (*Saussurea lappa*) Root Fractions: *In Vitro* Assay and Characterization of its Active Compound

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ABSTRACT

Inflammation is a physiological response involved in various pathological conditions. Natural products such as *Saussurea lappa* (*S.lappa*) offer potential anti-inflammatory treatments with fewer side effects. This study aimed to evaluate the anti-inflammatory activity of the fractions of *S. lappa* root, and identify the active compound. *S.lappa* fractions were obtained by liquid-liquid fractionation of the crude ethanol extract using n-hexane and ethyl acetate. The antiinflammatory activity of the fractions was evaluated *in vitro* using the erythrocyte membrane stabilization method. The potentially active compound in the ethyl acetate fraction was identified by liquid chromatography-mass spectrometric (LC-MS) analysis. Results showed that the n-hexane and ethyl acetate fractions *of S.lappa* exhibited anti-inflammatory activity *in vitro*. The ethyl acetate fraction showed a higher anti-inflammatory activity (IC_{50}= 1.113 ppm), than the n-hexane fraction with an IC_{50} of 1.206 ppm. LC-MS analysis identified costunolide as the potentially active anti-inflammatory compound in the ethyl acetate fraction of *S. lappa* root extract. Therefore, *S. lappa* root has shown promising anti-inflammatory properties that could be harnessed for therapeutic purposes.

Keywords: *Saussurea lappa*, Anti-inflammatory activity, Costunolide, LC-MS.

Introduction

Inflammation is the body's physiological response to cell or tissue damage caused by exposure to infection, harmful chemicals, or other noxious stimuli. The goal of inflammation is to inactivate the pathogen or harmful stimulus and induce tissue repair. However, chronic inflammation is implicated in numerous pathological conditions such as atherosclerosis, rheumatism, arthritis, asthma, inflammatory bowel disease, and others.[1,2]

Nonsteroidal anti-inflammatory drugs (NSAIDs) act by interfering with the synthesis of prostaglandins, which are important mediators of inflammation, through non-selective blockade of cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2). NSAIDs are widely used to treat several inflammatory conditions, but their long-term administration is associated with many side effects, such as ulceration and bleeding of the gastrointestinal tract,liver and kidney damage.[3]

The search for natural products with anti-inflammatory activity and the advantages of less toxic side effects and more curative effects is currently gathering attention. Natural products have numerous pharmacological activities and low toxicity. Therefore, they are becoming a potential source for the development of natural anti-inflammatory drugs.[4]

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In recent years, great progress has been made in the discovery and development of treatment options for chronic diseases associated with inflammation and the use of natural products to alleviate inflammatory diseases. Current studies have found that natural products with anti-inflammatory activity include polysaccharides, flavonoids, polyphenols, alkaloids, terpenes, natural pigments, plant volatile oils, quinones, and other compounds.[5]

Quts al Hindi (*Saussurea lappa*) or Indian wood is a plant that has been widely utilized as natural medicine. Quts al Hindi has been shown to have anti-ulcer, anti-convulsant, anti-cancer, hepatoprotective, anti-arthritic, and anti-viral activities.[6] Studies have shown that the ethanol extract of Quts al Hindi (*S.lappa*) has anti-inflammatory activity. The extract showed an inhibitory effect (50%) on neutrophil chemotaxis factors induced by cytokines. Another study also found that the bioactive compounds of *S. Lappa* include allantolactone, caryophyllene, costalic acid, costunolide, dehydrocostuslactone, and cyanopicrin. The anti-inflammatory effect of cynaropicrin, a sesquiterpene lactone from *Saussurea lappa* is exhibited through the release of tumour necrosis factor alpha (TNF-α) and nitric oxide (NO) and by lymphocyte proliferation. Cynaropicrin in the inflammatory response inhibits tumour necrosis factor-alpha (TNF-α) and NO, and CD4+ and CD8+ lymphocyte proliferation through conjugation with sulfhydryl groups on target proteins.[7,8] Previous research has investigated the anti-inflammatory activity of *S. lappa* extract using the erythrocyte membrane stabilization method. The results showed that the extract has anti-inflammatory activity with an IC_{50} value of 1,762 µg/mL.[7-9]

Therefore, the present study was conducted to further investigate the anti-inflammatory activity of fractions of Quts al Hindi (*S. lappa*) roots. The fractionation process allows for the isolation of individual compounds that contribute to the anti-inflammatory effect of the extract, which are subsequently analyzed using liquid chromatography-mass spectrometry (LC-MS). LC-MS provides a comprehensive chemical profile of the fractions, enabling detailed characterization of the compounds. By gaining a deeper understanding of the bioactive metabolites and their underlying mechanisms of action, there is an increased potential for the discovery of new lead compounds for anti-inflammatory drug development.

