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PRE-CLINICAL STUDIES ON THE EFFICACY OF IXORA CULTIVAR LEAVES CRUDE EXTRACT AS AN ANTI-INFLAMMATORY AGENT

SDG 3: Good Health and Well-Being

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Abstract

This study evaluated the phytochemical composition, acute toxicity, dermal safety, and anti-inflammatory potential of *Ixora cultivar* (locally known as santan) leaves collected from Marinduque, Philippines. The plant specimen was authenticated by the National Museum of the Philippines, Botany Division, as *Ixora cultivar* (family Rubiaceae). Crude ethanolic extraction, qualitative phytochemical screening, quantitative flavonoid determination, oral median lethal dose (LD50) testing, dermal irritation testing, and carrageenan-induced paw-edema testing were conducted through the Department of Science and Technology (Region IV-B) and the University of the Philippines Los Baños Institute of Plant Breeding. Phytochemical screening identified fats and oils, saponins, hydrolysable tannins, 2-deoxysugars, unsaturated steroids/triterpenes, and flavonoids (leucoanthocyanins and a γ-benzopyrone-nucleus compound), with an average total flavonoid content of 1.978%. Oral administration in female ICR mice yielded a median lethal dose (LD50) of 16.7687 ± 1.0316 g/kg, classifying the extract as practically non-toxic. Topical application on albino rabbits produced only a slight, transient erythema at the 4th hour that resolved within 24 hours, with no edema observed, indicating a non-irritant dermal profile. In the carrageenan-induced paw-edema assay, however, topical application of the crude extract at 0.5%, 1%, and 2% concentrations did not reduce paw swelling relative to the distilled-water negative control (0%

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protection); instead, all three concentrations produced larger average foot-volume differences than the negative control, corresponding to protection values of −48.72%, −33.33%, and −32.48%, respectively — in marked contrast to the positive control, ketoprofen, which achieved 72.65% protection against edema. Taken together, the findings establish that *Ixora cultivar* leaves are practically non-toxic and non-irritant and contain phytochemical classes with documented anti-inflammatory potential in the literature, but the crude ethanolic extract, under the topical protocol and concentrations tested, did not demonstrate measurable anti-inflammatory activity. The researchers recommend isolation and purification of the extract's active constituents to evaluate their independent and synergistic effects, and to explore alternative formulations, concentrations, or routes of administration before further pursuing *Ixora cultivar* as a candidate anti-inflammatory agent.

Keywords: Ixora cultivar; phytochemical screening; median lethal dose (LD50); pre-clinical toxicity; anti-inflammatory efficacy

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Introduction

An estimated 50,000 of the more than 422,000 flowering plant species reported worldwide are used for medicinal purposes (Ashour, Mohamed, El Aziz, Sadek, & Melad, 2019). In both low- and middle-income and industrialized countries, plant-based therapy remains an important part of healthcare, often serving as the only therapeutic option available to certain populations in emergencies or resource-limited settings. Because of this continued reliance on medicinal plants, it is essential that their use be properly regulated for safety, efficacy, and quality — principles that underlie modern pharmaceutical practice and consumer protection, and that are reflected in the Philippine Food and Drug Administration's Administrative Order No. 172 s. 2004, "Guidelines on the Registration of Herbal Medicines."

The pantropical genus *Ixora* is highly diverse, with several species endemic to the Philippines whose basic biology remains poorly characterized (Banag, Thrippleton, Alejandro, Reineking, & Schumann, 2015). Among its popular dwarf cultivars — typically under one meter in height — is the santan (*Ixora cultivar*) commonly grown as an ornamental in Marinduque province. Unlike *Ixora coccinea* and other related species, *Ixora cultivar* has not previously been characterized in the published literature for its phytochemical composition or healing properties, despite anecdotal use as a folk wound treatment.

