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Analgesic and Anti-inflammatory Activities of *Urena lobata* L. Leaf Extracts

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ABSTRACT

Pulutan (*Urena lobata* L.) is a medicinal plant that has been traditionally used to treat fever, infection, and inflammation. However, pre-clinical studies on its activity as an analgesic and anti-inflammatory agent are still limited. The present study was aimed at investigating the analgesic and anti-inflammatory activities of *U. lobata* leaf extracts, as well as predicting the biological properties of the active components. Eight (two control and six test) groups of male Wistar rats were used in this study. Water and ethanol were used to extract the leaves of *U. lobata*, and each prepared in doses of 125, 250, and 500 mg/kg body weight (BW) for the treatments. The anti-inflammatory and analgesic activities of the extracts were evaluated. Following active compound identification using UHPLC-MS/MS, several compounds were analyzed for drug-likeness and ADME-T properties. The results revealed that both aqueous and ethanolic extracts of *U. lobata* at 125 and 250 mg/kg BW significantly ($p < 0.05$) inhibited paw edema with an area under curve (AUC) volume of 10 and 5%, respectively, compared to the control group. For analgesic activity, the aqueous extract of *U. lobata* was able to significantly ($p<0.05$) increase the AUC for pain threshold by 30-100 % compared to the control group. However, the activity of the ethanolic extract was lower than the aqueous extract. The drug-likeness analysis indicated that all the phytoconstituents were within the range established by Lipinski's rule of five. Stigmasterol, ß-sitosterol, and mangiferin were predicted to have better ADME-T properties than that of the other compounds.

Keywords: Analgesic activity, Inflammation, Phytoconstituents, Pulutan, *Urena lobata*

INTRODUCTION

Inflammation is the underlying etiology of both degenerative and non-degenerative diseases. An inflammatory reaction is a physiological response of the body to protect itself against physical trauma, chemical agents, and microbial infection. During inflammation, some pro-inflammatory mediators such as prostaglandins, histamine, and leukotrienes are released, which causes the appearance of the inflammation cardinal signs: dolor (pain), tumor (swelling), rubor (red), and calor (heat). Inflammatory cells like macrophages express the COX-2 enzyme, which catalyzes the conversion of arachidonic acid to prostaglandins (PGs) (Zambre *et al.,* 2007). Inflammation plays a role in self-defense against injury factors. However, an uncontrolled and chronic inflammatory response would have a negative impact in form of hypersensitivity, autoimmune and degenerative diseases (Price, 2006).

Opioid analgesics, steroids, and non-steroidal anti-inflammatory drugs are used to treat both pain and inflammation, despite their adverse drug reactions. Medicinal plants can be used as an alternative source to substitute for them. Some herbs have been screened for their potential analgesic and anti-inflammatory activity. However, only a few of them have been reported. Therefore, exploring herbs to determine their active compounds would contribute to the current scientific knowledge (Sen *et al*., 2010).

Pulutan (*Urena lobate* L.), belonging to the Malvaceae family, is a plant that has medicinal properties. It has been discovered that *U. lobata* ethanolic extract has anti-inflammatory properties (Babu *et al*., 2016). According to preclinical research, active ingredients of *U. lobata*, such as

quercetin and mangiferin, have antioxidant, antibacterial, and diabetes-related properties (Adewale *et al*., 2007; Purnomo *et al*., 2021). Flavonoids and terpenoids are the main active compounds in herbs that have activity as an anti-inflammatory. Meanwhile, some alkaloids have analgesic and anti-inflammatory activities (Rivera *et al*., 2006). Investigations into the analgesic and anti-inflammatory activities of *U. lobata* have not been fully explored. To promote the development and discovery of oral medications, it is necessary to study these bioactivities as well as the pharmacokinetic profile and toxicity predictions of the active compounds in *U. lobata*.

Therefore, the present study was conducted to examine the bioactivity, pharmacokinetic profile, and toxicity predictions of the active substances in *U. lobate* to support the development and discovery of drugs taken orally.

MATERIALS AND METHODS

Source of plant material

The *Urena lobata* leaf powder was obtained from Balai Materia Medika Batu Malang, Indonesia. The powder was identified and authenticated in the UPT Laboratorium Herbal Materia Medika Batu Malang with an ID number 074/027/101.8/2015.

