RESEARCH PAPER

Evaluation of In Vitro Anti-Inflammatory Activity of Nelumbo nucifera

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ABSTRACT

BACKGROUND/OBJECTIVES:

Inflammation represents a major contributing factor to many chronic diseases. However, the long-term toxicity of anti-inflammatory drugs may impair their function. Nelumbo nucifera, a medicinal herb used extensively in indigenous medicine by various cultures, represents a rich source of various bioactive compounds with potential anti-inflammatory properties. The aim and objectives of this research were to determine the in vitro anti-inflammatory properties of leaf extract of Nelumbo nucifera by employing HRBC membrane stabilisation, egg albumin denaturation assay, and BSA denaturation assay.

MATERIALS/METHODS:

Leaves of Nelumbo nucifera were taken, and cold maceration was done to obtain ethanolic extracts. In vitro studies of the ethanolic extract of Nelumbo nucifera leaves for anti-inflammatory activity were done by three methods: (1) HRBC membrane stabilisation, (2) heat-induced egg albumin denaturation, and (3) bovine serum albumin denaturation. The concentration of the sample was varied from 100 to 500 µg/mL, and standard diclofenac sodium was used for comparison.

RESULTS:

The result showed that N. nucifera extract had a significant concentration-dependent anti-inflammatory effect on the three types of assays. At the highest concentration (500 µg/mL), the percentage protection offered by diclofenac was 65.3% in the HRBC membrane stabilisation method, and the lotus extract offered 55.6% protection. Diclofenac sodium and N. nucifera extract offered significant dose-dependent inhibition of protein denaturation. At a concentration of 500 µg/mL, diclofenac inhibited protein denaturation by 84.6%, and the lotus extract inhibited protein denaturation by 76.9%. The result showed that at 500 µg/mL, the albumin denaturation inhibition was maximised by diclofenac (87.5%), and the lotus extract exhibited a promising result of 75.0% inhibition. All the above results had statistical significance ($p<0.05$)

CONCLUSION:

The ethanolic extract of Nelumbo nucifera exhibited strong anti-inflammatory activity in vitro by means of membrane stabilisation and inhibition of albumin and protein denaturation. The results suggest its potential use as a natural compound, replacing synthetic anti-inflammatory drugs, and justify further studies in vivo and in clinical experiments.

Keywords: Nelumbo nucifera, Anti-inflammatory, HRBC, Albumin denaturation, protein denaturation, Medicinal plant, Diclofenac, In vitro assays

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1. INTRODUCTION

Inflammation is a biological defence mechanism that protects the body from pathogens, toxins, and injuries. Though beneficial in acute stages, chronic inflammation contributes to the development of many diseases, including arthritis and cardiovascular disorders [1]. They are effective; however, NSAIDs induce adverse effects such as gastrointestinal discomfort and renal complications most of the time [2]. These adverse side effects have therefore made the investigation of plant-based alternatives with fewer side effects prominent.

Nelumbo nucifera Gaertn, or commonly referred to as sacred lotus, finds its extensive use in the traditional medicine of Asia. It is reported to possess bioactive phytoconstituents such as alkaloids, flavonoids, and phenolic compounds [3]. The present work examines anti-inflammatory potential using in vitro assays for determination of cellular membrane stabilisation and inhibition of protein denaturation.

Inflammation is a result of the body's immune reaction to any harmful stimulus, like the damaged cells, pathogens, or any other irritant. While acute inflammation is somewhat beneficial, chronic inflammation can lead to diseases such as arthritis, cancer, and cardiovascular disorders [1]. The commonly resorted-to drugs to treat inflammation are NSAIDs, but due to their long-term consumption, side effects include gastrointestinal ulcers, renal impairment, and cardiovascular problems. This calls for safer alternatives. Nelumbo nucifera Gaertn. (commonly known as the Indian lotus) is one of the most valued plants from ancient traditional medicine systems. The different parts of the plant are rich in flavonoids, alkaloids, and phenolic acids, accountable for anti-inflammatory, antioxidant, and hepatoprotective properties [3]. The current study was carried out to study the in vitro anti-inflammatory activity of N. nucifera by using established models and comparing with standard diclofenac.

