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RESEARCH ARTICLE

Assessment of *In Vitro* Antioxidant and Analgesic Activity of Ethyl Acetate Fraction of *Urena lobata* Leaves

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Abstract

Background: The primary aim of this study is to identify the most active fraction from *Urena lobata* leaves and subsequently evaluate the analgesic and antioxidant accomplishments of the identified potent extract. The focus is on determining the fraction with the highest efficacy for potential therapeutic applications. **Methods:** *U. lobata* leaves were consecutively extracted with solvents like n-hexane, chloroform, ethyl acetate, and methanol. The concentrated fractions underwent initial phytochemical screening. The fraction exhibiting the highest activity, as determined by the carrageenan model, was further evaluated for its analgesic potential through the hot plate and acetic acid-induced writhing methods. In-vitro antioxidant activity was evaluated through DPPH and FRAP assays. **Results:** The preliminary phytochemical analysis of *U. lobata* leaf fractions exposed the presence of steroids, triterpenes, alkaloids, carbohydrates, tannins, and flavonoids. In the carrageenan-induced hind paw edema model, the ethyl acetate fraction of *U. lobata* leaf (ULEAF) demonstrated the highest anti-inflammatory effects, comparable to the standard drug Diclofenac sodium. Evaluation of analgesic potential using the hot-plate method and acetic acid-induced writhing test showed dose-dependent efficacy, with 500 mg/kg ULEAF exhibiting effects similar to ibuprofen. *In vitro,* antioxidant assays indicated potent radical scavenging and reducing power in the ethyl acetate fraction. **Conclusion:** In conclusion, *U. lobata* leaf fractions exhibited potent anti-inflammatory properties, with the ethyl acetate fraction demonstrating notable analgesic and antioxidant activity. These findings support the plant's therapeutic potential for further exploration in pharmaceutical development.

Keywords: Analgesic Activity, Antioxidant Properties, Ethyl Acetate Fraction, Inflammatory Conditions, Phytochemical Analysis, *Urena lobata*

1. Introduction

In the past decade, herbal products have become popular in traditional medicine. Various plants and plant-based items are recognized for their medicinal properties, owing to the diverse chemical compositions and biological activities they possess. This versatility enables their application in treating a range of diseases. Nowadays, herbal products are pivotal in discovering novel lead compounds for human disease treatment, highlighting their increasing significance in the field of healthcare[1].

Urena lobata, commonly known as *Caesar* weed, has diverse traditional uses in folk medicine. Its use in traditional medicine is documented in various ethnobotanical studies and herbal remedy compilations, underscoring its historical significance in folk healing

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practices. Its leaves produce a variety of secondary metabolites, including alkaloids, flavonoids, saponins, and tannins. These compounds contribute to the plant's therapeutic properties, making it valuable in treating diverse ailments. *U. lobata* has been traditionally employed as a diuretic, febrifuge, and anti-rheumatic agent. The plant's applications extend to wound healing, toothache relief, and managing conditions like gonorrhoea. In folk and traditional remedies[2], all parts of the plant are utilized for their medicinal value, addressing concerns such as gonorrhoea, leucorrhea, hematemesis, trauma, bleeding, colds, fevers, pain, numbness associated with rheumatism, wounds, toothaches, and inflammation. While these traditional uses provide valuable insights. The plant has a wide range of pharmacological properties, such as antioxidant[3,4], antimicrobial[5], antidiarrheal[6], Immunomodulatory[7], anxiolytic, antidepressant and anti-inflammatory[8], antidiabetic[9], anti-bacterial[10], antifertility[11], cytotoxic and anti-proliferative[12], anti-hyperglycemic and anti-nociceptive[13], wound healing[14], antiproliferative[15] and anti-arthritic activity[16].

In light of this, the current study aimed to investigate the phytochemical and pharmacological characteristics of the plant by creating various solvent fractions from the leaves of *U. lobata*. The primary goal was to pinpoint the most potent fraction of the plant with anti-inflammatory properties. Subsequently, the chosen fraction underwent further assessment for its analgesic and antioxidant activities.

