EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 12
Issue: 1
Year: 2025

PHYTOCHEMICAL INVESTIGATION AND ASSESSMENT OF *ANONNA CHERIMOLA* MILL. LEAVES FOR *IN-VITRO* ANTI-INFLAMMATORY ACTIVITY

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Article Received on 26/11/2024 **Article Revised on 15/12/2024** **Article Accepted on 05/01/2025**

ABSTRACT

Aim: Inflammation is the immune system response to harmful stimuli, such as pathogens, damaged cells, toxic compounds, or irradiation and acts by removing injurious stimuli and initiating the healing process. *Annona cherimola* Mill. Leaves belonging to the family *Annonaceae* contains biologically active compounds such as flavonoids, terpenoids, saponins, tannins, alkaloids, anthraquinones and essential oils etc. which are essential for anti-inflammatory activity. *Annona cherimola* Mill. Leaves has been widely used in Mexican traditional medicine as a natural remedy for treatment of various ailments, including cough, fever and headache as well as gastrointestinal and skin diseases. However, the impact of *Annona cherimola* Mill. Leaves on anti-inflammatory activity by in-vitro studies has not been reported till date. Therefore, the present study was aimed for phytochemical investigation and assessment of *Annona cherimola* Mill. Leaves for *in-vitro* anti-inflammatory properties. **Methods and Materials:** The *in-vitro* anti-inflammatory activity was evaluated by using HRBC membrane stabilization method and Protein denaturation Method. **Results**: Phytochemical investigation of *Annona cherimola* Mill. Leaves revealed that ethanolic extract contains alkaloids, carbohydrates, phenols, flavonoids, glycosides, saponins, proteins and tannins. The *in-vitro* results of HRBC membrane method showed significant anti-inflammatory activity at 400µg/mL i.e. 70.88% which was comparable to that of the standard Diclofenac sodium (400µg/mL) which showed 82.20% stabilization and the protein denaturation method revealed ethanolic extract of *Annona cherimola* Mill. Leaves showed maximum inhibition of 69.92% at 400 µg/ml concentration when compared to standard Diclofenac sodium i.e. 85.47% at a concentration of 400 µg/ml. **Conclusion:** These findings suggest that the bioactive compounds in the plant could be developed for therapeutic applications targeting inflammatory conditions.

KEYWORDS: *Annona cherimola* Mill., In-vitro anti-inflammatory activity, Diclofenac sodium.

INTRODUCTION

Inflammation is the immune system response to harmful stimuli, such as pathogens, damaged cells, toxic compounds, or irradiation[1] and acts by removing injurious stimuli and initiating the healing process.[2]

Based on visual observation, the ancients characterized inflammation by five cardinal signs, namely redness (rubor), swelling (tumor), heat (Calor; only applicable to the body's extremities), pain (dolor) and loss of function (function-laesa). The first four of these signs were named by Celsus in ancient Rome (30-38 B.C.) and the last by Galen (A.D 130-200).[3]

The classical description of inflammation accounts for the visual changes seen. Thus, the sensation of heat is caused by the increased movement of blood through dilated vessels into the environmentally cooled extremities, also resulting in the increased redness (due to the additional number of erythrocytes passing through the area). The swelling (oedema) is the result of increased passage of fluid from dilated and permeable blood vessels into the surrounding tissues, infiltration of cells into the damaged area and in prolonged inflammatory responses deposition of connective tissue. Pain is due to the direct effects of mediators either from initial damage or that resulting from the inflammatory response itself and the stretching of sensory nerves due to oedema. The loss of function refers to either simple loss of mobility in a joint due to the oedema and pain or to the replacement of functional cells with scar tissue.[4]

Inflammation is an organism's protective response to the presence of several agents such as pathogenic microorganisms (bacteria, fungi, and viruses) that cause a disturbance to tissue homeostasis. Especially bacteria

can release damage-associated molecular patterns or molecular associated microorganism patterns, which are detected by tissue resident macrophages. These immune cells differentiate and initiate an inflammatory response. The inflammation's primary purpose is to eliminate invading microorganisms or damaged cells and also to promote tissue repair and regeneration, mediated by conventional inflammatory cytokines.[5]

