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EVALUATION OF ANTI-INFLAMMATORY ACTIVITY OF POLYHERBAL EXTRACTS BY USING INVITRO MODELS

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ABSTRACT

1. Introduction: Inflammation is a natural response of the body to injury, infection, or harmful stimuli. However, prolonged inflammation may contribute to chronic diseases. Although non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used to manage inflammation, their long-term use may cause adverse effects. Therefore, medicinal plants are being investigated as potential sources of natural anti-inflammatory agents. The present study evaluates the in-vitro anti-inflammatory activity of a polyherbal extract containing turmeric (Curcuma longa), Tulsi (Ocimum tenuiflorum), black pepper (Piper nigrum), and Aloe vera.

2. Objectives: The main objective of this study is to evaluate the in-vitro anti-inflammatory activity of a polyherbal extract prepared using turmeric, Tulsi, black pepper, and Aloe vera by identifying its phytochemical constituents and assessing its activity through protein denaturation inhibition, membrane stabilization, hypotonicity-induced hemolysis, and antiproteinase assays at different concentrations. The study also aims to compare the extract's activity with standard preparations and determine the concentration showing maximum anti-inflammatory activity.

3. Methodology: The study was conducted using extracts of turmeric, Tulsi, black pepper, and Aloe vera. Preliminary phytochemical analysis was performed to identify the chemical constituents, followed by thin-layer chromatography (TLC) to separate and characterize the components. The anti-inflammatory activity was evaluated at concentrations ranging from

100 to 800 µg/mL. Different in-vitro methods were used, including protein denaturation inhibition, hypotonicity-induced hemolysis, antiproteinase activity, heat-induced hemolysis, and membrane stabilization assays. Standard preparations, including aspirin and Nishamalaki, were used for comparison. Laboratory equipment and appropriate chemicals were used to conduct the experiments.

4. Results: Preliminary phytochemical analysis revealed the presence of alkaloids, flavonoids, tannins, and saponins in turmeric, Tulsi, and black pepper, while Aloe vera showed the presence of saponins. TLC analysis showed different retention factor (Rf) values for the selected plant extracts. The polyherbal extract demonstrated promising in-vitro anti-inflammatory activity in the experimental assays. The maximum inhibition observed was 81% in the protein denaturation assay at 100 µg/mL, 89% in the hypotonicity-induced hemolysis assay at 400 µg/mL, 40.22% in the antiproteinase assay at 800 µg/mL, 66.8% in the heat-induced hemolysis assay at 800 µg/mL, and 77.5% in the membrane stabilization assay at 800 µg/mL. The results indicated protective effects against protein denaturation and red blood cell membrane damage. However, the activity varied across the different experimental methods and concentrations.

5. Conclusion: The study indicates that the polyherbal extract containing turmeric, Tulsi, black pepper, and Aloe vera possesses promising preliminary in-vitro anti-inflammatory activity. The phytochemical constituents present in these plants may contribute to the observed effects. The findings suggest that this formulation may be a potential source of natural anti-inflammatory agents. However, further studies involving statistical analysis, toxicity evaluation, identification of active constituents, and in-vivo experiments are necessary to establish its safety, efficacy, and therapeutic potential.

KEYWORDS: Polyherbal extract, anti-inflammatory activity, turmeric, Tulsi, black pepper, Aloe vera, phytochemical analysis, membrane stabilization.

INTRODUCTION

INFLAMMATION

The inflammation term is taken from the Latin word “inflamer” (to burn) (de Oliveira).[1,2] Inflammation is a complex biological process that is essential for the body's defense mechanisms against infection and injury. It is a complex response to harmful stimuli, such as pathogens, irritants, or damaged cells, aimed at removing the stimulus and initiating the healing process.[3]

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 of stress.

Inflammation of tissue is due to response to stress. It is defensive response that is characterized by redness, pain, heat, and swelling and loss of function in the injured area. Inflammation is one of the body's nonspecific internal systems of defense, the response of a tissue to an accidental cut is similar to response that results from other type of tissue damage, caused by burns due to heat, radiation, bacterial or viral invasion.[4]

Inflammation dilutes, destroys, or walls off harmful agents that have entered the body. It activates a sequence of biological events to heal the damage. The most common causes of inflammation are infections, burns and trauma, and many types of immune reactions.[5]

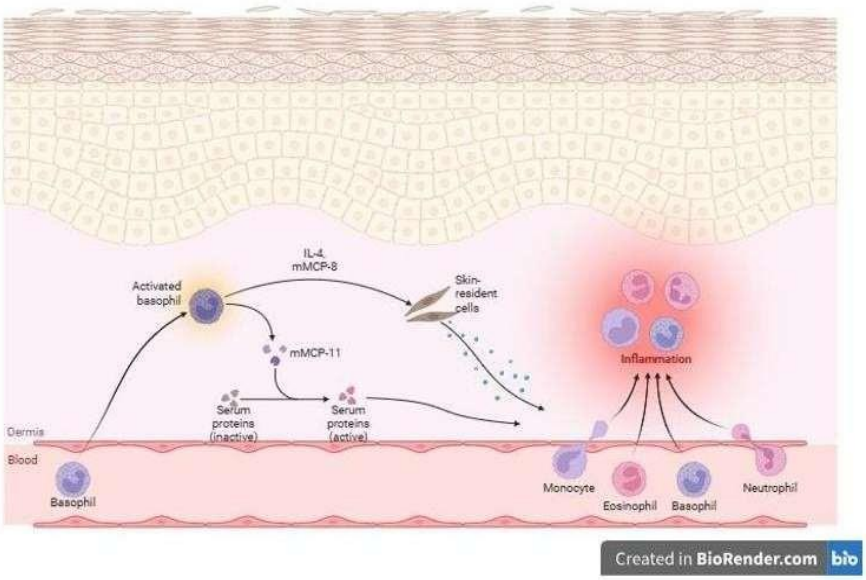


Figure 01: Basophil mediated skin inflammation.

CLASSIFICATION OF INFLAMMATION

The inflammatory response is triggered through two phases: (a) acute and (b) chronic, and each is apparently mediated by a different mechanism.[6]

1. ACUTE INFLAMMATION

Acute inflammation is a rapid and short-term response to injury, infection or tissue damage, it usually lasts for a few hours to days.[7] These immune responses which involved in acute

inflammation can be divided into vascular and cellular.[8]

The responses which occur in microvasculature normally appear in few minutes following tissue injury or microbial infection in the presence of other inflammatory stimuli named vascular events.

The occurrence of these processes is rapid and eventually will lead to vasodilation and subsequently makes the vessels become more permeable. These processes will result in entry of inflammatory mediators and produces interstitial Edema.[9]

Cellular events encompass the successive capture, trundling, and firming an adhesion to the microvascular endothelium.[10] These events in the mobilization pathway are arranged by cell adhesion molecules (CAMs). These CAMs include intracellular adhesion molecules (ICAM)-1, ICAM-2, integrins, and selectin. The selectin group of CAM contains three families; P-selectin and E-selectin produced by endothelial cells and L-selectin produced by white blood cells.[11]

Ex: cut wound, appendicitis.

2. CHRONIC INFLAMMATION

Chronic inflammation is a prolonged inflammatory response that leads to a progressive shift in the type of cells present at the site of inflammation.[8] It usually last for weeks, months or years. The inflammation of chronic events is distinguished by mononuclear cell infiltration (e.g., monocyte and lymphocytes), fibroblasts proliferation, collagen fibers, and connective tissue formation, which ultimately result in 2-mm granuloma.[12] With chronic inflammation, the tissue degeneration is normally mediated by nitrogen species, proteases, and other reactive oxygen species released from infiltrated inflammatory cells.[13] Certainly, genomic alterations in p53 were approved as causes for many chronic inflammatory diseases (e.g., inflammatory bowel diseases and rheumatoid arthritis) in addition to cancers.[14-15]

CAUSES OF INFLAMMATION

1. **Microbial infection:** (bacteria, viruses, fungi)

Ex: Bacterial wound infection causes redness and swelling

2. **Physical injuries:** (trauma, burns, radiation)

Ex: A Sprained ankle became swollen and painful

3. **Chemical agents:** (acids, toxins)

Ex: Acid burn on the skin cause inflammation

4. **Allergic reaction**

Ex: Hay fever cause nasal inflammation

5. **Tissue necrosis:** (cell death)

Ex: Inflammation after heart attack

6. **Autoimmune diseases**

Ex: In Rheumatoid arthritis, the joint becomes inflamed

CARDINAL SIGNS OF INFLAMMATION

Inflammation is a common clinical condition[16,17] and is a response of a tissue to injury. The cardinal signs of inflammation are:

- Swelling (tumor)
- Heat (Calor)
- Redness (rubor)
- Pain (dolor)
- Loss of function (function laesa)

And occurs due to the movement of plasma fluids, proteins, and inflammatory cells from the lumen of the vascular system out into the tissues. However, if untreated, it may lead to a host of diseases, such as hay fever, periodontitis, atherosclerosis, rheumatoid arthritis, and even cancer (e.g., gallbladder carcinoma).