2. Materials and Methods

2.1 Experimental Animals

Wistar rats (Female) with 200 – 250 gms body weight were used for this study. The study was conducted afterwards obtaining approval from the CCSEA "Reg no: 921/PO/ReBi/S/05/ CPCSEA" as per Protocol no: PIPH 12/22 and has been confirmed from the records by IAEC. (IAEC/PIPH 12/22). The rats were housed in clean polypropylene cages at 23 – 25 °C with a natural 12-hour light-dark cycle, comparative humidity of 50 – 60 %, and access to food and water.

2.2 Chemicals

All chemicals employed were of the highest purity and analytical grade. L-Ascorbic acid and Potassium Ferricyanide were procured from SRL Pvt. Ltd., while DPPH and trichloroacetic acid were acquired from Sigma Chemical Co, USA.

2.3 Collection and Verification of Plant Material

The plant material was authenticated by Dr. M. S. Khyade and Dr. S. D. Jadhav, professors at the Department of Botany, S. N. Arts, D. J. Malpani Commerce, and B. N. Sarda Science College, Sangamner, Maharashtra. A voucher specimen (SC-139) was deposited in the department for reference. Following collection, the crude drug was subjected to shade drying, followed by coarse powdering and sieving through a 100-mesh sieve. The powdered material was then stored in airtight containers for subsequent use.

2.4 Preparation of Fractions

U. lobata leaves were finely powdered. The yield of the extracts obtained from plant species largely relies on the polarity of the solvent used. The plant material underwent successive extraction with n-hexane, chloroform, ethyl acetate, and methanol using a Soxhlet apparatus. Upon completion of extraction, the solvent was evaporated, leaving concentrated fractions that were subsequently air-dried. These fractions were then stored in a refrigerator at 4°C for further utilization in phytochemical investigation and pharmacological screening.

2.5 Preliminary Phytochemical Analysis of Fractions

Preliminary phytochemical screening of prepared *U. lobata* solvent fractions was carried out as per methods mentioned by Kokate[17].

2.6 Selection of Fraction for Further Study

The selection of the fraction for further investigation was based on the carrageenan-induced hind paw edema model. Paw inflammation was induced in female Wistar rats (150 – 200 g) following Winter *et al.,* method[18]. Rats were divided into six groups, and a subcutaneous injection of 0.1 ml 1% w/v carrageenan was made into the right hind paw. Test groups received 100 mg/kg of the respective fraction orally one hour before carrageenan injection, with the standard group

given Diclofenac sodium 10 mg/kg (p.o.). Paw volume was measured at 15, 30, 60, 90, and 120 minutes using a Digital plethysmograph (Orchid Scientific Pvt. Ltd.).

2.7 Evaluation of Analgesic Potential of Selected Fraction

2.7.1 Hot-plate Method

The analgesic activity of the selected fraction, based on the aforementioned test, was assessed using the hot-plate method. Twenty-five female Wistar rats were grouped into five (n = 5). Three test groups received the selected fraction at doses of 100, 250, and 500 mg/kg/b.w. orally, while the control group received 0.5 ml dis. water per orally. The standard group received ibuprofen (25 mg/kg, orally). Pain stimulus was induced on a hot plate (55 ± 0.5 °C). The reaction time for each group was recorded at 30, 60, 90, and 120 minutes post-treatment. Paw licking or jumping off the plate was measured a response to the pain encouragement, and a 20-second cut-off period was applied to prevent accidental paw damage. The response time of both the extract and standard was compared with that of the control group[19].

2.7.2 Acetic Acid-induced Writhing Test

The analgesic action of the ethyl acetate fraction was assessed using the acetic acid-induced writhing method[20]. Female rats, with each study group comprising five rats, received test groups (100, 250, and 500 mg/kg b.w. p.o.) and control treatments at appropriate doses. The Standard group received ibuprofen (25 mg/kg, orally). 30 minutes later, acetic acid (0.7%, 10 ml/kg, intraperitoneal) was directed to encourage stomach contractions or writhing[21]. After a 5-minute interval, the count and recording of writhes for each group were conducted over 10 minutes. The writhes observed in the test groups at various dose levels and the standard was then compared with those in the control group.

2.8 *In Vitro* Anti-oxidant Activity

The antioxidant activity of the ULEAF was assessed using various *in vitro* methods, including the DPPH assay and ferric-reducing power assay. Each assay was performed three times (in triplicate), and the average values obtained were utilized for analysis.

