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In-vivo Studies of the Anti-inflammatory Effects of *Spirulina platensis*

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Known as the superfood, *Spirulina platensis*, a blue-green algae, has stood out in the natural world as a source of nutrition for Man for hundreds of years owing to its high content of protein, carbohydrates, vitamins and minerals. In addition, it is also known to possess several pharmacological properties of which one of the most prominent is its efficacy against inflammation. The objective of the present in-vivo studies therefore was to assess the anti-inflammatory activity of the crude ethanolic extract of *Spirulina platensis* employing Carrageenan Induced Rat Paw Edema (acute model) and Cotton Pellet Induced Granuloma (chronic model) tests. Two doses at 250 mg/kg and 500 mg/kg body weight were administered by gavage to the rats in both the experiments. The highest percentage inhibitions of inflammation by *Spirulina platensis* at 41.10% and 22.89% were recorded in the acute and chronic models, respectively. In both the tests inhibitions were induced dose-dependently. Our findings thereby confirm that *Spirulina platensis* possesses significant anti-inflammatory activity.

Keyword: *Spirulina*, Anti-inflammatory, Carrageenan, Edema, Granuloma.

1. Introduction

The blue-green algae *Spirulina platensis*, is a microscopic and filamentous cyanobacterium. Due to its abnormally high levels of chlorophyll, it was initially placed in the plant kingdom but was later shifted to the bacterial kingdom based on new understanding of its physiology, genetics and biochemical properties. Its cells form long strands which look similar to a coiled spring; thus the name *Spirulina,* meaning 'little spring'[1,2].

The major use of *Spirulina* is as a food source and its use dates back hundreds of years ago to the Mayans of Central America who used to cultivate it in water. However, the first written accounts of the algae go back to the year 1524 and later on it first became available in the market in processed form in 1979. Today *Spirulina* itself poses as one of the most valuable algae in the world, with an annual production of 2000 tonnes and an industry of US $40 million[1,2].

Most of the earlier studies were focused on the nutritional values of *Spirulina* because of its excellent dietary contents. It is naturally low in cholesterol, calories, fat and sodium and consists of large quantities of carbohydrate (19%), nine important vitamins and at least fourteen minerals. But its most noteworthy nutritive property can be attributed to a staggering 60% protein content, which is a percentage higher than in any food. Consequently, it has been successfully used in the malnutrition of children and has even been

used as an adequate dietary supplement for a segment of the HIV inflicted population in Africa[2,3,4,5].

In the 1980s, the focus shifted slowly from the nutritional aspects of *Spirulina* towards its efficacy against different diseases, and from then on a plethora of work has been done on its pharmacological properties. One of the most widely assessed properties is its hypolipidemic effects in which it has been shown to have excellent cholesterol reducing properties and subsequent cardioprotective properties. Its antioxidant properties have also been studied and some of its key constituents include useful antioxidants like beta carotene and Vitamin E. It has also been shown to stimulate adaptive response from the immune system in humans and stimulate both the Natural Killer cells and the production of interferon. This coupled with its antioxidant properties make it a potent anticancer substrate. This anticancer property has been attributed to one its components, phycocyanine. Moreover, the production of interferons makes it a potent antiviral drug and it has been shown to be efficacious against enveloped viruses like Herpes Simplex virus and HIV[6-21].

Apart from all of these, the anti-inflammatory properties of the phytoconstituent phycocyanin from *Spirulina* have been extensively studied in animal studies. In addition, the efficacy of Gamma Linolenic Acid (GLA) present in *Spirulina* as an anti-inflammatory agent has also been hypothesised. The published works to date on the evaluation of the anti-inflammatory activities of *Spirulina* have mostly been conducted in conjunction with model rats of other diseases, such as diabetes, hepatitis, arthritis etc. Our work aims to determine the anti-inflammatory properties of the crude extract of the algae employing two different methodologies in tandem on standard animal models and thus establish a confirmed basis to its widespread claim as an anti-inflammatory agent[22-28].