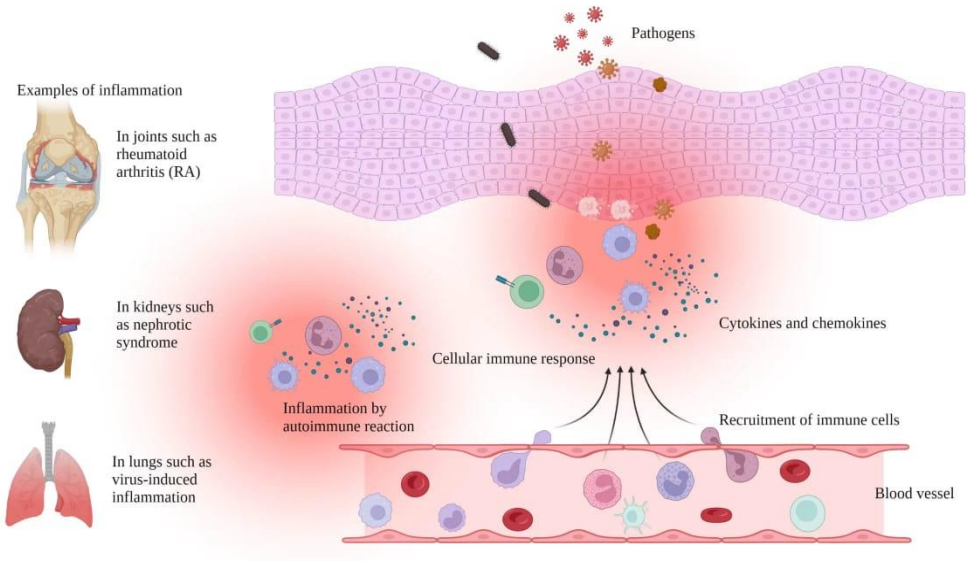

Figure-1: Inflammation Process.

The triggering of inflammation leads to the release of inflammatory mediators which can either be cell-derived or plasma-derived. Cell-derived mediators include: vasoactive amines, cytokines, nitric oxide, prostaglandins, thromboxane A_2 (T_xA_2), prostacyclin, leukotrienes and platelet- activating factor. They are released from inflammatory cells such as leukocytes, platelets and vascular endothelial cells.[6]

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. Protein denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compounds, such as strong acid or base, a concentrated inorganic salt, an organic solvent or heat. Most biological proteins lose their biological function when denatured. Denaturation of protein is a well- documented cause of inflammation.[7-8]

Table 1: Inflammatory Mediators and Their Actions.

Mediators	Source(s)	Actions
Cell Derived		
Histamine	Mast cells, basophils, platelets	Vasodilation, increased vascular permeability, endothelial activation
Prostaglandin	Leukocytes, mast cells	Vasodilation, pain, fever
Serotonin	Platelets	Vasodilation, increased vascular permeability
Leukotrienes	Leukocytes, mast cells	Increased vascular permeability, chemotaxis, leukocyte adhesion and activation
Platelet-activating factor	Leukocytes, Mast cells	Vasodilation, increased vascular permeability, leukocyte adhesion, chemotaxis, degranulation, oxidative burst
Cytokines (TNF, IL-1, IL-6)	Macrophages, endothelial cells, mast cells	Local: Endothelial activation (expression of adhesion molecules). Systemic: Metabolic abnormalities, hypotension(shock), Fever
Plasma-Protein Derived		
Complement products (C5a, C3a, C4a)	Plasma (produced in the liver)	Leukocyte chemotaxis and activation, vasodilation (mast cell stimulation)
Kinins	Plasma (produced in the liver)	Increased vascular permeability, smooth muscle contraction, pain and vasodilation
Proteases activated during coagulation	Plasma (produced in the liver)	Endothelial activation, leukocyte recruitment

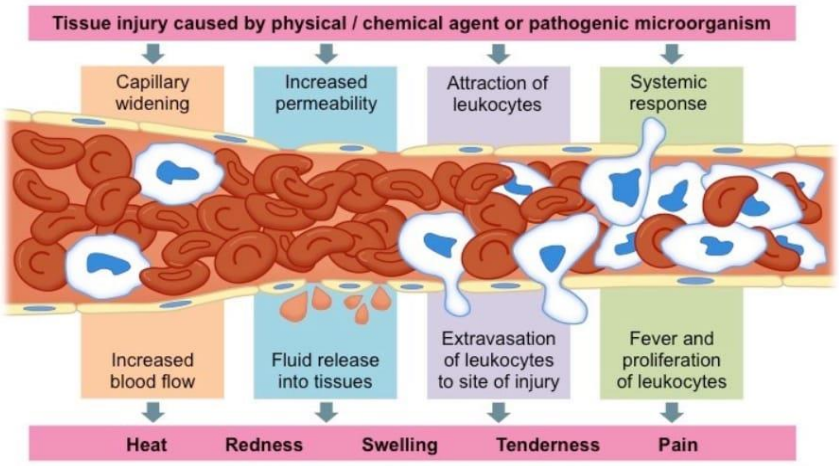

Figure-2: Tissue injury caused by physical/ chemical agent or pathogenic.

