***Original Research Article***

***Nymphaea nouchali, bioactivity and bio-safety substantiation***

# Abstract

*Nymphaea nouchali* is a species of perennial aquatic flowering plants with large, round and mostly floating leaves and rhizomes. For generations, this plant has been used in traditional medicinal practices in Southern Africa for various conditions including management of inflammatory conditions and dysmenorrhea. However, the biosafety and efficacy of the plant with regards to common pharmacological endpoints has never been systematically, scientifically validated. The aim of this present study therefore was to qualitatively determine the pharmacologically relevant metabolites prevalent in *N. nouchali*, to confirm the biosafety using laboratory animals as well as to determine the antioxidant activity, as well as its anti-inflammatory activities. The metabolomic screening was carried out using classical wet chemistry techniques, OECD guidelines were used to determine the acute oral toxicity profile of the plant. The egg albumin denaturation assay was used to determine anti-inflammatory activity with diclofenac as a standard. The anti oxidancy was evaluated with ascorbic acid as a standard using the DPPH assay method The phyto-screening confirmed the presence of numerous biomedically relevant secondary metabolites including phenols alkaloids and flavonoids. The biosafety studies using rat models confirmed that *N. nouchali* is nontoxic with an LD50 above 5000 mg/kg body weight. The lyophilized hydroethanolic extract demonstrated significant anti-inflammatory activity approximately 50% of diclofenac at all concentrations and high antioxidant activities (87.77% DPPH scavenging activity) comparable to ascorbic acid. It was therefore concluded that the plant is nontoxic to humans, possesses numerous relevant bioactive secondary metabolites and has significant antioxidant and anti-inflammatory activity. Our results support the continued use of plant extracts as an adjunct therapy for managing symptoms of dysmenorrhea and inflammatory conditions in traditional medical practices in Southern Africa.

Keywords: *Nymphaea nouchali*, anti-inflammatory, anti-oxidant activity phenols, secondary metabolites,

# Introduction

## *Nymphaea nouchali*

*Nymphaea nouchali* belongs to the water lily family, ancient aquatic plants that are important in many cultures around the world for different religious activities and traditional celebrations [1]. The plant has roots and branches that stay underwater and flowers that open during the day. The leaves are wide and can be flat or round with slightly turned up edges to help keep the plant floating [2]. These leaves can either be fully submerged in water or just rest on the surface**.** They can grow to over 20cm in diameter, and they can extend on a stem up to 1.5m long from the rhizome. Attached to the stems are thick, dark, sponge-like roots that grow under the water. The plants produce beautiful flowers that widely range in color, hues from purple, blue with red edges, lavender as well as pink. The large flowers may grow as lone units or in clusters.



Figure 1: Aquatic Nymphaea nouchali plants in lake Chivero, Zimbabwe.

In Zimbabwean traditional medicine, *N. nouchali* is widely used in the management of dysmenorrhea, diabetes, swelling kidney problems and hepatic diseases [3]. Infusions from the ribosomes and stems of the plants are also used as diuretics and emollients treating urinary tract diseases [4]. The flowers are reported to be utilized as a heart tonic due to their astringent action and are claimed to ‘purify’ blood serum as well as calm coughs. Their uses as aphrodisiacs as well as treating nausea, dizziness, worm infection and skin burns make them an important cardinal herb in folk medicine [5]. The seed extracts are used to treat fever and as a remedy for topical keratinization conditions like eczema. The plant rhizomes of *N. nouchal*i are commonly used to relieve pain and they are believed to be one of the most potent treatments in the management of menstruation irregularities and other dysmenorrhea symptoms [6].

## Secondary Metabolites and inflammation.

Inflammation is a natural response by the body to protect itself when there is injury or infection. It involves various cells, like white blood cells and special types of immune cells, which release signals that cause swelling and attract more immune cells to the affected area [7].This process helps remove harmful substances and signals the start of the healing process. In ancient writings Hippocrates, was among the first writers to record inflammation as part of healing processes and coined related terms still in use today in relation in inflammation including edema(swelling) and erysipelas (redness). Inflammatory conditions can either be classified as acute or chronic [8] Various secondary metabolites have pharmacological effects that modulate inflammatory mechanisms either in causing or managing the condition.

### Phenolic compounds

Phenolic compounds are the largest group of secondary metabolites that include flavonoids, condensed tannins, and gallo tannins. These compounds are believed to block precursor molecules that trigger inflammation in the body [9]. Many scientific studies confirm that phenolics help protect the body by reducing inflammation, fighting infections, easing asthma symptoms, lowering blood pressure, protecting the heart, preventing allergies and cancer. Their anti-inflammatory effects work by neutralizing harmful molecules called radicals and blocking the production of substances in the body that cause inflammation, such as certain proteins (cytokines), enzymes like inducible nitric oxide synthase (iNOS), and COX-2 [7] [10].