2. Materials and Methods

2.1 Plant Collection and Preparation

Spirulina was purchased from the Bangladesh Council of Scientific and Industrial Research, Dhaka, Bangladesh, where it is cultivated for market production and research purposes. Following collection, the dry algae was powdered using mixer grinder and a weighed amount of 500g of the powder was passed through the sieve number 20 in efforts to collect the fine powder. Cold solvent extraction was used to extract the powder with ethanol (95%v/v, 1L) for three times in a week. After collection, the filtrate was concentrated in a rotary vacuum evaporator (Bibby RE-200, Sterilin Ltd., UK) under reduced pressure at a temperature of 50°C. The dry extract produced was dark green in colour and weighed 78.6 g (yield ratio 15.7% w/w). It was refrigerated for future use[29].

2.2 Experimental Animals

Anti-inflammatory activities were studied using Wistar albino rats (*Rattus norvegicus)* of either sex weighing between 180 and 200g. The animals were procured from the Animal House of the Pharmacy Department of Jahangirnagar University (Dhaka, Bangladesh). For a week prior to the experiment they were acclimatized to standard laboratory conditions maintained at a temperature of 25±2°C, relative humidity of 55 ±5%, and light and dark cycles of 12:12 hours. They were housed in standard cages which were cleaned frequently and were provided with standard diet of rodent pellets and water *ad libitum*. The experiments were carried out in conformity with the UK Home Office regulations (UK Animals Scientific Procedures Act 1986) and the 'Principles of Laboratory Animal Care' (National Institutes of Health publication no.86-23, revised 1985) and were approved by the Ethics Committee on Animal Research, North South University.

2.3 Chemicals

Carrageenan was the product of Sigma Aldrich (USA). Diclofenac sodium and Ibuprofen were purchased from Novartis (Bangladesh) Ltd. Sterile normal saline was used as control. All the other chemicals used in the experiments were of analytical grade.

2.4 Evaluation of In Vivo Anti-inflammatory activity

2.4.1 Carrageenan Induced rat paw Edema Method

Carrageenan induced rat paw edema experiment was conducted to assess the effect of *Spirulina* in acute inflammation. The Wistar albino rats were divided into four groups (n=6) as follows-

- Group I- Control group received 0.9% saline solution (0.5 ml, *p.o.)*
- Group II- Standard group received Diclofenac sodium (positive control, 20 mg/kg, *p.o.*)
- Group III-Test group received *Spirulina* (250 mg/kg, *p.o.*)
- Group IV- Test group received *Spirulina* (500 mg/kg, *p.o.*)

The rats, following overnight fasting, with water given *ad libitum*, were given their respective drug or saline solutions by gavage. Half an hour after receiving treatment, edema was induced by subcutaneously injecting carrageenan (1%, 0.05ml) in the sub plantar tissue of the right hind paw of each animal. Plethysmometer (Model 7141, UGO Basile, Italy) was used to measure the paw volume (i.e. inflammation) before (0h) and at 1h, 2h, 3h, 4h, 6h after carrageenan injection. Having served as the reference paw, the left paw did not undergo inflammation. The average paw volumes of the treatment groups were compared to the control group. The percentage inhibition of the paw edema was calculated using the formula[30]:

$$PPE = (V_C - V_T/V_C) \times 100$$

Where, V_C and V_T represent mean paw volume of control and treated animal respectively.

2.4.2 Cotton Pellet Induced Granuloma Method

As a chronic inflammatory model, the cotton pellet induced granuloma experiment was carried out by grouping (n=6) the experimental rats in the following manner-

- Group I- Control group received 0.9% saline solution (0.5 ml, *p.o.*)
- Group II- Standard group received Ibuprofen (positive control, 20 mg/kg, *p.o.*)
- Group III-Test group received the *Spirulina* (250 mg/kg, *p.o.*)
- Group IV- Test group received the *Spirulina* (500 mg/kg, *p.o.*)

Sterile cotton pellets weighing 40mg each were used to induce granuloma in the Wistar albino rats. The rats were anesthetized with ketamine (60mg/kg) and the fur was shaved. One cotton pellet was inserted in each axilla and all the incisions were surgically stitched. The control vehicle along with the treatment drugs were administered to the rats orally every day for seven days. Standard pellet diet was continued for a week and the rats had access to food and water *ad libitum*. On the eight day, the animals were anesthetized and the cotton pellets were removed surgically. They were cleaned to separate the extraneous tissues. The moist cotton pellets were weighed and dried at 60° C for 18 hours and then reweighed. Granuloma formation was measured by the increment in the weight of the dried pellets. Percentage inhibition of granuloma formation was calculated using the formula[31,32]:

$$PGF = (DW_C - DW_T/DW_C) \times 100$$

Where, DW_C and DW_T represent the mean weight of the dried cotton pellets of the control and treatment groups, respectively.

