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Original Research Article

Inhibitory Potential of Pulutan (*Urena lobata*) Leaf Extract on Inducible Nitric Oxide Synthase as Anti-inflammatory Agent: *In Vitro* and *In Silico* Approaches

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ABSTRACT

Urena lobata is an herbal remedy traditionally used to cure certain ailments. Its flavonoid and phenol-rich content has been shown to have free radical scavenging activity. Nitric oxide (NO) is an active gaseous substance that is released by cells and plays a role in inflammation. This study examined the ability of *U. lobata* leaf extract to induce NO production and suppress inducible NO synthase (iNOS). The leaves of *U. lobata* were extracted with ethanol. The phytoconstituents of the extract were identified, their physicochemical and pharmacokinetic properties were predicted, and their anti-inflammatory activity was evaluated. The structures of the phytoconstituents were determined using the molecular docking approach. Griess technique was used to evaluate NO generation in IL-1β-treated hepatocyte culture media containing the extract. An *in silico* approach was employed to investigate the suppression of iNOS by phytoconstituents from *U. lobata*. The results showed that stigmasterol and gossypetin were identified in the extract and had a strong inhibitory effect on iNOS based on the inhibition constant. However, it has lower potency than β-sitosterol. The ethanol extract of *U. lobata* (IC_{50} = 163.03 µg/mL) had a lower potency than gossypin (IC_{50} = 100.31 µg/mL) but was comparable to gossypetin (IC_{50} = 164.18 µg/mL) for inhibiting NO generation. The ethanol extract of *U. lobata* demonstrated anti-inflammatory action by lowering NO level, but at a lower concentration than gossypin and gossypetin. The study revealed that *U. lobata* ethanol extract inhibited NO production through active substances, like gossypetin, mangiferin, β-sitosterol, and stigmasterol, thereby suppressing iNOS activity.

***Keywords*:** *Urena lobata*, inflammation, molecular docking, nitric oxide

Introduction

Urena lobata is a plant prevalent in Asia that has long been used to treat influenza, fever, and other infectious disorders.[1] In Indonesia, *U. lobata* is referred to as Pulutan, and preliminary research has shown antibacterial and anti-inflammatory properties.[2,3] Other studies have shown that flavonoids and phytosterols in *U. lobata* function as free radical scavengers (including superoxide and hydroxyl radicals) and lipid peroxidation inhibitors.[2,4] Gossypetin is a gossypin-derived aglycone found in *U. lobata* leaves.[5] The substances are classed as flavonoids and have anti-inflammatory properties.[6,7] Pro-inflammatory mediators are free radicals generated by cells infiltrated into damaged tissues. Inflammatory cells release mediators, which contribute to several inflammatory reactions, including vasodilation, tissue damage, increased vascular permeability, and discomfort.[8] Pro-inflammatory mediators comprise free radicals (~~NO~~-reactive nitrogen species, reactive oxygen species), leukotrienes, prostanoids (PGs), and inflammatory cytokines (IL-1β and IL-6). Anti-inflammatory drugs are those that inhibit their capacity for activation.[8,9]

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Nitric oxide (NO) is an active gaseous substance produced by cells that is responsible for the inflammatory response. Excessive NO release causes systemic vasodilation, which results in tissue damage and septic shock. When IL-1β is present, inducible NO synthase (iNOS) is induced in hepatocytes, resulting in the stimulation of NO.[10] The level of NO generation is considered one of the markers of inflammatory responses.[11] Hepatocytes in the liver produce a significant amount of NO. Primary cultured rat hepatocytes produce NO with greater effectiveness than the hepatocyte-derived cell line.[12] Thus, IL-1β treated rat hepatocytes are often employed as an *ex vivo* model.[12,13] The current study aimed to examine the effects of *U. lobata* leaf extract on NO production and iNOS inhibition using *in vitro* and *in silico* approaches.

Materials and Methods

Source of animals and ethical approval

Male Wistar rats (180-200 g) were provided by Charles River Laboratories Japan Inc., Yokohama, Japan. Before the experiment, the rats were kept in cages at 21-23°C and acclimated. The protocol for the current investigation was authenticated by the Ethical Research Commission of Ritsumeikan University in Kusatsu, Japan (BKC2019-034).

Plant collection and identification

The Laboratory of UPT Materia Medika Batu in Malang, Indonesia provided the simplicia of *U. lobata* leaves, under certificate number 074/027/101.8/2015. The herb was collected at three to four months old. Simplicia was identified by Dr. Husain from Laboratory of UPT Materia Medika Batu in Malang.