MECHANISM /PATHOPHYSIOLOGY OF INFLAMMATION

Inflammation is a host defense mechanism of the body, and it's an essential immune response that enables the body to survive during infection or injury and maintains tissue homeostasis in noxious conditions.[18,19] Inflammation usually considered as a pathological process can be beneficial in combating noxious infection or injury.[20,21]

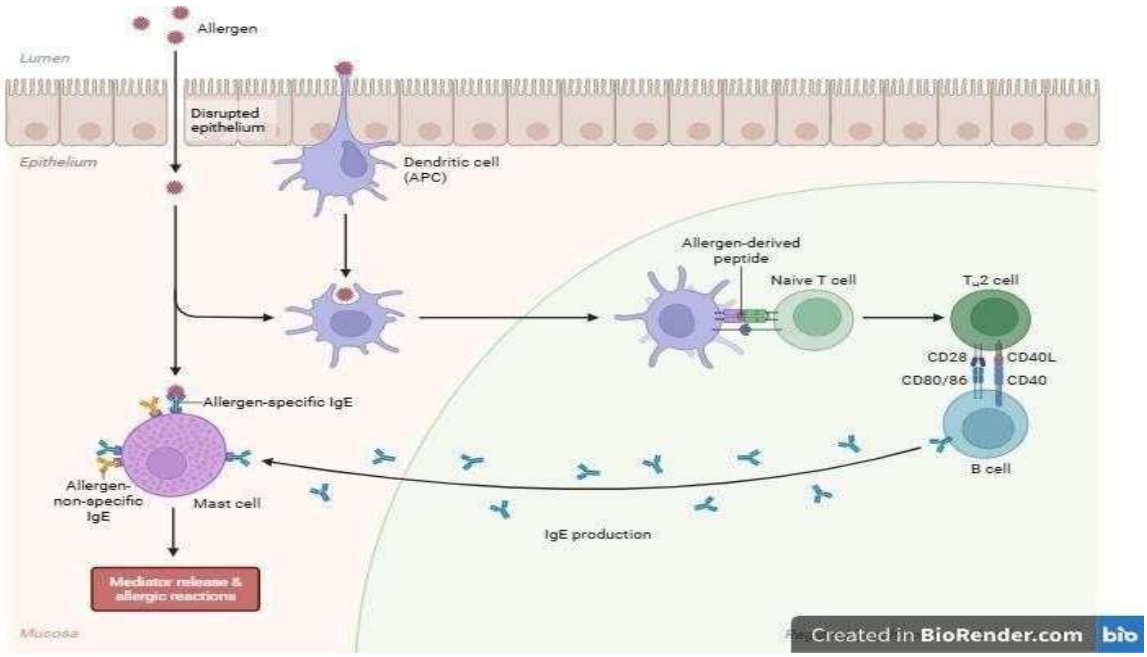


Figure 02: Mechanism / Pathway of Inflammation.

Inflammation has role in healing, restorative and aggressive processes.[20,22] However, such acute or chronic inflammatory processes lead to inflammatory disorders either on its response or by factors/enzymes that aggravate the inflammatory process.[20,23]

Plenty of cytokines participate in enzyme activation (such as phospholipase), mediator release, fluid extravasation and vasodilation, cell migration, and finally tissue damage which generally have been named inflammation and such elaborative and uncontrolled processes lead to inflammatory disorders which require progressive anti-inflammatory therapy wherein routinely used synthetic drugs have predisposed to severe side effects.[18,19,24,21,23]

INFLAMMATORY MEDIATORS

A variety of chemical mediators from circulation system, inflammatory cells, and injured tissue actively contribute to and adjust the inflammatory response. The released chemical mediators include vasoactive amines such as histamine and serotonin, peptide (e.g., bradykinin), and eicosanoids (e.g., thromboxanes, leukotrienes, and prostaglandins).

VASOACTIVE AMINES AND PEPTIDE

1. HISTAMINE

Histamine is released in a quantity of few pictograms from basophils to maintain acute-phase response during inflammation events.

2. SEROTONIN

Serotonin is produced via decarboxylation of tryptophan, and it is stored in the granule. In murine, the serotonin is available in basophilic granules, while in humans, it is present in platelets. Four serotonin receptors, namely 5-HTl, 5-HT2, 5-HT3, and 5-HT4, were documented to mediate its biological functions.

3. BRADYKININ

Bradykinin is a nano peptide created from plasma Kinin–Kallikrein system. Two or more distinct receptors are present for bradykinins which have been titled B1 and B2. Similar to histamine and serotonin, it can increase the synthesis of prostaglandins and produces pain locally.

4. PROSTAGLANDINS

Prostaglandins (prostaglandin E2 and prostaglandin b) are substantially encompassed in conserving the inflammatory process by increasing the vascular permeability and strengthening the outcome of other inflammatory mediators such as kinin, serotonin, and histamine and thus contributing to the redness, increased blood flow, and plasma exudation in the area of acute inflammation which leads to edema.

5. CYTOKINES

In addition to many stromal cells, fibroblasts, and endothelial cells, every cytokine can be released from many cells' types. Cytokines have important effects in the activity of many cells. However, they are of particular importance because of their significance in regulating the immune system. The function of cytokines in the manner of development in inflammatory disease as a result to bacterial infection or exposure to lipopolysaccharide (LPS) was investigated deeply in animals, pigs, cattle, and mice. In addition, the production of cytokines induces the release of acute-phase response.[25]

6. NITRIC OXIDE

Nitric oxide (NO) is a gaseous short lived free radical has been implicated as a mediator of inflammation and modulation of biosynthesis or activity of NO results in amelioration of acute inflammation and experimental arthritis model (47,48). NO is generated via the oxidation of the terminal guanidine nitrogen atom of L-arginine by the enzyme Nitric Oxide Synthase (NOS).[26]

7. LEUKOTRIENE

LTs are enzymatically generated from arachidonic acid (AA) released from membrane phospholipids by phospho-lipase A2 (PLA2) enzymes. LTs are mainly produced by immune cells, including neutrophils, mast cells, macrophages (Mϕ) and dendritic cells (DCs), because these cells express 5-lipoxygenase (5-LO) and 5-LO-activating protein (FLAP), both of which are required for LT biosynthesis. LTs are classified into leukotriene B4 (LTB4), which contains di-hydroxyl groups, and the cysteinyl LTs (CysLTs; LTC4, LTD4 and LTE4), which contain amino acids. Although LTs are involved in inflammatory and immune responses as potent lipid mediators, it is not fully understood how LTs regulate chronic inflammation through these receptors.[27]

8. PLATELET

Platelets are involved in hemostasis, wound healing, and inflammation. Under physiological conditions, platelets circulate in a quiescent state, protected from untimely activation by inhibitory mediators released from intact endothelial cells, including nitric oxide (NO) and prostaglandin I2(PGI2, prostacyclin). In addition, ectoADPase(CD39) removes extracellular ADP by converting it to adenosine. Endothelial dysfunction and changes in release of antiplatelet factors may lead to increased platelet activation followed by their interaction with neutrophils and monocytes, and increased platelet adhesion and aggregation.[28]

ROLE OF ARACHIDONIC ACID PATHWAY

Prostaglandins (PGs) are the end products of fatty acid metabolism, and are produced via the COX pathway. They have long been known to act as major physiological and pathological mediators in a number of therapeutic areas including inflammation, pain, pyrexia, cancer and neurological diseases. Arachidonic acid (AA), an unsaturated 20 carbon fatty acid embedded in cell membranes as a phospholipid ester, is the precursor of PG synthesis. Free AA is released in response to a wide variety of stimuli, and subsequently converted via the COX pathway and lipoxygenase (LOX) enzyme catalysis to lipid mediators collectively known as eicosanoids. In the COX pathway, the two known COX isoforms (COX-1 and COX-2) catalyse the first committed step in the biosynthesis of PGs and thromboxane's (TxA). The initial COX reaction converts AA to prostaglandin G2 (PGG2), and the subsequent peroxidase reaction reduces PGG2 to prostaglandin H2 (PGH2), which is then converted by various cell-specific isomerases and synthases to five biologically active primary PGs: prostaglandin D2 (PGD2), prostaglandin E2 (PGE2), prostaglandin F2 (PGF2),

prostacyclin (PGI2) and thromboxane A2 (TxA2). These products act as secondary messengers by interacting with prostanoid G protein-coupled and other receptors.

