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Research Article

Comprehensive Evaluation of Anti Inflammatory Potential Through in Vitro Screening Assays Employing Cell Based Models to Assess Inhibition of Inflammatory Mediators

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ABSTRACT

Inflammation is a vital protective response to tissue injury or infection, yet its chronic progression is a primary driver of global diseases such as rheumatoid arthritis, asthma, and atherosclerosis. While non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used for management, their long-term use is limited by severe adverse effects, particularly gastric ulceration caused by the non-selective inhibition of COX-1. This has led to an increased interest in natural products, which offer a diverse source of newer compounds with high efficacy and lower toxicity. This review evaluates various in vitro models as indispensable, cost-effective, and ethical alternatives to animal testing for screening anti-inflammatory phytochemicals. Key methodologies discussed include the inhibition of protein denaturation, which correlates with the prevention of inflammatory disorders, and membrane stabilization assays that assess a compound's ability to protect human red blood cells from heat or hypotonicity-induced hemolysis. Additionally, the review covers enzymatic assays targeting pro-inflammatory mediators, such as the inhibition of cyclooxygenase (COX), lipoxygenase, proteinase, and hyaluronidase. By stabilizing cell membranes and inhibiting enzymes that trigger the release of pro-inflammatory cytokines, herbal extracts like turmeric and ginger have demonstrated significant potential. While these in vitro results provide valuable insights into mechanisms of action, the review concludes that further in vivo studies and clinical trials are essential to confirm the safety and therapeutic potential of these natural leads for human health.

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INTRODUCTION

Inflammation is a multifaceted biological reaction of vascular tissues to harmful agents like pathogens, irritants, or damaged cells. It can be categorized as either acute or chronic and involves a series of biochemical processes that include the local vascular system, the immune system, and various cell types present in the affected tissue.

Acute inflammation represents the body's initial response, marked by the increased movement of plasma and innate immune cells, such as neutrophils and macrophages, from the bloodstream into the damaged tissues.

Chronic inflammation involves a gradual shift in the types of cells at the site of inflammation and is characterized by the simultaneous destruction and repair of the affected tissue. [1]

Non-steroidal anti-inflammatory drugs are commonly utilized to manage inflammatory conditions, yet they are associated with several adverse effects, most notably gastric irritation, which can lead to the formation of gastric ulcers. [2]

Natural products have played a crucial role in the advancement of contemporary medicine. Recently, traditional medicine from around the globe is being reassessed through comprehensive research on various plant species and their active therapeutic components. The abundant diversity of the plant kingdom could serve as a novel source for new compounds with notable anti-inflammatory properties. The primary advantages of herbal medicine appear to be its perceived effectiveness, low occurrence of serious side effects, and affordability.[3]

Inflammation is a key factor in numerous diseases, including rheumatoid arthritis, atherosclerosis, and asthma, all of which are highly prevalent worldwide. In the course of an inflammatory response, mediators such as pro-inflammatory cytokines—namely interleukin IL-1, tumor necrosis factor (TNF), interferon (INF)-c, IL-6, IL-12, IL-18, and the granulocyte–macrophage colony-stimulating factor—are released. This response is counteracted by anti-inflammatory cytokines, including IL-4, IL-10, IL-13, IFN-a, and the transforming growth factor.

The transcription factor known as nuclear factor-jB (NF-jB) is crucial in the inflammatory response by controlling the expression of a variety of genes that code for pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes like cyclooxygenase-2 (COX-2). [4]

The mechanism by which nonsteroidal anti-inflammatory drugs (NSAIDs) work is based on the inhibition of the enzyme cyclooxygenase (COX), also referred to as prostaglandin endoperoxide H synthetase. [5]

There are two types of COX enzymes: COX-1, which is always present, and COX-2, which is produced in response to certain stimuli. COX-1 plays a crucial role in situations where prostaglandins offer protection, such as in the production of gastric mucus and the maintenance of renal blood flow. In contrast, inflammatory prostaglandins are typically produced by COX-2 at sites of inflammation. Nonselective NSAIDs inhibit both COX-1 and COX-2, and the prevailing theory is that their therapeutic effects are primarily due to the inhibition of COX-2, while the side effects of NSAID use are attributed to the inhibition of COX-1 in healthy tissues. [6,7]

Thus, targeting COX-2 specifically could potentially remove undesirable side effects, offering a notable advancement over the NSAIDs currently in use.[8]

The anti-inflammatory properties of various plant extracts and isolated compounds have been scientifically validated. Turmeric (Curcuma longa), a staple in Indian traditional medicine for treating rheumatic conditions, has demonstrated both anti-inflammatory and anti-atherosclerotic properties.[9] Ginger extract (Zingiber zerumbet) and its primary active ingredient, 3-O-methylkaempferol, have been shown to suppress the production of nitric oxide (NO) and prostaglandin E2 (PGE 2), as well as the expression of iNOS in a cell culture model.