Microorganism
Inflammation can be classified as either acute or chronic.

Acute Inflammation
Acute inflammation is associated with increased vascular permeability, capillary infiltration and emigration of leukocytes. An injury or illness can involve acute or short-term inflammation.

Acute inflammation can result from

- Exposure to a substance such as a bee sting or dust.
- An infection.
- An injury.

Features
a) Accumulation of fluid and plasma at the affected site;
b) Intravascular activation of platelets; and
c) Polymorphonuclear neutrophils as inflammatory cells
Symptoms of acute inflammation last a few days. Subacute inflammation lasts 2–6 weeks.

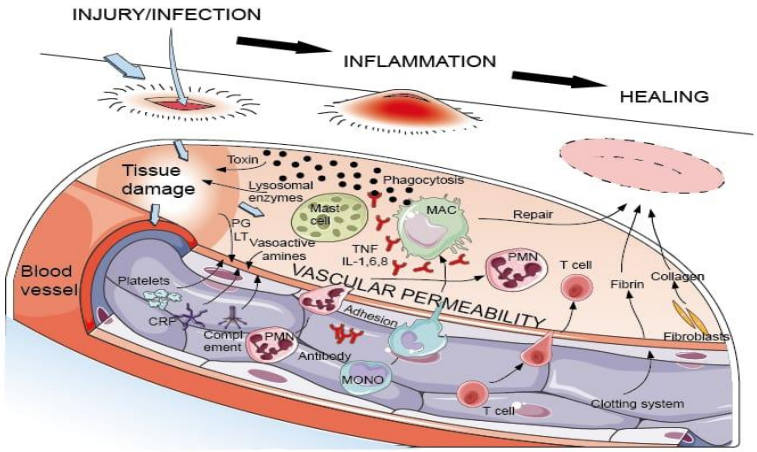

Figure- 3: Acute Inflammation.

Chronic Inflammation
Chronic inflammation is associated within filtration of mononuclear immune cells, macrophages, monocytes, neutrophils, fibroblast activation, proliferation (angiogenesis) and fibrosis. Chronic inflammation can continue for months or years.

It either has or may have links to various diseases, such as:

- Diabetes
- Cardiovascular disease (CVD)
- Arthritis and other joint diseases
- Allergies
- Chronic obstructive pulmonary disease (COPD)
- Rheumatoid arthritis

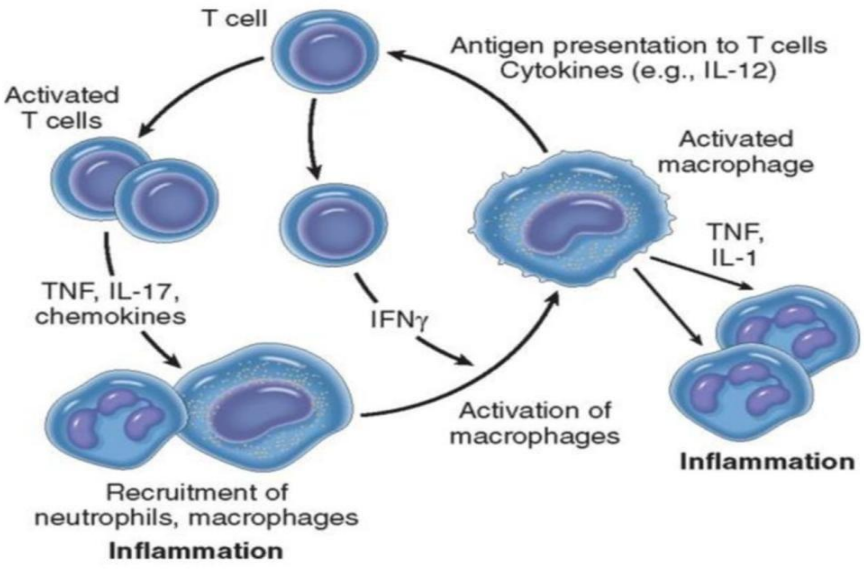

Figure- 4: Chronic Inflammation.