Source of animal

The Wistar rats (*Rattus norvegicus*) used for the study were obtained from the Pusat Veteriner Farma (Pusvetma) Surabaya.

Ethical approval

Ethical approval for this study was obtained from the Commission of Ethical Research at Brawijaya University, Malang, Indonesia with approval ID number 002-KEP-UB-2020.

Preparation of plant extract

Extraction from *Urena lobata* leaf powder was carried out by boiling 50 g of leaf powder in 250 ml of hot water at 90ºC for 30 minutes. The same quantity of *U. lobata* leaf powder was digested in 250 ml of ethanol for six hours using a water bath shaker, and the process was repeated three times with different solvents. A rotary evaporator was used to condense both extracts at the end.

Identification of active substances in Urena lobata leaf extracts

UHPLC (ACCELLA 1250, Thermo Scientific) and the detector, MS/MS Triple Q (Thermo Finnigan), were used in a qualitative analysis to identify the active components from both the aqueous and ethanolic extracts of *U. lobata* leaves. The mobile phase used was 0.1 % formic acid in methanol and water. Ten active molecules from the phytosterol, flavonoid, and alkaloid families were identified.

Prediction of biological activities

The structures of the identified compounds were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Using the Swiss ADME website (http://www.swissadme.ch/) for drug-likeness analysis, the physicochemical characteristics of Lipinski's rule of five were investigated. The pharmacokinetic properties (absorption, distribution, metabolism, excretion, and toxicity [ADME-T]) were analyzed using the pkCSM website (https://biosig.unimelb.edu.au/pkcsm).

Animal groupings and treatments

An *in vivo* experiment was conducted using male Wistar rats (180-200 g). The rats were divided into eight groups, each consisting of four rats. Within those eight groups, two control and six test groups were created. Each received an oral dose of 125, 250, and 500 mg/kg body weight (BW) of *U. lobata* aqueous extract (AUL) or ethanolic extract (EUL), respectively.

Anti-inflammatory activity assay

The extracts were administered orally an hour before testing. Following intraplanar injection of 1% carrageenan, anti-inflammatory activity was examined, and paw edema was monitored using a plethysmometer 6 h later. The data were presented as area under curve (AUC) of paw edema using ibuprofen 20 mg/kg as a reference drug.

Analgesic activity assay

One hour before the test, the extracts were administered orally. Analgesic activity was examined by an analgesymeter for 4h. Ibuprofen (20 mg/kg p.o.) was used as a reference drug while the pain threshold was being monitored. An AUC of pain threshold was used to display the data.

Statistical analysis

The data were presented as mean ± SD. Statistical analysis was performed by one-way ANOVA (SPSS version 25). Statistical significance was determined using the least significant difference (LSD) test and $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Bioactive substances present in Urena lobata leaf extracts

According to the results (Figure 1 and Table II) of the UHPLC-MS/MS analysis of the aqueous and ethanolic extracts of *U. lobata* leaves, the bioactive compounds were stigmasterol, gossypetin, and β-sitosterol with a lesser concentration of mangiferin and chrysoeriol.

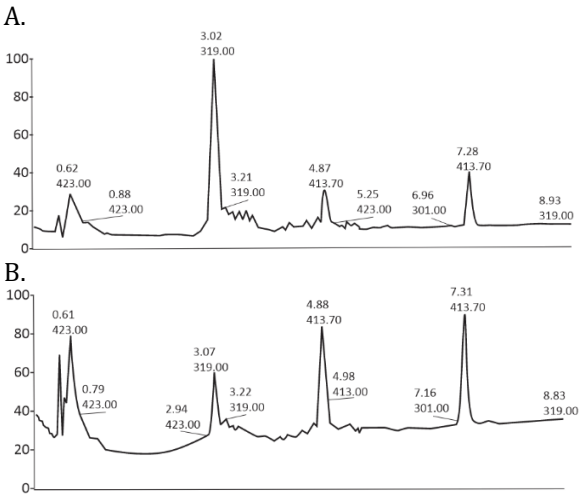

Figure 1. Chromatogram of active compound *U. lobata* (A). Water extract (B). Ethanolic extract