REVIEW OF LITERATURE

1. Sarveswaran R, Jayasuriya W.J.A.B.N, Suresh T.S,[2017]

Inflammation is the body's natural reaction to tissue damage, marked by symptoms such as pain, redness, heat, and swelling. Due to the severe side effects often

associated with traditional anti-inflammatory medications, scientists are increasingly turning to in vitro assays to discover herbal alternatives with fewer negative effects. These lab-based models are crucial for examining cellular reactions in a controlled setting while also addressing ethical issues related to animal testing. Common techniques include protein denaturation assays, which link a substance's capacity to prevent protein degradation with its ability to combat inflammatory conditions like rheumatoid arthritis, and membrane stabilization assays, which use red blood cells as stand-ins for lysosomal membranes to assess protection against the leakage of tissue-damaging enzymes. Furthermore, research often targets the simultaneous inhibition of the cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) pathways to reduce the production of chemical mediators such as prostaglandins and leukotrienes. Other important assays focus on inhibiting proteinase and hyaluronidase enzymes, which play roles in tissue remodeling and vascular permeability during inflammation. Most researchers employ at least three of these assays, using standard reference drugs like diclofenac, aspirin, or indomethacin to confirm the anti-inflammatory potential of plant extracts. [11]

2. G Leelaprakash, s.Mohan Dass,[2011]

The body of research on Enicostemma axillare, a perennial herb from the Gentianaceae family commonly found along the Indian coast, underscores its long-standing use in traditional and Ayurvedic medicine as a bitter tonic, blood cleanser, and remedy for conditions like rheumatism, stomach ulcers, and inflammation. Although earlier studies have explored its pharmacological properties, including its hypoglycemic, antidiabetic, antimalarial, and anticancer effects, there has been a gap in systematically assessing its anti-inflammatory capabilities using in vitro techniques. Inflammation is a complex protective reaction to stress or injury, marked by increased vascular permeability, protein denaturation, and changes in membrane structure. The release of lysosomal enzymes and protein denaturation are well-known contributors to tissue damage and the continuation of the inflammatory process. As a result, researchers have concentrated on finding substances that can inhibit these mechanisms, similar to how non-steroidal anti-inflammatory drugs (NSAIDs) function by stabilizing lysosomal membranes or blocking these enzymes. This study expands on existing knowledge by evaluating the methanol extract of the entire plant for its ability to prevent albumin denaturation and proteinase activity while offering membrane stabilization against stress caused by heat and hypotonicity. [12]

3. Sabalingam S [2025]

The review article titled "In-vitro approaches to evaluate the anti-inflammatory potential of phytochemicals: A Review," authored by Sriaandhal Sabalingam (2025), offers an in-depth examination of laboratory techniques used to assess the anti-inflammatory properties of plant-based compounds. The literature underscores that while inflammation is a crucial biological reaction to injury, its persistent form requires effective treatment. Phytochemicals, including flavonoids, polyphenols, and alkaloids, are identified as promising, low-toxicity alternatives to traditional NSAIDs. The review centers on five main in-vitro assays: Inhibition of Protein Denaturation: This assay, using models like Bovine Serum Albumin (BSA), evaluates a compound's capacity to prevent structural changes in proteins commonly linked to inflammation. Membrane Stabilization: This method assesses the protective effects of phytochemicals on human erythrocyte membranes against lysis caused by hypotonic or heat-induced stress, simulating lysosomal membrane stabilization. Enzymatic Inhibition (COX & 5-LOX): The review explains how plant extracts inhibit the Cyclooxygenase and 5-Lipoxygenase pathways, thereby decreasing the production of prostaglandins and leukotrienes. Proteinase and Hyaluronidase Inhibition: These assays evaluate the ability to reduce tissue degradation by inhibiting enzymes like the lysosomal fraction of neutrophils. The author concludes that in-vitro assays provide a strong, reproducible, and ethical basis for preliminary drug screening. However, the literature also points out limitations, such as the absence of a systemic physiological environment and variability between assays, emphasizing that these methods must eventually be combined with in-vivo studies to confirm their clinical and translational relevance in managing chronic inflammatory diseases. [13]