Features

Depending up on the tissues and causative organism, there may be difference in chronic inflammation, but there are some basic similarities amongst various types of chronic inflammation.

a) Mononuclear cell infiltration
b) Tissue destruction or necrosis
c) Proliferative changes

The symptoms will depend on the disease, but they may include pain and fatigue.[9-10]

Anti-inflammatory Drugs

CLASSIFICATION OF ANTI-INFLAMMATORY DRUGS

I. Steroidal Anti-inflammatory Drugs

- Natural Opium: Alkaloids Morphine, Codeine
- Semisynthetic Opiates: Diacetyl Morthine (Heroin) Ethyl Morphine, Pholcodeine. Many others like Hydromorphone, oxymorphone, Hydrocodone, Oxy codone
- Synthetic Opiodis: Pethidine (mepiridine), Fentanyl, Methadone, Dextropropoxyphene, Ethoheptazine. Many others like Alphoprodine, Anileridine, Dextromoramide, Alfentanil, Sufentanil.

II. Non-steroidal Anti-inflammatory Drugs Classification

A. Analgesics and Anti-inflammatory

- Salicylates: Aspirin, Salicylamide, Benoxylate, Difulnisal
- Pyrazolone derivatives: Phenyl butazone, Oxyphenbutazone
- Indole derivatives: Indomethacin, Sulindac
- Propionic Acid Derivatives: Naproxen, Ketoprofen, Fenoprofen, Flubiprofen

B. Analgesic but poor anti-inflammatory

- Para-Aminophenol derivatives : Paracetamol
- Pyrazolone derivatives: Propyphenazone
- Benzoxazocine derivative: Nefopam

Some of these are also used to treat conditions such as cancer or inflammatory bowel disease or to prevent organ rejection after a transplant. But when "chemotherapy" types of medications (such as methotrexate or cyclophosphamide) are used to treat inflammatory diseases, they tend to have lower doses and less risk of side effects than when they're prescribed for cancer treatment.[11]

Side effects

- At higher doses, NSAIDS raise risk of having heart stroke and stomach bleeding.
- Stomach pain, heartburn, gas, bloating, nausea and vomiting, diarrhoea and constipation.
- Stomach ulcers
- A tendency to bleed more, especially when taking aspirin.
- Headaches and dizziness
- Feeling lightheaded
- Ringing in the ears
- Allergic reactions such as rashes, wheezing, and throat swelling
- Liver or kidney problems
- High blood pressure
- Leg swelling
- Balance issues

Most commonly used drugs in the treatment of inflammation are NSAIDS.

Mechanism of action of NSAIDS

During inflammation arachidonic acid liberated from membrane phospholipids is converted to prostaglandins (PGs), catalyzed by the enzyme cyclo-oxygenase (COX). These prostaglandins produce hyperalgesia-they sensitize the nerve endings to pain caused by other mediators of inflammation like bradykinin and histamine.

NSAIDs inhibit the PG synthesis by inhibiting the enzyme cyclo-oxygenase and is the major mechanism responsible for pharmacological effects of aspirin.

Aspirin is an irreversible inhibitor of COX (acetylates COX) while the others are reversible competitive COX inhibitors. There are two forms of cyclo-oxygenase, viz. COX-1 and COX-2. COX-1 is found in most of the normal cells (constitutive) and is involved in maintaining tissue homeostasis. COX-2 is induced in the inflammatory cells by cytokines and other mediators of inflammation. This COX-2 catalyses the synthesis of prostanoids which are the mediators of inflammation. Most NSAIDs inhibit both COX-1 and COX-2 while some newer agents like celecoxib and rofecoxib selectively inhibit only COX-2.[12]

Inflammation is currently treated by NSAIDs. Unfortunately, these drugs cause increased risk of blood clot resulting in heart attacks and strokes. Therefore, the developments of potent anti- inflammatory drugs from the natural products are now under considerations. Natural products are a rich source for the discovery of new drugs because of their chemical diversity. A natural product from medicinal plants plays a major role in curing many diseases associated with inflammation. The conventional drug available in the market to treat inflammation produces various side effects.

Due to these side-effects, there is a need for the search of newer drugs with less or no side-effects. There are hundreds of phytoconstituents reported to have many pharmacological activities although most of these reports are of academic interest and very few find entry in clinical trials.[13] The plant is reported to contain alkaloids, flavonoids, glycosides, saponins, tannins, carbohydrates, proteins, phenolic compounds, phytosterols and amino acids.[14] Flavonoids, terpenoids, saponins, tannins, alkaloids, anthraquinones and essential oils play an important role in the treatment of inflammatory diseases.[15]

List of plants that show Anti-inflammatory activity are.

