International Journal of Environmental Sciences
ISSN: 2229-7359
Vol. 11 No. 23s, 2025
https://theaspd.com/index.php

Evaluation of Anti-Inflammatory Activity of Celosia Cristata Linn. Extract Invitro Study

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Abstract
Inflammation is a complex biological response to harmful stimuli, and its dysregulation is implicated in various chronic diseases. The present study aims to evaluate the in vitro anti- inflammatory activity of Celosia cristata Linn. extract, a traditionally used medicinal plant known for its ethnopharmacological properties. Methanolic extract of the aerial parts of Celosia cristata was prepared and subjected to anti-inflammatory screening using protein denaturation assay and membrane stabilization method, two well-established in vitro models. The extract was tested at various concentrations and compared against standard anti-inflammatory drug, diclofenac sodium. The results demonstrated that the plant extract exhibited dose-dependent inhibition of protein denaturation and erythrocyte membrane lysis, indicating significant anti-inflammatory potential. The observed activity is attributed to the presence of bioactive phytoconstituents such as flavonoids, tannins, and saponins identified in the preliminary phytochemical analysis. These findings suggest that Celosia cristata Linn. possesses promising anti-inflammatory activity and may serve as a potential source for the development of natural anti-inflammatory agents.
Keywords: *Celosia cristata L, Antioxidant activity, Methanolic extract, Free radicle, Oxidative stress*

1. INTRODUCTION

Inflammation is a complex process, which is frequently associated with pain and involves occurrences such as: the increase of vascular permeability, increase of protein denaturation and membrane alteration. When cells in the body are damaged by microbes, physical agents or chemical agents, the injury is in the form stress. Inflammation of tissue is due to response to stress. It is a defensive response that is characterized by redness, pain, heat, and swelling and loss of function in the injured area. Loss of function occurs depends on the site and extent of injury. Since inflammation is one of the body's nonspecific internal systems of defense, the response of a tissue to an accidental cut is similar to the response that results from other types of tissue damage, caused by burns due to heat, radiation, bacterial or viral invasion. (1) Inflammation plays an important role in the pathophysiology of conditions encountered by anesthesiologists and critical care practitioners on a daily basis. Surgery, sepsis, major trauma, burns, adult respiratory distress syndrome and ischemia–reperfusion injuries have major inflammatory components. Our understanding of the basic immunology of inflammation has progressed significantly during the past 10 years. However, these advancements in knowledge have not translated into widespread application in the clinical setting. (2) Historically, anti-inflammatory drugs had their origins in the serendipitous discovery of certain plants and their extracts being applied for the relief of pain, fever and inflammation. When salicylates were discovered in the mid-19th century to be the active components of Willow Spp., this enabled these compounds to be synthesized and from this, acetyl-salicylic acid or Aspirin™ was developed. Likewise, the chemical advances of the 19th–20th centuries lead to development of the non-steroidal anti-inflammatory drugs (NSAIDs), most of which were initially organic acids, but later non-acidic compounds were discovered. There were two periods of NSAID drug discovery post-World War 2, the period up to the 1970's which was the pre-prostaglandin period and thereafter up to the latter part of the last century in which their effects on prostaglandin production formed part of the screening in the drug-discovery process. Those drugs developed up to the 1980-late 90's were largely discovered empirically. 6

following screening for anti-inflammatory, analgesic and antipyretic activities in laboratory animal models. (3)

1.2 Natural products and medicinal plants for treatment: -

Due to the adverse effects of NSAIDs it is a dire need of hour to move towards natural anti- inflammatory products. New natural medicinal plants and their metabolites with strong anti- inflammatory activity and insignificant detrimental impacts has been exploring by the world scientific community due to its bioavailability and cost efficiency.

Many plants have been identified which consists of compounds and molecules with anti- inflammatory properties. Their study gained more interest due to their active potential and beneficial effects. These active molecules help the body to defend against inflammatory related pathogens.

1.3 Inflammation

Inflammation is caused by disrupted tissue homeostasis which leads to a biological reaction. This reaction is a tissue destroying process which arises from the utilization of blood derived products (plasma proteins, leukocytes and fluid). Any trauma or injury, exposure of foreign particles leads to the activation of inflammatory responses.

Inflammation has a primary function to isolate or destroy the foreign particles, removal of damaged tissues leading to restoring of tissue homeostasis.