2.8.1 DPPH Radical Scavenging Activity (DPPH Assay)

The DPPH scavenging activity of the ethyl acetate fraction was conducted following the procedures outlined by Ulewicz *et al.*, and Al-Laith *et al*[22,23]. In brief, 2 mg of respectively test sample was dissolved in two ml of methanol just before creating stock solutions with a concentration of 1 mg/ml. These stock solutions were then diluted to generate a series of test concentrations ranging from 0.1 to 50 µg/ml. Subsequently, 1 ml of each test sample dilution was mixed with 1 ml of a 0.1 mM methanolic solution of DPPH, and the mixture was kept in the dark for 30 minutes. Absorbance was measured at 517 nm using a UV-visible spectrophotometer (UV-1900). The methanolic solution of the ethyl acetate fraction of *U. lobata* leaf served as the test, and the DPPH (0.1 mM) methanolic solution served as the control. Ascorbic acid was employed as the standard antioxidant in this method. The percentage of DPPH scavenging activity was determined accordingly.

$$\text{Percentage of (\%)} = \frac{Absorbance\ of\ Control - Absorbance\ of\ Test}{Absorbance\ of\ Control} \times 100$$

A reduction in absorbance of the reaction mixture indicates stronger DPPH radical scavenging activity.

2.8.2 Ferric Reducing Antioxidant Power (FRAP) Assay

For the determination of reducing power, 1 millilitre of the methanolic solution of the ethyl acetate fraction (final concentration 10 to 100 µg/ml) was combined with 2.5 ml PBS buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide [$K_3Fe(CN)_6$] (10 g/l). Following a 20-minute incubation at 50°C, 2.5 ml CCl_3COOH (100 g/l) was added, and the mixture was then centrifuged for 20 minutes at 3000 rpm. After that, 2.5 ml of the supernatant solution, 2.5 ml distilled water and 0.5 ml $FeCl_3$ (1 g/l) were mixed. The absorbance of the resultant solution was determined at a wavelength of 700 nm using a UV-visible spectrophotometer. Ascorbic acid, within the concentration range of 10 – 100 µg/ml, served as the standard. A blank solution, containing the same reaction mixture excluding the extract, was used

for reference. An increase in absorbance indicated a stronger reducing power[24].

3. Statistical Analysis

The study findings were reported as mean ± SEM (standard error of the mean) and were subjected to statistical analysis employing ANOVA (Analysis of Variance), followed by post hoc analysis to establish the significance levels. Statistical significance was deemed to be achieved at a minimum threshold of $P < 0.05$.

4. Results

4.1 Preliminary Phytochemical Evaluation of *U. lobata* Fractions

The percentage yields of the extracts were 8.0, 7.0, 6.4, and 7.3 % w/w for hexane, chloroform, ethyl acetate, and methanolic extract respectively. Results of preliminary phytochemical tests for fractions are shown in Table 1 which indicates the existence of steroids, triterpenes, alkaloids, carbohydrates, tannins, and flavonoids in different fractions.

4.2 Selection of Fraction by Carrageenan-induced Hind Paw Edema Method

The administration of carrageenan to the hind paw resulted in a significant increase in paw volume, as indicated in Figure 1, compared to the Control group (1.63 ± 0.24 mm at 15 min, 1.75 ± 0.25 mm at 30 min, 2.13 ± 0.12 mm at 60 min, 1.75 ± 0.25 mm at 90 min, and 1.75 ± 0.25 mm at 120 min). Treatment with ULEAF led to significantly lower paw edema compared to control rats at all-time points (0.25 ± 0.25 mm, 0.25 ± 0.25 mm, 0.50 ± 0.289 mm, 0.25 ± 0.25 mm, and 0.30 ± 0.23 mm, respectively). Similarly, treatment with the standard drug, Diclofenac sodium, also resulted in significantly lower paw edema compared to control rats at all-time points (0.00 ± 0.00 mm, 0.25 ± 0.25 mm, 0.25 ± 0.25 mm, 0.25 ± 0.25 mm, and 0.02 ± 0.03 mm, respectively).