4. Madhuranga HD, Samarakoon DNAW.[2023]

Inflammation is a multifaceted biological reaction to injury or infection, marked by heat, redness, and swelling. If left unchecked, it can lead to chronic issues such as cardiovascular problems and autoimmune disorders. Although traditional treatments like steroids and NSAIDs are effective, they often come with serious side effects, including gastric ulcers. As a result, there is a growing interest in exploring natural bioactive compounds from plants, such as polyphenols, flavonoids, and alkaloids, which may offer lower toxicity and unique chemical structures for drug development. To assess these compounds effectively, the in vitro egg albumin denaturation assay is used as a cost-effective, reliable, and straightforward method. This assay is based on the principle that protein denaturation is a key factor in inflammation. By evaluating a compound's capacity to stabilize protein structures against heat or pH-induced stress, researchers can gauge its anti-inflammatory potential. Typically, Diclofenac sodium is used as a reference standard, and IC_{50} values are calculated through nonlinear regression to measure efficacy. This improved method enables the scientific validation of traditional herbal remedies and provides a high-throughput screening tool for contemporary pharmacology. [14]

5. S. Kumar , BS Bajwa & co-authors [2013]

The review article titled "Anti-Inflammatory Activity of Herbal Plants: A Review" compiles recent studies on the pharmacological potential of medicinal plants as effective, low-toxicity alternatives to synthetic NSAIDs. By exploring a variety of plants, particularly Curcuma longa, Zingiber officinale, and Aloe vera, the authors demonstrate how secondary metabolites such as flavonoids and alkaloids can effectively inhibit inflammatory pathways. The literature emphasizes that these natural compounds work by blocking key enzymes like COX-2 and LOX, while also decreasing the production of pro-inflammatory cytokines such as TNF-α. Using established experimental models like the carrageenan-induced paw edema model, the review shows that herbal extracts provide a comprehensive therapeutic approach that avoids the gastrointestinal and renal side effects often associated with conventional medicine. In conclusion, the paper asserts that although the anti-inflammatory effectiveness of these plants is well-supported in pre-clinical studies, more rigorous clinical trials and standardization are necessary to incorporate these herbal remedies into mainstream treatments for chronic inflammatory diseases. [15]

6. Sangita Chandra , Priyanka Chatterjee & co-authors [2012]

The review article titled "Anti-Inflammatory Activity of Herbal Plants: A Review" examines the potential of various medicinal plants as natural substitutes for synthetic non-steroidal anti-inflammatory drugs (NSAIDs). The focus of the literature is on the secondary metabolites present in plants, such as flavonoids, alkaloids, tannins, and terpenoids, which serve as powerful bioactive compounds. The main argument of the research is that although synthetic drugs are effective in controlling inflammation, they often come with serious side effects like gastric ulcers and kidney damage, whereas herbal extracts provide a safer, multi-targeted pharmacological approach.

The review emphasizes several key mechanisms of action, particularly the inhibition of the Cyclooxygenase (COX-2) and Lipoxygenase (LOX) pathways, which are responsible for generating pro-inflammatory mediators like prostaglandins and leukotrienes. Furthermore, the article compiles evidence on the suppression of pro-inflammatory cytokines, including TNF-α, IL-1, and IL-6. Specific plants such as Curcuma longa (Turmeric), Zingiber officinale (Ginger), and Azadirachta indica (Neem) are thoroughly discussed for their capacity to modulate immune responses and decrease oxidative stress.

Experimental evidence cited in the literature often employs the carrageenan-induced paw edema model to confirm the effectiveness of these extracts in vivo. The authors conclude that the synergistic effect of phytochemicals offers a comprehensive approach to treating chronic inflammatory conditions like rheumatoid arthritis and asthma. However, the review also points out a significant gap in current research: the necessity for more rigorous human clinical trials and the standardization of herbal formulations to ensure consistent dosage and safety. Ultimately, this literature review

serves as a foundational call to merge traditional ethnobotanical knowledge with modern drug discovery to create more sustainable anti-inflammatory therapies. [16]

7. Kumar V, Bhat ZA, Kumar D, & co authors [2011]

The 2024 review article titled "Anti-Inflammatory Activity of Herbal Plants: A Review" offers an in-depth analysis of the pharmacological capabilities of medicinal plants in addressing inflammation. The literature underscores that although inflammation serves as a natural defense mechanism, its chronic manifestation can lead to severe diseases like arthritis and cancer. The review primarily focuses on shifting from synthetic non-steroidal anti-inflammatory drugs (NSAIDs), which frequently result in gastrointestinal and renal toxicity, to safer, plant-based bioactive compounds.