Plant extraction
The herbal material (50 g) was extracted in a 1:5 ratio (w/v) in 96% ethanol (250 mL), and placed on a water bath shaker at 50ºC for 4 h. The procedure was repeated twice using fresh ethanol (96%). The extract was evaporated at 60ºC and 600 mmHg with a rotary evaporator and dried in the oven at 40ºC.

Identification of phytoconstituents of Urena lobata leaf extract
The phytochemicals in the extract were assessed by ultra-high-performance liquid chromatography and MS/MS detector. The mobile phase consisted of formic acid (0.1%) mixed with methanol and water. The identification included the ten target phytoconstituents of *U. lobata* leaf extract, including alkaloids, flavonoids, and phytosterols.

Prediction of physicochemical and pharmacokinetic properties of the phytoconstituents
The Swiss-ADME (https://biosig.lab.uq.edu.au/pkcsm/) and pkCSM online tools were employed to assess the physicochemical and pharmacokinetic properties of the phytoconstituents of *U. lobata* leaf extract.

Prediction of anti-inflammatory activity of the phytoconstituents
Five phytoconstituents from *U. lobata* were chosen to predict anti-inflammatory efficacy. They were retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov) using canonical SMILES. The structure was examined using the web-based application PASSOnline (http://www.way2drug.com/PASSOnline/) to predict anti-inflammatory activity. The prediction was presented as a probability score as active (Pa) or inactive (Pi). The Pa score of more than 0.7 indicated a high activity in both the *in vitro* and *in vivo* studies.

Determination of the structures of the phytoconstituents using the molecular docking approach
The computational analysis was carried out using an MSI OS; 12th Gen Intel(R) Core (TM) i7-12650H 2.70 GHz; 16 GB RAM; and NVIDIA GeForce RTX 3050. The structures of the phytoconstituents were acquired from PubChem (https://pubchem.ncbi.nlm.nih.gov), and the macromolecular protein targets were obtained from Protein Data Bank (https://www.rcsb.org/). Grid boxes were positioned using the native ligand coordinates. The dimensions for iNOS (PDB ID: 4NOS) were set to 14.1819 Å x 12.9908 Å x 16.3576 Å (x = -1.0311, y = 29.4965, z = 33.5714) and protoporphyrin is used as reference standard. The molecular docking was conducted by the PyRx 0.8 program. The outcomes of the molecular docking simulations were then visualized with Discovery Studio Visualizer V21.1.0.20298.

Culturing of primary hepatocytes
Using collagenase perfusion, hepatocytes were extracted from rat livers.[13] The dispersed cells were placed back in WE medium enriched with foetal calf serum (SAFC Biosciences Inc., Lenexa, Kansas, USA) and cultivated at $1.2x10^6$ cells/dish. Hepatocyte cultures were incubated at 37°C for 2 h before the media was replenished, followed by an overnight 37°C incubation and observation until the following day (day one).

Nitric oxide assay
After primary cell cultivation, *U. lobata* extracts were used to treat hepatocytes with 10 µL of rat IL-1 *β* (PeproTech, Rocky Hill, NJ, USA) for 8 h. The Griess method,[14] was applied to compute the concentration of nitrite, a stable NO metabolite, in the culture medium. The concentration of NO in the presence and absence of IL-1*β* was set to 100% and 0%, respectively. Gossypin (Sigma-Aldrich Corp.) and gossypetin were employed as positive controls to suppress NO-induced IL-1*β*. Gossypin (15.04 mg) was heated in 2 N HCl and the presence of gossypetin was confirmed. The 50% inhibitory concentration (IC_{50}) was evaluated three times for each time point, using at least three distinct concentrations.[15]

$$\% \text{ Inhibition} = \frac{A_{540}\text{ control} - A_{540}\text{ sample}}{A_{540}\text{ control}} \times 100\ \%$$

Statistical analysis
Data were expressed as mean ± SD and analyzed using one-way analysis of variance (ANOVA). Differences between means were analyzed using the least significant difference (LSD) test, with a p-value < 0.05 indicating statistical significance. The IC_{50} score was computed by fitting a linear regression curve with SPSS version 16.0.