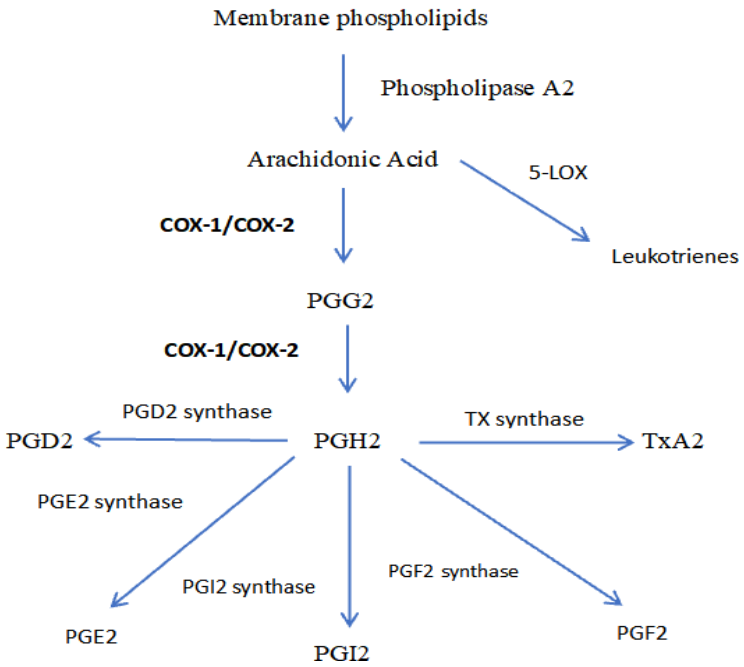


Figure 03: Role of Arachidonic acid pathway.

CLASSIFICATION OF NON-STEROIDAL ANTI-INFLAMMATORY DRUG[29]

NSAIDs can be classified on the basis of their chemical and pharmacological properties, and COX selectivity.

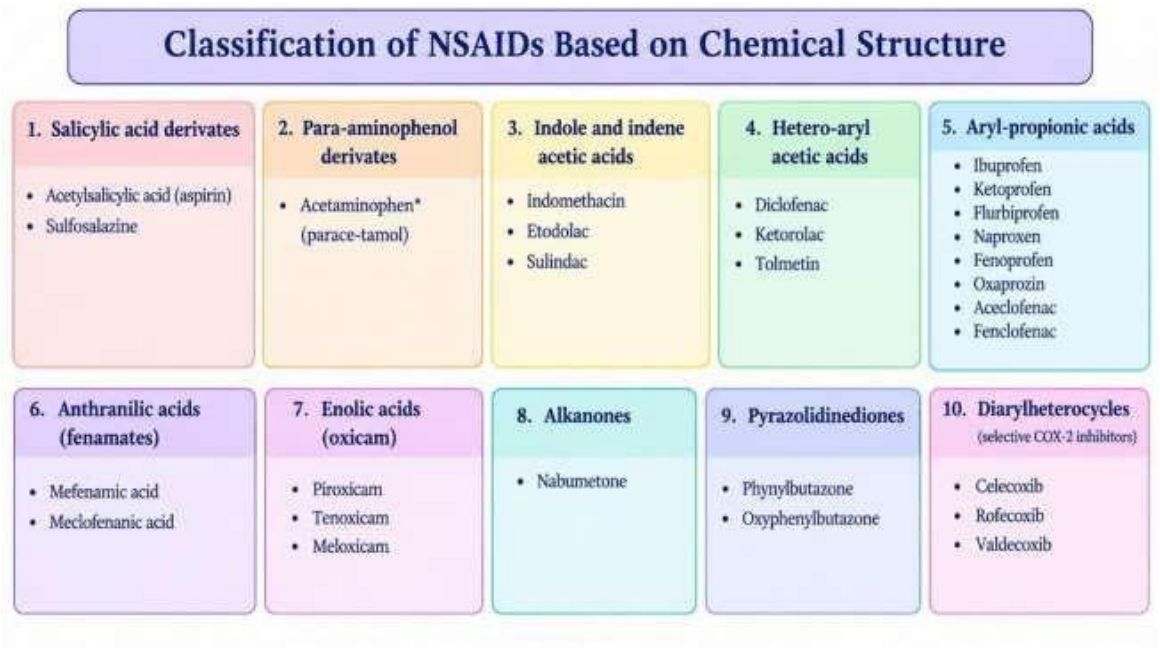


Figure 04: Classification of NSAID's based on chemical structure.

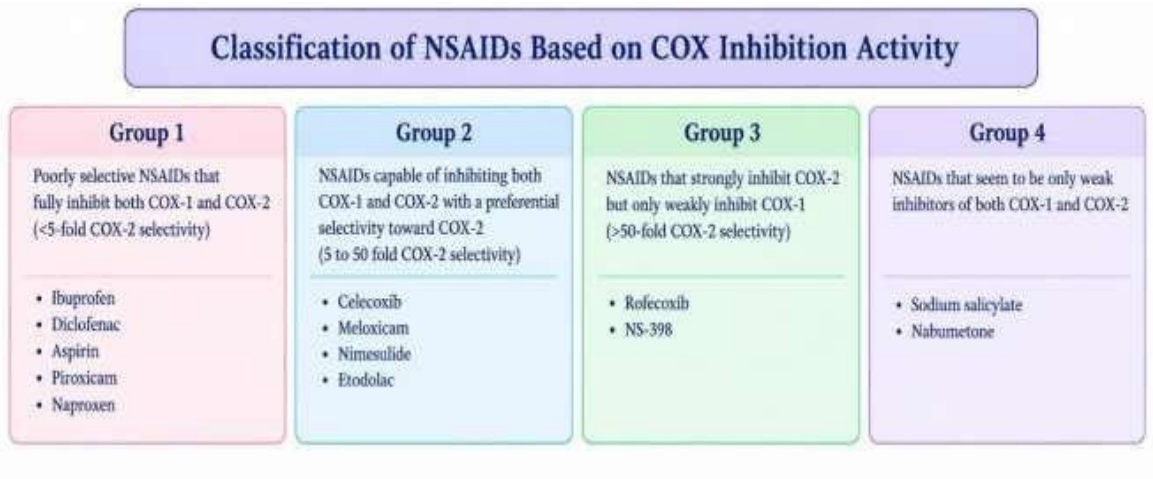


Figure 05: Classification of NSAID's based on COX inhibition activity.

HERBAL DRUGS

1. TULASI

Family: Lamiaceae.

Scientific name: *Ocimum tenuiflorum L.*

Synonym: Ocimum sanctum L.

It possesses significant anti-inflammatory activity due to the presence of bioactive compounds such as eugenol, ursolic acid, Rosmarinic acid, and flavonoids.

Therapeutic Uses

- Reduces swelling, redness, and pain.
- Protects tissues from inflammatory damage
- Useful in inflammatory conditions such as arthritis, respiratory disorders, and skin inflammation.



Figure 06: Tulasi.

2. ALOE VERA

Family: Asphodelaceae (formerly placed in Liliaceae)

Scientific name: *Aloe vera (L.) Burm.f.*

Synonyms: Aloe barbadensis Mill., Aloe indica Royle.

The gel obtained from its leaves contains several bioactive compounds such as polysaccharides, glycoproteins, vitamins, enzymes, and phenolic compounds that contribute to its anti-inflammatory activity.

Therapeutic Uses

- Relief of skin inflammation, burns, and wounds.
- Management of sunburn and minor skin irritations.
- Reduction of inflammation in ulcers and gastrointestinal disorders.



Figure 7: Aloe vera plant.

3. TURMERIC

Family: Zingiberaceae.

Scientific name: *Curcuma longa L.*

Synonyms: Curcuma domestica Valeton.

Its major active Constituentcurcumin, possesses significant anti-inflammatory activity.

Therapeutic Uses

- Decreases swelling, redness, and pain at inflamed sites.
- Helps to manage inflammatory conditions such as arthritis, inflammatory bowel disease, and skin inflammation.
- Protects tissues from damage caused by chronic inflammation.



Figure 08: Turmeric.