2.5 Statistical Analysis

The obtained data was statistically analysed and expressed as the mean ± SEM by one-way analysis of variance (ANOVA) and Dunnett's *t*-test as the test of significance was used. The minimum level of significance was considered to be P value <0.05.The SPSS 17.0 statistical software was used to carry out all the statistical tests.

3. Results

3.1 Carrageenan induced paw edema

Table 1 represents the decrease in the carrageenan induced paw edema of the experimental rats after receiving treatment. From what can be observed in the control group, a local edema was produced after the carrageenan was injected in the rat paw. The edema volume increased progressively and the maximum paw volume was attained at 3h after injection. The highest percentage inhibition of edema was observed for Diclofenac sodium at 46.63% ($p<0.001$). *Spirulina* produced significant results with greatest percentage inhibition being 41.10% for 500mg/kg ($p<0.001$) and 12.27% for 250mg/kg ($p<0.001$) at 3h.The anti-edematous responses declined for all the treatment groups after the 3rd hour. *Spirulina* at 500mg/kg significantly inhibited inflammation from 2h to 4h and the effect was comparable to the standard. Dose dependent inhibition of rat paw edema volume was observed for the extract treated animals.

Table 1 : The effect of Spirulina on paw edema volume in the carrageenan induced rat paw edema test

Group No.	Treatment Group	Decrease in carrageenan induced paw edema volume (mL)					
		0h	1h	2h	3h	4h	6h
I	Control	0.68±0.11	1.23±0.03	1.45±0.03	1.63±0.02	1.51±0.02	1.34±0.03
II	Standard	0.68±0.01	1.12±0.03 (8.94%)	1.17±0.03*** (19.31%)	0.87±0.02*** (46.63%)	0.86±0.01*** (43.05%)	1.16±0.04** (13.43%)
III	Extract 250g/kg	0.69±0.01	1.20±0.01 (2.44%)	1.35±0.03 (6.90%)	1.43±0.04*** (12.27%)	1.39±0.04** (7.95%)	1.26±0.03 (5.97%)
IV	Extract 500g/kg	0.69±0.01	1.15±0.03 (6.50%)	1.28±0.04** (11.72%)	0.96±0.01*** (41.10%)	1.08±0.01*** (28.48%)	1.23±0.02 (8.21%)

Each value represents the mean±SEM, n=6,. * p<0.05, ** p<0.01, *** p<0.001. Statistical significant test for comparison between groups was done by ANOVA, followed by Dunnett's test. Percentage inhibitions of the treatment groups II, III, IV are expressed in comparison to control group I.

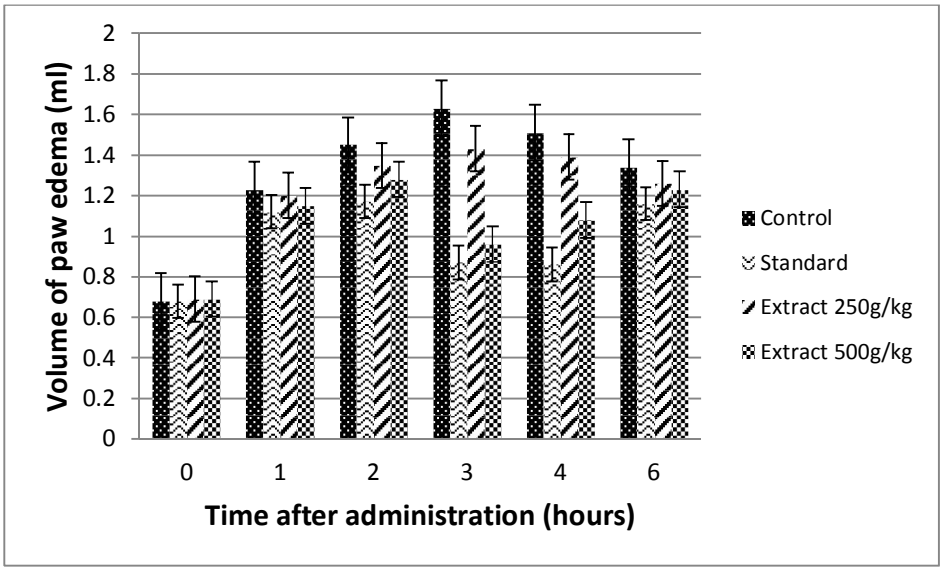

Fig 1: Comparative study of the effect of Spirulina on paw edema volume in the carrageenan induced rat paw edema test