- Cassia angustifolia
- Coriandrum sativum
- Foeniculum vulgare
- Glycyrrhiza glabra
- Hibiscus rosa-Sinensis
- Cissus quadrangularis
- Plumbago zeylanica
- Terminalia bellarica
- Terminalia chebulla

PLANT PROFILE OF *ANNONA CHERIMOLA* MILL

Botanical Name : *Annona cherimola* Mill.
Synonym : Custard Apple
Family : Annonaceae[16]

Figure- 5: *Annona cherimola* fruit and leaves.

BOTANICAL DESCRIPTION

Domain : Eukaryote
Kingdom : Plantae
Phylum : Tracheophyta
Class : Magnoliopsida
Order : Magnoliales
Family : Annonaceae[16]
Genus : Annona
Botanical name : *Annona cherimola* Mill.

VERNACULAR NAMES

English : Cherimoya, Cherimolla, Custard apple
Telugu : Hanuman phalamu
Hindi : Hanuman phal, Marytiphal
Kannada : Hanuman Phala

GEOGRAPHICAL DESCRIPTION

Annona cherimola Mill. Leaves is a fairly dense, fast-growing, woody, briefly deciduous but mostly evergreen, low-branched, spreading tree or shrub, 5 to 9 m (16 to 30

ft) tall. Mature branches are sappy and woody. The attractive leaves are single and alternate, 2-ranked with minutely hairy petioles 6 to 12.5 mm long; ovate to elliptic or ovate-lanceolate, short blunt-pointed at the apex; dark green and slightly hairy on the upper surface, velvety on the underside; 7.5 to 15 cm long, 3.8 to 8.9 cm wide. The fragrant flowers are borne solitary or in groups of 2 or 3 on short hairy stalks along the branches have 3 outer greenish, fleshy, oblong, downy petals to 3 cm long and 3 smaller pinkish inner petals.

CULTIVATION

Soil type: The cherimoya tree performs well on a wide range of soil types from light to heavy but seems to do best on a medium soil of moderate fertility. In Argentina, it makes excellent growth on rock-strewn loose sandy loam 60 to 90 cm above a gravel subsoil. The optimum pH ranges from 6.5 to 7.6.

CHEMICAL CONSTITUENTS

Several phytochemical surveys have been published including the random sampling approach which involved some plant accessions collected from all parts of the world. The major phytochemical constituents such as flavonoids, tannins, unsaturated sterols, terpenoids, essential oils etc. have also been reported.[17-18]

Several research works have identified the phytochemicals and bioactive compounds that can be extracted from the plant parts verifying their antioxidant, anti-degenerative, anti-cancer, chronic diseases and anti-microbial properties. Acetogenins, polyphenols, and terpenes are important components of *Annona cherimola* Mill. Leaves. Although they are widely present in different plant organs like seeds (polyphenols), leaves (terpenes) and both leaves and fruits (acerogenins) of cherimoya. *Annona cherimola* Mill. Leaves contains lutein, is a carotenoid Lutein contains anti-inflammatory qualities.

In the ethnopharmacological perspective, Annona *cherimola* Mill. Leaves has been widely used in Mexican traditional medicine as a natural remedy for treatment of various ailments, including cough, fever and headache as well as gastrointestinal and skin diseases.[19] The Mexican tradition has also handed down the use of *Annona cherimola* Mill. leaves as a natural remedy for diabetes.[20]

Activities Reported

Anti-protozoal, anti-hyperlipidemic, anti-hyperglycemic, anti-diabetic, anti-degenerative, anti-cancer, chronic diseases, hepatoprotective and anti-microbial properties.

MATERIALS AND METHODS

Collection of plant material

Annona cherimola Mill. leaves powder was procured from Sri Venkateshwara University, Tirupati and authenticated.

Preparation of plant extract

The powder obtained was subjected to successive Soxhlet extraction method, 10gm of powdered leaves was added to 150 ml of the selected solvent (ethanol). The extraction was made at solvent boiling point for 48 hrs to ensure optimal compound extraction until the sample ran out. Percentage yield of corresponding extract was calculated.