4. BLACK PEPPER

Family: Piperaceae.

Synonyms: *Pipernigrum L.*

Phytochemical Constituents: Piperine, piperidine, piperettine, volatile oils (β-caryophyllene, limonene, pinene, sabinene), flavonoids, lignans, and phenolic compounds.

Therapeutic uses

- Help to manage arthritis, Joint pain, other inflammatory conditions.
- Antioxidant activity.
- Stimulates secretion of digestive enzymes.



Figure 09: Black pepper.

MATERIALS AND METHDOLOGY

Materials

i. **Plant extracts:** Aloe Vera extract, Turmeric extract, Tulsi extract, Black pepper extract.
ii. **Standard drug:** Nishamalaki, Aspirin.
iii. **Equipment's:** UV visible spectrophotometer, centrifuge, incubator
iv. **Apparatus:** Water bath, ice bath, Test tubes, syringe, petri plates, motor and pestle, micro pipettes, weighing balance, Beakers, Volumetric flask, Glass rod, Measuring

cylinder, Spatula, Funnel, Filter paper, Holder, China dish, Iodine flask.

v. **Chemicals:** RBC suspension, Alsever's solution, phosphate buffer, Hyposaline, Isotonic buffer, Trypsin, Tris HCL buffer, casein solution,70% Perchloric acid.

vi. **Personnel protective equipment's (PPE):** Apron, gloves, head cap, mask, sanitizer, etc. should be used while carrying out any procedure in the laboratory. This should be compulsory worn to avoid health risks, hazards, staining, contamination, etc.

METHDOLOGY

1. Preparation of plant extract.
2. Preliminary phytochemical analysis of the extract
3. Thin layer chromatography
4. Preparation of sample drug
5. Preparation of 10% HRBC suspension
6. Inhibition of protein denaturation assay.
7. Hypotonicity induced hemolysis.
8. Anti proteinase assay.
9. Heat induced hemolysis.
10. Membrane stabilization method

PROCEDURE

Procedure for Plant Extraction:

Collection of Plant Materials.

↓

Authentication of Plants.

↓

Washing with Tap Water or Rinsing with Distilled Water.

↓

Shade Drying (at Room Temperature).

↓

Grinding into Coarse Powder.

↓

Weighing of Plant Powder.

↓

Addition of Suitable Solvent (Ethanol/Methanol/Water).

↓

Maceration (24–72 hours with intermittent shaking).

↓

Filtration (Muslin Cloth / Whatman Filter Paper).

↓

Concentration of Extract (Water Bath/Rotary Evaporator).

↓

Collection of Crude Extract.

↓

Storage at 4°C in Airtight Containers.

↓

Ready for Anti-inflammatory Assays.



Figure 10: Plant Extraction.

Preliminary phytochemical analysis of the extract

1) **Alkaloids Detection Test**

a. **Mayer's Test:** The extract was combined with Mayer's reagent (a solution of potassium mercuric iodide). The formation of a cream or white precipitate indicated the presence of alkaloids.

b. **Drangendraff's test:** The extract was treated with Dragendorff's reagent (a potassium bismuth iodide solution). An orange-red precipitate suggested alkaloids' presence.



Figure 11: Results of Mayer's Test and Drangendraff's test.

1) Tannins Detection Test

Ferric Chloride Test: The extract was treated with 2-3 drops of a 5% ferric chloride solution. The development of a blue, green, or black color indicated the presence of phenolic compounds.



Figure 12: Results of Ferric Chloride Test.

1) Saponins Detection Test

Foam Test: The extract was shaken vigorously with distilled water. The presence of a stable foam layer (at least 1 cm high) after 15 minutes indicated the presence of saponins.



Figure 13: Results of Foam Test.

3. Flavonoids Detection Test

Lead Acetate Test: The extract was treated with several drops of lead acetate solution. The presence of a yellow precipitate confirmed the existence of flavonoids.



Figure 14: Results of Lead acetate test.

Thin layer chromatography

Mobile phase and Reagents used in thin layer chromatography: -

Table 01: Mobile phase and Reagents used in TLC.

Sl.no	Extract	Mobile phase	Ratio of MP	Detecting reagent
01	Turmeric	CHCl3: Methanol	97:3	Anisaldehyde Sulfuric acid reagent.
02	Tulsi	Acetonitrile: Methanol	50:50	Anisaldehyde Sulfuric acid reagent.
03	Aloe vera	Toluene: Methanol	90:10	Ethanolic Potassium hydroxide solution.
04	Black pepper	Toluene: Ethyl acetate	70:30	Dragendroff's reagent.

Procedure for Thin layer chromatography

A silica gel TLC plate was prepared by drawing a pencil line approximately 1 cm from its bottom edge. Using a capillary tube, a small spot of turmeric extract was applied to the baseline. A small quantity of the mobile phase was poured into the developing chamber, which was closed for a few minutes to allow solvent-vapor saturation. The TLC plate was then placed vertically in the chamber, ensuring that the sample spot remained above the solvent level. The chamber was closed, and the solvent was allowed to travel up the plate. When the solvent front approached approximately 1 cm from the top, the plate was removed, and the solvent front was immediately marked with a pencil. The plate was allowed to dry in a suitable, ventilated area, and the separated yellow spots were observed under visible light. Finally, the retention factor (Rf) was calculated using the formula:

$$Rf\ value = \frac{Distance\ travelled\ by\ the\ solute\ spot}{\text{Distance travelled by the solvent front}}$$



Fugure 15: Thin layer chromatography.

PREPARATION OF SAMPLE DRUG

Stock solution

Table 02: Preparation of Stock solution.

Extract	Quantity (Extract in mg)	Solvent (Ethanol in ml)	Final Volume (ml)	Dose(mg/ml)
Turmeric	100	10	10	10
Tulsi	100	10	10	10
Black pepper	100	10	10	10
Aloe vera	100	10	10	10

Take 1ml from each iodine flask and then transfer into another flask and add 6ml of ethanol to dilute the extract and make up the volume to 10ml. (Dose of each extract is 2.5mg/ml)

Sub-stock solution

1ml of the stock solution and make up the volume to 10ml by using 9ml of ethanol.

$C_1V_1=C_2V_2$

Where,

C_1 = Initial concentration

V_1 = Volume taken from the stock

C_2 = Final concentration

V_2 = Final volume

Calculation,

$10mg/ml \times 1ml = C_2 \times 10ml$

$$C_2 = \frac{10mg/ml \times 1ml}{10ml}$$

$C2 = 1mg/ml$



Figure 16: Stock solutions.

Preparation of different concentrations of each extract

Table 03: Preparation of different concentration of extract.

Concentration (µg/ml)	Sample drug (ml)	Solvent (Ethanol in ml)	Final volume (ml)
100	1	9	10
200	2	8	10
400	4	6	10
600	6	4	10
800	8	2	10



Figure 17: Different concentrations of each extract.

Preparation of 10% HRBC suspension

Human blood was collected in an appropriate anticoagulant tube following institutional biosafety procedures. The blood was centrifuged at approximately 1,500–2,000 rpm for 5–10 minutes using the centrifuge manufacturer's recommended settings. The supernatant plasma and buffy coat were carefully removed without disturbing the packed red blood cells (RBCs). The packed RBCs were then washed with 0.9% normal saline, centrifuged, and the supernatant was discarded. This washing procedure was repeated three times to remove residual plasma and other impurities. Finally, a 10% RBC suspension was prepared by resuspending 1 ml of washed, packed RBCs in 9 ml of normal saline to obtain a final volume of 10 ml.



Figure 18: Preparation of 10% HRBC suspension.

PROCEDURE FOR ASSAY

1. Inhibition of protein denaturation assay

The test extract and standard drugs, Nishamalaki and Aspirin, were prepared at concentrations of 100, 200, 400, 600, and 800µg/ml, and 0.05 ml of each sample was taken in separate tubes. A control was prepared using ethanol as the vehicle without the test extract. Then, 0.45 ml of 0.5% (w/v) bovine serum albumin (BSA) solution was added to each tube, and the samples were incubated at 37°C for 20 minutes. The temperature was then increased to 57°C for 3 minutes, after which the samples were allowed to cool. Subsequently, 2.5 ml of phosphate buffer was added to each tube, and the absorbance was measured at 660 nm using

a UV-Visible spectrophotometer. The results obtained for the test extract were compared with those of the standard drugs, Nishamalaki and Aspirin, to evaluate the in-vitro anti-inflammatory activity.[32]

$$\% \text{ inhibition} = \frac{Absorbance\ of\ control - Absorbance\ of\ test/Standard}{\text{Absorbance of control}} \times 100$$



Figure 19: Inhibition of protein denaturation assay.