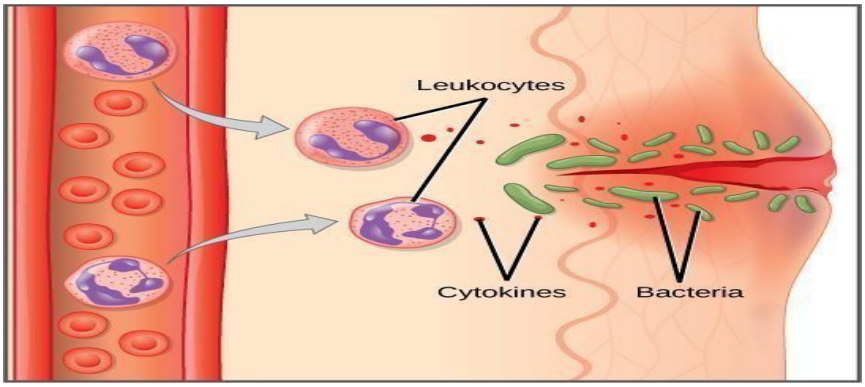

Fig.1 Causes of Inflammation.

2. MATERIALS AND METHODS

2.1 In vitro:

- Celosia cristata Linn. plant is extracted.
- Various concentrations of extract (control) are prepared.
- Acetyl salicylic acid is used as a reference standard.

2.2 Parameters:

- Inhibition of protein denaturation assay
- Membrane stabilization method
- Hypotonic solution induced haemolysis
- Heat induced haemolysis
- Assay of cyclooxygenase and 5-lipooxygenase inhibition
- Anti- cyclooxygenase activity
- Anti-lipoxygenase activity
- Assay of proteinase inhibition
- Hyaluronidase inhibition assay

2.3 Inhibition of protein denaturation assay:

Protein denaturation causes protein molecules to lose their biological capabilities. Protein denaturation has been linked to the development of inflammatory diseases such cancer, diabetes, and rheumatoid arthritis. Therefore, a substance's ability to stop protein denaturation may also aid in stopping inflammatory illnesses.

Either egg albumin or bovine serum albumin (BSA) is utilised as a protein in this experiment. By maintaining the reaction mixture in a water bath at 70°C for 10 minutes, protein denaturation is brought about.

2.4 Preparation of reference drug (Positive Control):

As reference medications, NSAIDs (Acetyl salicylic acid, diclofenac sodium, ibuprofen, or indomethacin) and one steroid (prednisolone) were used. Prednisolone was finely ground into powder. A digital analytical balance (Adam PW 254) was used to weigh the prednisolone medication powder, which was added to 20.0 ml of distilled water after being measured at about 0.2 g. A vortex was used to thoroughly combine the liquids. Ibuprofen underwent a similar process.

2.5 Serial dilutions:

The serial dilution of the C. cristata extract and the reference medications (prednisolone and ibuprofen) were carried out from 1000 g/ml to 0.01 g/ml. Each sample had a total volume of 5.0 ml. 2.8 ml of phosphate-buffered saline (pH 6.4) and 0.2 ml of fresh hen's egg albumin were used to create reaction solutions. The reaction mixtures were then gently blended with 2 ml of C. cristata flower extract from each respective concentration. Prednisolone and ibuprofen, which served as positive controls in this study, underwent a similar approach. Distilled water was also utilized as a negative control.

2.6 Inhibition of protein denaturation:

After 15-20 minutes of incubation at 37°C + 2°C in a water bath, the temperature was raised to 70°C, where the reaction mixture was kept for 5 minutes. The reaction mixture was then left to cool for 15 minutes at room temperature. A colorimeter was used to measure the reaction mixture's absorbance at 660 nm before and after denaturation for each concentration (1 g/ml, 100 g/ml, 10 g/ml, 1 g/ml, 0.1 g/ml, and 0.01 g/ml). The mean absorbance was calculated after three iterations of each test. Using the following formula, the percentage of protein inhibition was calculated with respect to the control.
The experiment is done in triplicate, and the following equations are used to calculate the percent inhibition for protein denaturation:
% Denaturation inhibition = (1-D/C) X 100.

Where C is the absorbance of the negative control (without the test sample or reference drug) and D is the absorbance of the test sample.