4.3 Evaluation of Analgesic Potential

4.3.1 Hot-plate Method

Significantly higher response times were observed in rats exposed to thermal stimulus on the hot plate following treatment with the standard analgesic agent,

Table 1. Preliminary phytochemical test for fractions

Chemical constituents	Chemical test	n-hexane fraction	Chloroform fraction	Ethyl acetate fraction	Methanol fraction
Alkaloids	Mayer's test	+	+	+	+
	Dragendorff's test	+	+	-	+
Saponin	Foam forming test	-	-	+	+
Tannins	Ferric Chloride test	-	-	-	+
	Dilute nitric acid test	-	-	+	+
Proteins	Million's test	-	-	+	-
	Biuret test	-	-	-	-
Flavonoids	Shinoda test	+	+	+	+
	Lead Acetate test	+	+	+	+
Glycoside	Killer Killani test	-	-	-	-
Carbohydrate	Molisch's test	-	-	+	-
	Fehling's test	+	+	-	-
Triterpenes	Vanillin- Sulphuric Acid test	+	+	+	+
Amino Acids	Ninhydrin test	-	-	+	-
Sterols	Liebermann- Burchard's test t	+	+	+	+
	Salkowski's test	+	+	+	+
Phenols	Ferric Chloride test	+	+	+	+

'+' indicates present and '-' indicates absent.

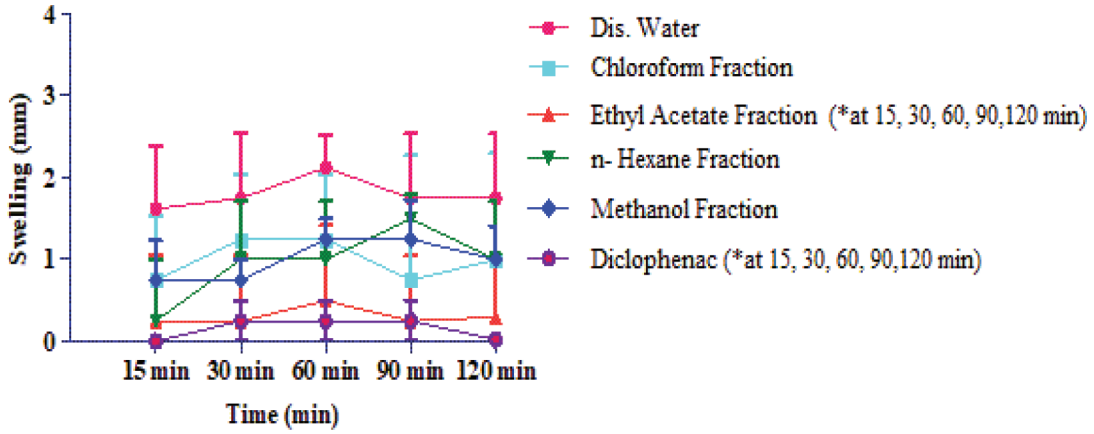


Figure 1. Effect of different fractions of *U. lobata* leaf on carrageenan-induced hind paw edema.
Values are expressed as mean ± SEM (n=5). Analyzed using Graph Pad Prism 5.0 by two way ANOVA followed by Bonferroni Post hoc analysis. *(P<0.05) when compared to distilled water treated rats, (p < 0.05) **p < 0.01, ***p < 0.001.

Table 2. Evaluation of the analgesic effect of ULEAF by hot plate method

Time (min)	Control Group	Standard Group	Treatment 100mg/kg	Treatment 250mg/kg	Treatment 500mg/kg
15	4.80 ± 0.37	8.80±0.20***	6.60 ± 0.50	7.60 ± 0.40*	8.40 ± 0.24**
30	5.00 ± 0.31	9.80±0.58***	7.60 ± 0.67	8.20 ± 0.37***	9.40 ± 0.24***
60	5.40 ± 0.51	11.00±1.14***	9.20 ± 0.49**	10.20±0.73***	11.40±2.54***
90	4.88 ± 0.73	14.20±0.20***	9.20 ± 1.07**	9.60 ± 0.81***	11.00 ± 0.32***

Values are presented as mean ± SEM (standard error of the mean) with n = 5. Statistical analysis was performed using Graph Pad Prism 5.0, employing two way ANOVA followed by Bonferroni Post hoc analysis. *(P<0.05) when compared to distilled water treated rats, (p < 0.05) **p < 0.01, ***p < 0.001.

ibuprofen. Importantly, a dose-dependent analgesic activity of ULEAF was evident, as highlighted in Table 2.

