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In Vitro Metabolite Profiling and Anti-Inflammatory Activities of *Rhodomyrtus Tomentosa* with Red Blood Cell Membrane Stabilization Methods

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Abstract

Background: *Rhodomyrtus Tomentosa* (Karamunting) is one of South Kalimantan's biodiversity. In previous research on asthma and coal dust exposure models, nebulization with an ethanol extract of *R. tomentosa* leaves has been shown to reduce inflammatory cells. This study aimed to investigate the anti-inflammatory activity on the stabilization of red blood cell membranes and to identify the chemical compounds present in extracts of *R. tomentosa* leaves.

Methods: Anti-inflammatory effect of *R. tomentosa* leaves was evaluated by *in vitro* red blood cell membrane stabilization methods. Nonsteroidal anti-inflammatory sodium diclofenac was used as the reference drug. The content of chemical compounds in the ethanol extract of *R. tomentosa* leaves was identified using Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS).

Results: The results of inhibition of red blood cells membrane lysis showed the n-hexane fraction (concentration 25 ppm), ethyl acetate fraction (concentration 50 ppm), and a fraction of water (concentration 50 ppm) with an inhibition level of 54.5%, 81.8%, 63.6%, respectively. The ethyl acetate fraction showed the highest inhibition of hemolysis. This result is not significantly different from the standard anti-inflammatory sodium diclofenac (90.09%). Oleanonic acid and ursonic acid were two similar metabolites in subfractions ethyl acetate 1, 2, and 3, which may have anti-inflammatory properties.

Conclusions: *R. Tomentosa* leaves can have potency as an anti-inflammatory by increasing the red blood cell membrane stability equal to lysosome cells, depending on the concentration.

Keywords: Anti-inflammatory, Oleanonic acid, Ursonic acid, *Rhodomyrtus tomentosa*.

Introduction

Inflammation is one of main defense mechanisms in the body. A process that is responded to by the immune system by recognizing and eliminating harmful stimuli such as infections, harmful chemicals, drugs, viruses, and bacteria (1). The chronic inflammatory response causes permanent damage to healthy tissues and organs. Damaged cells or tissues will respond by releasing inflammatory mediators like

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histamine, cytokines, and serotonin to prevent further damage and start the healing process. Chronic inflammation is implicated in developing various cardiovascular, respiratory, metabolic, neurodegenerative, and cancer diseases (2).

Prostaglandin is lipids synthesized from arachidonic acid by cyclooxygenase (COX) isoenzymes and play an important role in inflammatory response (3). One of the common medications for inflammation is nonsteroidal anti-inflammatory drugs (NSAIDs). These drugs block the conversion of arachidonic acid to prostaglandins by inhibiting the COX enzyme (4). These anti-inflammatory drugs are still problematic because they can cause gastrointestinal, cardiovascular, and other toxicity for prolonged use. Hence, it is an opportunity to find an alternative medicine for inflammation and use safer natural nonsteroidal anti-inflammatory drugs. Herbal medicine is one of the most potential drug resources, and Indonesia has a large biodiversity (5,6).

The biochemical process involved during the inflammatory reaction was controlled by anti-inflammatory agents like NSAIDs by stabilizing the membrane of lysosomes. The lysosomal membrane is relatively identical to the red blood cell membrane. Exposure to a harmful condition like hypotonic causes lysis of the red blood cell membrane (6,7).

There will be an increased susceptibility of cells to secondary damage by lipid peroxidation induced by free radicals if there is damage to the red blood cell membrane (7). Due to the stability of the membrane, after inflammation, serum proteins and fluids are prevented from leaking into the tissues during the increased permeability. The mechanism of a plant extract's anti-inflammatory activity was measured by its ability to inhibit hypotonicity impact by membrane stabilization of the red blood cell (6).

Rhodomyrtus tomentosa, one of South Kalimantan native flowering plants, exhibits a wide range of therapeutic properties, including promising effects as an anti-inflammatory. Previous studies have proven nebulization with ethanol extract from *R. tomentosa* leaves can reduce inflammatory cells in asthma and coal dust exposure models (8). Flavonoids, terpenoids, steroids, and alkaloids are organic chemicals found in the leaves of *R. tomentosa* that are believed to have anti-inflammatory properties (9). We have not encountered any study in the literature on the ethanol extract fraction of *R. tomentosa* leaf as an anti-inflammatory by inhibiting red blood cell membrane lysis, especially in Indonesia during our literature research. In the effort to find new natural biological anti-inflammation, it is necessary to evaluate the anti-inflammatories activities of *R. tomentosa*.