Results and Discussion

Phytoconstituents of Urena lobata leaf extracts
The extract yield from *U. lobata* leaves was 4.1%. Figure 1 and Table 1 show the target phytoconstituent of *U. lobata* leaf ethanol extract. From the chromatogram peak height, stigmasterol and mangiferin were the most prevalent phytoconstituents in *U. lobata* leaf extract. The extracts also contained *β*-sitosterol, gossypetin, and chrysoeriol, in low concentrations. The procedure employed is a semi-qualitative analysis that compares the molecular weights of active substances from a library or database.

Prediction of the anti-inflammatory activity of Urena lobata phytoconstituents
The phytoconstituents of the extract were identified and analyzed. The results are presented in Table 2. Gossypin and gossypetin had a high probability of being active (Pa score greater than 0.700). The high probability score for both compounds suggests they are pharmacologically active compounds, particularly as anti-inflammatory agents. Phytoconstituents, such as chrysoeriol, gossypin, and gossypetin from *U. lobata* demonstrated high probability score in predicting anti-inflammatory activity. They are categorized as flavonoids, and some research has shown that they have anti-inflammatory properties. Flavonoids act as anti-inflammatory agents by inhibiting lipoxygenase, cycloxygenase, aldose reductase, xanthin oxidase, and phosphodiesterase. Inhibiting these enzymes will reduce the synthesis of cytokines that promote inflammation. The generation of pro-inflammatory cytokines will result in an inflammatory response. NF-Kß activation leads to the release of several pro-inflammatory cytokines, including IL-6, IL-8, COX-2, and TNF-α.[16]

Table 1: Phytoconstituent identification of *Urena lobata* leaf extract

Active substance	MW(KDa)	Ethanol extract
Mangiferin	423	+
Quercetine	303	-
Kaempferol	286	-
Hypolaetin	302	-
Gossypetin	318	+
Luteolin	286	-
Apigenin	270	-
β-Sitosterol	415	+
Stigmasterol	413	+

Note : MW = Molecular weight
(-) : not identified (+) : identified

Table 2: Activity prediction of phytoconstituents from *Urena lobata* leaf extract as an anti-inflammatory agent

Phytoconstituent	Anti-inflammation	
	pa	pi
Gossypin	0.765	0.009
Chrysoeriol	0.627	0.026
Gossypetin	0.712	0.014
Mangiferin	0.479	0.063
β-sitosterol	0.482	0.004
Stigmasterol	0.542	0.045

Physicochemical and pharmacokinetic properties of Urena lobata phytoconstituents

Table 3 shows the predicted physicochemical properties. The number of hydrogen bond acceptors and donors, molecular weight, and lipophilicity characteristics of all phytoconstituents in *U. lobata* agree with Lipinski's rule of five. The predicted pharmacokinetic characteristics (Table 4) showed that only chrysoeriol had a high intestinal absorption rate after oral dosing. Meanwhile, mangiferin and gossypin demonstrated good skin permeability based on the value. All phytoconstituents did not show blood-brain barrier (BBB) permeability or glycoprotein (P-gp) activity. Nevertheless, chrysoeriol and gossypetin inhibited both CYP3A4 and CYP2D6. Stigmasterol and *β*-Sitosterol showed excellent total clearance.

Table 3: Physicochemical properties of phytoconstituents in *Urena lobata* leaf extract

Phytoconstituent	Physicochemical properties					Lipinski requirement
	MW	Log P	Torsion	HBA	HBD	
	≤ 500	≤ 5	≤ 5	≤ 10	≤ 5	
Mangiferin	422.34	-0.37	2	11	8	yes
Gossypetin	318.24	1.81	1	8	6	yes
Gossypin	480.38	0.01	4	13	8	yes
Chrysoeriol	300.26	3.10	2	6	3	yes
β-Sitosterol	414.71	9.34	6	1	1	yes
Stigmasterol	412.69	8.56	5	1	1	yes

MW: molecular weight; Log P: Lipophilicity; Torsi: rotatable bonds; HBA: H-bond acceptors; HBD: H-bond donors.

Table 4: Pharmacokinetic properties of phytoconstituents in *Urena lobata* leaf extract

Phytoconstituent	Absorption		Distribution		Metabolism		Excretion
	Intestinal absorption	Skin Permeation (cm/s)	BBB Permeant	P-gp substrate	CYP2D6 inhibitor	CYP3A4 inhibitor	Cl tot (mL/min/kg)
Mangiferin	Low	-9.14	No	No	No	No	2.223
Gossypetin	Low	-6.96	No	No	Yes	Yes	2.014
Gossypin	Low	-9.22	No	No	No	No	1.862
Chrysoeriol	High	-5.93	No	No	Yes	Yes	3.954
β-Sitosterol	Low	-2.20	No	No	No	No	4.246
Stigmasterol	Low	-2.74	No	No	No	No	4.149

BBB: blood-brain barrier; P-gp: permeability glycoprotein; Cltot: clearance total.