2. Hypotonicity-induced hemolysis

The polyherbal extract was used as the test drug, while Nishamalaki and Aspirin were used as standard drugs. An isotonic solution containing 154 mM NaCl in 10 mM sodium phosphate buffer was prepared, and the pH was adjusted to 7.4. Then, 50 µl of stock red blood cell (RBC) suspension was mixed with 5 ml of hypotonic solution containing the ethanolic polyherbal extract at concentrations of 100, 200, 400, 600, and 800 µg/ml. A drug-free solution containing ethanol was used as the control. The mixtures were incubated at room temperature for 10 minutes and then centrifuged at 5,000 rpm for 5 minutes. The absorbance of the supernatant was measured at 540 nm using a UV-Visible spectrophotometer. The anti-inflammatory activity of the polyherbal extract was compared with that of the standard drugs, Nishamalaki and Aspirin.[30] The percentage inhibition of haemolysis was calculated using the following formula:

$$\% \text{ inhibition} = \frac{Absorbance\ of\ control - Absorbance\ of\ test/Standard}{\text{Absorbance of control}} \times 100$$



Figure 20: Hypotonicity-induced hemolysis.

3. Anti proteinase assay

The polyherbal extract was used as the test drug, while Nishamalaki and Aspirin were used as standard drugs. A reaction mixture was prepared by mixing 0.06 ml of trypsin, 1 mL of 20 mM Tris-HCl buffer (pH 7.4), and 1 ml of the test drug at different concentrations of 100, 200, 400, 600, and 800 µg/ml. A control was prepared without the test extract using ethanol as the vehicle. The reaction mixtures were incubated for 10 minutes, followed by the addition of 1 ml of 0.65% (w/v) casein solution and further incubation for 20 minutes. The reaction was then stopped by adding 2 ml of 2 M perchloric acid ($HClO_4$). The resulting cloudy suspension was centrifuged at 3,000 rpm for 15 minutes, and the absorbance of the supernatant was measured at 280 nm using a UV-Visible spectrophotometer. The percentage inhibition of trypsin activity was calculated using the following formula, and the results were compared with those of the standard drugs to evaluate the in-vitro anti-inflammatory activity of the polyherbal extract.[33]

$$\% \text{ inhibition} = \frac{Absorbance\ of\ control - Absorbance\ of\ test/Standard}{\text{Absorbance of control}} \times 100$$



Figure 21: Anti proteinase assay.

4. Heat-induced hemolysis

The polyherbal extract was used as the test drug, while Nishamalaki and Aspirin were used as standard drugs. Five milliliter portions of phosphate-buffered saline containing the ethanolic crude polyherbal extract at concentrations of 100, 200, 400, 600, and 800 µg/mL were prepared in duplicate tubes. A control was prepared without the extract using ethanol as the vehicle. Then, 50 µl of red blood cell (RBC) suspension was added to each tube and mixed gently by inversion. One set of tubes was incubated at 54°C for 20 minutes in a water bath, while the other set was maintained at 0–5°C in an ice bath. After incubation, the mixtures were centrifuged at 5,000 rpm for 5 minutes, and the supernatants were collected. The absorbance of the supernatants was measured at 560 nm using a spectrophotometer. The percentage inhibition of haemolysis was calculated using the specified formula, and the results were compared with those of the standard drugs to evaluate the in-vitro anti-inflammatory activity of the polyherbal extract.[30]

$$\% \text{ inhibition} = \frac{Absorbance\ of\ control - Absorbance\ of\ test/Standard}{\text{Absorbance of control}} \times 100$$



Figure 22: Heat-induced hemolysis.

5. Membrane stabilization method

The polyherbal extract was used as the test drug, while Nishamalaki and Aspirin were used as standard drugs. The extract was prepared at concentrations of 100, 200, 400, 600, and 800 µg/ml. For each concentration, 1 ml of phosphate buffer, 2 ml of hyposaline solution, and 0.5 ml of 10% human red blood cell (HRBC) suspension containing an equal volume of Alsever's solution were added. The mixtures were incubated at 37°C for 30 minutes and then centrifuged at 3,000 rpm for 20 minutes. A control was prepared without the extract using ethanol as the vehicle. After centrifugation, the haemoglobin content of the supernatant was measured at 560 nm using a spectrophotometer. The percentage inhibition of haemolysis was

calculated using the following formula, and the results were compared with those of the standard drugs to evaluate the in-vitro anti-inflammatory activity of the polyherbal extract.[46]

$$\% \text{ inhibition} = \frac{Absorbance\ of\ control - Absorbance\ of\ test/Standard}{\text{Absorbance of control}} \times 100$$



FIGURE 23: Membrane stabilization method.

RESULT

1. Phytochemical analysis

Table 04: Phytochemical test result.

Phytochemical constituents	Test	Turmeric	Tulsi	Black Pepper	Aloe vera
Alkaloids	Mayer's test	+	+	+	-
	Dragendroff's test	+	+	+	-
Flavonoids	Lead acetate test	+	+	+	-
Tannins	Ferric chloride test	+	+	+	-
Saponins	Foam test	+	+	+	+

Phytochemical analysis revealed the presence of alkaloids, flavonoids, tannins, and saponins in turmeric, tulsi, and black pepper, while aloe vera showed saponins only.

2. Thin Layer Chromatography

Table 05: Thin layer Chromatography result.

Extracts	Retention factor value (cm)
Turmeric	0.43, 0.56, 0.73
Tulsi	0.83
Black pepper	0.58
Aloe vera	0.72

TLC analysis showed distinct Rf values for all four plant extracts. Turmeric exhibited three spots, indicating multiple constituents, while tulsi, black pepper, and aloe vera showed single

spots.

3. Protein denaturation method

Table 06: Sample drug result for Protein denaturation method.

Concentration(µg/ml)	Absorbance (nm)	% inhibition (%)
100	0.1316	81
200	0.1724	76
400	0.1885	74
600	0.4511	36
800	0.2935	59

Table 07: Standard drug result for Protein denaturation method.

Concentration(µg/ml)	Absorbance (nm)	% inhibition (%)
100	0.2289	69
200	0.1290	27.52
400	0.2837	60
600	0.4573	36
800	0.3795	47
200(Aspirin)	0.725	83

Control of hypotonic solution= **0.71 nm**

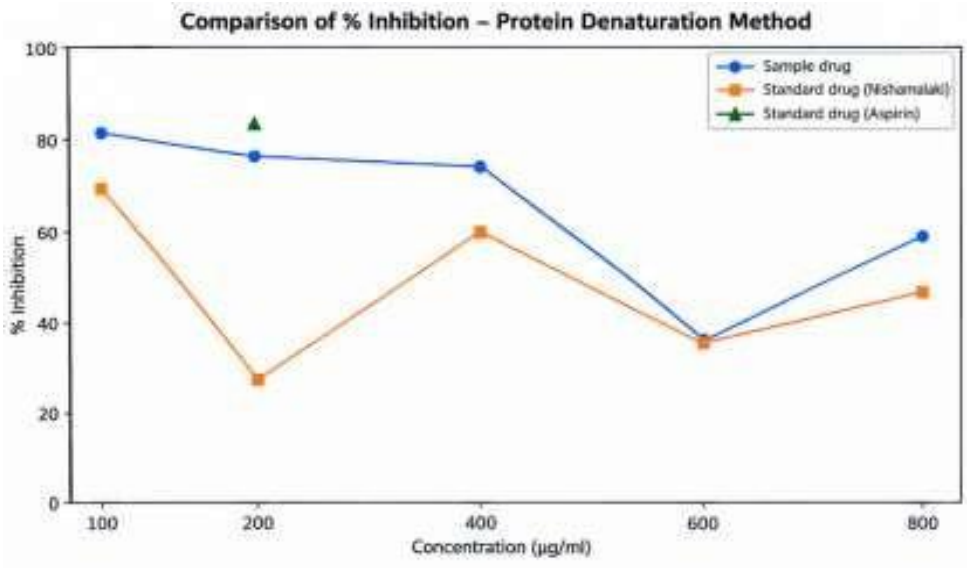


Figure 24: Graph of Protein denaturation method.

The protein denaturation method showed significant anti-inflammatory activity. The standard drug exhibited maximum inhibition of 69% at 100µg/ml, while the sample drug showed maximum inhibition of 81% at 100 µg/ml. Aspirin showed the highest inhibition of 83% at 200 µg/ml, indicating its strong protein denaturation inhibitory activity.