Predicted bioactivity of Urena lobata leaf extracts

The physicochemical characteristics and pharmacokinetics of the active ingredient from *U. lobata* as determined by an *in silico* analysis (Table II and III). To determine whether a molecule has the potential to be developed into a medication, Lipinski's rule of five is applied (drug-likeness). This rule predicts that a molecule has a high likelihood of being developed into a drug if there are no more than two exceptions to the following conditions: there are no more than five H-bond donors (HBD) and 10 H-bond acceptors (HBA), the molecular weight (MW) is smaller than 500, and the calculated LogP is smaller than 5 (Lipinski *et al*., 1997; Naglah *et al*., 2020). The predicted drug analysis result is shown (Table II). All of the active ingredients in *U. lobata* satisfy Lipinski's rule of five in terms of molecular weight, torsion, number of hydrogen bond acceptors and donors, and lipophilicity.

Lipinski's rule of five is the most frequently applied rule used in drug-likeness analysis. The rule has four parameters with different criteria. A compound with a molecular weight ≤ 500 g/mol has strong permeability to the blood and intestinal barriers, which lengthens the time it takes for it to pass through the lipid bilayer. The profile of hydrogen bond donor ≤ 5 and hydrogen bond acceptor ≤ 10 indicates that the compound can be effectively absorbed. The value exceeding the criterion indicates that the compound is soluble in a polar solvent such as water through hydrogen bonds (Pollastri, 2020). The logP parameter is related to the lipophilicity or hydrophobicity of a compound. Because the drug molecule is retained for a long time in the lipid bilayer membrane and is widely distributed in the body, a chemical with a logP value >5 causes severe toxicity. In contrast, a chemical that has a negative logP value is hydrophilic and may be difficult for the molecule to be absorbed. If a compound has a logP value of 5 and is not negative, it is said to have a high absorption and permeation profile since it can cross the hydrophilic outer layer of the membrane and enter the hydrophobic lipid bilayer (Rahmania *et al*., 2015).

The skin permeation of a molecule is expressed as a skin permeability coefficient (Kp), which is determined by molecular size and lipophilicity (Table III). If the log Kp value is negative, it indicates that the molecule is less skin permeable, and if it is less than -2.5 cm/h, it means that the molecule can hardly penetrate the skin (Testa and Kraemer, 2007). Mangiferin, in this instance, had a low skin permeant value and might not be skin absorbable. This property is crucial when developing certain drug delivery systems, especially if the drug is intended to be transdermal.

The pharmacokinetic profile of a molecule as a good drug candidate would also be dependent on its passive gastrointestinal absorption and blood-brain barrier (BBB) permeation. Except for chrysoeriol, five of the active substances were shown to have low BBB penetration and gastrointestinal absorption, but chrysoeriol had high intestinal absorption. The likelihood that a substance will be actively transported through a biological membrane depends on whether it can act as a substrate for the permeability glycoprotein (P-gp), a crucial member of the ATP-binding cassette transporters (ABC-transporters). ABC transporters are present in the gastrointestinal wall as well as the brain (Lipinski *et al*., 1997). The major role of P-gp is to protect the central nervous system (CNS) from xenobiotics (Pires *et al*., 2015). The interaction of chemicals with cytochromes P450 (CYP), in addition to P-gp, is crucial.

Table I. Active compounds in *U. lobata* leaf extracts

No	Active compounds	Molecule weight	Aqueous extract	Ethanolic extract
1	Stigmasterol	413	(++)	(+++)
2	B-Sitosterol	415	(+)	(+)
3	Mangiferin	423	(+)	(++)
4	Quercetine	303	(-)	(-)
5	Kaempferol	286	(-)	(-)
6	Hypolaetin	302	(-)	(-)
7	Gossypetin	318	(+++)	(+)
8	Luteolin	286	(-)	(-)
9	Apigenin	270	(-)	(-)
10	Chrysoeriol	300	(+)	(+)

Note : (-) : negative; (+): weak; (++): moderate; (+++); strong

Table II. Physichochemical properties of active compounds in *U. lobata* leaf extracts