Related *Ixora* species have a documented history of traditional use elsewhere in Asia. The National Institute of Science Communication and Information Resources (New Delhi, 1992), as cited in Dontha, Hemalatha, and Manthripragada (2015), reported that *Ixora* species in India have traditionally been used for hepatoprotective, chemoprotective, antimicrobial, antioxidant, anti-nociceptive, anti-mitotic, and anti-inflammatory purposes: root decoctions for nausea, hiccups, and anorexia; powdered roots for sores and chronic ulcers in Indo-China; and poultices of fresh leaves and stems for sprains, eczema, boils, and contusions. *Ixora chinensis* cultivar leaves have similarly been used for headache, stomachache, and as a remedy for tuberculosis, while *I. coccinea* leaves have shown anti-inflammatory, anti-diarrheal, anti-asthmatic, antiulcer, and anti-nociceptive activity.

Motivated by these regional precedents and by an informal observation in which a 7–14-day topical application of the crude leaf extract appeared to progressively dry and fully heal a slow-healing wound in a consenting individual, the researchers undertook the present pre-clinical study — the first of its kind for *Ixora cultivar* — to establish a scientific basis for its anti-inflammatory potential and to lay groundwork for a cost-effective, community-derived natural anti-inflammatory product from santan leaves.

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Materials and Methods

All procedures followed standard pre-clinical trial protocols involving laboratory analysis of crude plant extracts and experimentation in mice and rabbits.

Plant Collection and Authentication

Samples of santan were collected in Marinduque and submitted to the National Museum of the Philippines, Botany Division, for taxonomic identification. The species was confirmed as *Ixora cultivar* (family Rubiaceae). Following authentication, leaves were collected and transported to the Department of Science and Technology (DOST), Chemical and Energy Division, for crude extraction, phytochemical analysis, oral LD50 testing, dermal irritation testing, and anti-inflammatory testing. Quantification of total flavonoid content was performed at the University of the Philippines Los Baños, Institute of Plant Breeding.

Crude Ethanolic Extraction

Air-dried santan leaves (1.2 kg) were pulverized using a Wiley mill and macerated in 8.0 L of 96% ethyl alcohol for 48 hours. Extraction of the 1.2 kg air-dried sample yielded 6.8 L of ethanolic extract, which was concentrated to 66.0 g of semi-solid crude extract.

Qualitative Phytochemical Screening

A 500 g portion of semi-dried leaves was macerated in 2 L of 95% ethyl alcohol for 48 hours with occasional agitation, filtered, and concentrated under vacuum at 60°C in a rotary evaporator to a syrup consistency, then further dried in a water bath at the same temperature. The resulting extract was subjected to standard qualitative tests, summarized below.

Table 1. Summary of qualitative phytochemical screening procedures

Phytochemical class	Test procedure and positive indicator
Fats and oils	Filter-paper spot test: extract-moistened filter paper dried and examined for a greasy translucent appearance.
Alkaloids	Extract treated with 2M HCl, heated, cooled, salted with NaCl, filtered; filtrate tested with Dragendorff's reagent (orange precipitate) and Mayer's reagent (white precipitate).
Tannins	Alcoholic extract re-extracted with hot water, filtered, and treated with ferric chloride TS; a blue-black color indicates hydrolysable tannins, a brownish-green color indicates condensed tannins.
Saponins	Alcoholic extract dissolved in distilled water, shaken vigorously, and observed for a persistent “honeycomb” froth exceeding 2 cm in height after 10 minutes; 5% Na2CO3 was added to test for free fatty acids.

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Phytochemical class	Test procedure and positive indicator
Unsaturated steroids and triterpenes (Liebermann–Burchard)	Extract dissolved in chloroform, treated with acetic anhydride, then concentrated sulfuric acid; a bluish-green to dark-green, red, pink, purple, or violet color indicates a positive result.
2-deoxysugars (Keller–Kiliani)	Defatted sample treated with chloride reagent and concentrated sulfuric acid; a reddish-brown interface color turning blue or purple indicates a positive result.
Flavonoids	Defatted extract taken up in 80% ethanol, divided into three portions (control; +HCl with warming, Bate-Smith and Metcalf test; +HCl with magnesium turnings) and observed for color change.
Anthraquinones	Aqueous filtrate extracted with benzene, divided into two portions; one treated with ammonia solution and compared with the untreated control for a red coloration in the ammoniacal layer (Bornträger's test).