2.7 Membrane stabilization method:

Lysosomal membrane lysis, which releases the enzyme components that cause a number of illnesses, may take place during inflammation. Non-steroidal anti-inflammatory medicines (NSAIDs) work by stabilizing the lysosomal membranes or by preventing the release of lysosomal enzymes, respectively. When red blood cells are exposed to harmful substances, hemolysis and the oxidation of hemoglobin may result in the lysis of the red blood cell membranes.
Hypotonic medium, heat, methyl salicylate, and phenyl hydrazine are harmful chemicals. The suppression of hypotonicity and heat-induced lysis of red blood cell membrane will be used as a gauge of the mechanism of anti-inflammatory effect because human red blood cell membranes are comparable to lysosomal membrane.
The excessive fluid buildup in red blood cells brought on by a hypotonic solution leads the membrane to rupture. The hemolysis of red blood cells finally occurs.
Red cell membrane damage makes the cell more vulnerable to secondary damage from lipid peroxidation caused by free radicals. In the lysosomes of neutrophils that have been activated, there are bacterial enzymes and proteases.
Following extracellular release, lysosomal component leakage causes further tissue inflammation and injury.
Lysosome membrane stabilization is therefore crucial for regulating the inflammatory response. This will result in the prevention of constituent leakage.
Through heat-induced hemolysis and hypotonic solution-induced hemolysis utilizing human erythrocytes, rat erythrocytes, or mouse erythrocytes, the membrane stabilizing action of the extracts can be identified. Because the erythrocyte membrane is similar to the lysosomal membrane, extracts' effects on erythrocyte stabilization also apply to lysosomal membrane stabilization.
The erythrocyte suspension was initially prepared by all staff for testing the aforementioned techniques.

2.8 Hypotonic solution induced hemolysis:

By mixing 154 mM NaCl with 10 mM sodium phosphite solution, which served as the solution's buffer, the isotonic solution with a pH buffer of 7.4 was created. The control sample was combined with drug-free solution, while the stock RBC suspension was mixed with 50 l of the hypotonic solution containing the C. cristata ethanolic extract at concentrations of 50, 100, 200, 400, and 800 g/ml.
The entire mixture was centrifuged at 5000 rpm for five minutes after incubation at room temperature for ten minutes. At 540 nm, the absorbance of the supernatant was measured using a UV spectrophotometer. The reference standard was 200 g/ml of acetyl salicylic acid (ASA).

The following equations determine the degree of stabilization or protection of the red blood cell membrane.

$$\% \text{ protection} = 100 - \frac{\text{Optical density of drug treated sample}}{\text{Optical density of control}} \times 100$$

2.9 Heat induced heamolysis:

This procedure uses heat to separate the sample via incubation. The reaction mixture (2 ml) is made up of either 5 ml of isotonic buffer containing 2.0 mg/ ml of various extractives and 10% erythrocyte suspension, or 1 ml of test material at various concentrations (100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, 500 µg/ml & 600 µg/ml).
In the control test tube, 1 ml or 30 µ L of vehicle is inserted in place of the test sample. 50 - 400µg/ml Standard medications include acetyl salicylic acid (aspirin) or diclofenac sodium. By inversion, this reaction mixture is gently agitated.
All of the centrifuge tubes holding the reaction mixture are incubated in a water bath at 60°C, 56°C, or 54°C for 30 or 20 minutes, respectively.
The aforesaid reaction combination has been duplicated by some personnel, and the other pair is kept at 0 to 5 degrees Celsius in an ice bath.
The tubes are cooled under running water at the conclusion of the incubatory period. The reaction mixture was centrifuged for 5 minutes at 3000 rpm, 10 minutes at 2500 rpm, 26 minutes at 1300 rpm, or 18 minutes at 18 rpm, and the supernatants' absorbance was measured at 560 nm. For each test sample, the experiment is carried out three times.

The percentage inhibition of haemolysis is calculated as follows: Percentage inhibition of haemolysis = $\frac{\text{Absorbance control} - \text{Absorbance test}}{\text{Absorbance control}} \times 100$

% Inhibition of haemolysis = $100 \times [1-(OD1-OD2)/ (OD3-OD1)]$.