Yield of the Extract

The yield of the extracts was calculated by using the formula

$$\text{Total Yield (\%)} = \frac{\text{Total mass of extract}}{\text{Total mass of sample}} x100$$

Preliminary Phytochemical Screening

Standard screening tests of the Ethanolic extract of *Annona cherimola Mill.* leaves was carried out for various plant constituents. The crude extract was screened for the presence or absence of secondary metabolites using standard procedures.[21]

In vitro methods

- **HRBC Membrane Stabilization Method**

Human red blood cell (HRBC) membrane stabilization method was used for the study of *In-vitro* anti-inflammatory activity. The blood was collected from blood bank and mixed with equal volume of Alsever solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid and 0.42% NaCl). Then it was centrifuged at 3,000 rpm and the packed cells were washed with isosaline (Nacl, pH 7.2) and a 10% suspension was made. To 0.5ml of test samples, 1ml of phosphate buffer (0.15 M. pH 7.4), 2 ml hyposaline (0.36% NaCl) and 0.5 ml of HRBC suspension were added. The solution was incubated at 37°C for 30min and centrifuged at 3,000 rpm for 20 mins. The content of the supernatant solution was absorbed spectrophotometrically at 560 nm. Control was taken without the test sample. Diclofenac (100 to 500 µg/ml) was used as reference standard.[22]

$$\text{Percentage (\%) of stabilization} = 100 - \frac{\text{optical density of the drug treated sample}}{\text{optical density of the control}} x100$$

- **Protein Denaturation method**

The reaction mixture (5ml) consisted of 0.2ml of egg albumin (from fresh hen's egg), 2.8ml of phosphate buffer saline (PBS, pH 6.4) and 2ml of varying concentrations of plant extract so that final concentrations become 50, 100, 150, 200, 250µg/ml. Similar volume of double distilled water served as a control. Then the mixture was incubated at (37±2) °C in incubator (Universal) for 15 min and then heat at 70°C for 5 min. After cooling, their absorbance was measured at 660nm (Systronic 2202) by using vehicle as blank. Diclofenac sodium at the final concentration of 50, 100, 150, 200, 250µ/ml was used as reference drug and treated similarly for determination of absorbance and

viscosity. The percentage inhibition of protein denaturation was calculated using the following formula

Percentage (%) of inhibition = $1 - \frac{Vt}{Vc} x100$

Where, V_t = absorbance of test sample, V_C = absorbance of control.[19]

RESULTS

PRELIMINARY PHYTOCHEMICAL SCREENING

The ethanolic extract of *Annona cherimola* Mill**.** Leaves gave positive results for alkaloids, carbohydrates, phenols, flavonoids, glycosides, saponins, proteins and tannins.

Effect on Human Red Blood Cell (HRBC) - Membrane Stabilization Assay

In the present study, we have evaluated the effect of ethanolic extract of *Annona cherimola* Mill. Leaves on stabilization of HRBC membrane. It was found that the different concentrations ethanolic extract of *Annona cherimola* Mill. Leaves have an ability to stabilize the RBC membrane and can inhibit haemolysis. The effect of ethanolic extract of *Annona cherimola* Mill. Leaves on stabilization of RBC membrane is shown table-3 and fig-6. The maximum percentage of stabilization was shown 70.88 ± 0.72 at concentration of 400µg/mL. Ethanolic extract of *Annona cherimola* Mill. Leaves possesses significant anti-inflammatory activity when compared with standard Diclofenac sodium (82.20 ± 0.54) at 400µg/mL concentration.

Table 2: Effect of Ethanolic extract of *Annona cherimola* Mill. Leaves on HRBC Membrane Stabilization Assay.

Sl.no	Concentration (µg/mL)	% stabilization of plant extract	% stabilization of standard (Diclofenac sodium)
1	100	18.96 ± 0.58	23.56 ± 0.40
2	200	44.68 ± 0.60	49.23 ± 0.65
3	400	70.88 ± 0.72	82.20 ± 0.54

Values are expressed as Mean ± SD; n=6.