Quantitative Determination of Total Flavonoid Content

Total flavonoid content was determined following the method of Fattahi et al. (2014). A 1.0 g fresh sample was macerated with 10 mL of methanol using a mortar and pestle and filtered. A 0.20 mL aliquot of the methanolic extract was diluted with 2.0 mL of distilled water, treated with 0.30 mL of 5% sodium nitrite, and after 5 minutes with 0.30 mL of 10% aluminum chloride, then allowed to stand for 1 minute at room temperature. Sodium hydroxide (1.0 mL) was added and mixed by vortex. Absorbance of the resulting solution was read at 510 nm using a UV-Vis spectrophotometer, with total flavonoid content calculated by interpolation from a catechin standard curve.

Anti-inflammatory Activity: Carrageenan-Induced Paw Edema Assay

Anti-inflammatory activity was assessed using the carrageenan-induced edema method in ten (10) female Sprague-Dawley rats weighing 117–190 g. Edema was induced by injecting 0.05 mL of a 1% carrageenan solution into the plantar tissue of the right or left hind paw, with initial foot volume recorded using a plethysmometer paw-volume meter, which measures displacement of water in a calibrated cell as the paw is immersed. Rats were divided into groups of two (2) and treated topically as follows: distilled water (negative control), ketoprofen 2% solution, pH 7.49 (positive control), and santan crude extract at 0.5%, 1%, and 2% concentrations. Final foot volume was measured 30 minutes after topical treatment, and percent protection against edema formation was calculated relative to the negative control using the formula: Percent inhibition (%) = [1 − (Test difference ÷ Negative-control difference)] × 100.

Modified Dermal Irritation Test

A dark brown, pasty crude extract of santan (approximately 130 g, pH 5.13) was used to assess dermal irritation on three (3) male albino rabbits, divided into control and experimental groups. A patch containing the Ixora crude extract was applied to the experimental group, and a distilled-water patch was applied to the control group. Patches were removed after 4 hours, and skin reactions (erythema/eschar formation and edema) were scored at the 4th hour and at 24 hours, 2, 3, 7, and 14 days post-exposure using a standard dermal-scoring scale.

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Modified Acute Oral Toxicity Test (LD50)

The median lethal dose (LD50) — the dose expected to cause death in 50% of test animals, expressed in mg/kg or g/kg of body weight — was determined in female ICR mice (23–30 g). A dark brown, pasty crude extract (approximately 130 g, pH 5.13) was prepared as a 300 mg/mL suspension in distilled water. Following preliminary dose-range finding, three increasing log doses were administered orally to three groups of ten (10) mice each. Mortality and other adverse behavioral signs (toxidrome) were observed and recorded at close intervals for the first 2 hours post-administration, then daily through 24–48 hours and up to 14 days.

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Results

Plant Authentication

The plant specimen collected from Boac, Marinduque, was identified by the National Museum of the Philippines, Botany Division, as *Ixora cultivar* (family Rubiaceae), Specimen 01, verified by Wilfredo F. Vendivil, Ph.D., Curator II (Control No. 249-2014, dated May 7, 2014).

Qualitative Phytochemical Screening

The ethanolic extract of santan leaves tested positive for fats and oils, hydrolysable tannins, saponins, 2-deoxysugars, unsaturated steroids/triterpenes, and two flavonoid classes (leucoanthocyanins and a γ-benzopyrone-nucleus compound), and tested negative for alkaloids, free fatty acids, and anthraquinones (Table 1).

Table 1. Results of the qualitative phytochemical analysis of the ethanolic extract

Qualitative test	Result	Indication
Filter paper test	Greasy appearance (+)	Presence of fats and oils
Dragendorff's test	No orange precipitate (−)	Absence of alkaloids
Mayer's test	No white precipitate/turbidity (−)	Absence of alkaloids
Ferric chloride test	Blue-black color (+)	Presence of hydrolysable tannins
Froth test	Froth > 2 cm, persistent (+)	Presence of saponins
Sodium carbonate test	No stable, dense froth (−)	Absence of free fatty acids
Keller-Kiliani test	Reddish-brown interface color turning blue (+)	Presence of 2-deoxysugars
Liebermann-Burchard test	Bluish-green to dark-green color (+)	Presence of unsaturated steroids and triterpenes
Bate-Smith & Metcalf test	Strong red color (+)	Presence of flavonoid (leucoanthocyanins)
Wilstatter “Cyanidin” test	Strong red color (+)	Presence of γ-benzopyrone-nucleus flavonoid
Bornträger's test	No reaction (−)	Absence of anthraquinones

Total Flavonoid Content

Across three trials of the first batch of santan leaves, the average total flavonoid content was 1.978% (Table 2).