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Materials and Methods

Chemicals and reagents

EDTA, DMEM (Gibco), 96% ethanol (Sigma-Aldrich, Singapore), n-hexane (Sigma-Aldrich, Singapore), ethyl acetate (Sigma-Aldrich, Singapore), Diclofenac Sodium (Merck, Indonesia), distilled water (Sigma-Aldrich, Singapore), $Na_2HPO_4.2H_2O$ (Merck, Indonesia), NaCl (Merck, Indonesia), chloroform (Sigma-Aldrich, Singapore), acetic acid (Sigma-Aldrich, Singapore), concentrated sulfuric acid (Sigma-Aldrich, Singapore), HCl (Merck, Indonesia), iron (III) chloride (Merck, Indonesia).

Plant collection, extraction, and fractionation

The roots of Quts al Hindi (*S. lappa)* were collected from UD. Juragan Jamu Yogyakarta. The plant material was identified and authenticated with a voucher specimen number 035/SKD/0106/022.The plant material was dried, pulverized, and a powdered sample (500 g) was macerated in 96% ethanol at room temperature for 72 hours with periodic stirring. After removal of the extract, the extraction process was repeated until the sample was exhaustively extracted. The combined extracts were concentrated *in vacuo* using a rotary evaporator. The concentrated extract was fractionated by solvent-solvent partitioning using n-hexane and ethyl acetate in order of their increasing polarity.

Determination of anti-inflammatory activity

The anti-inflammatory activity of the extract was determined *in vitro* using the red blood cell membrane stabilization method. Briefly, a red blood cell suspension was made and divided into three treatment groups, namely, negative control, positive control (diclofenac sodium), and test solutions with concentrations of 25, 50, 75, 100, and 125 ppm. The test solution consisted of 2 mL of hypotonic saline, 1.0 mL of 0.15 M sodium phosphate buffer (pH 7.4), 0.5 mL (10% v/v) of red blood cell suspension, and 1.0 mL of test sample and standard solution. The mixture was incubated at 37ºC for 30 min, and then the solution was centrifuged at 3000 rpm for 30 min. The absorbance of the solution was measured at 560 nm.[10,11] The percentage of haemolysis inhibition was calculated using the formula:

$$\% \text{ inhibition} = \frac{A1-A2}{A1} \text{x } 100\%$$

Where A1 = absorbance of the control solution, A2 = absorbance of the test solution/standard solution.

The IC_{50} values, which represent the concentration required to inhibit 50% of the erythrocyte membrane hemolysis, were calculated from the regression equation obtained from the plot of the absorbance versus the concentration of the test samples.

LC-MS analysis

The LC-MS analysis of the ethyl acetate fraction of *S.lappa* was conducted using a Thermo HPLC-DIONEX ULTIMATE-TSQ Quantum Access MAX Triple Quadrupole Mass Spectrometer. For sample preparation, 5 mg of the fraction was dissolved in 5 mL of methanol (HPLC grade) and filtered through a nylon syringe filter with a pore size of 0.22 µm. The filtered sample was injected into the LC-MS system at an injection volume of 5 µL. The chromatographic separation was achieved using a Thermo Scientific™ Hypersil GOLD™ C-18 HPLC column. The mobile phase consisted of water with 0.1% formic acid (Solvent A) and acetonitrile with 0.1% formic acid (Solvent B). The flow rate was set at 0.3 mL/min. The gradient elution program started with 60% A and 40% B for 1 minute, followed by a linear gradient to 5% A and 95% B over 8 minutes, which was maintained for 1 minute before returning to the initial conditions of 60% A and 40% B for the final minute. The detection was performed in positive ion mode (MS+), with a mass range of 150 to 1000 m/z.

Statistical analysis

Data were presented as mean ± standard error of mean (SEM) of three independent experiments. The IC_{50} values were determined from the nonlinear regression equation of the concentration-response curve. Graphpad statistical package was used for the analysis.

Results and Discussion

Anti-inflammatory activity of S. lappa root

A previous study on the *in vitro* anti-inflammatory activity of *S. lappa* extracts using the red blood cell membrane stabilization method[9] provided an important basis for exploring the potential anti-inflammatory activity of this plant. The study showed that *S. lappa* extract has anti-inflammatory activity with an IC_{50} value of 1,762 µg/mL, which is an early indication of anti-inflammatory activity of the extract. As a result, additional studies were conducted to evaluate the antiinflammatory activity of fractions derived from the roots of *S. lappa*. The fractionation process allowed the separation of individual compounds responsible for the anti-inflammatory effect.