Were,
OD1 = Optical density of unheated test sample OD2 = Optical density of heated test sample OD3 = Optical density of heated control sample

2.10 Assay of cyclooxygenase and 5-lipooxegenase inhibition:

The enzymes cyclooxygenase and 5-LOX are used in the body's two primary metabolic pathways to break down arachidonic acid (AA).
Prostaglandins and thromboxane are produced by the COX route, whereas eicosanoids and leukotrienes are produced by the 5-LOX system.
It has been proposed that blocking both pathways could provide synergistic effects and obtain the best anti-inflammatory efficacy by preventing the formation of prostaglandins and leukotriene. Other invitro assay for cyclooxygenase activity and inhibitors characterization methods are:

Oxygraph Assay Peroxidase Assay

Kinetic Assay PGE2
ELISA Assay
Endpoint Assay

Consequently, the simultaneous inhibition of the COX and 5-LOX pathways may result in a broader array of anti-inflammatory actions.

2.11 Anti- cyclooxygenase activity:

The colorimetric COX (ovine) inhibitor screening assay kit is used to determine the results of spectrophotometer is used to assess the colour change in the chromogenic test, which is based on the oxidation of TMPD during the reduction of PG-G2 (prostaglandin-G2) to PG- H2.

Indomethacin or asprin are used as reference drugs, and other chemicals are added in accordance with the manufacturer's instructions. In brief, the assay mix consists of test substances in varying quantities. The plate is shaken briefly and incubated at 25°C for 5 minutes.

Arachidonic acid (20 l) and TMPD (20 l) are added to each well to start the reaction. The plate is shaken briefly and incubated at 25°C for 5 minutes. At 590 nm, the absorbance is measured using a microplate reader. There are three duplicates of each reaction.

2.12 Anti-lipoxygenase activity:

Linoleic acid was used as the substrate and lipoxidase as the enzyme in studies on anti- lipoxygenase action. As an enzyme, human recombinant lipoxygenase or soybean lipoxygenase can be employed. The procedure for analysing this test is summarised below. 20 ml of soybean lipoxygenase solution (167 U/ml) and 160 ml of sodium phosphate buffer (100 mM, pH 8.0) are combined in a reaction mixture, which is then incubated at 25 °C for 10 min. 10 l of the substrate, which is a solution of sodium linoleic acid, are added to start the reaction.

Using a UV-vis spectrophotometer, the absorbance is measured at 234 nm over the course of three minutes, once each minute.

As a positive reference drug, nordihydroguaiaretic acid (NDGA), indomethacin, or quercetin are employed. The control is made by leaving out the medication or plant extract from the mixture above. A dose-response curve was plotted to establish the half maximal inhibitory concentration (IC50) values. IC50 is defined as the concentration sufficient to obtain 50% of a maximum scavenging capacity. All the reactions are performed in triplicates.

The percentage of inhibition is calculated as:

$$\% \text{ inhibition} = \frac{\text{Abs Control} - \text{Abs extract}}{\text{Abs control}} \times 100$$

30

2.13 Assay of proteinase inhibition:

It has been proven that proteinase is responsible for the tissue damage caused by inflammatory responses. In neutrophil lysosomal granules, proteinases are found in large quantities. As a result, proteinase inhibitors produce at a high level. The anti-inflammatory action of protinease inhibitors depends on direct inhibiton of neutral proteinases released from PMNs in the inflammatory locus.

Proteinase or trypsin are examples of enzymes that can be employed in this assay, whereas casein and bovine serum albumin are examples of proteins. The reaction mixture (2 ml) contains Diclofenac sodium at concentrations ranging from 100 to 600 µg/ml, 0.06 mg of proteinase or trypsin, and 1 ml of 20 mM Tris HCl buffer (pH 7.4). After the mixture has been incubated at 37°C for 5 minutes, 1 ml of either 4% (w/v) or 0.8% (w/v) bovine serum albumin is added.

An extra 20 minutes of incubation are given to the mixture. To stop the process, 2 ml of either 5% or 70% trichloroacetic acid (TCA) is added. Cloudy solution is centrifuged at 3000 or 2500 rpm for 10 or 5 minutes, respectively, and the absorbance of the supernatant is assessed at 210 or 217 nm, respectively,

against a buffer used as a blank. Three duplicates of the experiment are run.
The percentage inhibition of proteinase inhibitory activity is calculated using the following equation.
Percentage inhibition = (Abs control –Abs sample) X100/ Abs control.