Materials and Methods

Study Design

This research is an experimental laboratory. The Pharmacology Laboratory of the Faculty of Medicine, Lambung Mangkurat University, performed fractionation and sub-fractionation. Anti-inflammatory analysis of red blood cells was carried out in the "Biochemistry and Biomolecular Laboratory of the Faculty of Medicine, Lambung Mangkurat University". Metabolite profiling analysis was conducted at the Forensic Laboratory, Indonesian Police Criminal and Investigation Agency, Bogor Regency, West Java. This study was approved by the Ethics Committee of the Faculty of Medicine Lambung Mangkurat University with approval code "No. 476/KEPK-FK ULM/EC/X/2022".

Sample Collection and Identification

R. tomentosa (Karamunting) plants were collected from the Palam Banjarbaru area, South Kalimantan. The Biology laboratory of the Faculty of Mathematics and Natural Sciences, Lambung Mangkurat University, carried out plant authentication with a certificate of test results No. 073/LB.LABDASAR/III/2021.

Sample Preparation

R. tomentosa leaves were extracted using the maceration method. A total of 330 grams of

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mashed *R. tomentosa* leaf was placed in a maceration jar, 1.5 L of 70% ethanol was added and stored for 72 hours. After 72 hours, the filtrate was separated, and an additional 1.5 L of 70% ethanol was added; this procedure was repeated up to three times. In the next step, the filtrate was collected and evaporated with a rotary evaporator at a water of 50 ºC. The concentrated ethanol extract from *R. tomentosa* was obtained and then tested for anti-inflammatory activities.

Fractionation and Subfraction of R. Tomentosa leaf ethanol extract

The liquid fractionation method was used to fractionate *R. Tomentosa* leaf ethanol extract. The *R. tomentosa* ethanol extract weighed as much as 5 grams, and 5 mL of 70% ethanol was added and stirred until the extract dissolved. 50 mL of distilled water followed by 25 mL of n-hexane was added and shaken until some of the extraction was extracted in the n-hexane solvent. This process was repeated until the n-hexane was colourless. The remaining n-hexane was collected and dried.

The n-hexane solvent-fractionated water component added 25 mL of ethyl acetate, and extracts were obtained. This process was repeated until the ethyl acetate was colourless. The ethyl acetate was collected and dried. The number of compounds and their polarity contained in each fraction were seen by Thin Layer Chromatography.

The column chromatography method was used to sub-fractionate the ethyl acetate fraction of the *R. tomentosa* leaf. In five mL of the first eluent, 10 mg of the ethyl acetate fraction was dissolved, and five g of silica gel 60 (0.063-0.200 mm) was added, stirred until dry, and the ethyl acetate fraction was utterly mixed with silica gel 60. Then 45 mL of the first eluent was added with 35 g of silica gel 60, stirred until dispersed, and put into the chromatography column. Meanwhile, the first eluent was added continuously to ensure no air was trapped. The column faucet was closed when the eluent surface equals the silica gel 60 surfaces. The initial 100 mL ethyl acetate fraction was collected in the vial for five minutes at a rate of 30-32 drops per minute. The process was continued with the following eluents, namely: 50 mL n-hexane (1): ethyl acetate (4); 50 mL n-hexane (1): ethyl acetate (9); 50 mL n-hexane (1): ethyl acetate (9): ethanol (1); and 100 mL ethyl acetate (1): ethanol (1). The Thin Layer Chromatography technique was used to elute each subfraction. Subfractions with similar stain appearances were combined to create several subfraction groups.