The red blood cell membrane stabilization method is used because the cell structure is similar to lysosomes, where red blood cells have a membrane that envelops hemoglobin; when the membrane is ruptured, the haemoglobin is released. When an injury occurs, the lysosomal membrane will release substances like the phospholipase enzyme. Red blood cells that are stable against hypotonic solution-induced disturbances can be used as a measure to assess the stabilization of the lysosomal membrane to hypotonic stress-induced destabilization of the membrane.[12]The extent of haemolysis that arises on the red blood cell membrane induced by hypotonic solution was used as a measure of the anti-inflammatory activity of fraction of the roots of qutsh al hindi plant (*S.lappa*).

The anti-inflammatory activity of the n-hexane and ethyl acetate fractions of *S.lappa* ethanol extract is presented in Table 1. A lower absorbance value indicates a smaller amount of haemoglobin released, which in turn indicates a more stable membrane and a higher anti-inflammatory activity. On the other hand, a higher absorbance value indicates a higher amount of haemoglobin released, a less stable membrane, and a lower anti-inflammatory activity.

As shown in Table 1 and Figure 1, the n-hexane and ethyl acetate fractions of *S.lappa* ethanol extract exhibited a percentage haemolysis inhibition similar to that of the positive control (diclofenac sodium). In addition, the fractions exhibited a dose-dependent increase in the stability of the red blood cell membrane.

The inhibitory effect of the *S. lappa* fraction is believed to be due to the presence of chemical constituents that stabilize red blood cell membranes. Other studies have reported that secondary metabolites, such as flavonoids, act as anti-inflammatory agents and exhibit their anti-inflammatory activity through the inhibition of inflammatory mediators like histamines and bradykinins. The anti-inflammatory activity of flavonoids is also exhibited through the reduction of the expression of nitric oxide synthase (NOS) isoforms, cyclooxygenase, and lipoxygenase, which are important enzymes of the inflammatory pathway.[13] Additionally, flavonoids can inhibit phosphodiesterase and exert anti-inflammatory effects by inhibiting cytokine biosynthesis, which mediate the attachment of leukocytes to wound sites.[14]

The n-hexane fraction of *S. lappa* ethanol extract showed a higher percentage hemolysis inhibition than the ethyl acetate fraction at the same concentrations, particularly at 100 ppm and 125 ppm. However, the ethyl acetate fraction demonstrated a slightly lower IC_{50} value (1.113 ppm) compared to the n-hexane fraction (IC_{50} = 1.206 ppm), indicating that the ethyl acetate fraction was more effective in inhibiting inflammation at lower concentrations. Compared to previous studies, the anti-inflammatory activity of the n-hexane and ethyl acetate fractions of *S. lappa* showed a higher anti-inflammatory activity than the *S. lappa* extract with an IC_{50} value of 1.762 ppm, and diclofenac sodium, which had an IC_{50} value of 1.861 ppm.[9]

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Table 1: Absorbance value and percentage of erythrocyte membrane stability of ethyl acetate and n-hexane fractions of *S.lappa*

Sample	Concentration (ppm)	Ln C	Absorbance			Mean	% Inhibition	IC_{50} (ppm)
			1	2	3			
Ethyl Acetate Fraction	125	4.83	0.109	0.119	0.119	0.116	86.230	1.113
	100	4.61	0.113	0.118	0.113	0.115	86.349	
	75	4.32	0.121	0.121	0.128	0.123	85.317	
	50	3.91	0.136	0.136	0.138	0.137	83.730	
	25	3.22	0.141	0.141	0.14	0.141	83.254	
n-Hexane fraction	125	4.83	0.127	0.137	0.137	0.134	84.087	1.206
	100	4.61	0.129	0.13	0.129	0.129	84.603	
	75	4.32	0.137	0.135	0.135	0.136	83.849	
	50	3.91	0.143	0.142	0.142	0.142	83.056	
	25	3.22	0.145	0.145	0.148	0.146	82.619	
Positive Control (Diclofenac sodium)	125	4.83	0.189	0.081	0.091	0.120	85.675	1.837
	100	4.61	0.198	0.095	0.095	0.129	84.603	
	75	4.32	0.142	0.143	0.143	0.143	83.016	
	50	3.91	0.152	0.142	0.152	0.149	82.302	
	25	3.22	0.152	0.151	0.158	0.154	81.706	
Negative Control	100	4.61	0.273	0.253	0.254	0.146	69.087	-