2.14 Hyaluronidase inhibition assay:
The hyaluronidase Inhibition Assay is done by the hyaluronidase inhibitor screening assay kit which uses a two -step turbidimetric reaction to measure hyaluronidase activity by the amount of hyaluronic acid that is hydrolyzed. One of the enzymes involved in tissue remodeling during inflammation is hyaluronidase. Hyaluronic acid is degraded in both human and animal tissues. Hyaluronic acid has a significant role in the connective tissues' extracellular matrix.
By reducing the viscosity of hyaluronic acid, the enzyme is known to play a role in allergic reactions, inflammation, and increasing vascular membrane permeability. Hyaluronic acid is the substrate in this test, and the assay is started after the substrate has been added.

31 Samples of plant extract (5 mg) are dissolved in 250 µL of dimethylsulphoxide. The samples are produced by dissolving in sodium phosphate buffer (200 mM, pH 7) at different concentrations (100, 200, 300, 400, and 500 µg/mL). Sample solution (25 µL) is combined with hyaluronidase (4U/mL, 100 L), which is then incubated at 37 °C for 10 minutes.
To activate the enzyme, some researchers have added calcium chloride at concentrations of
2.5 mM in 1.2 mL or 12.5 mM in 50 mL, and the mixture is once again incubated at 37·C for 20 minutes. Some researchers didn't include CaCl2 in their experiments.
The reaction is then started by adding the substrate, a 100 µL, 0.03% solution of hyaluronic acid in 300mM sodium phosphate with a pH of 5.4, and incubating it for 45 minutes at 37·C. The remaining undigested hyaluronic acid is subsequently precipitated using acid albumin solution (0.1 percent bovine serum albumin in 24 milligrams of sodium acetate, 1 millilitre). At 600 nm, absorbance is measured following a 10-minute incubation period at room temperature. The absorbance reading without any enzyme is used as a reference point to determine the level of maximal inhibition. To confirm the effectiveness of the assay, either quercetin or indomethacin is employed as the positive control. The test is carried out three times.
The percentage of hyaluronidase inhibition is determined using the following formula: Percentage inhibition = Abs sample/ Abs control X100.
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3 RESULTS:

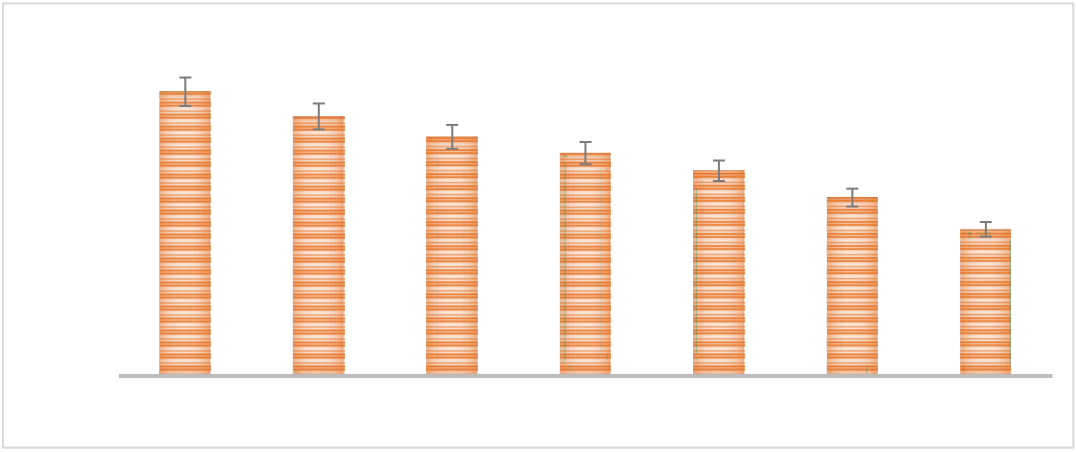
3.1 HRBC membrane stabilization method

In the present study, the observation revealed the increase in absorbance of the control group when compared the different test concentrated treated groups as well as standard groups. There was significant ($p < 0.0001$) decrease in absorbance of the different test concentrations treated groups such as 5, 10, 50, 100,250 and 500 µg/ml with respectively Test-1, Test-2, Tes- 3, Test- 4 of CC when compared to vehicle treated group.
The percentage of HRBC membrane stabilization of CC compound was identified as 8.45, 15.67, 21.39, 27.61, 37.06 and 48.25% with respectively Test-1 to Test-4 samples in dose dependent manner, whereas, standard diclofenac showed 55.22, 67.41, 68.65 and 73.63%respectably protecting HRBC in hypotonic solution.
Anti-inflammatory Effect of Standard drug (Aspirin) by using HRBC Membrane Stabilization method