### Flavonoids

Over 4,000 different types of flavonoids, which are grouped into categories such as flavonols, flavones, anthocyanidins, isoflavonoids, catechins, and flavanones have been identified [11]. A number of Flavonoids have been reported to have antimicrobial and antiviral effects. They have confirmed antiulcerogenic antioxidant and anti-inflammatory activities as well as antihypertensive and antineoplastic pharmacological effects. One specific flavonoid called quercetin has been studied for its ability to reduce inflammation by targeting key molecules involved in the inflammatory process by blocking enzymes including cyclooxygenase (COX) and lipoxygenase, as well as proteins such as cytokines and nuclear factor kappa B (NF-κB), which play major roles in causing inflammation [7]. Animal studies on Wistar rats have shown that flavonoids can significantly reduce both early stage swelling and later tissue growth associated with inflammation. The structure of certain flavonoids especially those with chemical groups like catechol or guaiacol seems to enhance their anti-inflammatory effects [12].

### Coumarins.

Coumarins are secondary metabolites that are characterized by a benzopyrone structure and possess a wide range of biological activities, including antioxidant, anti-cancer, anti-inflammatory, and anti-microbial properties [13]. Due to these pharmacological effects, coumarin derivatives may be especially useful for treating conditions like high-protein edema (swelling caused by fluid buildup). Some coumarins have been demonstrated to neutralize harmful superoxide anion radicals, while others can block key pathways involved in inflammation, such as the lipoxygenase and cyclooxygenase pathways that process arachidonic acid [14]

### Saponins

Saponins are a diverse group of metabolic glycosides that are characterized by their ability to saponify or form stable foams in water. They have a characteristic bitter taste and have potential toxicity to lower organisms.  They are classified under two main groups: steroid glycosides and triterpene glycosides [15].These compounds are known for their wide range of biological activities, including anti-inflammatory effects. Studies have shown that saponins extracted from about 50 different plants can reduce inflammation in various experimental models involving mice and rats. For example, certain saponins were tested for their ability to reduce ear swelling caused by TPA (a chemical irritant). Out of these, five saponins were particularly effective in reducing the swelling in a dose-dependent manner [16]. The mechanisms behind the anti-inflammatory effects of saponins include both indirect and direct actions. Some saponins, like saiko saponins have demonstrated ability to mimic the anti-inflammatory activity of corticosteroids indirectly, whereas others, such as Seiko saponin d and ginsenosides, act directly by mimicking corticosteroid activity [17]. Additionally, certain saponins like glycyrrhizin prevent the breakdown of glucocorticoids (natural anti-inflammatory hormones), while others inhibit enzymes responsible for producing or releasing inflammatory mediators.

### Antioxidants and inflammation

Various metabolites as discussed above have antioxidants effect, and they work by neutralizing harmful molecules called free radicals, which can cause damage to cells. This damage, known as oxidative stress, happens when there’s an imbalance between free radicals and the body’s defense system [18] [19] [20]. Free radicals, such as reactive forms of oxygen, nitrogen, and sulfur, try to stabilize themselves by attacking important parts of our cells including proteins, lipids, and DNA. Leading to protein peroxidation, lipid peroxidation and DNA damage respectively. This reported cell damage is linked to over hundreds of diseases, including cancer, diabetes, heart problems, neurological disorders like Alzheimer’s, and inflammatory conditions. [6] Oxidative stress can harm the lining of blood vessels, including those in the uterus, and make menstrual pain worse [21]. Naturally the body has repair mechanisms based on repair enzymes that fix damaged DNA, as well as antioxidant enzymes including SOD, catalase, and glutathione peroxidase. From both internal and external sources, the body stores antioxidant molecules inside and outside cells such as glutathione, vitamin C, vitamin E, and other compounds which help in combating free radical damage [6]. However, these natural antioxidant mechanisms can be inefficient for severe and/or continued oxidative stress. Based on this idea, there has been a strong demand of therapeutic antioxidant agents with limited toxicity to enhance the antioxidant capacity of the body [22]. Endogenous and exogenous antioxidants counter the imbalances between reactive oxygen species (ROS) production and detoxifies the products. The inflammatory cells generate more soluble inflammatory mediators such as cytokines, arachidonic acid, and chemokines. These mediators act through active inflammatory cells in the area of infection and they release more reactive species. These can stimulate signal transduction cascades. In addition to alterations in transcription factors, like signal transducer and activator of transcription 3, nuclear factor kappa B (NF-𝜅B), activator protein-1, NF-E2 related factor-2, nuclear factor of activated T cells, and hypoxia-inducible factor-1𝛼 (HIF1-𝛼) [23].They also mediate vital cellular stress reactions and initiation of cyclooxygenase-2(COX-2), inducibility of nitric oxide synthase (iNOS), and high expression of inflammatory cytokines, including tumor necrosis factor-𝛼 (TNF-𝛼), interleukin-1𝛽 (IL-1𝛽), IL-6, and chemokines (CXC chemokine receptor 4)( V.R [24]. To add the changes in the expression of specific microRNAs, they have also been exhibited to have a role in oxidative stress-induced inflammation. This inflammatory/oxidative environment triggers an unhealthy circle, which can harm healthy stromal cells and neighboring epithelial cells, which after a long period of time may trigger carcinogenesis [21]

# MATERIALS AND METHODS

## Materials, Equipment and Facilities

Materials, Equipment, Facilities and all chemicals, associated reagents, equipment and facilities for the *in-vivo* laboratory animal toxicity investigations and the bioactivity assays were obtained from the University of Zimbabwe, Faculty of Medicine and Health Sciences laboratories.