2. MATERIALS AND METHODS

The Cold Maceration Process:

The cold extraction method was used on dried and powdered Nelumbo nucifera leaves. A measured amount of 10 grams of the plant powder was taken and dissolved in 100 mL of ethanol solution prepared in a 70:20:10 proportion. This ethanolic mixture was packed in a sealed glass container and was incubated for 72 hours. This process was meant to enable the extraction of the bioactive components.

After the period of maceration, the mixture was filtered through sterile filter paper in order to harvest the liquid extract from the plant residue. The extracted liquid was concentrated using a rotary evaporator at a temperature of 40°C in order to drain the excess solvent. The last step of drying the extracts was conducted at room temperature to evaporate all the solvent. The weight of dried extracts was measured.

Extraction yield can be calculated using the following equation:

Yield=Weight Before Extraction-Weight After Extraction

The total extract yield computed was 0.56 grams.

2.1 Preparation of HRBC Suspension:

Human red blood cells were employed to evaluate the membrane-stabilising ability of the test extracts. A sample of blood was collected from a healthy volunteer in a sterile setting, ensuring the donor had not taken any anti-inflammatory drugs for the past 10 days. The blood was mixed with an equal amount of Alsever’s solution, acting as an anticoagulant and preservative, maintaining cell viability, thereby preventing blood cells from degrading or dying.

The blood-Alsever's solution mixture was then centrifuged at a speed of 3000 rpm for a period of 5 minutes. The supernatant fluid rich in plasma and preservatives was carefully aspirated, and the red blood cells (RBCs) were washed three times with isotonic saline (0.85% NaCl with a pH of 7.2) to ensure that there were no traces of plasma proteins left in the RBCs. A 10% suspension of RBCs was then made in isotonic saline for further experimental work.

2.2 HRBC Membrane Stabilisation Assay:

The human red blood cell membrane stabilisation technique has been used to assess the potential anti-inflammatory properties of the extract. The test is carried out under hypotonic conditions that induce red blood cell lysis. Any compound that inhibits lysis is considered to have anti-inflammatory properties.

Different concentrations of the plant extract (100-500 μg/mL) were prepared. To each concentration to be tested, 1 mL of the plant extract solution, 1 mL of phosphate buffer solution with a pH of 7.4, 2 mL of hypotonic saline solution (0.25% NaCl), and 0.5 mL of 10% HRBC solution were added. The mixture was kept in a water bath at 37°C for 30 minutes.

After incubation, centrifugation of samples at a speed of 3000 rpm for a period of 10 minutes was done. Then, absorbance was measured using a spectrophotometer set to a wavelength of 560 nm. The inhibition percentage of hemolysis in each of the samples under test was measured using the percentage inhibition formula below:

Where AbsControl is the absorbance of control, while AbsSample is the absorbance of a test sample.

HRBC Protection Percentage = 100 - (AbsSample/AbsControl) X 100

2.3 Protein (Egg Albumin) Denaturation Assay:
Inflammation processes involve protein denaturation, and this assay is used to screen compounds that can prevent proteins from being damaged by heat. Egg albumin protein was used here for the assay.

A mixture for each reaction contained various concentrations of the plant extract ranging from 100 to 500 µg/mL, 0.2 mL of normal egg albumin, and phosphate-buffered saline (PBS at pH 6.4), which was used to make the total volume 5 mL.. The mixtures were left at 37°C for 15 minutes for the interaction between the plant extract and the protein. The mixtures were then denatured by heating at 72°C for 5 minutes.

Then, after cooling to room temperature, the absorbance of each solution was determined at a wavelength of 660 nm. A lower value for absorbance represents a higher stability of proteins. The percentage inhibition of protein denaturation is given by:

Protein Denaturation Inhibition % = (Control Absorbance – Sample Absorbance / Control Absorbance) x 100.

Diclofenac sodium served as the standard drug for comparison purposes.

2.4 Bovine Serum Albumin (BSA) Denaturation Assay:
To further confirm the anti-inflammatory activity, another model of protein denaturation using bovine serum albumin (BSA) was also performed. BSA is a pure protein and a good substrate for the study of heat-induced protein denaturation.

In the BSA denaturation assay, conducted in this setup, a mixture of 1 mL of plant extracts and 1 mL of 5% BSA solution of five different concentrations ranging from 100 µg/mL to 500 µg/mL was taken. To this mixture, 1N hydrochloric acid is added, and the pH is maintained at 6.3. The mixture of different concentrations ranging from 100 µg/mL to 500 µg/mL was incubated at 37°C for a period of 20 minutes. After that, in order to increase albumin denaturation, it is incubated at a temperature of 57°C for a period of 10 minutes.