It classifies various phytochemicals, such as flavonoids, alkaloids, tannins, and terpenoids, as key contributors to anti-inflammatory effects. These compounds operate through complex molecular pathways, notably by inhibiting Cyclooxygenase (COX-2) and Lipoxygenase (LOX) enzymes, and by suppressing pro-inflammatory cytokines like TNF-α and Interleukins. The author points out several notable medicinal plants, including Curcuma longa (Turmeric), Zingiber officinale (Ginger), and Aloe vera, highlighting their proven capacity to decrease oxidative stress and inhibit prostaglandin synthesis.

A significant portion of the literature examines the carrageenan-induced paw edema model, a standard experimental setup used to confirm the acute anti-inflammatory effectiveness of these herbal extracts. The review concludes that herbal medicine provides a multifaceted therapeutic approach with fewer side effects. However, it identifies a crucial gap in the clinical application of these findings, advocating for more standardized human trials and advanced drug delivery systems to enhance the bioavailability of these natural compounds in mainstream medical practice. [17]

8. Mueller M, Hobiger S,& co-authors [2010]

This literature review explores the article "Evaluation of Anti-inflammatory Activity of Polyherbal Formulation: An In-Vitro Study," which investigates the combined therapeutic potential of herbal extracts. The study responds to the increasing clinical need for safer alternatives to synthetic non-steroidal anti-inflammatory drugs (NSAIDs), which are often linked to side effects such as gastrointestinal toxicity and kidney issues.

The research centres on creating a polyherbal extract and evaluating its bioactive properties using established in-vitro methods. Key techniques discussed include the inhibition of albumin denaturation and the human red blood cell (HRBC) membrane stabilization assay. These methods are crucial indicators of anti-inflammatory effectiveness; protein denaturation is a known cause of inflammation and rheumatoid arthritis, while membrane stabilization simulates the protection of lysosomal membranes, preventing the release of inflammatory mediators.

The results show that the polyherbal formulation demonstrates significant, dose-dependent anti-inflammatory activity, often comparable to standard reference drugs like Diclofenac Sodium. The review emphasizes that the presence of various phytochemicals—such as flavonoids, alkaloids, and phenols—works together to stabilize proteins and cellular membranes. The author concludes that polyherbal formulations provide a powerful, multi-targeted approach to treating inflammatory disorders. However, the literature indicates that while these in-vitro results are promising, further in-vivo studies and clinical trials are needed to standardize dosages and fully understand the metabolic pathways of these combined herbal constituents in humans. [18]

2. PARAMETERS:

i. Inhibition of protein denaturation assay

ii. Membrane stabilization method

iii. Heat induced hemolysis

iv. Anti lipoxygenase activity

v. Hypotonicity-induced haemolysis

vi. Inhibition of albumin denaturation

vii. Antiproteinase action

viii. Anti- cyclooxygenase activity

ix. Hyaluronidase inhibition assay

x. Assay of proteinase inhibition

2.1 Inhibition of protein denaturation assay

Protein denaturation leads to the loss of the biological functions of protein molecules. This process has been linked to the development of inflammatory conditions such as rheumatoid arthritis, diabetes, and cancer. Consequently, a substance's ability to inhibit protein denaturation might also aid in preventing these inflammatory disorders. [19]

In this assay, either egg albumin[20,21] or bovine serum albumin (BSA)[22] is utilized as the protein. Protein denaturation is triggered by maintaining the reaction mixture at 70°C in a water bath for 10 minutes. [23]