Anti-inflammatory activity on red blood cells

The anti-inflammatory potency of various *R. Tomentosa* extracts was evaluated using *in vitro* sheep red blood cell membrane stabilization techniques. The isotonic solution was made by combining 154 mM NaCl and 10 mM sodium phosphate solution with a pH of 7.4. Fifthy µl of stock sheep red blood cells cell suspension was mixed with the hypotonic solution containing *R. Tomentosa* n-hexane, ethyl acetate, and water fractions at 12.5, 25, 50, and 100 ppm concentrations. The drug-free solution was used as a control. After 10 minutes of incubation at room temperature, the whole mixture was spun at 5000 rpm for 5 minutes. The absorbance of the supernatant was then measured at 540 nm with a UV spectrophotometer. As the standard, 200 g/ml of diclofenac sodium drug was used. The percentage inhibition of red blood cells membrane lysis was calculated according to the equation:

% Red blood cells membrane stability = 100x[1-(OD2 - OD1/OD3 - OD1)]

with OD1 = Test sample in isotonic solution; OD2 = Test sample of hypotonic solution and OD3 = Control sample in hypotonic solution

Identification of ethyl acetate subfraction metabolite of R. Tomentosa leaf ethanol extract using UPLC-MS

The content of chemical compounds in the ethyl acetate 1, 2, and 3 ethanol extract from *R. tomentosa* leaves was identified using "Ultra Performance Liquid Chromatography-Mass Spectrometry" (UPLC-MS) based on the modified Hakim et al. procedure (10). A total of one mg of the subfraction sample was weighed

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and dissolved in one ml of methanol. Up to five µl of subfraction in methanol was entered into the UPLC-MS/MS column (replication was done four times) through the C18 column (1.8 m 2.1x100 mm) HSS. With a gradient elution system, the eluent was a mixture of (A) water: formic acid (99.9: 0.1) and (B) acetonitrile: formic acid (99.9: 0.1), eluted with 0.2 ml/min flow rate. A Q-ToF analyzer will then separate the ions produced by the detector. Chromatograms with polar compounds will appear first, followed by compounds with lower polarity. The Q-ToF-MS detector then read the separation results to produce a chromatogram peak. The chromatogram peaks were then interpreted using the *Masslynx* application.

Statistical Analysis

The data on the percentage of inhibition of red blood cells membrane lysis for each concentration of the ethyl acetate, n-hexane, and water sub-fractions were calculated as mean ± standard deviation. The calculated data were tested for normality by Shapiro-Wilk and the homogeneity test using the Levene Test. Statistical analyses were performed using the one-way ANOVA, Least Significance Different (LSD), and the Kruskal-Wallis test. SPSS version 24 with a p-value significant level <0.05. The result of data interpretation from the separation process using UPLC-MS is the name of the compound and the percentage of the area taken for the five most prominent components of the percentage.

Results

Inhibition of hemolysis by R. Tomentosa extracts

Table 1 displays the inhibition percentage of red blood cell membrane lysis after the administration of *R. tomentosa* leaf. The ethanol extract in n-hexane, ethyl acetate, and water fractions was compared to diclofenac sodium (a standard nonsteroidal anti-inflammatory drug).

The results of inhibition of red blood cells cell membrane lysis showed statistically significant ($p<0.05$) in the n-hexane fraction (concentration 25, 50 and 100 ppm), ethyl acetate fraction (concentration 12.5 ppm), and a fraction of water (concentration 12.5, 25 and 100 ppm). This result showed that the anti-inflammation potency of R. tomentosa leaves was underneath diclofenac sodium drugs as a control in the above concentration. Meanwhile, the result is not significantly different from the standard anti-inflammatory sodium diclofenac (90.09%) in the n-hexane fraction (concentration 25 ppm), ethyl acetate fraction (concentration 50 ppm), and a fraction of water (concentration 50 ppm) with an inhibition level of 54.5%, 81.8%, 63.6% respectively.

Table 1. Percentage of red blood cells membrane lysis inhibition following administration of *R. tomentosa* leaf ethanol extract compared with diclofenac sodium.

No	Concentration (ppm)	Fraction		
		n-Hexane	Ethyl acetate	Water
1	12.5	36.40±5.66*	27.20±5.48*	39.10±8.53*
2	25	54.40±8.98	54.40±6.77	27.30±6.73*
3	50	18.20±9.27*	81.80±9.95	63.60±5.66
4	100	36.40±8.98*	72.70±7.85	45.50±7.62*

% Positive control red blood cells membrane stability (diclofenac sodium): 90,9±5,75
*Significantly different from the positive control, p-value <0.05.

Profiling metabolite subfraction of ethyl acetate ethanol extract of R. Tomentosa leaves

Analysis of the metabolite profile of the ethyl acetate subfraction (SFEA 1,2,3) of the ethanol extract from *R. tomentosa* leaves in this study used "Ultra Performance Liquid Chromatography-Mass Spectrometry" (UPLC-MS). UPLC was a liquid chromatography development technique

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used to segregate different components in a mixture with a molecular level of up to two microns of analyte particles.