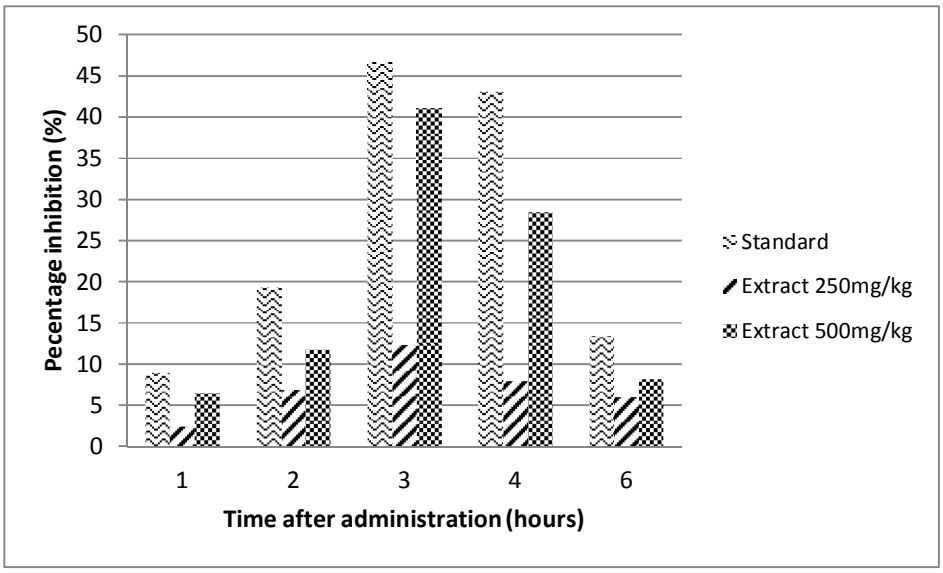

Fig 2: Comparative study of the effect of Spirulina on the percentage inhibition of carrageenan induced rat paw edema

3.2 Cotton pellet induced granuloma

The results of the cotton pellet induced granuloma experiment are displayed in Table 2. The mean dry weight of the sterile cotton pellets of the treatment groups were compared to the control group. The standard drug Ibuprofen drew the highest percentage reduction in dry weight of 36.9% ($p<0.001$), followed by *Spirulina* 500mg/kg at 22.89% ($p< 0.001$). *Spirulina* at 250mg/kg produced the least inhibition of 6.75% ($p<0.01$) and thus certifying its dose-dependent activity in the inhibition of chronic inflammation.

Table 2: The effect of Spirulina on granuloma formation in the cotton pellet granuloma test

Group No.	Treatment Group	Weight of dry cotton pellet granuloma (mg)	Percentage Inhibition (%)
I	**Control**	175.33±1.71	---
II	**Standard**	110.67±2.46	36.9***
III	**Extract 250mg/kg**	163.5±2.38	6.75**
IV	**Extract 500mg/kg**	135.2±1.66	22.89***

Each value represents the mean±SEM, n=6. * p<0.05, ** p<0.01, *** p<0.001. Statistical significant test for comparison between groups was done by ANOVA, followed by Dunnett's test. Percentage inhibitions of the treatment groups II, III, IV are expressed in comparison to control group I.