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Table 2. Total flavonoid content of the first batch of santan leaves

Trial	Absorbance	Flavonoid weight (mg)	Flavonoid content (mg/g fresh weight)	% Flavonoid
1	0.259	2.060	20.604	2.060
2	0.244	1.941	19.411	1.941
3	0.243	1.933	19.311	1.933

Average total flavonoid content: 1.978% (mean of the three trials; average flavonoid content ~19.78 mg/g fresh weight).

Anti-inflammatory Activity

Following carrageenan injection, the negative control (distilled water) group showed an average foot-volume difference of 0.2925 mL 30 minutes after treatment, corresponding to 0% protection by definition. The positive control, ketoprofen, achieved 72.65% protection against edema. In contrast, santan crude extract at 0.5%, 1%, and 2% concentrations produced average foot-volume differences of 0.4350, 0.3900, and 0.3875 mL, respectively — all larger than the negative control — corresponding to protection values of −48.72%, −33.33%, and −32.48% (Table 3). While the magnitude of the negative protection values decreased as concentration increased from 0.5% to 2%, none of the tested concentrations achieved a positive (edema-reducing) effect.

Table 3. Summary of anti-inflammatory activity of distilled water, ketoprofen, and santan crude extract

Group	Test sample	n (rats)	Average foot-volume difference (mL)	% Protection aga edema
1	Negative control (distilled water)	2	0.2925	0
2	Ketoprofen (positive control)	2	0.0800	72.65
3	Santan crude extract, 0.5%	2	0.4350	−48.72
4	Santan crude extract, 1%	2	0.3900	−33.33
5	Santan crude extract, 2%	2	0.3875	−32.48

Dermal Irritation

In the distilled-water control group, all three male albino rabbits showed a very slight erythema score (1) at the 4th hour, which resolved to a score of 0 by 24 hours and remained at 0 through day 14. No edema was observed at any time point (Table 4).

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Table 4. Dermal reaction scores of the distilled-water control group in male albino rabbits

Rabbit no.	Sex	4th hr	24 hr	Day 2	Day 3	Day 7	Day 14	Edema (all timepoints)
1	M	1	0	0	0	0	0	0
2	M	1	0	0	0	0	0	0
3	M	1	0	0	0	0	0	0

Acute Oral Toxicity

Toxidrome and mortality varied by dose (Table 5). At 10 g/kg, all ten mice displayed transient abnormal behavior (decreased motor activity, increased respiratory rate, grooming, piloerection, hyperemia, ptosis) but recovered within 24 hours, with no deaths through day 14. At 14.142 g/kg, the same toxidrome was observed along with soft stool and ataxia; four mice died within 24 hours, and one additional mouse died by 48 hours (5/10 total), with the remaining five mice recovering. At 20 g/kg, one mouse died within 6 hours, and eight more died within 24 hours (9/10 total), with mortality unchanged through day 14.

Table 5. Behavioral observation (toxidrome) after oral administration to female ICR mice

Dose (g/kg)	No. of mice	Observation
10	10	Toxidrome (decreased motor activity, increased respiratory rate, grooming, piloerection, hyperemia, ptosis, decreased respiratory rate, loss of pinna reflex) observed at 15 minutes; all mice recovered by 24 hours with no deaths through day 14.
14.142	10	Toxidrome as above plus soft stool and ataxia, observed at 10 minutes; 4 deaths within 24 hours, 1 additional death by 48 hours (5/10 total); remaining 5 mice recovered and survived to day 14.
20	10	Toxidrome as above, observed at 10 minutes; 1 death within 6 hours, 8 additional deaths within 24 hours (9/10 total); mortality unchanged through day 14.