According to data derived from the interpretation of chemical content analysis performed with UPLC-MS, SFEA 1, 2, and 3 have different metabolite profiles characterized by variations in the types of compounds identified in each subfraction, but the same metabolites were also present. Tables 2 contain a list of the five major chemicals in SFEA 1, 2, and 3. Meanwhile, Figure 1 displays the spectra of each compound subfraction 1, 2, and 3.

UPLC-MS analysis showed that the same significant compounds from the three subfractions of the ethanolic extract of *R.tomentosa* leaves were oleanonic acid, ursonic acid, and 1-[1-(Adamantan-1-yl)ethyl]-3-benzylthiourea and the amount was different for the three subfractions.

Table 2. Five Compounds contained in the subfraction ethyl acetate 1, 2 and 3 (SFEA 1, 2 and 3).

SFEA 1						
%	**Peak**	**m/z**	**RM**	**Prediction 1**	**Prediction 2**	**Prediction 3**
25.46	16.81	387.2895	C21H34N6O	"N-Cyclopropyl-1'- {[2-(di methylamino)-5-pyrimi dinyl] methyl}-1,4'-bipi peridine-3-carboxamide	"N-Cyclopropyl-1'-[4-(isopropyl amino)-2-pyrimi dinyl]-1,4'-bipi peridine-3-carbo xamide	-
14.94	18.88	455.3526	C30H46O3	Oleanonic acid	Ursonic acid	-
11.40	17.47	455.3526	C30H46O3	Oleanonic acid	Ursonic acid	-
7.63	17.75	455.3526	C30H46O3	Oleanonic acid	Ursonic acid	-
7.22	13.82	329.2049	C20H28N2S	1-[1-(Adamantan-1-yl) ethyl]-3-benzylthiourea	-	-
SFEA 2						
21.75	6.42	450.2701	C17H35N7O7	-	-	-
17.75	13.84	329.2049	C20H28N2S	1-[1-(Adamantan-1-yl) ethyl]-3-benzylthiourea	-	-
12.22	10.31	375.1076	C15H14N6O6	2'-Deoxy-1-(5nitro-2-pyridinyl) inosine	Methyl 4-(2-azido-3-nitrophenyl)-2,6-dimethyl -5-nitro-1,4-dihydro-3-pyridine carboxylate	-
8.77	18.44	792.5620	C41H73N7O8	1,1-Dimethylethyl 4-[11-[[(2S)-5-(1,1-dimethylethoxy)-2- [4-[5-[[(1,1-dime thylethoxy) carbo nyl]amino]pentyl]-1H-1,2,3-triazol-1-yl]-1,5-dioxo pentyl]amino]-1-oxoundecyl]-1-piperazine carboxylate	-	-
8.19	7.30	139.1119	C9H14O	Isophorone	2,6-Nona dienal	-
SFEA 3						
32.07	15.52	455.3522	C30H46O3	Oleanonic acid	Ursonic acid	-
17.13	16.11	705.5089	C41H64N6O4	-	-	-
9.42	16.57	531.4083	C30H58O5S	"Didodecyl 3,3'-sulfinyl dipropanoate	-	-
6.97	13.84	329.2050	C20H28N2S	"1- [1-(Adaman tan-1-yl) ethyl] -3-benzylthio urea	-	-
6.13	11.15	455.3521	C26H42N6O	"3-Butyl-1-sec-butyl-1- {[5-(4-ethyl-1-pipera zinyl)-3-methyl-1-phenyl-1H-pyrazol-4-yl] methyl} urea"	"3-Butyl-1- {[5-(4-ethyl-1-pipera zinyl)-3-methyl-1-phenyl-1H-pyrazol-4-yl] methyl}-1-isobutyl urea"	"1-{[5-(4-Ethyl-1-piperazinyl)-3-methyl-1-phenyl-1H-pyrazol-4-yl] methyl}-1-isobutyl-3-(2-methyl-2-propanyl) urea