Lipinski's rule of five and physicochemical characteristics are used to analyze phytoconstituents with the potential to be developed as drugs. This rule indicates that the molecule has a high possibility of being transformed into a pharmaceutical if only two rule violations are found in the following specifications: The molecular weight (MW) is less than 500; the calculated Log P is less than 5; there are no more than five H-bond donors (HBD) and ten H-bond acceptors (HBA).[17,18] When the molecular weight is less than 500 g/mol, it indicates that the molecule has good blood and intestinal barrier permeability. The log P parameter represents the substance's lipophilicity. Phytoconstituents have a favourable absorption and penetration profile if their log P is less than 5 and not negative because they can pass through the membrane's hydrophilic outer layer and enter the hydrophobic lipid bilayer.[19] The presence of hydrogen bond donors less than 5 and acceptors less than 10 indicates that the molecule can be well absorbed. The score exceeded the criterion, indicating that the chemical is soluble in a polar solvent via hydrogen bonds.[20] All phytoconstituents had low absorption in the gastrointestinal tract except chrysoeriol. These findings indicate that chrysoeriol is more absorbed in the gastrointestinal tract, making it simpler to distribute in the systemic circulation. This prediction was made using the BOILED-Egg method, which is a descriptive graphical approach that separates chemicals that are well and poorly absorbed by the gut.[21] All phytoconstituents did not pass through the BBB and did not interact with P-glycoprotein. Many organs rely on efflux transporters, such as P-gp to transfer drugs.[22] Permeability glycoprotein is widely distributed in the gut epithelium, pumping xenobiotics back into the gut lumen and from capillary endothelial cells in the brain back into the capillaries.[22] P3A4 cytochrome (CYP3A4) and P2D6 cytochrome (CYP2D6) are the two primary isoforms relating to the metabolism of drugs.[21]

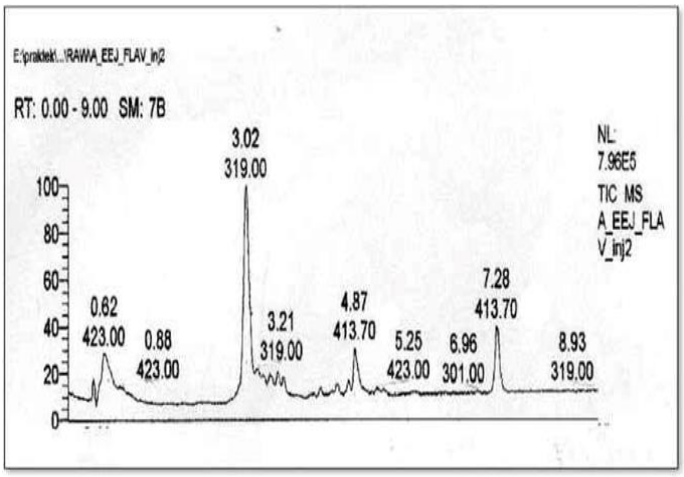

Figure 1: Chromatogram of *Urena lobata* leaf extract identified by ultra-high-performance liquid chromatography and MS/MS detector

Table 4 shows that, practically all active drugs except chrysoeriol and gossypetin had no effect on or inhibited CYP2D6 or CYP3A4. Furthermore, it was predicted that the chemicals will block the metabolism of P_{450} enzymes in the body. Clearance total indicates the excretion profile; clearance is a pharmacokinetic parameter that measures the rate of irreversible drug removal from the body. The clearance rate for all phytoconstituents is poor, with scores less than 5 mL/min/kg. A medicine has a high clearance rate if its score is greater than 15 mL/min/kg. A moderate clearance rate is 5-15 mL/min/kg.[23]

Molecular docking of phytoconstituents of Urena lobata on iNOS

Table 5 shows the molecular docking of phytoconstituent of *U. lobata* leaf extract with iNOS. *Urena lobata*'s *β*-sitosterol and stigmasterol showed reduced inhibition constant (Ki) and free energy binding. They have strong anti-inflammatory properties against iNOS. Protopirin's performance as a reference standard outperformed stigmasterol and *β*-sitosterol concerning Ki value and free energy binding. Considering the amino acid interaction of iNOS with phytoconstituents of *U. lobata* (Table 6), *β*-sitosterol had two amino acid interactions (194 TRP and 197 ALA) compared with the reference standard. Meanwhile, stigmasterol and mangiferin only had one (197 ALA).