Table 6. Mortality ratio of mice administered the extract orally

Group	Dose (g/kg)	No. of mice	Day 1	Day 2	Day 3	Day 7	Day 14
I	10	10	0/10	0/10	0/10	0/10	0/10
II	14.142	10	4/10	5/10	5/10	5/10	5/10

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Group	Dose (g/kg)	No. of mice	Day 1	Day 2	Day 3	Day 7	Day 14
III	20	10	9/10	9/10	9/10	9/10	9/10

Based on these data, the median lethal dose (LD50) of the crude extract administered orally to female ICR mice was 16.7687 ± 1.0316 g/kg. Necropsy after 14 days revealed no significant abnormal findings in the vital organs of the test animals, although decreased body weight was noted among surviving mice. Applying the standard toxicity rating scale (Table 7), an oral LD50 above 15 g/kg falls within Category IV, "practically non-toxic," requiring no signal word on labeling.

Table 7. Toxicity rating scale and labeling requirements

Category	Signal word required on label	LD50 oral (mg/kg)	LD50 dermal (mg/kg, ppm)	Probable oral lethal dose
I — highly toxic	DANGER – POISON (skull and crossbones)	< 50	< 200	A few drops to a teaspoon
II — moderately toxic	WARNING	51–500	200–2,000	Over 1 teaspoon to 1 ounce
III — slightly toxic	CAUTION	Over 500	Over 2,000	Over 1 ounce
IV — practically non-toxic	None required	15 g/kg and above	—	—

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Discussion

Phytochemical Profile

The positive test for fats and oils is consistent with the role these compounds play in plant defense against pests and microorganisms and in adaptation to environmental stress, and with reported anti-inflammatory, anti-cancer, antibacterial, and anti-parasitic activity of plant fats and oils in human health (Reszczyńska & Hanaka, 2020; Konda et al., 2020).

Hydrolysable tannins, detected via the ferric chloride test, are water-soluble polyphenols with documented antibacterial, antioxidant, anti-tumor, antiseptic, and anti-inflammatory properties that make them of pharmaceutical and nutraceutical interest (Singh & Kumar, 2019).

Saponins, indicated by the froth test, are glycosides with reported hemolytic, anti-inflammatory, antibacterial, antifungal, antiviral, insecticidal, anti-cancer, and cytotoxic activity (Ashour et al., 2019), and have also been associated with cholesterol-lowering effects in animal and human studies (Moghimipour & Handali, 2015).

The two flavonoid classes detected — leucoanthocyanins and a γ-benzopyrone-nucleus compound (a hydroxyflavan bearing hydroxyl groups at the 3 and 4 positions; Drabkin, 2013) — are consistent with the antibacterial, anti-allergic, antioxidant, and anti-inflammatory activities widely reported for flavonoid-rich plant extracts (Mishra, Kumar, & Pandey, 2013). Friesenecker et al. (1994), as cited in Panche, Diwan, and Chandra (2016), found that oral administration of a purified flavonoid fraction suppressed leukocyte adhesion in a hamster ischemia–reperfusion injury model, implicating flavonoids in modulating leukocyte-mediated inflammatory responses. The 1.978% average flavonoid content recovered here is broadly consistent with the diversity of bioactive terpenoid and flavonoid constituents reported for the related species *I. coccinea*, whose essential oil has been shown to be dominated by triterpenes (62.60%) and monoterpenes (31.73%), with ursolic acid, oleanolic acid, and lupeol as major triterpene constituents (Obuzor, 2011).

Taken together, the phytochemical profile of Ixora cultivar leaves is favorable on paper for anti-inflammatory activity, which motivated the direct pharmacological testing that follows.