4.3.2 Acetic Acid-induced Writhing Test

In the acetic acid test, there was a significant reduction in the writhing response after ULEAF treatment (7.80 ± 0.20, 7.00 ± 0.316 and 5.804.4 ± 0.374 in 100, 250 and 500 mg/kg ULEAF Vs 9.40 ± 0.40 in Control animals), as illustrated in Figure 2. Notably, the analgesic activity observed with 500 mg/kg ULEAF closely approached that of ibuprofen (4.4 ± 0.25). Furthermore, others of ULEAF also exhibited a dose-dependent effect on the reduction of the writhing response.

4.4 *In Vitro* Anti-oxidant Activity

4.4.1 DPPH Assay

Various concentrations of ULEAF demonstrated notable radical scavenging activity, as represented in Figure 3. However, the maximum scavenging activity was somewhat lower than that obtained with Ascorbic acid.

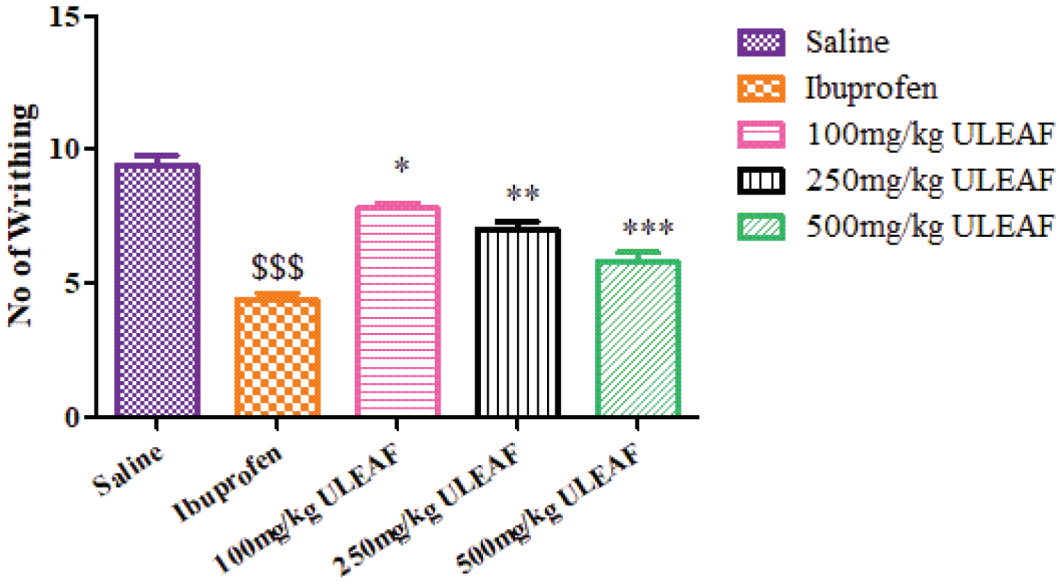
4.4.2 FRAP Assay

The ethyl acetate fraction of *U. lobata* leaf displayed substantial reducing power, as indicated by the absorbance of the reaction solutions (Figure 3). A higher value of absorbance in the reaction mixtures signified greater reducing power.

5. Discussion

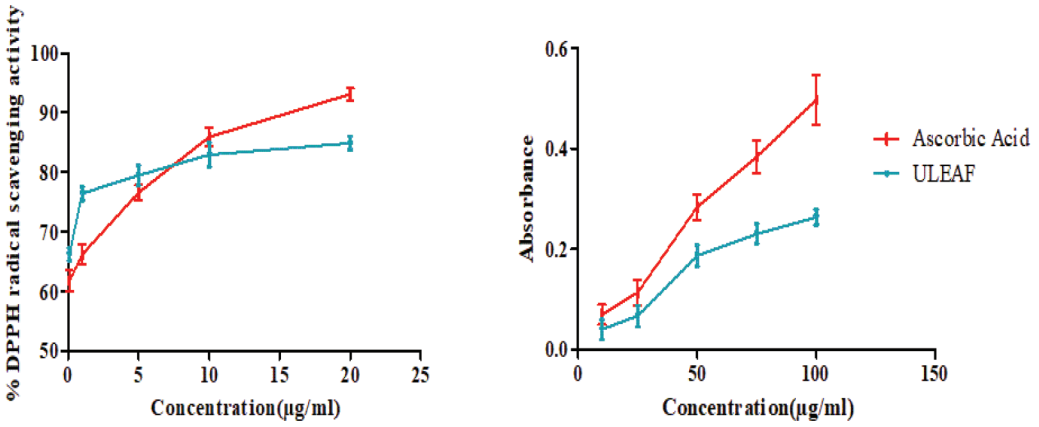
The preliminary phytochemical test for fractions unveiled the presence of steroids, triterpenes, alkaloids, carbohydrates, tannins, and flavonoids in n-hexane, chloroform, ethyl acetate, and methanol fractions.