Table 5: Molecular docking of phytoconstituents of *Urena lobata* leaf extract with inducible nitric oxide synthase

Active compounds	Est. Free Energy of Binding (Kcal/mol)	Est. Inhibition Constant Ki (µM)
Protoporphyrin*	-12.60	0.0006
Chrysoeriol	-9.80	0.1773
Gossypin	-9.50	12780
Mangiferin	-9.40	36890
Gossypetin	-9.20	325.55
β-Sitosterol	-10.80	0.0119
Stigmasterol	-10.60	0.0167

*: reference standard

Other phytoconstituents had no similar amino acid interaction with the reference standard. According to the molecular docking investigation, chemicals from *U. lobata* leaf extract had an inhibitory effect against iNOS. This was determined by assessing the inhibition constant, binding free energy values, and amino acid interactions. Low binding free energy indicates a strong interaction between a ligand and its protein target, as well as a greater likelihood of biological activity.[24] *β*-sitosterol, demonstrated the lowest inhibition constant and binding free energy. Protoporin, a reference standard, serves to prevent iNOS, which inhibits NO generation. The level of NO generation is thought to be one of the factors that suggest inflammatory responses.[11] When NO is produced during inflammation, it causes systemic vasodilation, which results in tissue damage and septic shock.[25] Molecular docking techniques are a frequent way to shorten the length of a drug discovery project by predicting the chance of a ligand binding to a protein target with a known structure. This prediction is made mostly by evaluating the binding free energy value,[26] which represents the ligand's affinity to bind to a protein target, with a lower score indicating a higher affinity.[27] Furthermore, inhibition constants might indicate a compound's bioactivity and toxicity.[28] However, *in silico* approaches must be confirmed by *in vitro* and *in vivo* studies.

Inhibitory activity of Urena lobata in nitric oxide production induced by IL-1β

Figure 2 and Table 7 show the effect of IL-1*β* on *U. lobata*'s NO production. *Urena lobata* extract administered at 75, 150, and 300 µg/mL significantly reduced ($p < 0.05$) NO generation by 30, 40, and 90%, respectively, compared to the control group. Gossypin at 80 and 160 µg/mL significantly ($p < 0.05$) reduced NO generation by 50 and 80%, respectively, while gossypetin at 100 and 200 µg/mL significantly reduced ($p < 0.05$) it by 40 and 60%, respectively. Gossypetin dose of 50 µg/mL did not significantly reduce ($p > 0.05$) NO levels.

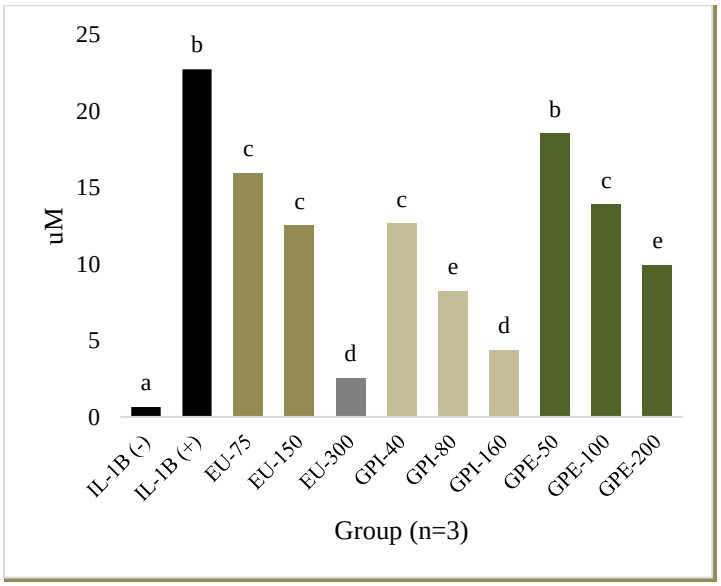

Figure 2: Effect of nitric ~~acid~~ oxide (NO) production of *Urena lobata* leaf extract on hepatocytes culture induced by IL-1*ß*. Different alphabets on the bars indicate a significant difference at $p < 0.05$ with the LSD test.