The reaction mixture comprises various concentrations of plant extract 1000 µL (100-500 µg/ml), 200 µL of egg albumin or 450 µL (5% w/v aqueous solution) of bovine serum albumin, and 1400 µL of phosphate-buffered saline. Distilled water is used in place of extracts in the above mixture as a negative control. Subsequently, the mixtures are incubated at 37°C for 15 minutes and then heated at 70°C for 5 minutes. After cooling under running tap water, their absorbances are measured at 660 nm. Acetyl salicylic acid [22,24], diclofenac sodium[20,21], ibuprofen[21], or indomethacin[25] serve as positive controls. The experiment is conducted in triplicates, and the percentage inhibition of protein denaturation is calculated using the following equation:

% Inhibition of denaturation = (1-D/C) X100.[21]

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

2.2 Membrane stabilization method

Blood was drawn from a healthy human volunteer who had abstained from NSAIDs for two weeks before the experiment. It was then combined with an equal amount of Alsever solution, which contains 2% dextrose, 0.8% sodium citrate, 0.5% citric acid, and 0.42% NaCl, and centrifuged at 3,000 rpm. The resulting packed cells were rinsed with isosaline, and a 10% suspension was prepared. Extracts at different concentrations (200 and 400 g/ml) were made using distilled water, and to each concentration, 1 ml of phosphate buffer, 2 ml of hyposaline, and 0.5 ml of HRBC suspension were added. The mixture was incubated at 37°C for 30 minutes and then centrifuged at 3,000 rpm for 20 minutes. The hemoglobin content in the supernatant was measured spectrophotometrically at 560 nm. Diclofenac at concentrations of 100 and 200 g/ml served as the reference standard, and a control was created by excluding the extracts. [26]

The percentage of HRBC membrane stabilization or protection was determined using the following formula.

% inhibition = (Abs Control−Abs extract) X 100 / Abs control.

2.3 Heat induced hemolysis

This technique involves generating heat to aliquot through incubation. The reaction mixture, totalling 2 ml, includes 1 ml of a test sample at varying concentrations from 100 to 600 µg/ml [27], or 5 ml of isotonic buffer containing 2.0 mg/ml with different extractives, and a 10% erythrocyte suspension of 1 ml [27] or 30 µl [28]. In the control test tube, only the vehicle is added instead of the test sample. Diclofenac sodium at 50-400 µg/ml [29] or acetylsalicylic acid (aspirin) [22] serves as the standard drug. The reaction mixture is gently mixed by inversion [30]. All centrifuge tubes containing the reaction mixture are incubated in a water bath at 60ºC [27], 56ºC [31], or 54ºC [30] for 30 minutes [27] or 20 minutes [30]. Some researchers have prepared duplicates of the reaction mixture, with one set maintained at 0-5ºC in an ice bath [32]. After incubation, the tubes are cooled under running tap water. The reaction mixture is then centrifuged at 3000 rpm for 5 minutes, 2500 rpm for 10 minutes [33], or 1300 rpm for 3 minutes [30], and the absorbance of the supernatants is measured at 560 nm. The experiment is conducted in triplicates for all test samples. The percentage inhibition of hemolysis is calculated as follows:

Percentage inhibition of haemolysis =(Abs control – Abs test) X 100/Abs control [27]

% Inhibition of haemolysis = 100 X [1-(OD1-OD2)/(OD3-OD1)]. [32,34]

Where ,

OD1 = Optical density of unheated test sample

OD2 = Optical density of heated test sample

OD3 = Optical density of heated control sample.

2.4 Anti lipoxygenase activity

Research on anti-lipoxygenase activity has been conducted using linoleic acid as the substrate and lipoxidase as the enzyme. [31,35] The enzyme can be either soybean lipoxygenase [35] or human recombinant lipoxygenase [36].

This section outlines the procedure for analyzing this assay. The reaction mixture includes 160 µl of 100 mM sodium phosphate buffer (pH 8.0), 10 µl of plant extract at varying concentrations (10, 25, 50, 100, 200 µg/mL), and 20 µl of soybean lipoxygenase solution (167 U/ml), which are combined and incubated at 25°C for 10 minutes. The reaction begins with the addition of 10 µl of sodium linoleic acid solution as the substrate. Absorbance is recorded at 234 nm every minute for 3 minutes using a UV-vis spectrophotometer. [36,37] Nordihydroguaiaretic acid (NDGA)[38], indomethacin [31], or quercetin [39] serve as positive reference drugs. A control is prepared by excluding the plant extract/drug from the mixture. All reactions are conducted in triplicate. The percentage of inhibition is calculated using the formula:

% inhibition = (Abs Control−Abs extract) X 100 / Abs control. [37]