The Carrageenan-induced hind paw edema model, commonly employed to assess the anti-inflammatory potential of pharmacological substances, demonstrated a significant decrease in paw volume with the administration of the ethyl acetate fraction of *U. lobata*. Based on this notable result the ethyl acetate fraction was selected for further investigation.



Values are presented as mean ± SEM (standard error of the mean) with n = 5. Statistical analysis was performed using Graph Pad Prism 5.0, employing Two-Way ANOVA followed by Bonferroni Post hoc analysis. *indicates statistical significance (P<0.05) compared to rats treated with distilled water, **p < 0.01, ***p < 0.001.

Figure 2. Analgesic effect of ULEAF by acetic acid-induced writhing test in rats.



The value is presented as mean ± SEM with a sample size of n = 3. Statistical analysis was conducted using GraphPad Prism 5.0.

Figure 3. Effect of ULEAF on DPPH and FRAP assay.

The analgesic potential of ethyl acetate fraction from *U. lobata* was assessed through two acute pain models: heat-induced pain and chemically induced pain. The pain pathway, characterized by its multisynaptic nature, offers opportunities for pain modulation at various action sites[25]. While NSAIDs are recognized for their impact on nociceptors, they also exhibit central mechanisms of pain relief[26,27]. ULEAF demonstrated significant analgesic activity in both tests.

The hot plate method, employing thermal stimuli to induce pain through tissue damage and inflammation-triggered release of peripheral mediators, relies on polysynaptic reflexes initiated at the spinal level and regulated at supraspinal centres. In the hot plate test, ULEAF exhibited its maximum effect at 60 minutes, indicating a lower Tmax than the standard drug. It is noteworthy that even at Tmax, the analgesic activity of ULEAF is lower than that of the standard drug.

A similar trend was observed in the acetic acid-induced writhing test, where the maximum effect of ULEAF was inferior to that of the standard drug. Despite the lower efficacy, this study provides crucial information that ULEAF contains one or more active

constituents with analgesic potential, warranting further identification and development.

The ethyl acetate fraction of *U. lobata* leaves exhibited significant antioxidant activity. In the study, the antioxidant potential was assessed by measuring the scavenging activity against the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. The DPPH radical possesses an unpaired electron, leading to its characteristic absorbance maxima at 517 nm, along with a deep purple color. Antioxidants donate an electron to the DPPH radical, converting it to a colorless form. A lower absorbance observed after incubation with the ULEAF indicates robust antioxidant activity. This finding aligns with similar observations reported in earlier studies, confirming the consistency of the plant's DPPH scavenging activity. The ability of the ethyl acetate fraction to effectively neutralize the DPPH radical underscores its potential as a potent antioxidant, highlighting the therapeutic potential of *U. lobata* in combating oxidative stress-related conditions[28].

The reducing power assay assesses the capacity of a substance to reduce the Fe^{3+}/ferricyanide complex, transforming it into the ferrous form of Perl's Prussian blue with absorbance maxima at 700nm. In the study, the ethyl acetate fraction of *U. lobata* leaves demonstrated robust reducing properties. The increased formation of Perl's Prussian blue and higher absorbance at 700nm indicates the strong reductive capabilities of the ULEAF. This observation suggests that the ethyl acetate fraction possesses potent reducing power, emphasizing its potential as an effective agent in redox reactions and supporting its application in antioxidant-related therapeutic contexts[29].

6. Conclusion

The ethyl acetate fraction of *U. lobata* leaf exhibited significant anti-inflammatory activity and potent analgesic effects in both thermal and chemically induced acute pain models in rats. Additionally, the extract demonstrated robust anti-oxidant activity, suggesting the presence of pharmacologically important chemical constituents. Further in-depth *in vivo* investigations are warranted to explore its therapeutic potential, especially in conditions such as rheumatoid arthritis and other inflammatory disorders.

7. Acknowledgments

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8. References

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