S. No.	Sample	Treatment	Absorbance (Mean + SEM)	% Inhibition of Haemolysis
1.	Control	HRBC suspension + Vehicle	0.403±0.001	-

2.	Test - 1	HRBC suspension + Aspirin 100 µg/ml	0.18 + 0.006***	55.22
3.	Test - 2	HRBC suspension + Aspirin 200 µg/ml	0.131 + 0.002***	67.41
4.	Test - 3	HRBC suspension + Aspirin 400µg/ml	0.126 + 0.001***	68.65
5.	Test - 4	HRBC suspension + Aspirin 800µg/ml	0.106 + 0.002***	73.63

*** p<0.001, when compared to control group



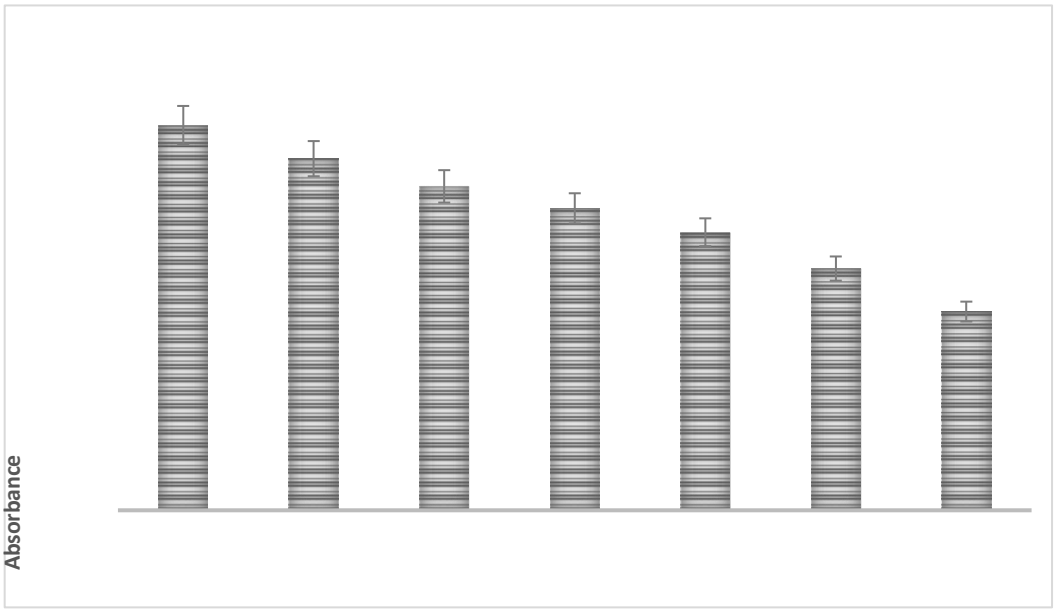
3.2 Protein (Egg Albumin) Denaturation Method

The standard drug diclofenac sodium treated groups showed a significant ($p < 0.001$) percentage inhibition of protein denaturation against heat as 85.08, 8.13, 91.52, 92.69, 93.56 and 94.44 % with respectively Test-1, Test-2, Tes-3, Test-4, Test-5 and Test-6 (5, 10, 50, 100,250 and 500 µg/ml) groups of standards (Table-3) as well as the test treated groups showed as 73.39, 75.14, 77.77, 81.87, 83.04and 88.01%with respectively Test-1 to Tet-6 groups

S. No.	**Sample**	**Treatment**	**Absorbance (Mean + SEM)**	**% Inhibition of protein denaturation**
1.	Control	Protein + PBS + Vehicle	0.342	-
2.	Test - 1	Protein + PBS + Diclofenac sodium 100µg/ml	0.025 + 0.0002***	91.52
3.	Test - 2	Protein + PBS + Diclofenac sodium 200µg/ml	0.029 + 0.0003***	92.69
4.	Test - 3	Protein + PBS + Diclofenac sodium 400µg/ml	0.044 + 0.000***	93.56
5.	Test - 4	Protein + PBS + Diclofenac sodium 800µg/ml	0.051 + 0.0001***	94.44

*** p <0.0001, when compared to control group

The inhibitory effect of different concentration of CC on protein denaturation at a concentration range of 100, 200, 400, 800, 1000 µg/ml and standard (aspirin) 100, 200, 400, 800, 1000 µg/ml showed significant inhibition of denaturation of egg albumin in concentration dependent manner. In this study, 4-benzylpiperidine showed maximum inhibition, 73.80% at 1000 µg/ml. Aspirin, a standard anti-inflammatory drug showed the maximum inhibition, 75.52% at the concentration of 1000 µg/ml.