## *Nymphaea nouchali* plant material collection and preparation

Plant material was collected from the Mberengwa area of Zimbabwe in the Midlands province, about 423 kilometers from the City of Harare. The plant material was authenticated as *Nymphaea nouchali* by the National Herbarium and Botanical Garden in Harare, Zimbabwe. To remove contaminants and debris, the leaves were thoroughly washed using distilled water. After they were then shade dried at room temperature to constant weight and moisture of about 4% for three weeks and then ground to a fine powder using an electric grinder. The phyto-extraction was done by adding 200g plant powder into 1000ml of 70% (v/v) hydro ethanolic mixture in a 2-litre sterile amber bottle and macerated for 3 days, with 3 minutes physical shaking twice a day. To obtain a filtrate from the solution, a muslin cloth was used which was further clarified by filtration using What man filter paper number 1. The filtrate was then evaporated 3 times under vacuum and low pressure using Rotavapor R-300, followed by freeze drying (lyophilization) under 140Pa pressure and -50 °C. The lyophilized extract was stored in an airtight sample bottle.

## Phytochemical Screening

In a 200ml flask, 5g of freeze-dried hydro-ethanolic extracts from *N. nouchali* were mixed with 50ml of distilled water. This mixture was investigated using various methods to check for the presence or absence of pharmacologically active secondary metabolites. Several specific tests were performed on the liquid extract to identify these compounds. All procedures were carried out as per published methods by Chifamba *et al.*, 2024 [25] with slight modifications.

### Detection for alkaloids by the Iodine test

The Iodine test was used to determine the presence of alkaloids. In this test, a few drops of iodine solution were slowly added along the sides of the test tube to 3ml of the lyophilized extract solution. The presence of alkaloids was then identified by the appearance of a blue colour, which disappears on boiling and reappears on cooling [26]

### Detection of tannins.

The Braymer’s test was used to detect the presence of tannins. To 1ml lyophilised extract solution, 2 mls of distilled water were added followed by 4 drops of a 10% Ferric chloride solution. The presence of tannins is confirmed by a blue green color [26].

### Detection of flavonoids by the ammonia test.

Flavonoids were detected by means of the ammonia test where 5ml dilute ammonia solution was added to 5ml of the lyophilised extract solution followed by a few drops of concentrated sulphiric acid. The emergence of a yellow colour indicates the presence of flavonoids [27].

### Detection of glycosides

For the detection of glycosides, 3ml of chloroform was added to 2mls of the extract followed by 3 drops of ammonia in a test-tube. Formation of a pink color indicates presents of the glycosides [28].

### Detection of phenolic compounds by the Ferric chloride test.

Phenolic compounds were detected using the ferric chloride test. In this assay, 1ml of the lyophilized extract solution was added to 2ml of distilled water followed by a few drops of ferric chloride. Formation of a blue-green color indicates presence of phenols [29].

### Detection of saponins by the simplified foam test

The simplified foam test was used to determine the presence of saponins where 2ml of the extract was added to 20ml distilled water. The mixture was shaken in a graduated cylinder for 15 minutes. The presence of saponins was confirmed by the formation of form with a head height of at least 1cm [30].

### Quantification of total flavonoids

The total flavonoid content of the lyophilized *N. nouchali* was estimated spectrophotometrically at 510 nm. In the test, 1mg of extract was dissolved in 2mL of distilled water. 0.5mL of 1M sodium nitrite was added together with 2ml of a 1M, NaOH solution to this solution. Distilled water was then added to make up to 10ml volume. The solution was shaken and allowed to stand at room temperature for 15 min and the absorbance was subsequently measured. The total flavonoid content was estimated as mg of quercetin equivalent (mg QE/g extract) on a dry weight basis using the standard curve [31]

Preparation of standard quercetin solution: About 10 mg of quercetin was dissolved into 10 ml of distilled water to make the stock solution. Then serial dilution was performed to prepare a different concentrated solution (6 ppm, 12ppm, 24ppm, 48ppm, 96ppm). Figure 2 shows the standard quercetin standard curve.

Figure 2: Standard curve of quercetin.

### Quantification of Total Phenolic and Tannins content.

To quantify phenolics and tannins, 10 mg of gallic acid was dissolved into 10 ml of distilled water. Then serial dilution was performed to this stock solution to prepare different concentrated solutions of 31.25 ppm, 62.5 ppm, 125 ppm, 250 ppm, 500 ppm, and 1000 ppm. The total phenol content of extracts was evaluated by the Folin-ciocalteu method as described by R. Rmos (2017) [31] [32]. About 1ml of sample extracts or standard at different concentrations were mixed with 2 ml of Folin–Ciocalteu reagent (10 times diluted), and incubated at room temperature for 3 minutes. After that, 10 ml of 20% sodium carbonate was added to the mixture and left for incubation at room temperature for an hour. The absorbance of the mixture was measured at 765 nm with a Shimadzu UV VIS-2600 spectrophotometer against a blank solution. The blank solution contained all the reagent mixture without extract or standard sample. Gallic acid standard curve was used to quantify total phenolic contents and the results were expressed as mg of gallic acid equivalent (GAE) per gram of dried weight. All determinations were performed in triplicate. The following gallic acid calibration curve (figure 3) was used to estimate the total phenolic content of *N. nouchali* crude extracts. The linear regression equation was y =0.0022x – 0.0043 (R2 = 0.9945).