The turbidity measurements were made after cooling the test sample to room temperature. The effectiveness of the test sample in protecting the protein molecules against denaturation was calculated using the percentage inhibition of denaturation. The formula for calculating percentage inhibition of denaturation is:

Albumin Denaturation Inhibition percentage = {Control Absorbance − Sample Absorbance / Control Absorbance} x 100.

Standard reference drug used is Diclofenac sodium.

3. RESULTS

3.1 HRBC Membrane Stabilisation:

Inflammation inhibiting capacity of the Nelumbo nucifera leaf extract was determined by the HRBC membrane stability method. This method has the following principle: the extracts that can protect the red blood cell membrane can also protect the lysosomal membranes by inhibiting the secretion of the inflammatory mediators.

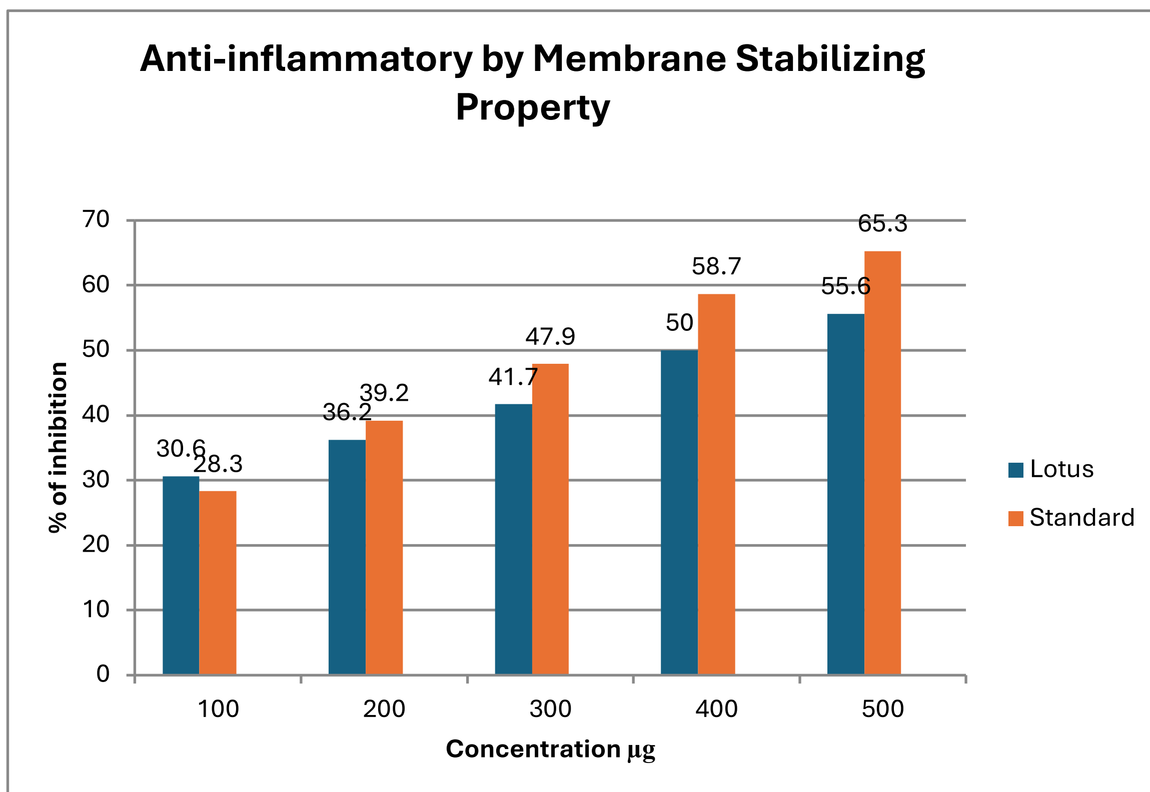
HRBC Protection Percentage = 100 - (AbsSample/AbsControl) X 100

Std Blank =0.46

Sample Concentration (µg)	**100**	**200**	**300**	**400**	**500**
Diclofenac	0.33	0.28	0.24	0.19	0.16
% inhibition	28.3	39.2	47.9	58.7	65.3