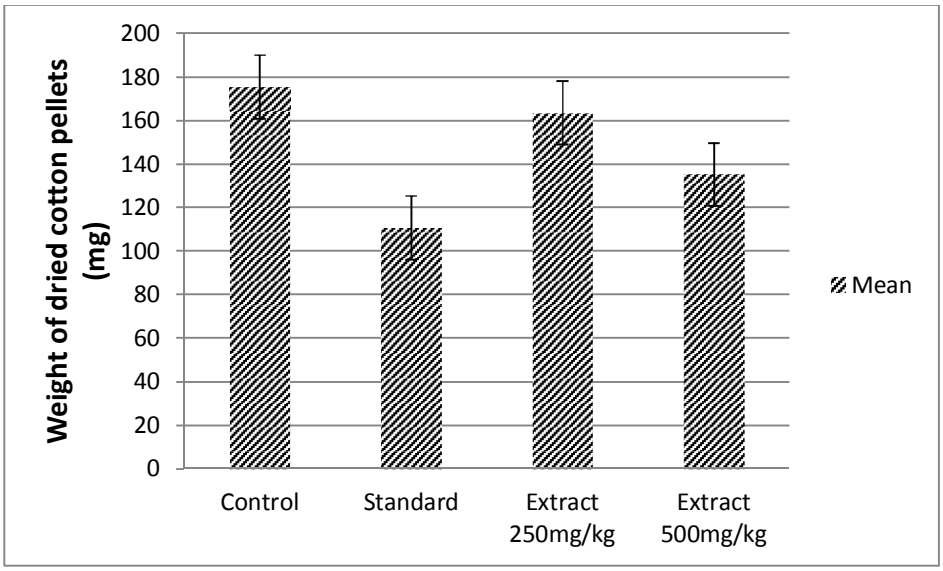

Fig 3: Comparative study of the effect of Spirulina on cotton pellet induced granuloma formation

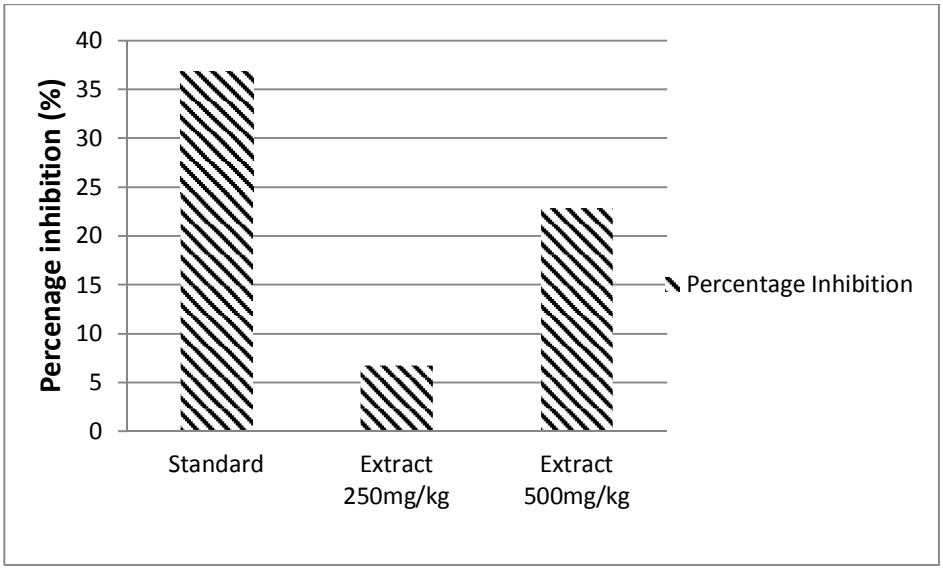

Fig 8: Comparative study of the effect of Spirulina on the percentage inhibition of cotton pellet induced granuloma formation

4. Discussion

An immunological defense mechanism, inflammation is brought about to respond to mechanical injuries, burns, microbial infections, allergens and other harmful stimulus. Being extremely regulated, it initiates a series of physiological responses that enables the immune system to efficiently remove the injurious stimuli and begin the healing process. However when left uncontrolled, the inflammation persists and aids in the development of many chronic pathological conditions, such as atherosclerosis, retinitis, multiple sclerosis, inflammatory bowel disease, rheumatoid arthritis, psoriasis etc. In this modern synthetic age, the use of anti-inflammatory agents may provide beneficial results in the treatment of inflammatory disorders. The uses of non-steroidal or steroidal anti-inflammatory drugs are common in the treatment of different inflammatory conditions. Due to the many side effects of the currently available anti-inflammatory drugs, there lies a serious setback in their clinical use. Hence the attention has now been drawn to the evaluation of the effectiveness of products obtained from natural sources. Not only do they present a greater prospect in terms of affordability but also in terms of fewer adverse effects[33-41].