Anti-inflammatory Activity: A Negative Finding

Despite this favorable phytochemical background, the carrageenan-induced paw-edema assay did not demonstrate anti-inflammatory activity for the crude extract under the conditions tested. All three tested concentrations (0.5%, 1%, 2%) produced average foot-volume differences greater than the negative control, yielding negative “protection” values, in clear contrast to ketoprofen's 72.65% protection. The narrowing of the negative protection value as concentration increased (−48.72% at 0.5% to −32.48% at 2%) suggests a dose-related trend toward reduced additional swelling, but this trend did not cross into a net edema-reducing effect within the concentration range and 30-minute observation window tested. Mendjiwa (2010) notes that inter-species extrapolation of drug effects assumes a high degree of correlation between animal and human response, though this correlation can be confounded by differences in dose, concentration, and route of administration across species — a caveat directly relevant to interpreting a negative topical result here. Plausible explanations for the lack of measurable protection include insufficient penetration of the active constituents through intact rat paw skin at a single 30-minute timepoint, a formulation or vehicle effect, or a genuine absence of anti-inflammatory activity in the unpurified crude extract at the concentrations and route tested. These results should be read as a negative finding for the crude extract under this specific protocol, not as a refutation of anti-inflammatory potential in the underlying phytochemical constituents identified in section 4.1.

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Dermal Safety

The distilled-water control group showed only a very slight, transient erythema (score 1) at the 4th hour that resolved by 24 hours, with no edema at any time point — a result the researchers attribute in part to the mildly acidic character of distilled water on prolonged skin contact (pH effects from absorbed atmospheric carbon dioxide; Deziel, 2018). The narrative description of the Ixora-extract group (not tabulated in the source manuscript; see section 3.5) similarly reported only slight redness resolving within 24 hours at an extract pH of 5.13, comparable to the control. On the data available, this supports a non-irritant dermal profile for the crude extract, though confirmation requires the missing experimental-group data table.

Acute Oral Toxicity and Safety Classification

Acute toxicity is defined as an unwanted effect occurring shortly after single or multiple administration of a substance, and the LD50 remains a standard parameter for the initial toxicological screening of chemical and pharmacological agents, alongside observation of toxidrome onset, duration, and recovery (Chinedu, Arome, & Ameh, 2013; McCallum & Padmanabhan, 2014). The oral LD50 obtained here, 16.7687 ± 1.0316 g/kg, places *Ixora cultivar* crude extract in Category IV ("practically non-toxic," LD50 ≥ 15 g/kg) of the standard toxicity rating scale, requiring no hazard signal word on labeling. The absence of significant necropsy findings in the surviving animals' vital organs, alongside dose-dependent but reversible toxidrome at sub-lethal-range doses, further supports a favorable safety margin for the crude extract at the doses relevant to topical or low-dose oral use.

Implications and Limitations

These findings support the safety of *Ixora cultivar* leaf extract — both orally (practically non-toxic) and dermally (non-irritant) — but do not, on their own, support its anti-inflammatory efficacy as a crude topical preparation under the single-timepoint, carrageenan-edema protocol used here. This distinguishes santan from related *Ixora* species with documented anti-inflammatory activity in the literature and indicates that any therapeutic anti-inflammatory claim for santan cannot yet be generalized from its phytochemical profile alone. Isolation and purification of the extract's individual active constituents, testing of alternative concentrations, vehicles, or routes of administration, and evaluation at additional post-treatment timepoints are needed before santan-derived anti-inflammatory products can be responsibly pursued.

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Conclusion

Based on the pre-clinical data obtained, the following conclusions are drawn:

1. The plant specimen collected from Boac, Marinduque was identified as Ixora cultivar (family Rubiaceae), Specimen 01, verified by Wilfredo F. Vendivil, Ph.D., Curator II, Botany Division, National Museum (Control No. 249-2014, dated May 7, 2014).
2. Ixora cultivar leaves contain phytochemical classes (fats and oils, hydrolysable tannins, saponins, 2-deoxysugars, unsaturated steroids/triterpenes, and flavonoids) with documented antioxidant and anti-inflammatory activity in the broader literature.
3. Ixora cultivar leaves contain an average of 1.978% total flavonoid content, a compound class with reported antioxidant and anti-inflammatory potential.
4. Topical application of the crude extract produced only slight, transient dermal irritation in rabbits, resolving within 24 hours and indicating a non-irritant profile.
5. In the carrageenan-induced paw-edema assay, the crude extract at 0.5%–2% concentrations did not reduce edema relative to distilled water and underperformed the positive control, ketoprofen; the extract's anti-inflammatory efficacy was not demonstrated under the tested topical protocol.
6. The oral LD50 of 16.7687 ± 1.0316 g/kg classifies the crude extract as practically non-toxic under the standard toxicity rating scale.

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