Blank = 0.36

Sample Concentration (µg)	**100**	**200**	**300**	**400**	**500**
lotus	0.25	0.23	0.21	0.18	0.16
% inhibition	30.6	36.2	41.7	50	55.6



From the above result, it has been identified that the standard drug as well as lotus extract has a concentration-dependent membrane-stabilising activity. In a concentration of 500 mcg/mL, 65.3% and 55.6% protection has been exhibited by the standard drug diclofenac and lotus extracts, respectively. The above result reveals that the lotus has an appreciable quantity of potency in anti-inflammation. On the other hand, from the above experiment, it has also been identified that the potency in anti-inflammation of lotus extracts has a slightly lower quantity compared to the potency of the standard drug diclofenac.

The stabilising activity of the HRBC membrane could thus be explained by the presence of flavonoids, alkaloids, and other phytochemicals known to have anti-inflammatory properties in Nelumbo nucifera.

3.2 Protein (Egg albumin) Denaturation Assay: Denaturation of proteins occurs due to the inflammation, and thus, it causes damage to the tissues. Agents that possess the capability to inhibit the denaturation process can also be used as anti-inflammatory agents.

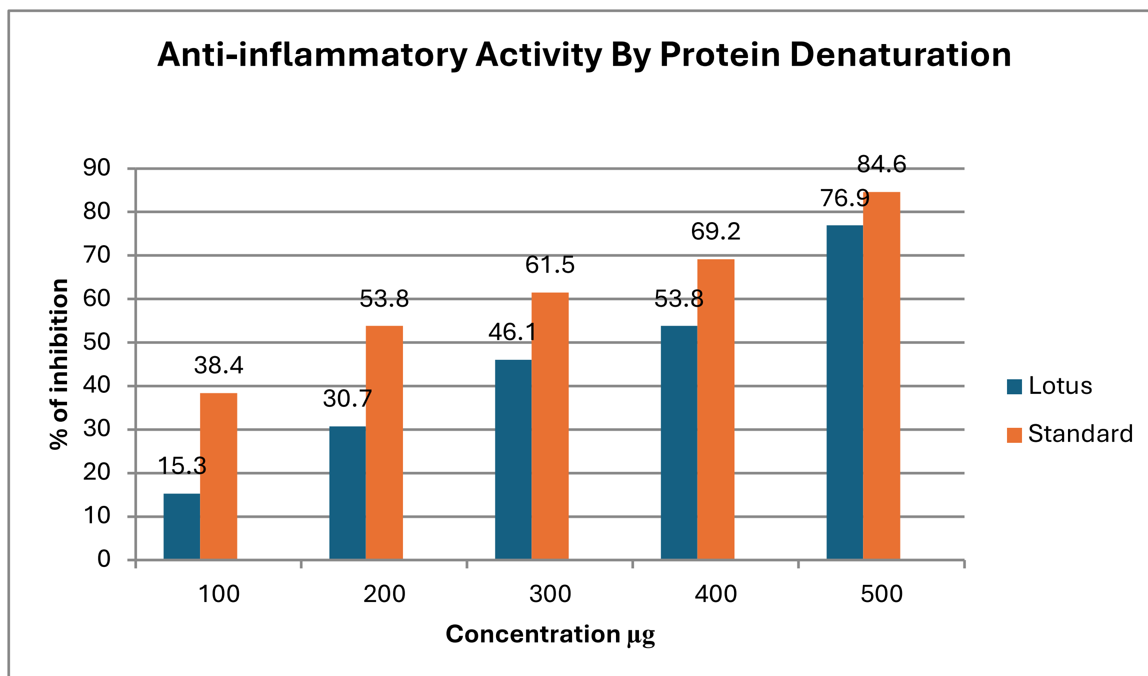
Protein Denaturation Inhibition % = (A control – A sample /A control) x 100.

Std Blank =0.13

Sample Concentration (µg)	**100**	**200**	**300**	**400**	**500**
Diclofenac	0.08	0.06	0.05	0.04	0.02
% inhibition	38.4	53.8	61.5	69.2	84.6

Blank =0.13

Sample Concentration (µg)	**100**	**200**	**300**	**400**	**500**
lotus	0.11	0.09	0.07	0.06	0.03
% inhibition	15.3	30.7	46.1	53.8	76.9



Both Diclofenac Sodium and Nelumbo nucifera extracts showed strong inhibition of protein denaturation in a dose-dependent manner. When compared to the highest concentration of 500µg/mL, the percentage inhibition caused by Diclofenac Sodium was 84.6% and that of the lotus extracts was 76.9% .