Figure 3: Standard curve of Gallic acid

## Anti-inflammatory Activity of *N. nouchali* using the Egg Albumin Denaturation Test

The anti-inflammatory effects of the lyophilized extract were evaluated using a modified version of the egg albumin protein denaturation method described by chifamba *et al* (2024) [25]. For this test, 0.4 mL of egg albumin, 10 mL of phosphate-buffered saline (PBS) with a pH of 7.4, and 5-mL solutions containing different concentrations of the freeze-dried *N. nouchali* extracts dissolved in 0.4% DMSO were used. The extract concentrations ranged from 50 to 8000 µg/mL in the total reaction mixture. The samples were first incubated at 37°C for 20 minutes, then heated at 70°C for another 30 minutes to force protein denaturation. After cooling, the mixture was filtered, and its absorbance was measured at 660 nm using a blank solution as reference. Negative controls included fresh egg albumin (0.4 mL), DMSO (0.5 mL), and PBS (3 mL). Diclofenac sodium served as the positive control at similar concentrations to compare results. The percentage inhibition, which indicates how well the extract prevents inflammation, was calculated using a standard formula given below.

*Equation 1*

*in*𝑓𝑙𝑎𝑚𝑎𝑡𝑖𝑜𝑛 𝑖𝑛ℎ𝑖𝑏𝑖𝑡𝑖𝑜𝑛 𝑝𝑒𝑟𝑐𝑒𝑛𝑡𝑎𝑔𝑒 𝑒𝑓𝑓𝑒𝑐𝑡 = × 100

## Anti Oxidancy Evaluation of N. nouchali using the DPPH Scavenging Assay

The ability of test samples from *N. nouchali* to scavenge DPPH radicals was assessed using the method described by Nariya *et al (*2013) [33]. First, 4 mg of DPPH was mixed in 100 mL of 95% methanol and kept in the dark. Then, 10 mg of ascorbic acid was dissolved in 10 mL of distilled water to create a stock solution of 1 mg/mL. This stock solution was diluted to make solutions of different concentrations: 2 ppm, 4 ppm, 8 ppm, 16 ppm, and 32 ppm. Next, 4 mL of the DPPH solution was combined with 1 mL of either the sample extracts or the standard at various concentrations. The mixture was shaken well and left at room temperature in the dark for 30 minutes. Absorbance was measured at 517 nm using a UV-Vis spectrophotometer, with a blank for comparison. The control sample had the same volume but no extract, using ascorbic acid as a standard reference. Methanol served as the blank. The IC50 value was calculated based on the percentage of inhibition. The DPPH radical scavenging activity was determined using the following formula:

*Equation 2*

*D*𝑃𝑃𝐻 𝑆𝑣𝑎𝑣𝑒𝑛𝑔𝑖𝑛𝑔 𝑝𝑒𝑟𝑐𝑒𝑛𝑡𝑎𝑔𝑒 𝑒𝑓𝑓𝑒𝑐𝑡= × 100%

## Acute Oral Toxicity Evaluation of *N. Nouchali*

The acute oral toxicity evaluation of *N. nouchali l*yophilized extract was done using a slightly modified OECD technical guideline 425 (The up and down test) (OECD, 2025) using 8 Sprague Dawley rats. The rats were acclimatized to the test environment for 10 days prior to the commencement of the test protocols. Commercial standardized rodent pellet from Nova feed (PVT) LTD and clean water were used to feed the animals. The animal habitat was kept at an average temperature of 25°C throughout the study with a relative humidity level of 45%. The animal welfare, observations and care were supervised by a qualified veterinary officer. Sequential ordered progressions of doses were orally administered to the animals at 48-hour intervals in our test. The test animals were divided into 2 groups, the control group (group 1) which received distilled water and the second group which received incremental doses of *N. nouchali* solution. The selected animals were marked so as to facilitate individual identification. The experimental animals were fasted for 20 hours with water prior to dosing. Initial starting doses were chosen based on related toxicological studies. The first animal received a dose of 250mg/kg body weight. This dose was below a randomly selected estimated LD50. The next dose was doubled, and the test animals were observed over a period of 48 hours. The *N. nouchali* extract was administered via an oral gavage in a water solution in 4 different sets of doses of: 250, 500, 1000, 2500 and 5000 mg/kg body weight. The rats were observed by a veterinary specialist for morbidity and mortality twice daily. The rats were observed for any visible changes and clinical signs and symptoms of toxicity every 1 hour, and up to 12 hours on day 1, and thereafter, once daily up to a maximum of 14 days. The animals were also weighed daily.