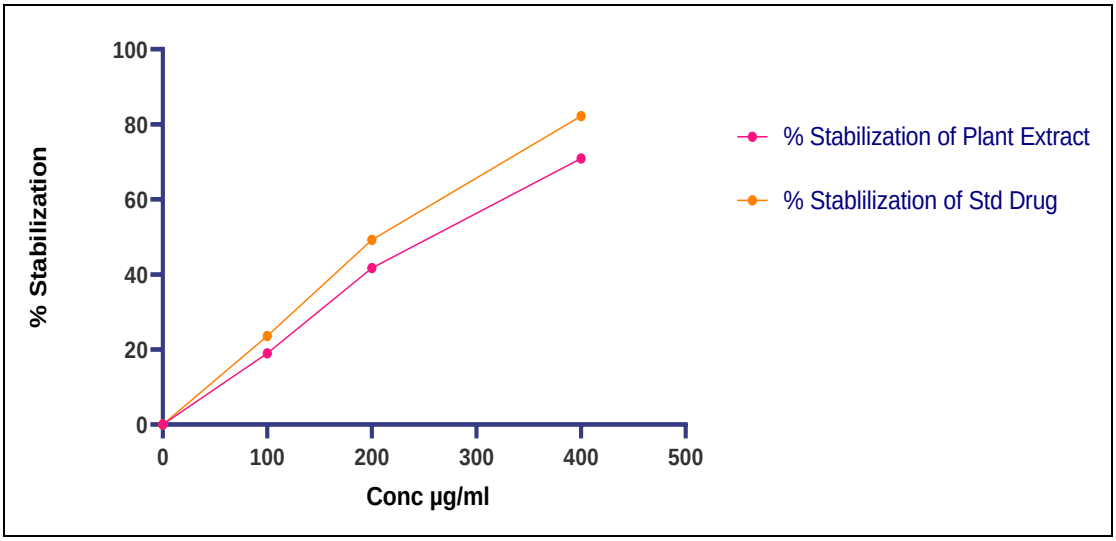

Figure 6: Effect of Ethanolic extract of *Annona cherimola* Mill. Leaves on HRBC Membrane Stabilization Assay.

Effect of Ethanolic Extract of *Annona cherimola* Mill Leaves on Protein Denaturation

In the present study, we have evaluated the effect of ethanolic extract of *Annona cherimola* Mill. Leaves on protein denaturation. It was found that the different concentrations ethanolic extract of *Annona cherimola* Mill. *Leaves* have an ability to inhibit protein denaturation. The inhibitory effect of different concentrations of ethanolic extract of *Annona cherimola* Mill. Leaves on protein denaturation was shown in table-4 and figure-7.

Ethanolic extract of *Annona cherimola* Mill. Leaves and standard (Diclofenac sodium) at a concentration of 100, 200 and 400 µg/ml showed significant inhibition of denaturation of albumin in concentration dependent manner. In our study, ethanolic extract of *Annona cherimola* Mill. Leaves showed maximum inhibition of 69.92% at 400 µg/ml concentration when compared to standard Diclofenac sodium which showed maximum inhibition of 85.47% at a concentration of 400 µg/ml.

Table 3: Effect of Ethanolic extract of *Annona cherimola* Mill. Leaves on Protein Denaturation.

Sl.no	Concentration (µg/mL)	% inhibition of plant extract	% inhibition of standard (Diclofenac sodium)
1	100	25.38 ± 0.46	35.65 ± 0.61
2	200	46.16 ± 0.58	54.74 ± 0.57
3	400	69.92 ± 0.68	85.47± 0.41

Values are expressed as Mean ± SD; n=6.