The activity of the lotus extract was moderate at lower concentrations and steadily increased as the concentrations were taken to higher doses. This gives a strong support to the hypothesis that certain substances like flavonoids and polyphenols in Nelumbo nucifera can act as helping agents in preventing the denaturation of proteins by heat and can thus show anti-inflammatory properties.

3.3 Albumin(Bovine serum) Denaturation Assay:

Protein denaturations, particularly albumin proteins, have been related to the inflammatory process due to their functionality as a biochemical mechanism. Substances that possess the ability to inhibit the occurrence of protein denaturations have been considered to possess great potential in anti-inflammatory drugs.

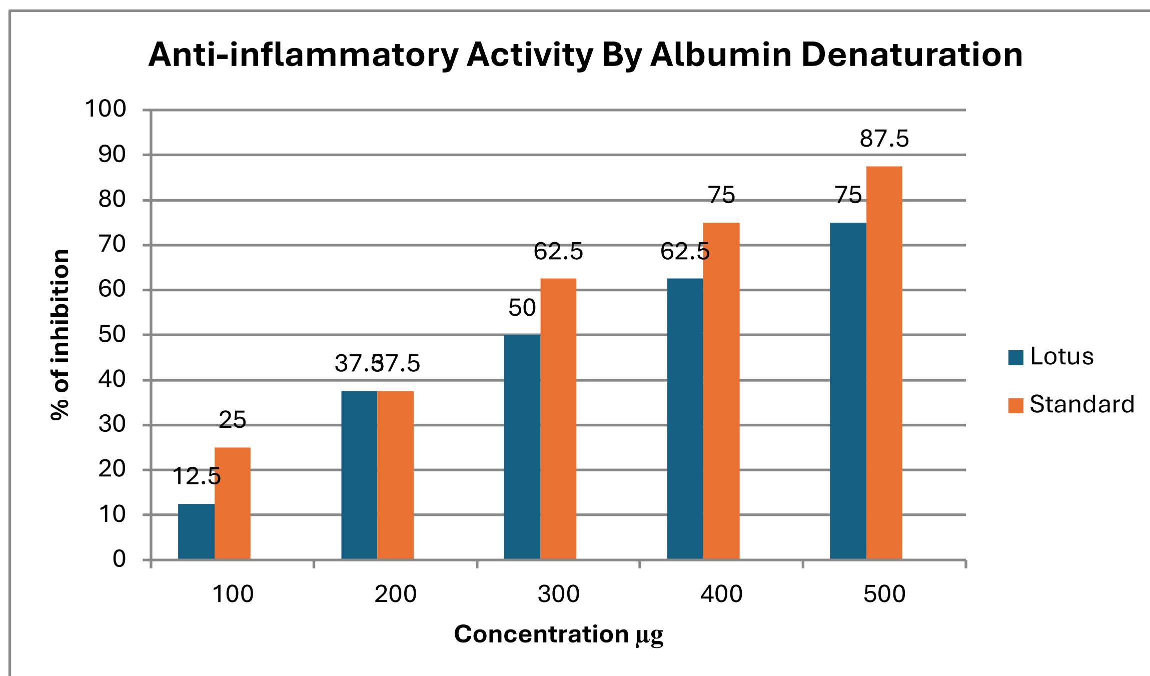
Albumin Denaturation Inhibition percentage (%) = (A control – A sample /A control) x 100.

Std Blank =0.08

Sample Concentration (µg)	**100**	**200**	**300**	**400**	**500**
Diclofenac	0.06	0.05	0.03	0.02	0.01
% inhibition	25.0	37.5	62.5	75.0	87.5

Blank = 0.08

Sample Concentration (µg)	**100**	**200**	**300**	**400**	**500**
Lotus	0.07	0.05	0.04	0.03	0.02
% inhibition	12.5	37.5	50	62.5	75



Data analysis revealed the concentration-dependent inhibition of albumin denaturations by the diclofenac sodium and Nelumbo nucifera extracts. At 500 µg/mL concentration, diclofenac sodium attained maximum inhibition of 87.5%, and the inhibition achieved by lotus extracts was at a promising level of 75.0%.