4. Hypotonicity induced hemolysis method

Table 08: Sample drug result for Hypotonicity induced hemolysis method.

Concentration (µg/ml)	Absorbance (isotonic solution) (nm)	Absorbance (hypotonic solution) (nm)	% Inhibition (%)
100	0.03	0.71	29
200	0.06	0.41	45
400	0.15	0.24	89
600	0.30	0.19	61
800	0.32	0.10	39

Table 09: Standard drug result for Hypotonicity induced hemolysis method.

Concentration (µg/ml)	Absorbance (isotonic solution) (nm)	Absorbance (hypotonic solution) (nm)	% Inhibition (%)
100	0.08	0.513	25
200	0.10	0.430	40
400	0.07	0.163	85
600	0.12	0.354	57
800	0.09	0.459	36
200 (Aspirin)	0.18	0.16	96

Control of hypotonic solution= **0.65 nm**

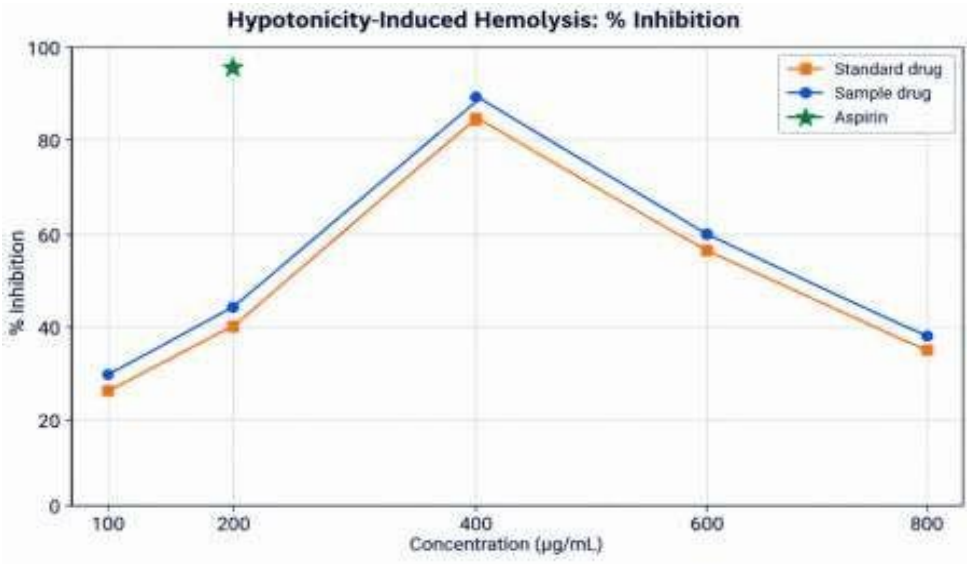


Figure 25: Graph of Hypotonicity induced hemolysis method.

The hypotonicity-induced hemolysis method demonstrated significant membrane-stabilizing activity. The sample drug showed maximum inhibition of 89% at 400 µg/ml, while the standard drug showed 85% inhibition at 400 µg/m. Aspirin exhibited the highest inhibition of 96% at 200 µg/ml, indicating strong protection against hypotonicity-induced hemolysis and potential anti-inflammatory activity.

5. Antiproteinase Assay

Table 10: Sample drug result for Antiproteinase Assay.

Concentration(µg/ml)	Absorbance (nm)	% inhibition (%)
100	2.459	26.8
200	2.551	31.56
400	2.621	35.06
600	2.629	36
800	2.719	40.22

Table 11: Standard drug result for Antiproteinase Assay.

Concentration(µg/ml)	Absorbance (nm)	% inhibition (%)
100	2.003	3.3
200	2.221	14.54
400	2.398	23.67
600	2.493	28.57
800	2.636	35.94
200 (Aspirin)	0.450	76.78

Control = **1.939 nm**

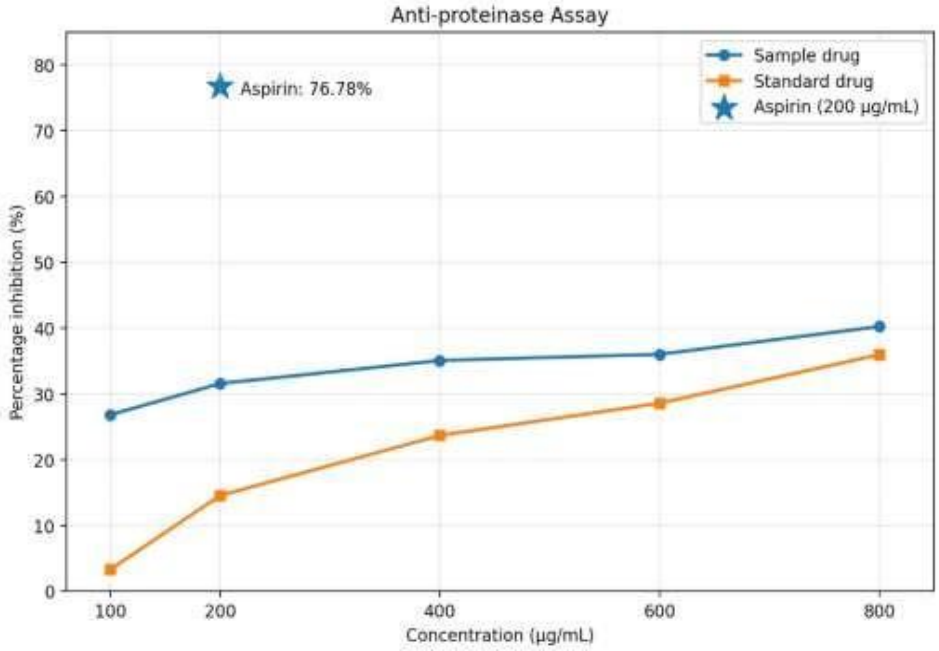


Figure 26: Graph of Antiproteinase Assay.

The antiproteinase assay showed concentration-dependent inhibitory activity. The standard drug exhibited maximum inhibition of 35.94% at 800 µg/ml, while the sample drug showed 40.22% inhibition at 800 µg/ml. Aspirin demonstrated the highest inhibition of 76.78% at 200 µg/ml, indicating stronger antiproteinase activity than the sample drug.

6. Heat induced hemolysis

Table 12: Sample drug heat treated result for Heat induced hemolysis.

Concentration(µg/ml)	Absorbance (nm)	% inhibition (%)
100	0.68	6.2
200	0.58	20
400	0.45	37.9
600	0.32	55
800	0.24	66.8

Table 13: Standard drug heat treated result for Heat induced hemolysis.

Concentration(µg/ml)	Absorbance (nm)	% inhibition (%)
100	0.692	4.5
200	0.651	10.2
400	0.592	18.34
600	0.443	38.89
800	0.324	55.31
200 (Aspirin)	0.192	73.51

Control heat treated= **0.725nm**

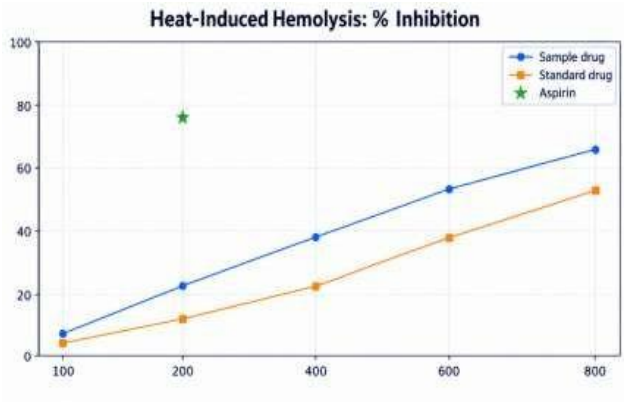


Figure 27: Graph of Heat induced hemolysis (Heat treated).

The heat-induced hemolysis method demonstrated concentration-dependent membrane-stabilizing activity. The sample drug showed maximum inhibition of 66.8% at 800 µg/ml, while the standard drug showed 55.31% inhibition at 800 µg/ml. Aspirin exhibited 73.51% inhibition at 200 µg/ml, indicating strong membrane-stabilizing and potential anti-inflammatory activity.

Table 14: Sample drug heat untreated result for Heat induced hemolysis.

Concentration(µg/ml)	Absorbance (nm)	% inhibition (%)
100	0.095	24
200	0.091	27.2
400	0.083	33.6
600	0.076	39.2
800	0.069	44.8

Table 15: Standard drug heat untreated result for Heat induced hemolysis.