3.3 Proteinase inhibitory action:

The inhibitory effect of different concentration of 4-benzylpiperidine on proteinase inhibitory action at a concentration range of 100, 200, 400, 800, 1000 µg/ml and standard (aspirin) 100, 200, 400, 800, 1000 µg/ml showed significant inhibition of proteinase inhibitory action in concentration dependent manner. In another study, it was reported that Enicostemma axillare methanol extract showed maximum inhibition of 53% at 500 µg/ml and aspirin showed 55% at 100 µg/ml [16]. In this study, 4-benzylpiperidine exhibited significant antiproteinase activity at different concentrations as shown in Table 2. It showed maximum inhibition of 57.77% at 1000 µg/ml and aspirin showed maximum inhibition of 77.77% at 1000 µg/ml.

Table 1. Anti-cyclo oxygenase activity and lipoxygenase activity:

Treatments	Concentration (µg/ml)	Absorbance (660 nm)	Inhibition %
Control		0.42±0.04	
C.cristata	00	0.24±	47.85
	00	0.20±	52.
	00	0.17±	59.
	00	0.14±	66.
Aspirin (sta d)	00	0.19±	54.
	200	0.17±0.02	59.52
	400	0.14±0.05	66.19
	800	0.12±0.01	69.09

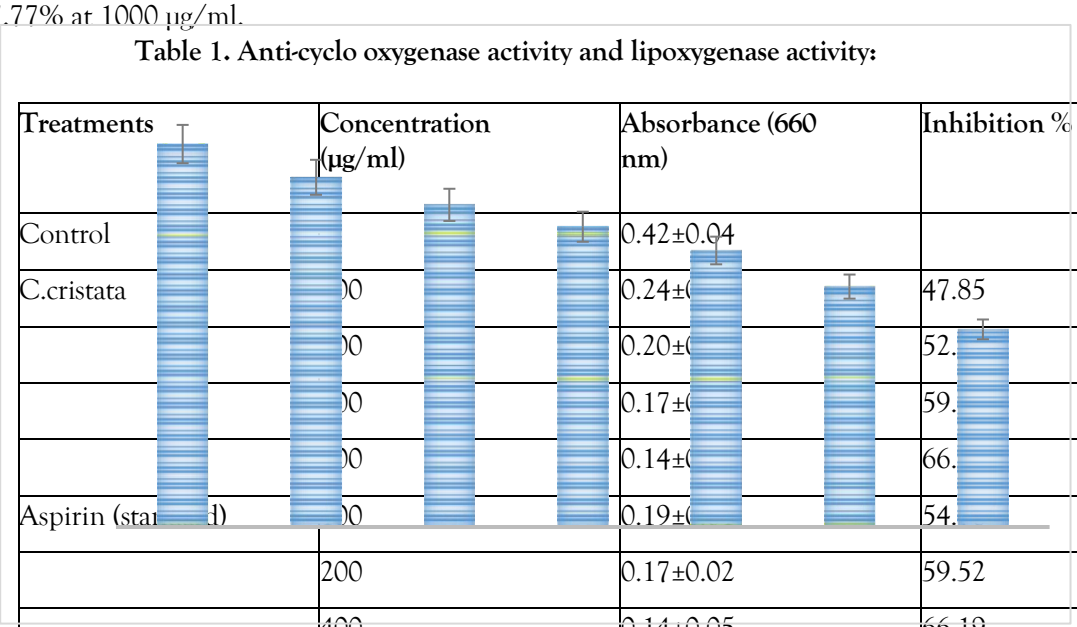


Table2. Assay of proteinase inhibition:

Treatments	Concentration (µg/ml)	Absorbance (660 nm)	Inhibition %
Control		0.45±0.09	
C.cristata	100	0.37±0.06	17.70
	200	0.34±0.01	24.44
	400	0.29±0.02	35.50
	800	0.22±0.06	51.11
Aspirin (standard)	100	0.21±0.06	54.76
	200	0.17±0.01	66.66
	400	0.15±0.01	73.33
	800	0.14±0.01	77.77

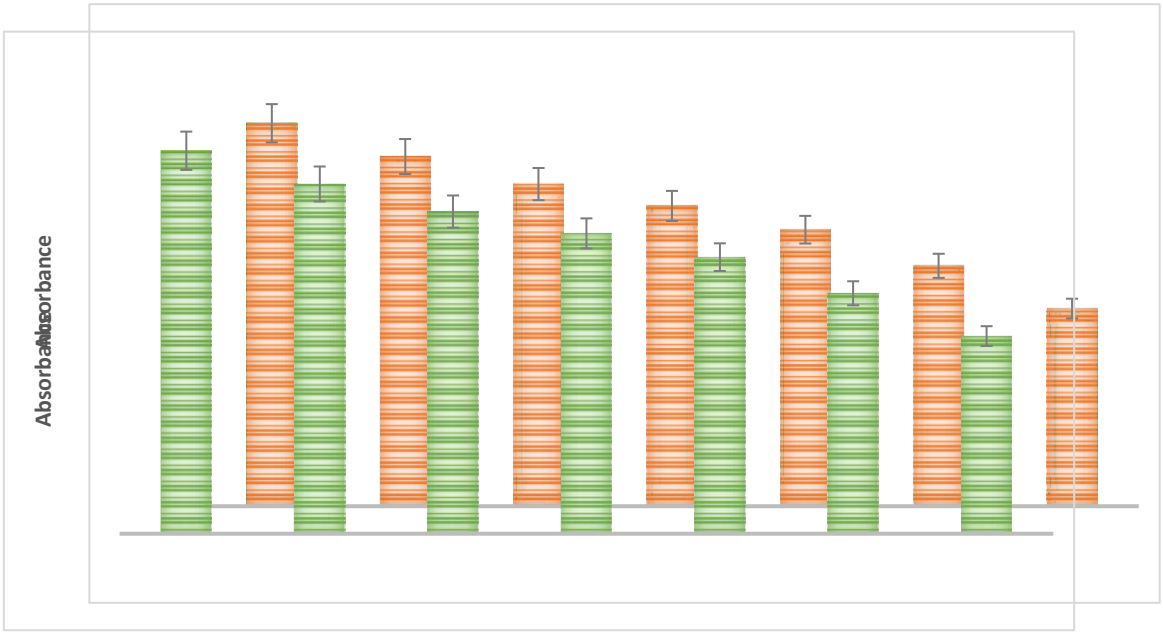


Table.3 Hyaluronidase inhibition assay:

Treatments	Concentration (µg/ml)	Absorbance (660 nm)	Inhibition %
Control		0.42±0.04	
C.cristata	100	0.20±0.03	47.85
	200	0.19±0.03	52.38
	400	0.17±0.01	59.52
	800	0.14±0.05	66.66
Aspirin (standard)	100	0.19±0.01	54.76
	200	0.17±0.02	59.52

	400	0.14±0.05	66.19
	800	0.12±0.01	69.09

4. CONCLUSION:

Healing with the medicinal plant is the old technique of the human beings themselves. From the very past, there is a connective link established between man and his search for medicine, this is proof from various findings such as from hand written documents and old herbariums collection. Bark, seeds, fruits, leaves and other plant parts are used as medicine for many years due to the experience to cure chronic diseases. Chinese Traditional Medicine, Ayurvedic Medicine and Unani Medicine are agreed that the potent bioactive chemical substances present in Celosia cristata. he presents study demonstrates that the methanolic extract of Celosia cristata Linn. possesses significant anti-inflammatory activity in vitro, as evidenced by its ability to inhibit protein denaturation and stabilize red blood cell membranes. The extract showed dose-dependent effects comparable to the standard drug, diclofenac sodium. These findings support the traditional use of Celosia cristata in the treatment of inflammatory conditions and suggest that its bioactive constituents—likely flavonoids, tannins, and saponins—contribute to the observed activity. Further in vivo studies and phytochemical investigations are recommended to isolate the active compounds and elucidate their mechanisms of action.

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