# Results and discussions

## Qualitative phytochemical analysis

From the phytochemical screening studies carried out, our research validated the presence of a significant quantity of primary and secondary metabolites that are relevant to biomedical applications with regards to pain. The most prevalent compounds in the plant were phenolics, particularly flavonoids. These results correlate with studies by Parimala and Shoba (2014) [6] who identified terpenes, tannins, flavonoids, coumarins and other compounds in *N. nouchali*. The abundance of medically significant phytochemicals supports the various applications of the plant in traditional medicine, particularly in treating conditions such as dysmenorrhea in traditional medicine. Table 1 below shows the phytoconstituents in *N. nouchali.*

Table 1: Qualitative screening of N. nouchali secondary metabolites.

|  |  |  |
| --- | --- | --- |
| Test | Presence in hydro-ethanolic extract | Presence in distilled water extract |
| Alkaloids | ++ | + |
| Phytosterols | +++ | ++ |
| Flavonoids | +++ | + |
| Saponins | +++ | + |
| Steroids | +++ | + |
| Coumarins | +++ | + |
| Phenolic compounds | +++ | + |
| Tannins | +++ | + |
| Carbohydrates | ++ | + |
| Glycosides | ++ | + |
| Terpenoids | +++ | + |

(-): Indicates the absence of the phytochemical

(+): Indicates the presence of the phytochemical

(++): Indicates moderate presence of the phytochemical

(+++): Indicates strong presence of the phytochemical

Plants generate a wide variety of organic compounds. Among these, the compounds that do not play a role in growth and development are known as secondary metabolites. Phenolics are primarily produced as a defensive response to adverse environmental conditions. These plant phenolics are predominantly acid derivatives of hydroxybenzoic and hydroxycinnamic acids. There has been growing interest in their potential medicinal applications, particularly as antioxidants and anti-inflammatory agents. [25]. Flavonoids are a class of polyphenolic compounds with subgroups like flavones, flavanols, flavanones and flavanols.

## Quantitative phytochemical analysis

The total phenols of the water lily were 16.51 mg GAE/g (table 2). According to multiple reports in the literature, phenolic compounds exhibit free radical inhibition, peroxide decomposition, metal inactivation or oxygen scavenging in biological systems and prevent oxidative disease burden. The amount of flavonoids in the extract was around was around 7.5 mg Quercetin equivalent/g on an average (table 3). Flavonoids are also important for human health. Like vitamins, these compounds are not produced endogenously by the body and must be supplied either through the diet or nutritional supplements [34]. Tannins show an outstanding array of biochemical and pharmacological actions including anti-inflammatory, antioxidant, anti-allergic and anti-carcinogenic activities. Various studies have illustrated the beneficial effects on blood cholesterol levels, cancer, and stimulation of the immune system [34].

Table 2: The total phenolic content in the extract.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Sample solution | Weight of the dry extract per ml (g) | absorbance | GAE conc. C µg/ml | GAEE conc.C mg/ml | TPC as QE A= (c.v)/m | Mean |
| 5mls | 0.005 | 0.053 | 0.02600 | 0.016562 | 16.562 | 26.76±0.77 |
| 5mls | 0.005 | 0.057 | 0.02782 | 0.016451 | 16.451 |
| 5mls | 0.005 | 0.054 | 0.02646 | 0.016524 | 16.524 |

Table 3: Total flavonoid content in the extract.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Sample solution | Weight of the dry extract per ml((g) | absorbance | GAE conc. C µg/ml | GAEE conc.C mg/ml | TPC as QE A= (c.v)/m | Mean |
| 1000 | 0.001 | 0.003 | 7.341 | 0.007341 | 7.341 | 7.478±0.1 |
| 1000 | 0.001 | 0.006 | 7.671 | 0.007671 | 7.671 |
| 1000 | 0.001 | 0.004 | 7.421 | 0.007421 | 7.421 |

## Antioxidant Assay

DPPH scavenging activity was 92.37% for ascorbic acid (the standard used) at 125µm/ml. While *N. nouchali* ethanolic extract was 87.77% at that same concentration (table 4), (figure 4). The antioxidant activity was determined from the absorbance obtained at specific concentrations. The IC50 values were 14.13 µg/ml and 19.76 µg/ml for ascorbic acid and *N. nouchali* ethanolic extract. Antioxidants exert their free radical scavenging activities via diverse mechanisms including reducing power, chelating transition metal, radical scavenging activities, and disintegrating radicals [35]. In our studies we used the DPPH radical. The DPPH is a methanol soluble compound characterized by a deep-violet colour that exhibits maximum UVR absorption at 515 nm. The test principles are based on the fact that reactive species are able to reduce DPPH to 2,2-diphenyl-1-hydrazine (DPPH-H) or a substituted analogous hydrazine (DPPH-R) characterized by colourless or pale-yellow colour [36]. This colour change can easily be tracked spectrophotometrically. Previous scholars established that the participating metabolites in antioxidant activities of *N. nouchali* include polyphenols phytosterols, as well as enzymes. These metabolites are able to inhibit the oxidation related colour change. [37]. The % inhibition was directly proportional to the extract concentration as shown in below.