In our investigation to confirm the role of *Spirulina* in suppressing inflammation in the absence of other pathological conditions, two experiments were used in tandem to assuredly verify the findings. Carrageenan induced paw edema, the most commonly used model, is a biphasic event. The model can be depicted in two phases – the early phase and subsequently the late phase. The early phase (1-2.5 h after carrageenan injection) is mediated mainly by serotonin, histamine, bradykinin and a spike in prostaglandin synthesis in the area of the damaged tissue. Edema and swelling are induced which can be observed from the injected rat paws. During the first 1.5 hours, the mediators histamine and serotonin are mostly released. The secretion of bradykinin follows next almost 2.5 hour after carrageenan injection. Ensuing this comes the late phase (2.5-4 h after injection) which is mediated in most part by an oversynthesis of prostaglandins and their successive release. *Spirulina* has been corroborated to reduce acute inflammation in both the phases in a pattern marked as dose-dependent. It has already been shown that *Spirulina* reverses age-related increase in pro-inflammatory cytokines in cerebellum, such as tumor necrosis factor-alpha (TNFα) and TNFβ. It could therefore be suggested that *Spirulina* might suppress the release of the pro-inflammatory mediators (serotonin, histamine, bradykinin) as well as the production and release of prostaglandin[38,42-48].

As a chronic model in this experiment, cotton pellet induced granuloma method is used to investigate the efficacy of the extract in transudative, exudative and proliferative phases. Subcutaneous implantation of cotton pellets directly elicits an acute inflammatory response. This action, being unable to draw a sufficient response to eliminate the inflammation causing mediators, calls in turn for a stronger chronic inflammatory reaction. The agents known to infiltrate in chronic inflammation are neutrophils and mononuclear cells. The dry weight of the separated cotton pellets can be taken to measure the amount of granuloma formed. Hence the effectiveness of *Spiruilna* to reduce granulomatous tissue formation during the chronic inflammation can be meaningfully associated with the decrease in the dry weight of the pellets as compared to the control group. It can therefore be deduced that *Spiruilna* decreases the number of fibroblasts, collagen and mucopolysaccharides, which are the agents in the proliferative stage of the chronic model. *Spiruilna* acts dose-dependently to reduce chronic inflammation and it can be mentioned that the higher dose of the extract (500mg/kg dose) played a more significant role to draw a more clinically noticeable response[49,50,51].

The anti-inflammatory activities of *Spirulina* can be attributed to one of its phytochemical constituents phycocyanin which inhibits pro-inflammatory cytokine formation such as TNFα, suppresses cyclooxygeanase-2 (COX-2) expression and reduces the production of prostaglandin E2. Cyclooxygenase is a key enzyme involved in the biosynthesis of prostaglandin and plays an important role in inflammation. With the discovery of inducible form of cyclooxygenase, COX-2, it has been postulated that prostaglandins that contribute to inflammatory process are derived exclusively from COX-2[52-56,23].

Another phytoconstituent β-carotene augments the anti-inflammatory activities. Studies have shown that β-carotene inhibited the production of nitric oxide and prostaglandin E2, and suppressed the expression of iNOS, COX-2, TNF-α and IL-1β. Such suppression of inflammatory mediators by β-carotene is likely to have resulted from its inhibition of NF-κB activation through blocking nuclear translocation of NF-κB p65 subunit. NF-κB is known to be ubiquitously expressed and to play a major role in controlling the expression of protein involved in immune, inflammatory and acute phase response. Without stimulation, NF-κB is in an inactive state bound to its inhibitor IκB in the cytoplasm. Various agonists, such as IL-1, TNF-α and TLR ligands, activate NF-κB. Then the NF-κB undergoes nuclear translocation, where it binds to and stimulates transcription of target genes. In addition, β-carotene suppresses the transcription of inflammatory cytokines including IL-1β, IL-6, and IL-12 in macrophage cell line stimulated by lipopolysaccharide (LPS) or IFNγ[57-59].

5. Conclusion

Our study confirms the claimed property of *Spirulina* in suppressing inflammation. The algae is also generally considered safe for human consumption on basis of its long historical use and documented safety profile in animal studies. However, occasional cases of side-effects have been reported and more vigorous clinical studies are essential in order to scientifically prove its usefulness as a safe anti-inflammatory natural product.

6. Ethical Approval

All authors hereby declare that "Principles of laboratory animal care (NIH publication No. 85-23, revised 1985) were followed, as well the laboratory animal care laws and protocols of national educational institutions.

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8. Conflict of Interest

Authors have no conflict of interest to declare.

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