Table 6: Amino acid interaction of inducible nitric oxide synthase (iNOS) with phytoconstituents of *Urena lobata*

Compound	Amino Acid Interactions						
	Conventional hydrogen bond	**Carbon hydrogen bond/pi donor hydrogen bond**	**Pi-Sigma**	**Pi-Alkyl/Alkyl**	**Pi-Anion**	**Pi-pi stacked**	**Unfavorable negative-negative**
Protoporphyrin*	200 CYS, 491 TYR		194 TRP	**197 ALA**	199 ARG		377 GLU
β-sitosterol	498 TYR		194 TRP	197 ALA, 199 ARG, 200 CYS, 355 MET, 369 PHE, 374 MET, 434 MET			
Stigmasterol			372 TRP	197 ALA, 199 ARG, 200 CYS, 355 MET, 369 PHE			
Chrysoeriol	190 THR, 370 ASN, 489 TYR		197 ALA	199 ARG, 200 CYS, 209 LEU, 469 TRP		194 TYR, 369 PHE	
Gossypin	242 SER, 372 TRP	200 CYS, 202 GLY, 371 GLY	197 ALA			194 TRP, 369 PHE	
Mangiferin	199 ARG, 201 LEU, 377 GLU, 489 TYR	200 CYS		197 ALA		194 TRP, 369 PHE	
Gossypetin	377 GLU	200 CYS	202 GLY			194 TRP, 369 PHE	

*: reference standard

Table 7: IC_{50} of *Urena lobata* leaf extract and reference standards in nitric ~~acid~~ oxide (NO) production

Sample	**n**	**IC_{50} (**μg/mL)
Urena lobata extract (EU)	3	163.03±6.80
Gossypin (GPI)*	3	100.31±2.01
Gossypetin (GPE)*	3	164.18±2.46

*: reference standard.

Urena lobata extract inhibited NO generation, but its IC_{50} value was lower than that of gossypin. Gossypin, as a reference standard, had the highest activity to reduce NO generation than gossypetin.
Gossypetin is an aglycone derived from gossypin that is found in *U. lobata* leaves. Aglycone is a non-sugar fragment of a glycoside, whereas a glycoside is a molecule that has a sugar group connected to a non-sugar by a nitrogen or oxygen atom. The hydrolysis of gossypin leads to the production of gossypetin and sugar. According to the hypothesis, the aglycone form is more soluble in lipids and more active than glycoside.[6] However, in this investigation, gossypin is more effective than gossypetin at inhibiting NO generation. Gossypin (log P = -0.83) is more hydrophobic than gossypetin (log P = 1.69), making it easier to interact with cells and their receptors.[17]
The ethanol extract from *U. lobata* inhibited NO generation less than gossypin, a reference standard. This could be due to the extract containing a large number of active compounds but at low concentration and purity.[6] The crude extract has a more complicated composition than the reference standard, which is a single chemical. As a result, the probability of interaction between active compounds increases and affects activity,[29] potentially modulating substance activity. The ethanol extract of *U. lobata* includes stigmasterol, *β*-sitosterol, mangiferin, and gossypetin, which have been demonstrated to inhibit iNOS through molecular docking. This suggests that the compounds can limit NO generation. Previous research on *U. lobata* leaf extract showed anti-inflammatory effects in rats induced by carrageenan.[30] Another study found that the n-butanol component of *U. lobata* had a potent anti-inflammatory effect by inhibiting protein denaturation.[31] According to toxicity studies, *U. lobata* has a low to moderate level of toxicity in zebrafish, making it safe for therapy.[32]

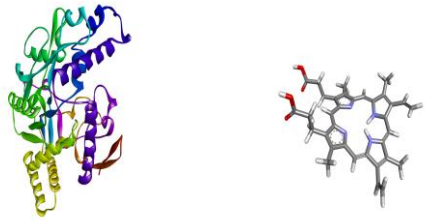

Figure 3: Crystal structure of iNOS (left) and chemical structure of protoporphyrin (right)

Conclusion

The findings from this study revealed that the ethanol extract of *U. lobata* inhibited NO production through active substances, such as gossypetin, mangiferin, *β*-sitosterol, and stigmasterol, which suppressed iNOS activity. However, their activity is lower than that of gossypin, which serves as the reference standard.

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.

Conflict of Interest

The authors declare no conflict of interest.

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