Table 4: Antioxidant activity of N. nouchali.

|  |  |  |
| --- | --- | --- |
| Concentration (µg/ml) | Ascorbic acid (%inhibition) | *N. Nouchali* (% inhibition) |
| 5 | 2.61 | 1.75 |
| 10 | 29.67 | 17.65 |
| 15 | 56.82 | 36.76 |
| 20 | 73.54 | 54.78 |
| 25 | 83.21 | 67.97 |
| 30 | 84.02 | 76.56 |
| 40 | 85.6 | 81.3 |
| 60 | 86.7 | 84.15 |
| 80 | 88.26 | 85.46 |
| 125 | 92.37 | 87.77 |

Figure 4: Inhibition of ascorbic acid and N. nouchali

These studies confirm that extracts of *N. nouchali* are rich in various antioxidant compounds, which can manage oxidative stresses.

## Anti-Inflammatory Test

The lyophilized extracts of *N. nouchali* demonstrated anti-inflammatory properties that are approximately half as effective as the standard medication Diclofenac for all concentrations(table 5)*.* Although various methods were employed to measure inflammation inhibition, the significant anti-inflammatory potential of *N. nouchali* is highlighted by the results from our egg albumin test assay at 1000µg/ml, especially considering that this extract was in its crude form. It is anticipated that using bioactive fractions and isolated compounds will further enhance this activity [38]. This suggests that with further development and improvement through more advanced extraction techniques the anti-inflammatory activity *N nouchali* will be greatly improved. [39]

Table 5: Anti-inflammatory activity of lyophilized N. nouchali extracts

|  |  |  |
| --- | --- | --- |
| Concentration(µg/ml) | Inhibition in protein denaturation (%) | |
|  | ***N. nouchali (%)*** | **Diclofenac sodium (%)** |
| 30 | 19.27 | 51.12 |
| 60 | 25.99 | 58.7 |
| 125 | 32.15 | 64.01 |
| 250 | 35.70 | 75.20 |
| 500 | 37.40 | 77.98 |
| 1000 | 43.33 | 87.02 |

## Acute oral toxicity evaluation

The acute toxicity study was carried out as per OECD technical guideline 425. The observations, results and interpretation were done by Vertinary personnel. Our findings showed that the extract was safe at doses up to 5000 mg/kg body weight. There were no fatalities, all the animals completed the study without any adverse health conditions symptoms or weight loss, and none were removed during the observation period (table 6). Our extracts were non-toxic, based on the toxicity classification by Loomis, T. A., & Hayes, A. W (1996) [40]; which categorizes substances with LD50 values from 500 to 5000 mg/kg as slightly toxic and those with LD50 values from 5000 to 15,000 mg/kg body weight as practically non-toxic. This means that using high concentrations of the extracts to get the desired benefits presents no toxicity effects.

Table 6: Acute oral toxicity study of N. nouchali behavioral Observations

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Observed parameter | Dose of *N. nouchali* in mg/kg body weight | | | | |
| **250** | **500** | **1000** | **2500** | **5000** |
| Food intake | Normal | Normal | Normal | Normal | Normal |
| Water intake | Normal | Normal | Normal | Normal | Normal |
| Death | No deaths | No deaths | No deaths | No deaths | No deaths |
| Breathing | Normal | Normal | Normal | Normal | Normal |
| Diarrhea | None | None | None | None | None |
| Urination | Normal | Normal | Normal | Normal | Normal |
| skin color | Normal | Normal | Normal | Normal | Normal |
| Drowsiness | None | None | None | None | None |
| Erection of fur | Not observed | Not observed | Not observed | Not observed | Not observed |

## Rat weight observations

Generally, unexpected fluctuations in body weight are a simple and sensitive reflection of toxicity after exposure of study animals to materials in toxicity studies (figure 5). In this study the extracts did not significantly alter the expected normal body weight growth pattern over the study period.

Figure 5: Observed rat weights. over investigation period

# Conclusion.

The lyophilized hydro-ethanolic extracts of *N. nouchali* were shown to possess considerable antioxidant and anti-inflammatory activities. The observed activities were attributable to the presence of secondary metabolites including phenolic compounds and flavonoids. These contribute to the underlying mechanisms behind the plant’s proven ability to manage various ailments in traditional medicine including dysmenorrhea. The lyophilized plant extracts were toxicologically safe at 5000mg/kg. Our biosafety and bioactivity studies therefore authenticate the use of *N. nouchali* as a potential anti-inflammatory and antioxidant remedy in traditional medicinal practices.

# Disclaimer AI.

The Authors hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

# Competing interests.

# We declare that we have no conflict of interest. The authors are entirely responsible for the research content and the compilation of this report.