Active compounds	Lipinski's						Drug-likeness
	MW	Log P	Torsion	HBA	HBD	No. of rule Violations	
	≤ 500	≤ 5	≤ 5	≤ 10	≤ 5	≤ 2	
Stigmasterol	412.69	8.56	5	1	1	0	yes
β-Sitosterol	414.71	9.34	6	1	1	0	yes
Mangiferin	422.34	-0.37	2	11	8	0	yes
Gossypetin	318.24	1.81	1	8	6	0	yes
Chrysoeriol	300.26	3.10	2	6	3	0	yes

MW=Molecular weight; LogP=Lipophylicity; Torsi=rotatable bonds; HBA=H-bond acceptors; HBD=H-bond donors;

Table III. Pharmacokinetic properties of active compounds in *U. lobata* leaf extracts

Active compounds	Absorption		Distribution		Metabolism		Excretion	Toxicity
	Intestinal absorption	Skin permeation	BBB permeant	P-gp Substrat	CYP2D6 inhibitor	CYP3A4 inhibitor	Cltot (log ml/min/kg)	LD50 (mol/kg)
Stigmasterol	Low	-2.74	No	No	No	No	0.618	2.540
β-Sitosterol	Low	-2.20	No	No	No	No	0.628	2.552
Mangiferin	Low	-9.14	No	No	No	No	0.347	2.396
Gossypetin	Low	-6.96	No	No	Yes	Yes	0.304	2.527
Chrysoeriol	High	-5.93	No	No	Yes	Yes	0.597	2.337

BBB= Blood Brain Barrier; P-gp= Permeability glycoprotein; Cltot=Clearance total

This superfamily of isoenzymes is a key player in drug biotransformation (Monatari and Ecker, 2015). CYP and P-gp could process small molecules synergistically to enhance the protection of tissues and organs (Szakács *et al*., 2008).

Cytochrome P450 is an enzyme mostly found in the liver and functions in the detoxification and oxidation of drugs and other xenobiotics, as well as the body's removal of these substances. Although cytochrome P450 primarily renders these drugs inactive, it is also known that some pharmaceuticals may be activated by this enzyme, and as a result, this enzyme frequently participates in the biotransformation of drugs. It is important to assess a drug candidate's capacity to act as a substrate for cytochrome P450, as well as P2D6 cytochrome (CYP2D6) and P3A4 cytochrome (CYP3A4), which are also the two main isoforms responsible for drug metabolism (Testa and Kraemer, 2007). From Table 3, it can be observed that almost all the active substances do not affect the CYP2D6 and CYP3A4 enzymes, apart from gossypetin and chrysoeriol. Two chemicals have the potential to increase drug levels in the blood and cause adverse drug reactions in addition to inhibiting the enzyme that metabolizes drugs. This

suggests that the P450 enzyme would metabolize the majority of the drugs tested in this study. Because β-sitosterol has a better overall clearance rate than other substances, it has a decreased risk of accumulation and toxicity. However, biological activity must be taken into account. Based on the LD-50 value, both mangiferin and chrysoeriol have higher toxicities than other compounds; hence, their safety needs to be evaluated. Most of the substances have a low value of intestinal absorption, except chrysoeriol, but because they are not P-gp substrates, they cannot cross the BBB. Chrysoeriol and gossypetin both inhibit CYP2D6 and CYP3A4, but not other drugs.

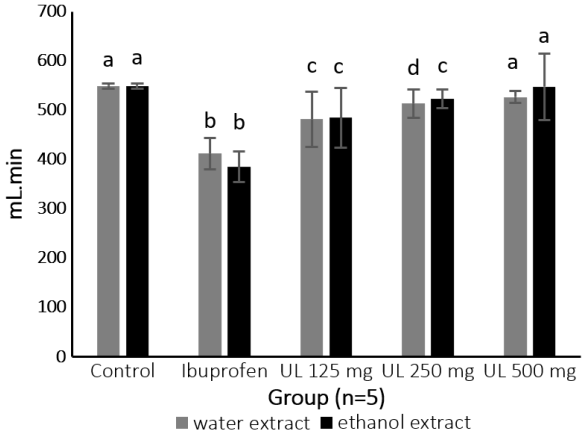

Figure 2. AUC of edema paw administrated U. lobata leaf extract; a,b,c etc. indicated the differences of potency ($p<0.05$, LSD test)