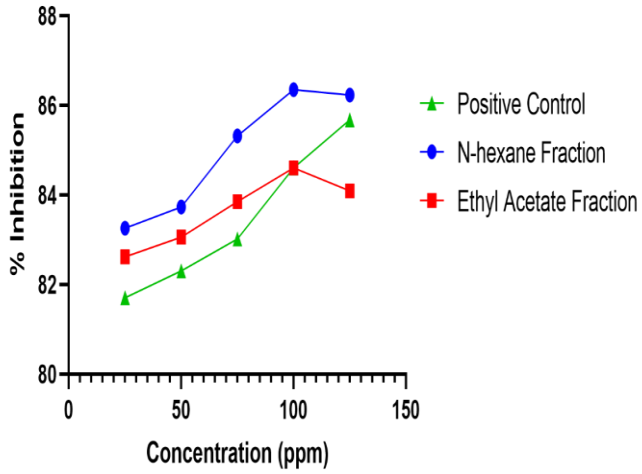

Figure 1: Percentage inhibition of erythrocyte membrane stability by fractions of *S. lappa* ethanol extract

LC-MS profile of S. lappa ethyl acetate fraction

The ethyl acetate fraction of *S. lappa* extract was subjected to LC-MS analysis to identify the potential bioactive compound(s) in the fraction that may be responsible for the anti-inflammatory activity. The LC chromatogram showed a prominent peak with a retention time (RT) of 4.35 min. MS analysis of this peak gave a molecular weight of 233.14 g/mol, which was consistent with the molecular formula $C_{15}H_{20}O_2$ (Figure 2). MS fragmentation pattern of the compound and comparison with data from literature identified the compound as a costunolide.[15] Costunolide is one of the compounds that have been identified in the roots of *S. lappa*.[16] Costunolide is a naturally occurring sesquiterpene lactone (Figure 3), and is reported to have several activities such as antioxidant, anti-inflammatory, anti-allergic, bone remodelling, neuroprotective, hair growth promoting, anticancer, and antidiabetic activities.[15]

Several studies have reported the anti-inflammatory activity of costunolide. The compound has been shown to attenuate carrageenan-induced paw edema, myeloperoxidase (MPO) activity, and N-acetylglucosaminidase (NAG) activity in rats.[17] One of the transcriptional regulators of proinflammatory gene expression is the transcription factor nuclear factor-kappaB (NF-κB). Costunolide abrogated NF-κB activation through blockade of IκBα phosphorylation in lipopolysaccharide (LPS)-stimulated RAW264.7 cells, thereby reducing the expression of proinflammatory markers, such as inducible nitric oxide synthase (iNOS), and nitric oxide (NO) production.[18]