Concentration(µg/ml)	Absorbance (nm)	% inhibition (%)
100	0.102	18.4
200	0.098	21.6
400	0.086	31.2
600	0.081	35.2
800	0.076	39.2
200 (Aspirin)	0.031	75.2

Control heat untreated = **0.125 nm**

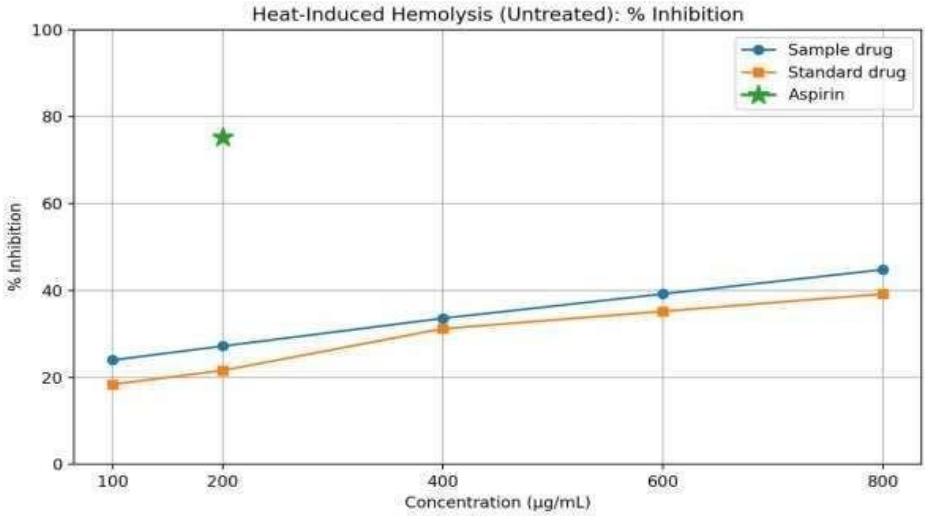


Figure 28: Graph of Heat induced hemolysis (Heat untreated).

The heat-induced hemolysis (untreated) method showed concentration-dependent membrane-stabilizing activity. The sample drug exhibited maximum inhibition of 44.8% at 800 µg/ml, compared with 39.2% for the standard drug at the same concentration. Aspirin showed 75.2% inhibition at 200 µg/ml, indicating stronger membrane-stabilizing and potential anti-inflammatory activity.

7. Membrane Stabilization assay

Table 16: Sample drug result for Membrane Stabilization assay.

Concentration(µg/ml)	Absorbance (nm)	% inhibition (%)
100	0.550	31.25
200	0.420	47.5
400	0.300	62.5
600	0.280	65
800	0.180	77.5

Table 17: Standard drug result for Membrane Stabilization assay.

Concentration(µg/ml)	Absorbance (nm)	% inhibition (%)
100	0.650	18.75
200	0.525	34.37
400	0.378	52.75
600	0.323	59.62
800	0.242	69.75
200 (Aspirin)	0.112	86

Control = **0.800 nm**

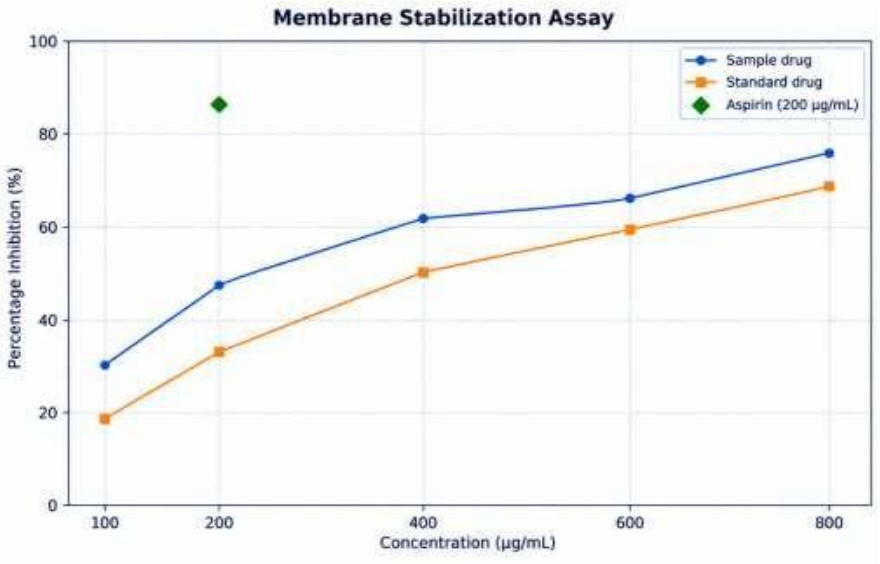


Figure 29: Graph of Membrane Stabilization assay.

The membrane stabilization assay showed that the sample drug exhibited concentration-dependent activity, with inhibition increasing from approximately 31% at 100 µg/ml to 77.5% at 800 µg/ml. The sample drug demonstrated higher inhibition than the standard drug at all tested concentrations. Aspirin (200 µg/ml) showed approximately 86% inhibition. These results suggest that the sample drug possesses significant in-vitro anti-inflammatory potential.

DISCUSSION

The present study evaluated the in-vitro anti-inflammatory activity of a polyherbal extract containing turmeric (*Curcuma longa*), Tulsi (*Ocimum tenuiflorum*), black pepper (*Piper nigrum*), and Aloe vera. Preliminary phytochemical analysis revealed alkaloids, flavonoids, tannins, and saponins in turmeric, Tulsi, and black pepper, whereas Aloe vera showed saponins. Thin-layer chromatography (TLC) revealed Rf values of 0.43, 0.56, and 0.73 for turmeric, 0.83 for Tulsi, 0.58 for black pepper, and 0.72 for Aloe vera. In the protein denaturation assay, maximum inhibition of 81% was observed at 100 µg/ml, indicating

potential anti-inflammatory activity.

The hypotonicity-induced hemolysis assay showed maximum inhibition of 89% at 400 µg/mL, suggesting significant membrane-protective activity. The antiproteinase assay showed inhibition ranging from 26.8% to 40.22%, with maximum activity at 800 µg/ml. The heat-induced hemolysis assay demonstrated concentration-dependent inhibition, reaching 66.8% at 800 µg/ml. In the heat-untreated hemolysis experiment, inhibition ranged from 24% to 44.8%, with maximum activity at 800 µg/ml. The membrane stabilization assay also showed concentration-dependent activity, with maximum inhibition of 77.5% at 800 µg/ml. Comparison with standard drugs indicated promising activity, although the extract was not superior to the standards in all assays.

The observed effects may be attributed to phytochemicals, particularly flavonoids, tannins, alkaloids, and saponins. Variations in inhibition across concentrations highlight the need for repeated experiments, statistical analysis, and further phytochemical investigations. Future studies should include toxicity testing, in-vivo investigations, and optimization of extract proportions to establish safety and efficacy. Overall, the polyherbal preparation demonstrated promising preliminary in-vitro anti-inflammatory activity, supporting further investigation of its potential as a natural anti-inflammatory agent.

REFERENCE

1. Isailovic N, Daigo K, Mantovani A, Selmi C. Interleukin-17 and innate immunity in infections and chronic inflammation. Journal of autoimmunity, Jun 1, 2015; 60: 1-1.
2. Todd I, Spickett GP, Fairclough L. Immunology. John Wiley & Sons, 2015 Oct 5.
3. Medzhitov, R. Origin and physiological roles of inflammation. Nature, 2008; 454(7203): 428-435.
4. Khandelwal K. Practical pharmacognosy. Pragati Books Pvt. Ltd., 2008 Sep 7.
5. Bhangare NK, Pansare TA, Ghongane BB, Nesari TM. Screening for anti-inflammatory and anti-allergic activity of Bharangi {Clerodendrum serrratum (Linn.) moon} in animals.
6. Uttara B, Singh AV, Zamboni P, Mahajan R. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. Current neuropharmacology, Mar. 1, 2009; 7(1): 65-74.
7. Graham JE, Robles TF, Kiecolt-Glaser JK, Malarkey WB, Bissell MG, Glaser R. Hostility and pain are related to inflammation in older adults. Brain, behavior, and immunity, Jul. 1, 2006; 20(4): 389-400.