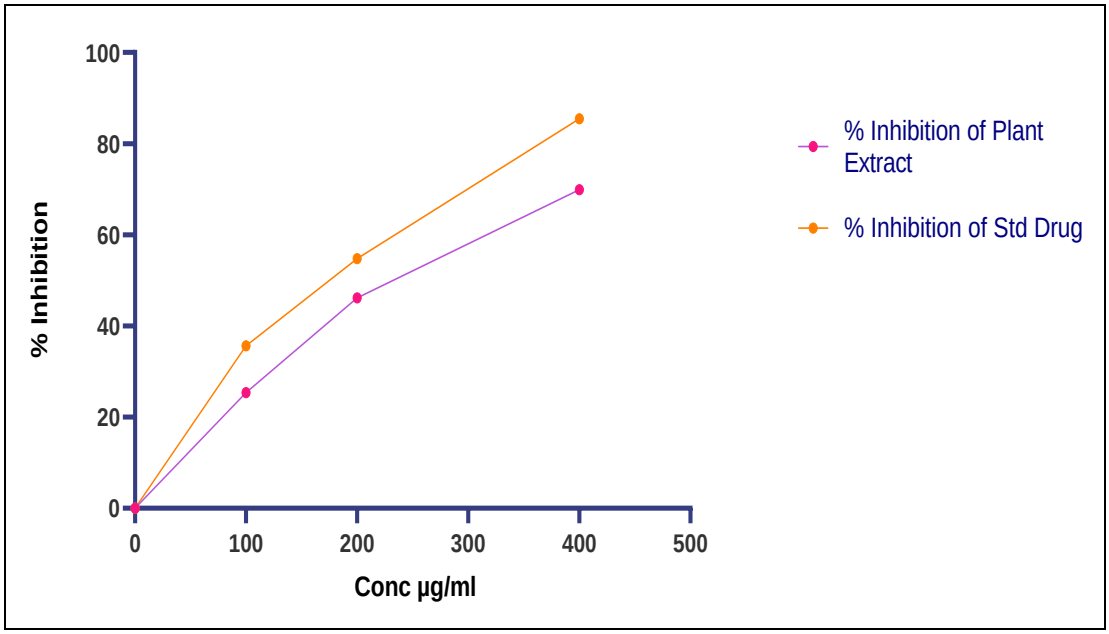

Figure 7: Effect of Ethanolic extract of *Annona cherimola* Mill. Leaves on Protein Denaturation.

DISCUSSION

Inflammation is a biological response of the immune system that can be triggered by a variety of factors, including pathogens, damaged cells and toxic compounds. These factors may induce acute and/or chronic inflammatory responses in the heart, pancreas, liver, kidney, lung, brain, intestinal tract and reproductive system, potentially leading to tissue damage or diseases such as cardiovascular diseases, chronic liver diseases, pancreatic cancer, kidney disorders, chronic obstructive pulmonary disease and joint diseases etc.[23]

Anti-inflammatory treatment of infections is challenging due to the heterogeneity of etiologic agents and complex immune interactions. Inflammation plays a central role in the pathophysiology of infections, being one of the principal body defensive mechanisms. Unfortunately, it also causes accompanying unpleasant symptoms. Anti-inflammatory medications such as NSAIDs are commonly used in infections to reduce unpleasant symptoms and to modify host responses but they exhibit some side effects. Hence, use of herbal plant sources and phytochemicals were greatly increased.[24]

The present study was carried out to investigate phytochemicals and evaluate the anti-inflammatory properties of *Annona cherimola* Mill. leaves by *In-vitro* methods such as HRBC membrane stabilization method and Protein denaturation Method.

The phytochemical investigation showed the presence of alkaloids, carbohydrates, phenols, flavonoids, glycosides, saponins, proteins and tannins which are essential for anti-inflammatory properties.

HRBC membrane stabilization method was employed, as the membrane of RBC is identical to the lysosomal membrane and its stability signifies the anti-inflammatory property.[25] Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extra cellular release.[26] On the basis of *In-vitro* evaluated results, the ethanolic extract of *Annona cherimola* Mill. Leaves at 400µg/mL concentration showed significant anti-inflammatory activity i.e. 70.88 % was comparable to that of the standard Diclofenac sodium (400µg/mL) showed 82.20 % stabilization.

The denaturation of proteins corresponds to proteins losing their secondary, tertiary, and quaternary structures, resulting in loss of biological function.[27] This process could be induced by the application of external stress, such as heat, or by exposure to chemicals, such as organic solvents.[28] Hence, to investigate the anti-inflammatory activity, the ability of protein extract to inhibit the heat-induced denaturation of albumin was evaluated. The ethanolic extract of *Annona cherimola* Mill. Leaves showed significant anti-proteinase activity ranging from 25.38 to 69.92% at different concentrations (100, 200, 400µg/ml). Dose dependent inhibitory activity was observed from plant extract i.e. the highest value was found to be 69.92% at 400 µg/ml concentration and it was significantly comparable to that of standard Diclofenac sodium (85.47%) at 400 µg/ml concentration.

CONCLUSION

In conclusion, the *In-vitro* anti-inflammatory studies of the ethanolic extract of *Annona cherimola* Mill. leaves demonstrated significant activity, attributed to the presence of saponins, phenols and flavonoids etc. The ethanolic extract exhibited notable stabilization in the HRBC (Human Red Blood Cell membrane stabilization) method and inhibition in the Protein Denaturation method, highlighting its potential as a natural anti-inflammatory agent. These findings suggest that the bioactive compounds in the plant could be developed for therapeutic applications targeting inflammatory conditions. Further in depth studies, including *in-vivo* models are recommended to confirm its efficacy and elucidate the underlying mechanisms.

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