Anti-inflammatory effect of Urena lobata leaf extracts

The results of the anti-inflammatory activity of *U. lobata leaf extracts* as determined by the area under the curve (AUC) (Figure 2). At concentrations of 125, 250, and 500 mg/kg BW, aqueous extract of *U. lobata* significantly ($p < 0.05$) reduced edema paw by 12, 6, and 4%, respectively, compared to the control group. Similarly, the inhibition percentages of the ethanolic extract from the *U. lobata* were 12, 5 ($p < 0.05$), and 0.2% ($p > 0.05$), respectively, compared to the control group. Because aqueous and ethanolic extracts of *U. lobata* leaves contain β-sitosterol, mangiferin, and stigmasterol, they were able to reduce edema paw, even though their activity was less than that of ibuprofen. Mangiferin is a well-known natural polyphenol derived from plants that has several pharmacological actions, including those that are anti-inflammatory, analgesic, antibacterial, antiviral, anti-diabetic, and antipyretic (Du *et al*., 2018; Purnomo *et al*., 2015). β-Sitosterol, a ubiquitious phytosterol in plants, is also found in *U. lobata* extracts (Purnomo *et al*., 2018).

Both *in vivo* and *in vitro* studies have demonstrated that phytosterols have immunomodulatory properties; as a result, they may be beneficial for both human and animal health (Bouic and Lamprecht, 1999; Prieto *et al*., 2006). Mangiferin and β-sitosterol can reduce COX-2 levels induced by lipopolysaccharides (Bhatia *et al*., 2008; Lee *et al*., 2012). The synthesis of prostacyclin (PGI2) and prostaglandins (PGE2) is reduced when COX-2 levels are reduced, resulting in the suppression of the inflammatory response (Bhatia *et al*., 2008).

In vivo studies have shown mangiferin and β-sitosterol to have COX-2 inhibitory activity (Bhatia *et al*., 2008; Lee *et al*., 2012). COX-2 plays a significant role in inflammatory pathogenesis, and inflammatory agents such as bacterial endotoxins and cytokines may cause COX-2 to be secreted. It is involved in the release of prostanoids during inflammation, making it a potential molecular target for analgesics and anti-inflammatory medications (Ricciotti and Fitzgerald, 2011). Based on an *in silico* investigation, previous research also suggested that mangiferin, stigmasterol, and β-sitosterol inhibited COX-2 activity (Wahyuningsih *et al*., 2022). Furthermore, mangiferin and β-sitosterol have inhibitory activity on COX-2. The expression of nuclear factor kappa B (NF-κB) and tumor necrotic factor (TNF-α) is known to be inhibited by mangiferin and β-sitosterol (Loizou *et al*., 2010; Lee *et al*., 2012; Saha *et al*., 2016; Sun *et al*., 2020). TNF-α is a pro-inflammatory cytokine with numerous physiological and pathological functions. TNF-α activates NF-κB, which is required for the expression of pro-inflammatory cytokines, the differentiation of innate immune cells, and the differentiation of inflammatory T-cells. NF-κB and TNF-α inhibition actions, which also reduce inflammation, aid in the healing process (Lawrence, 2009; Pozniak *et al*., 2014; Liu *et al*., 2017). Both the ethanolic and aqueous extracts of *U. lobata* leaf extract had relatively similar efficacy in reducing the levels of paw edema. A 500 mg/kg BW dose of aqueous and ethanolic extracts of *U. lobata*, however, was unable to reduce paw edema. Low receptor sensitivity is caused by active drug dose and duration increases (Dar *et al*., 2005). Additionally, the extract's anti-inflammatory efficacy will decrease with an increase in dose. The administration of *U. lobata* leaf extract had potency

as an anti-inflammatory agent. However, its efficacy is lower than ibuprofen as a reference drug. The herbs contain many active compounds that can interact with one another. This interaction may have perhaps resulted in an antagonist effect, therefore reducing its activity (Goodman and Gilman, 2006).