8. Nguyen TT. Systems biology approaches to corticosteroid pharmacogenomics and systemic inflammation. Rutgers The State University of New Jersey, School of Graduate Studies, 2012.
9. Porter, S. (2013) Tidy's Physiotherapy. Elsevier Health Sciences, Amsterdam.
10. Nourshargh S, Hordijk PL, Sixt M. Breaching multiple barriers: leukocyte motility through venular walls and the interstitium. Nature reviews Molecular cell biology, May 2010; 11(5): 366-78.
11. Springer TA, Anderson DC, Rosenthal AS, Rothlein R, editors. Leukocyte Adhesion Molecules: Proceedings of the First International Conference on:" Structure, Function and Regulation of Molecules Involved in Leukocyte Adhesion", Held in Titisee, West Germany, September 28-October 2, 1988.
12. Gleeson M, Bishop NC, Stensel DJ, Lindley MR, Mastana SS, Nimmo MA. The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. Nature reviews immunology, Sep. 2011; 11(9): 607-15.
13. Murakami M, Hirano T. The molecular mechanisms of chronic inflammation development. Frontiers in immunology, Nov. 15, 2012; 3: 323.
14. Ogrunc M, Di Micco R, Liontos M, Bombardelli L, Mione M, Fumagalli M, Gorgoulis VG, D'Adda Di Fagagna F. Oncogene-induced reactive oxygen species fuel hyperproliferation and DNA damage response activation. Cell Death & Differentiation, Jun. 2014; 21(6): 998-1012.
15. Niederhuber JE, Armitage JO, Doroshow JH, Kastan MB, Tepper JE. Abeloff's clinical oncology. Philadelphia: Elsevier, 2014.
16. Nadkarni AK. The Indian Materia Medica. Popular Press Bldg., 2007; 1: 53-55.
17. Nadkarni AK. The Indian Materia Medica. Popular Prakashan, 2005; 1: 296-297.
18. Vishal V, Sharma GN, Mukesh G, Ranjan B. A review on some plants having anti-inflammatory activity. J Phytopharmacol, 2014; 3(3): 214-21.
19. Adegbola P, Aderibigbe I, Hammed W, Omotayo T. Antioxidant and anti-inflammatory medicinal plants have potential role in the treatment of cardiovascular disease: a review. American journal of cardiovascular disease, Apr. 15, 2017; 7(2): 19.
20. Rhen T, Cidlowski JA. Antiinflammatory action of glucocorticoids—new mechanisms for old drugs. New England Journal of Medicine, Oct. 20, 2005; 353(16): 1711-23.
21. Medzhitov R. Inflammation: New adventures of an old flame. Cell., 2010; 140: 771-6.
22. Fullerton JN, Gilroy DW. Resolution of inflammation: A new therapeutic frontier. Nature Rev Drug Discovery, 2016; 15: 551-67.

23. Markiewski MM, Lambris JD. The role of complement in inflammatory diseases from behind the scenes into the spotlight. Am J Pathol, 2007; 171(3): 715-27.
24. Gessner DK. Ringseis R, Eder K. Potential of plant polyphenols to combat oxidative stress and inflammatory processes in farm animals. J Anim Physiol Anim Nutri., 2017; 101: 605-28.
25. Abdulkhaleq LA, Assi MA, Abdullah R, Zamri-Saad M, Taufiq-Yap YH, Hezmee MN. The crucial roles of inflammatory mediators in inflammation: A review. Veterinary world, May 15, 2018; 11(5): 627.
26. Kumar S, Bajwa BS, Kuldeep S, Kalia AN. Anti-inflammatory activity of herbal plants: a review. Int J Adv Pharm Biol Chem., Apr. 2013; 2(2): 272-81.
27. Sasaki F, Yokomizo T. The leukotriene receptors as therapeutic targets of inflammatory diseases. International immunology, Sep. 2019; 31(9): 607-15.
28. Zarbock A, Polanowska-Grabowska RK, Ley K. Platelet-neutrophil-interactions: linking hemostasis and inflammation. Blood reviews, Mar. 1, 2007; 21(2): 99-111.
29. Bacchi S, Palumbo P, Sponta A, Coppolino MF. Clinical pharmacology of non-steroidal anti-inflammatory drugs: a review. Anti-Inflammatory & Anti-Allergy Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Inflammatory and Anti-Allergy Agents), Jun. 1, 2012; 11(1): 52-64.
30. Anosike CA, Obidoa O, Ezeanyika LU. Membrane stabilization as a mechanism of the anti-inflammatory activity of methanol extract of garden egg (Solanum aethiopicum). DARU Journal of Pharmaceutical Sciences, Nov. 14, 2012; 20(1): 76.
31. Kudumela RG, McGaw LJ, Masoko P. Antibacterial interactions, anti-inflammatory and cytotoxic effects of four medicinal plant species. BMC complementary and alternative medicine, Jul. 3, 2018; 18(1): 199.
32. Yadav R, Mahalwal VS. In-vitro anti-inflammatory activity of oral poly herbal formulations. Pharma Innov J., 2018; 7(2): 272-6.
33. Modi CM, Bhatt PR, Pandya KB, Patel HB, Patel UD. Comparative evaluation of in vitro anti-inflammatory activity of different extracts of selected medicinal plants from Saurashtra region, Gujarat, India. Int. J. Curr. Microbiol. Appl. Sci., 2019; 8(5): 1686-98.
34. Padmanabhan P, Jangle SN. Evaluation of in-vitro anti-inflammatory activity of herbal preparation, a combination of four medicinal plants. International journal of basic and applied medical sciences, 2012; 2(1): 109-116.
35. Mital NM. In-vitro anti-inflammatory activity of traditionally reported poly herbal combination. Asian Journal of Pharmacy and Technology, 2023; 13(1): 1-3.

36. Balkrishna A, Ranjan R, Sakat SS, Sharma VK, Shukla R, Joshi K, Devkar R, Sharma N, Saklani S, Pathak P, Kumari P. Evaluation of polyherbal ayurvedic formulation 'Peedantak Vati'for anti-inflammatory and analgesic properties. Journal of ethnopharmacology, May 10, 2019; 235: 361-74.
37. Joshi GC, Bharkatiya M. Journal of Pharmacognosy and Phytochemistry, 2022; 11(4): 96–105.
38. Kunchanur M, Mannur VS, Koli R, Biradar P. Design and optimization of polyherbal tablets for anti-inflammatory therapy: A design of experiment approach with in vitro and in vivo evaluation. Journal of Holistic Integrative Pharmacy, Mar. 1, 2026; 7(1): 91-101.
39. Thirumalai V, Alex MM, Andrew RSM, Anirutha GR, Anju S, Anna KR, Ashima R. Evaluation of in-vitro anti-inflammatory property of novel polyherbal formulation containing *Sida cordifolia*, *Solanum xanthocarpum*, and *Psidium guajava*. Advances in Bioresearch, 2024; 15(5): 164–170.
40. Kantati YT, Kombate D, N'Bouke ED, Houssounoukpe MK, Kombate B, Kpoyizoun PK, Motto EA, Metowogo K, Lawson-Evi P, Eklu-Gadegbeku K. In vitro antioxidant, anti-inflammatory, and ex vivo neuroprotective activities of KF1 polyherbal formulation.
41. Patil VV, Patil VR. Evaluation of anti-inflammatory activity of Ficus carica Linn. leaves. Indian J Nat Prod Resour, Jun. 2011; 2(2): 151-5.
42. Esho BA, Samuel B, Akinwunmi KF, Oluyemi WM. Membrane stabilization and inhibition of protein denaturation as mechanisms of the anti-inflammatory activity of some plant species. Trends in Pharmaceutical Sciences and Technologies, Dec. 1, 2021; 7(4): 269-78.
43. Kumari CS, Yasmin N, Hussain MR, Babuselvam M. Invitro anti-inflammatory and anti-artheritic property of Rhizopora mucronata leaves. Int. J. Pharm. Sci. Res., 2015; 6: 482-5.
44. Chopade AR, Somade PM, Sayyad FJ. Membrane stabilizing activity and protein denaturation: A possible mechanism of action for the anti-inflammatory activity of Phyllanthus amarus. Journal of Krishna Institute of Medical Sciences University, 2012; 1(1): 67-72.
45. Cordero JA, Camacho M, Obach R, Domenech J, Vila L. In vitro based index of topical anti-inflammatory activity to compare a series of NSAIDs. European journal of pharmaceutics and biopharmaceutics, Mar. 1, 2001; 51(2): 135-42.
46. Vijender K, Bhat ZA, Dinesh K, Puja B, Sheela S. In-vitro antiinflammatory activity of leaf extracts of Basella alba linnVar. Alba. Int. J. Drug Dev. Res., 2011; 3(2): 176-9