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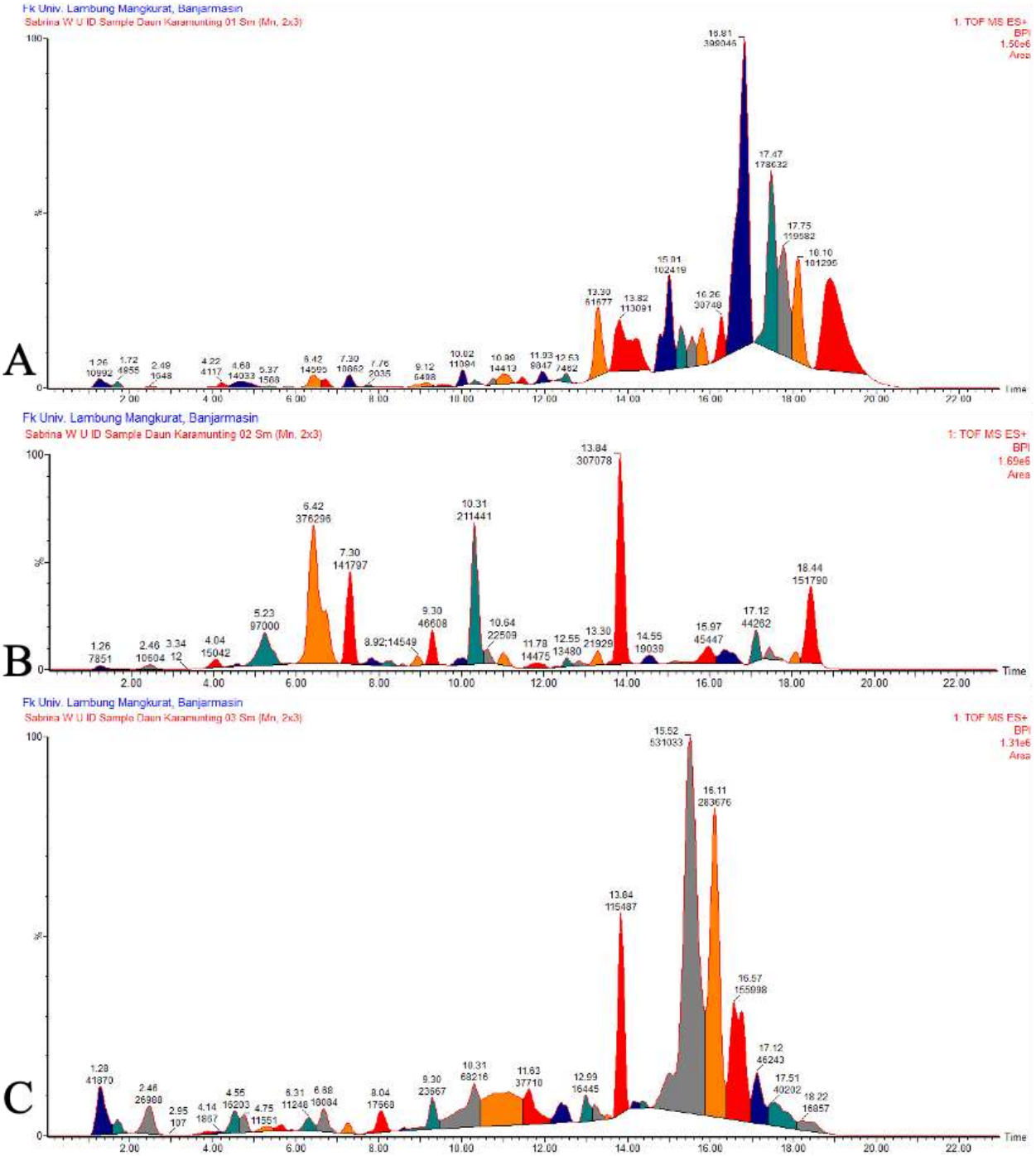

Fig. 1. Chromatogram UPLC-MS Ethyl acetate subfraction 1,2,3 ethanol extract of *R. Tomentosa* leaves, (A). SFEA 1, (B). SFEA 2, (C). SFEA 3.

Discussion

In this study, we demonstrated that the fraction of the ethanolic extract of the leaves of *R. tomentosa* showed an ability to inhibit the hemolysis of red blood cells in a hypotonic solution. From the three fractions of the ethanolic extract of *R. tomentosa*, the ethyl acetate fraction at a concentration of 50 ppm showed the highest inhibition of hemolysis with inhibitory activity of 80%. This result is close to the standard drug diclofenac sodium inhibition by 90%. It is demonstrated that the ethanolic extract of R. tomentosa leaves has biological membrane stabilizing capabilities that prevent stress-related plasma membrane breakdown.