Analgesic effect of Urena lobata leaf extracts

When compared to the control group, the administration of *U. lobata* aqueous extract at 125, 250, and 500 mg/kg BW significantly ($p<0.05$) increased the AUC of pain threshold by about 40, 70, and 100%, respectively (Figure 3). Meanwhile, ethanolic extract from *U. lobata* increased significantly ($p<0.05$) by 20, 50, and 50%, respectively. Except for the aqueous extract of *U. lobata* at a dose of 500 mg/kg BW, the activity was lower than ibuprofen. This observation might be a result of some active ingredients in *U. lobata*, which can act by inhibiting the release of pain mediators such as prostaglandin, bradykinin, and histamine (Goodman and Gilman, 2006; Ricciotti and Fitzgerald, 2011). β-sitosterol and mangiferin are active compounds in *U. lobata* leaf extract having activity as an analgesic.

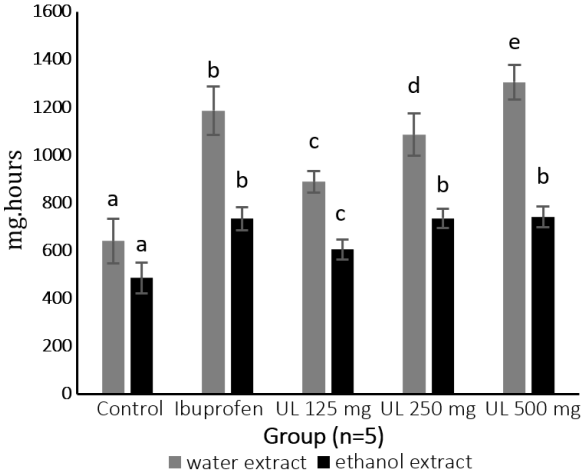

Figure 3. AUC of pain threshold administrated *U. lobata* leaf extract; a,b,c etc. indicated the differences of potency ($p<0.05$, LSD test).

The study of the analgesic activity of mangiferin showed anti-nociceptive responses in both peripheral and central areas (Dar *et al*., 2005). The peripherally active sensory afferent nerves are the source of nociceptive responses, neuropathic pain, and anti-inflammatory responses. This shows that the inhibition of several neuro-receptors, such as opioids, alpha-adrenergic, cholinergic, and cannabinoid receptors, may represent possible targets for drugs (Sawynok, 2003; Imran *et al*., 2017). The involvement of mangiferin and β-sitosterol in the opioid receptor cannot be ignored (Dar *et al*., 2005; Dighe *et al*., 2016). β-sitosterol showed central anti-nociceptive action, therefore exerting its effect through the central opioid receptor or promoting the release of endogenous opioid peptides (Dighe *et al*., 2016). Mangiferin and β-sitosterol can lower COX-2 levels, which in turn prevents the formation of both prostaglandins (PGE2) and prostacyclin (PGI2), a pain-mediating compound (Bhatia *et al.,* 2008; Lee *et al.,* 2012). Some of the aforementioned inflammatory mediators can activate pain receptors or nociceptors, which results in pain. Histamine and PGE2 release stimulate nociceptors (nociceptive afferent fibers), which are responsible for transmitting pain signals to the brain (Dar *et al*., 2005). As demonstrated in this study, stigmasterol, β-sitosterol, and mangiferin can inhibit COX-2 activity by molecular docking, therefore serving as potential anti-nociceptive.

An aqueous extract of *U. lobata* has a stronger analgesic effect than its ethanolic extract. This may indicate that the *U. lobata* active ingredients have low solubility and absorption in ethanol, which would reduce their bioactivity as analgesics. The formation of complex compounds as well as changes in active compound conformation would further reduce active substance solubility and therefore decrease its ability to prevent pain mediator release (Stevens *et al*., 2010). With a dose of 125 mg/kg BW, the ethanol extract of *U. lobata* exhibits the least analgesic efficacy. It is brought on by modest doses of herbs' inability to prevent the release of pain mediators and nociceptor activation.

CONCLUSION

The findings of this study reveal that the leaf extracts from *Urena lobata* have anti-inflammatory and analgesic properties. The range established by Lipinski's rule of five for drug-likeness analysis was applied to all of the phytoconstituents, and three constituents were expected to have better ADME-T characteristics.

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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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