The outcomes revealed that the activities of the lotus extracts are similar to those of diclofenac, particularly at an intermediate concentration of 200 µg/mL (37.5%) and 400 µg/mL (62.5%).

Evidently, thermostable phytochemicals are present within the extracts of the lotus plants, having the ability to safeguard proteins from denaturation.Moderate to strong inhibition shown in the lotus extract could have implications of being useful in the role of the latter as an anti-inflammatory drug, perhaps due to protein stabilisation, similar to other NSAIDs.

4. DISCUSSION

Inflammation is a biological process mediated by certain agents, in which acute inflammation is of a protective nature, whereas chronic inflammation is well known to cause a number of pathologies, which include rheumatoid arthritis, cardiovascular problems, and cancer [1, 2]. The anti-inflammatory activity of Nelumbo nucifera has been investigated in this research paper using three separate in vitro methods, which include membrane stabilisation, protein denaturation, and Denaturation of Bovine Serum Albumin.

The HRBC lytic activity was effectively prevented by the lotus extract in a highly potent manner in the HRBC assay, which reflects its lysosomal membrane protective activity. This is a very important aspect since the rupture of lysosomal membranes generates hydrolytic enzymes; hence, there is additional tissue damage and inflammation [4,5]. The protective index afforded by the strongest concentration of the lotus extract (500µg/mL) was 55.6% compared to 65.3% afforded by diclofenac.

Similarly, for the protein denaturation models involving egg albumin and BSA proteins, the extract had a good inhibition potential for protein denaturation triggered by heat. The experiments carried out above can be considered to have a pathological condition with respect to the misfolding of proteins triggered by inflammation and the generation of immune complexes [6]. The inhibition potential of Nelumbo nucifera for the denaturation of proteins for egg albumin and BSA models was 76.9% and 75.0%, respectively.

The afore-mentioned biological effects can be attributed to the phytoconstituents that exist in N. nucifera including flavonoids, alkaloids, and polyphenols. Additionally, flavonoids inhibit the COX and LOX pathways in N. nucifera, including compounds such as quercetin and kaempferol. They were observed to inhibit the pathways thereby lowering the production of pro-inflammatory mediators such as prostaglandins and leukotrienes [7, 8]. Furthermore, nuciferine was observed to inhibit NF-kappa B signaling. Other biological effects that were observed include inhibiting the production of pro-inflammatory markers.

Furthermore, the ability of the compounds from the lotus flower to suppress ROS could act synergistically towards decreasing the amount of ROS to avoid the oxidative stress presented during the process of inflammation [10].

Combining the ability to suppress inflammation and oxidative stress, N. nucifera is of great interest when exploring.

Various comparative studies on phytomedicines such as Curcuma longa (Turmeric) and Ocimum sanctum (Tulsi) have also revealed the similar effect on models of membrane stabilization and protein denaturations [12, 13]. All the above findings are consistent with the established finding and hence authenticate the therapeutic potential of N. nucifera.

It should, however, be noted that the results derived from in vitro may not always lead to success with in vivo due to various problems and challenges relating to bioavailability and metabolism within the body of a living organism. It is important that future studies aim at the application of animal models and a molecular approach based on pharmacokinetics concepts.

5. CONCLUSION

The results confirm the potent anti-inflammatory activity of the leaf extract of Nelumbo nucifera in vitro. The crude extract in various models, like HRBC membrane stabilisation assay, egg albumin denaturation method, and BSA denaturation assay, demonstrated concentration-dependent and statistically significant inhibition of inflammation.

At high concentrations, the result was similar in effectiveness to diclofenac sodium, a standard drug from the NSAID class. The mentioned properties could be attributed to the high phytochemical content of the plant, especially flavonoids and alkaloids, which are known to possess properties that modulate the cellular pathway and are antioxidants.

Notably, the results have a valid justification for the native folk medical practice of N. nucifera and mark it as a potential source for the formulation of anti-inflammatory medications from plants. In vivo testing should, however, be conducted to firmly establish its pharmacological and therapeutic properties.

With this, it can be concluded that the encouraging findings of this experiment provide the basis for improvement of N. nucifera from traditional medicine to a scientifically supported anti-inflammatory drug.

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