# References

|  |  |
| --- | --- |
| [1] | X. Xiong, Z. J., Y. Yang, Y. Chen, Q. Su, Y. Zhao, J. Wang, Z. Xia, L. Wang, L. Zhang and F. Chen, "Water lily research: Past, present, and future," *Tropical Plants,* vol. 2, no. 1, pp. 1-8, 2023. |
| [2] | M. Khan, "NUTRITIONAL COMPOSITION, PHYTOCHEMICAL AND ANTIOXIDANT ACTIVITY OF STEM OF (Nymphaea nouchali) AND (Nymphaea rubra," *Doctoral dissertation,* 2019. |
| [3] | M. S. Simmonds and M. J. Howes, "Plants used in the treatment of diabetes," in *Traditional medicines for modern times: antidiabetic plants*, S. A, Ed., New York, CRC Press, 2006. |
| [4] | F. R. Irvine and R. Trickett, "Waterlilies as food," *Kew Bull,* vol. 8, no. 3, 1953. |
| [5] | Y. Punjabi, V. Khilnani and P. Damle, "Antibacterial activity of flower extracts of Nymphaea nouchali," *Pharmacophore.,* 2014. |
| [6] | M. Parimala and F. G. Shoba, "Phytochemical analysis and in vitro antioxidant activity of hydroalcoholic seed," *Asian Pac J Trop Biomed.,* 2014. |
| [7] | O. Fawole, A.R.Ndhlala, S.O.Amoo, J.F.Finnie and J. Staden, "Anti-inflammatory and phytochemical properties of twelve medicinal plants used for treating gastro-intestinal alinimants in South Africa," *Ethnopharmacology,* vol. 123, pp. 237-243, 2009. |
| [8] | M.W, "Mediators of inflamationand inflamation process," *Journal of Allergy and clinical immunology ,* vol. 103, pp. 378-381, 1999. |
| [9] | Y.-H. Wang, B. Ge, X.-l. Yang, Li-NingYang, X.-X. Wang, X. Liu, J.-C. Shi and Y.-J. Wu, "Proanthocynanidins from grape seds modulates the nuclear factor -kappa B signal transduction pathways in rates with TNBS-induced recurrent ulcerative colitis," *International immunopharmacology,* vol. 11, pp. 1620-1627, 2011. |
| [10] | P. N. Diouf, T. Stevanoic and A. Cloutier, "Study on chemical composition, antioxidant and anti-inflammatory activities of hot water extract from Piecea bark and its proanthocynanidin-rich fractions," *food chemistry,* vol. 113, pp. 897-902, 2009. |
| [11] | L. Y-DI, C. M.Frenz, C. Mian-Hua, W. Yu-Rong, L. Feng-Juan, L. Cheng, L. Ning, Y. Hua, L. Bohlin and W. Chang-Lu, "Primary Virtual and in vitro Bioassay Screening of Natural Inhibitors from Flavonoids against COX-2," *Chinese Journal of Natural Medicines ,* vol. 9, pp. 0156-0160, 2011. |
| [12] | L. E. Pelzer, T. Guardia, A. a. o. jauarez and E. Guerreiro, "Acute and chronic antiinfammatory effects of plant flavonoids," *Farmaco,* vol. 53, pp. 421-424, 1998. |
| [13] | K.-H. Kang, C.-S. Kong, Y. Seo, M.-M. Kim and S.-K. Kim, "The anti-inflammatory effects of coumarins isolated from Corydalis heterocarpa in HT-29 human colon carcinoma cells," *Food and Chemical Toxicology ,* vol. 47, pp. 2129-2134, 2009. |
| [14] | K. Satoh, F. Nagai, M. Ono and N. Aoki, "Inhibition of Nab,kb-ATpase the extract of Stephania cephararantha HAYATA and bisbenzylisoquinoline alkaloid cycleanine,a major constituent," *Biochemical Pharmacology,* vol. 66, pp. 379-385, 2003. |
| [15] | H.Wang, J.Gao, J.Kou, D.Zhu and B.Yu, "Anti-infalammatory activities of triterpenoid saponins from Polygala japonica," *Phytomedicine,* vol. 15, pp. 321-326, 2008. |
| [16] | S.G.Sparg, M.E.Light and J. Staden, "Biological activities and distribution of plant saponins," *Journal of Ethnopharmacology ,* vol. 94, pp. 219-243, 2004. |
| [17] | M. Lacaille-Dubois and Wagner, "A review of the biological and pharmacological activities of saponins," *Phytomedicine,* vol. 2, no. 4, pp. 363-386, 1996. |
| [18] | P. Poudel, S. Petropoulos and F. Di Gioia, "Plant tocopherols and phytosterols and their bioactive properties," *Natural Secondary metabolites:From Nature,Through Science, to industry,* pp. 285-319, 2023. |
| [19] | J. Van Ee, "Soy constituents:modes of action in low-density lipoproteins management," *Nutrition Reviews ,* vol. 67, pp. 222-234, 2009. |
| [20] | R. O. Poyton, K. A. Ball and P. R. Castello, "Mitochondrial generation of free radicals and hypoxic signaling," *Trends in Endocrinology and Metabolism,* vol. 20, no. 7, pp. 332-340, 2009. |
| [21] | S. Reuter, S. C. Gupta, M. M. Chaturvedi, Aggarwal and B. B., "Oxidative stress, inflammation, and cancer: how are they linked?," *Free Radical Biology and Medicine ,* vol. 49, no. 11, 2010. |