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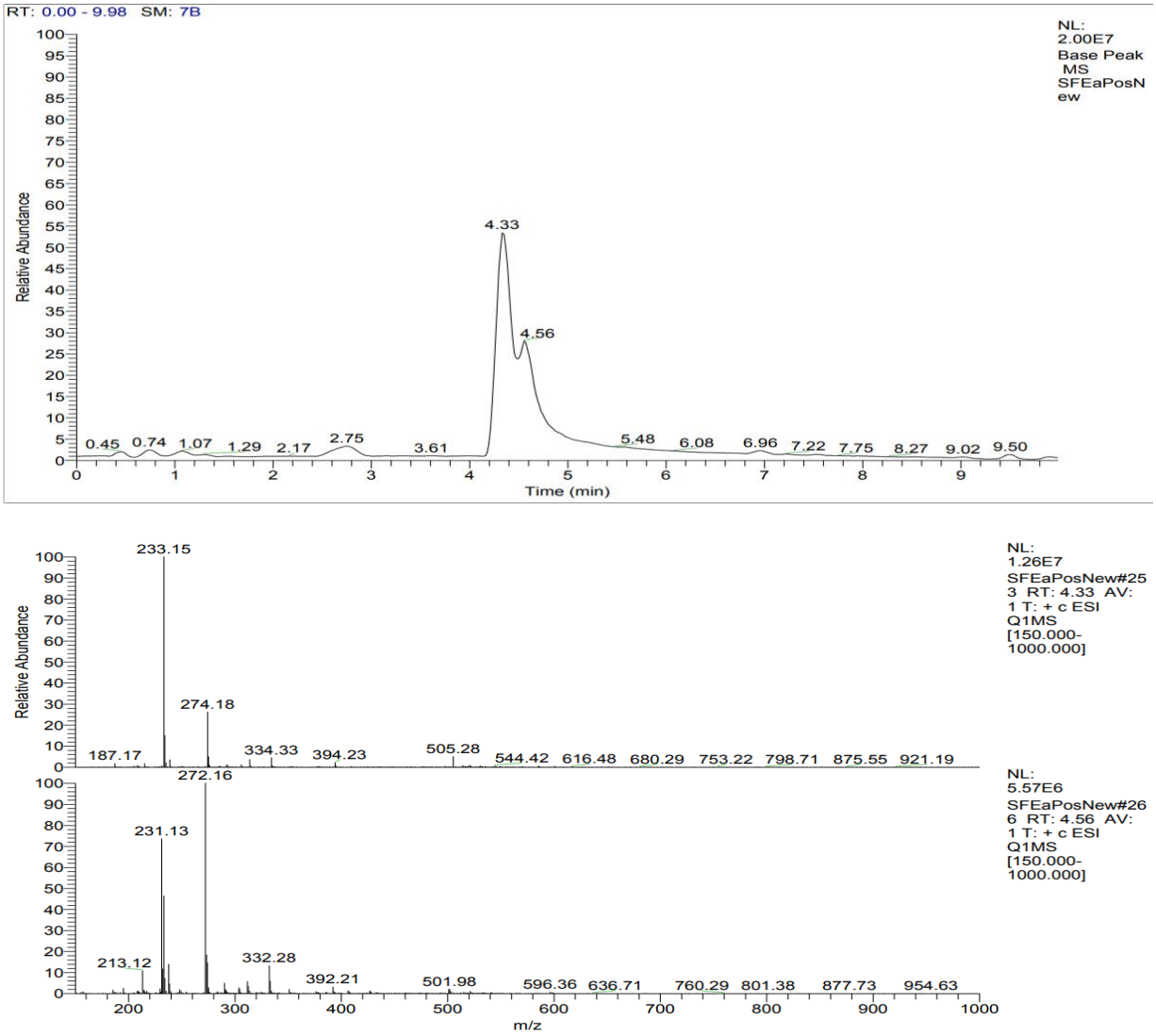

Figure 2: LC chromatogram and MS spectrum of ethyl acetate fraction of *S.lappa*ethanol extract

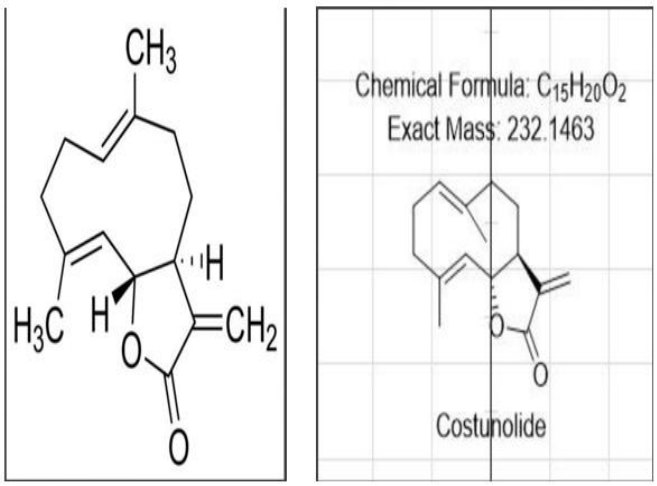

Figure 3: Chemical structure of Costunolide

A study has also shown that treatment with costunolide inhibited 5-fluorouracil (5-FU)-induced iNOS expression, cyclooxygenase-2 (COX-2), TNF-α, and nitric oxide (NO) production in a mouse model of intestinal mucositis by blocking NF-κB activation.[19] In addition, costunolide alleviated lung inflammation in a carrageenan-induced mouse pleurisy model, as evidenced by reduced accumulation of polymorphonuclear cells and reduced expression of TNF-α, intracellular adhesion molecule-1 (ICAM-1), P-selectin, and nitrotyrosine.[20]

Conclusion

The anti-inflammatory activity of fractions of *S. lappa* root was evaluated *in vitro*, and results revealed an effective anti-inflammatory activity of the n-hexane and ethyl acetate fractions of the plant roots. *S. lappa* fractions exhibited membrane-stabilizing effects by inhibiting hypotonicity induced lysis of erythrocyte membranes. LC-MS analysis showed that the ethyl acetate fraction contained costunolide, which has been shown to have anti-inflammatory activity.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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