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Red blood cells possess complex intracellular proteins playing sophisticated roles in physiological and pathophysiological processes (11). The cell membrane of red blood is relatively identical to the lysosomal membrane (6). Its stabilization indicates that the extract of *R. tomentosa* can also stabilize the lysosomal membrane. The stability of membranes can reduce serum protein and fluid leakage from tissues. The release of proteases and bactericidal enzymes, the lysosomal components of activated neutrophils, causes inflammation and additional tissue damage due to the release of extracellular enzymes. Thus, it is essential to block them to reduce the inflammatory response (12).

Generally, lysosomal membrane damage results in the release of phospholipase A2, which catalyzes the hydrolysis of phospholipids to generate inflammatory mediators. This cell membrane stability prevents cell lysis and the release of cytoplasmic contents, thereby limiting tissue damage and exacerbating the inflammatory response. Consequently, compounds with membrane stabilization-enhancing activity are anticipated to significantly protect the cell membrane against releasing such cell-damaging substances (13). Anti-inflammatory agents can mediate this mechanism and prevent increasing membrane permeability (14).

In a previous study, flavonoids as a compound of *R. tomentosa* exhibit anti-inflammatory effects in various experimental models. Flavonoid mechanism by the ability to block the cyclooxygenase and lipoxygenase enzymes of arachidonic acid so that the synthesis of prostaglandin is inhibited (15). Flavonoids had anti-inflammatory effects as a result of their membrane-stabilizing ability, which was reported by some studies. The production of free radicals, such as lipid superoxide and peroxides, is reported for membrane destabilization. Flavonoids are reported to act as an effective scavenger of free radicals (16).

UPLC-MS is a versatile technique for identifying and quantifying an unknown organic compound. Our result UPLC-MS study showed that the significant compounds from the three subfractions of the ethanolic extract of *R. tomentosa* leaves and was reported to possess anti-inflammatory potency were oleanonic acid and ursonic acid. Ethyl acetate subfraction 1,2,3 contains oleanonic acid (synonym 3-oxooleanolic acid), a pentacyclic triterpenoid with powerful anti-inflammatory properties. These metabolites can negatively downregulate various extra and intracellular target molecules associated with disease progression. Modified oleanonic acid inhibits hypotonic solution-induced hemolysis (17). The main anti-inflammatory effect of oleanonic acid is the NF, STAT3/6, and Akt/mTOR pathways inactivation (18). Research using rat models showed that edema in rat paws could be reduced by administering oleanolic acid IC50 17M. Oleanolic acid, which has a ketone at C3, implies an increased inhibitory effect on the model associated with 5-lipoxygenase activity and reduces the production of bradykinin and phospholipase A2, leukotrienes B4 from the rat's peritoneum (19). Reduced histamine release in mast cells, inhibiting lipoxygenase, cyclooxygenase, and phospholipase activity, decreased production of nitric oxide and reactive oxygen species, inhibition of signalling pathway activation, downregulation of inflammatory factor expression, and inhibition of elastase and complement activity are the primary anti-inflammatory mechanisms (20).

The second prediction of the ethyl acetate subfraction of the ethanolic extract of the leaves of *R. Tomentosa* was ursonic acid. These metabolites are natural products found in various plants. The biological activity of ursonic acid from Plantago inhibits prostaglandin biosynthesis and cyclooxygenase 2/cyclooxygenase 1. Another potential of this metabolite is that it can reduce the proliferation and survival of various cancer cells. This compound can also inhibit protozoan parasites that cause several tropical diseases. Ursonic acid has antihyperglycemic, anti-inflammatory, antiviral and antioxidant activities (21). LPS-induced RAW264.7 cells treated with ursonic acid showed inhibited nitric oxide production.

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Nitric oxide is a molecule that is strongly associated with inflammation and immunity (22).

The study concluded that the ethanolic extract of *R. Tomentosa* (Karamunting) leaves shows increased red blood cell membrane stability. That has similar physiological properties to lysosome cells. This experimental evidence indicates that *R. Tomentosa* leaves could have potential anti-inflammatory potency. The metabolites that may act as an anti-inflammatory are oleanonic acid and ursonic acid.

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