| [22] | A. S. Ravipati, L. Zhang, S. R. Koyyalamudi and e. al., "Antioxidant and anti-inflammatory activities of selected Chinese medicinal plants and their relation with antioxidant content," *BMC Complementary and Alternative Medicine,* vol. 12, p. 173, 2012. |
| [23] | S. P. Hussain and C. C. Harris, "Inflammation and cancer: an ancient link with novel potentials," *International Journal of Cancer,* vol. 121, no. 11, 2007. |
| [24] | V. R. Tandon, S. Verma, J. Singh and A. Mahajan, "Antioxidants and cardiovascular health," *Journal of Medical Education & Research ,* vol. 7, no. 2, 2005. |
| [25] | J. Chifamba, A. J. Addae, S. Zengeni, M. Pomerai and N. Kurebgaseka, "Lyophilised Aloe excelsa fractions, photo-protection and actinic damage retardation," *Journal of Complementary and Alternative Medical Research,* vol. 25, no. 5, pp. 37-52, 2024. |
| [26] | M. Bharudin, S. Zakaria and C. Chia, "Condensed tannins from acacia mangium bark: Characterization by spot tests and FTIR," *AIP Conference Proceedings,* vol. 1571, no. 1, pp. 153-157, 2013. |
| [27] | M. Hossain, K. AL-Raqmi, Z. AL-Mijizy, A. Weli and Q. Al-Riyami, "Study of total phenol, flavonoids contents and phytochemical screening of various leaves crude," *Asian Pacific Journal of Tropical Biomedicine,* vol. 3, no. 9, pp. 705-710, 2013. |
| [28] | R. Sheel, K. Nisha and J. Kumar, " Preliminary phytochemical screening of methanolic extract of Clerodendron infortunatum," *IOSR Journal of Applied Chemistry ,* vol. 6, no. 6, pp. 1771-1778 , 2014. |
| [29] | N. Jaradat, F. Hussen and A. Al Ali, " Preliminary phytochemical screening, quantitative estimation of total flavonoids, total phenols," *Journal of Materials and Environmental Science,* vol. 6, no. 6, pp. 1771-1778 , 2015. |
| [30] | M. El Aziz, A. Ashour and A. Melad, "A review on saponins from medicinal plants: chemistry, isolation, and determination," *Journal of Nanomedicine Research,* vol. 8, no. 1, pp. 282-288, 2019. |
| [31] | R. Rmos, I. Bezera, M. Ferreira and L. Soares, "Spectrophotometric quantification of flavonoids in herbal material, crude extract, and fractions from leaves of Eugenia uniflora linn," *Pharmacognosy Research,* vol. 9, pp. 253-260, 2017. |
| [32] | V. Singleton, R. Orthefer and R. Lamuela-Raventos, "Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent," *Methods in Enzymology,* vol. 299, pp. 152-178, 1999. |
| [33] | P. Nariya, N. Bhalodia, V. Shkla, A. R and N. M.B, "in vitro evaluation of antioxidant activity of Cordia dichotoma," *An international Quarterly Journal of Research in Ayurveda,* vol. 34, p. 124, 2013. |
| [34] | SakihamaY, C. MF, G. SC and Y. H, "Plant phenolic antioxidant and prooxidant activities:Phenolics -induced oxidative demage mediated by metals in plants," *Toxicology,* vol. 177, pp. 67-80, 2002. |
| [35] | J. a. A. P. Adjimani, "Antioxidant and free radical scavenging activity of iron chelators.," *Toxicology Reports,* vol. 2, pp. 721-728, 2015. |
| [36] | R. Costa, "Production of Lipid Nanoparticles for Topical Administration of Flavonoids," Universidade do Porto (Portugal), 2023. |
| [37] | M. N. Uddin, M. A. Samad, M. Zubair, M. Z. Haque, K. Mitra, T. Khan, M. Hossain, A. Syed and A. Afroze, "Potential bioactive phytochemicals, antioxidant properties and anticancer pathways of Nymphaea nouchali," *Asian Pacific Journal of Tropical Biomedicine,* vol. 10, no. 2, pp. 555-562, 2020. |
| [38] | M. Biozid, M. M. Rahman, M. N. Alam, M. A. Sayeed, A. I. Chowdhury, M. Faruk and M. J. Abedin, "In-vitro comparative study of anti-inflammatory and anti-arthritic effects of Flemingia stricta Roxb. and Nymphaea nouchali leaf.," *Int J Pharm Pharm Sci (International Journal of Pharmacy and Pharmaceutical Sciences),* vol. 7, no. 8, pp. 49-52, 2015. |
| [39] | M. S. Biozid, M. M. Rahman, M. N. Alam, M. A. Sayeed, A. I. Chowdhury, M. Faruk and M. J. & Abedin, "In-vitro comparative study of anti-inflammatory and anti-arthritic effects of Flemingia stricta Roxb. and Nymphaea nouchali leaf.," *Int J Pharm Pharm Sci (International Journal of Pharmacy and Pharmaceutical Sciences),* vol. 7, no. 8, pp. 49-52, 2015. |
| [40] | T. A. Loomis and A. W. Hayes, "Loomis’s essentials of toxicology," 4th ed ed., Academic Press, 1996. |