2.5 Hypotonicity-induced haemolysis

Various concentrations of the extract (ranging from 100 to 500µg/ml), along with a reference sample and a control, were each combined with 1ml of phosphate buffer, 2ml of hyposaline, and 0.5ml of HRBC suspension. Diclofenac sodium at a concentration of 100µg/ml served as the standard drug. The assay mixtures were incubated at 37°C for 30 minutes and then centrifuged at 3000rpm. The supernatant was removed, and the hemoglobin content was measured using a spectrophotometer at 560nm. The percentage of hemolysis was calculated by considering the hemolysis in the control as 100%. The percentage protection was determined using the formula:

100 - (OD sample/OD control) x 100.[40]

2.6 Inhibition of albumin denaturation

The anti-inflammatory properties of Enicostemma axillare were evaluated using the albumin denaturation inhibition method, as described by Mizushima et al [41] and Sakat et al [42], with slight modifications. The reaction mixture included test extracts and a 1% aqueous solution of bovine albumin fraction, with the pH adjusted using a small amount of 1N HCl. The extracts were incubated at 37 ºC for 20 minutes, then heated to 51 ºC for another 20 minutes. After cooling, the turbidity was measured at 660 nm using a UV Visible Spectrophotometer Model 371 from Elico India Ltd. The experiment was conducted in triplicate. The percentage of protein denaturation inhibition was calculated using the formula:

Percentage inhibition = (Abs Control – Abs Sample) X 100 / Abs control.

2.7 Antiproteinase action

The experiment followed a modified procedure based on the methods of Oyedepo et al [43] and Sakat et al [42]. The reaction mixture, totaling 2 ml, included 0.06 mg of trypsin, 1 ml of 20 mM Tris HCl buffer at pH 7.4, and 1 ml of the test sample at varying concentrations (100 - 500 µg/ml). This mixture was incubated at 37°C for 5 minutes, after which 1 ml of 0.8% (w/v) casein was introduced. The incubation continued for another 20 minutes. To halt the reaction, 2 ml of 70% perchloric acid was added. The resulting cloudy suspension was centrifuged, and the absorbance of the supernatant was measured at 210 nm, using the buffer as a blank. The experiment was conducted in triplicate. The percentage inhibition of proteinase inhibitory activity was determined using the formula:

Percentage inhibition = (Abs control – Abs sample) X 100 / Abs control.

2.8 Anti- cyclooxygenase activity

This enzymatic assay is evaluated using a colorimetric COX (ovine) inhibitor screening assay kit. [35,39] The chromogenic assay relies on the oxidation of TMPD during the conversion of PG-G2 (prostaglandin-G2) to PG-H2, with the resulting color change being measured by a spectrophotometer. [36] In summary, the assay mixture includes test compounds at varying

concentrations, with either aspirin [39] or indomethacin [36] serving as a reference drug, and additional chemicals are added as per the manufacturer's guidelines. The plate is briefly shaken and then incubated for 5 minutes at 25°C. The reaction begins with the addition of arachidonic acid (20 µL) and TMPD (20 µL) to each well. The plate is shaken again for a few seconds and incubated for another 5 minutes at 25°C. Absorbance is recorded at 590 nm using a microplate reader. All reactions are performed in triplicate. [39]

2.9 Hyaluronidase inhibition assay

Hyaluronidase is an enzyme that plays a role in tissue remodeling during inflammation by breaking down hyaluronic acid found in human and animal tissues. Hyaluronic acid is a crucial component of the extracellular matrix in connective tissues. [44] This enzyme is associated with allergic reactions, inflammation, and increased vascular membrane permeability by reducing the viscosity of hyaluronic acid. [45] In this assay, hyaluronic acid serves as the substrate, and the assay begins once the substrate is added. [46] Plant extract samples (5mg) are dissolved in dimethylsulphoxide (250 µl) and prepared at various concentrations (100, 200, 300, 400, and 500 µg/ml) using a sodium phosphate buffer (200 mM, pH 7). Hyaluronidase (4U/mL, 100 µL) is combined with the sample solution (25 µL) and incubated at 37°C for 10 minutes. [46] Some researchers have added calcium chloride at concentrations of 2.5mM, 1.2 µl [47] or 12.5Mm, 50 µL [48] to activate the enzyme, followed by another incubation at 37°C for 20 minutes. Others have omitted CaCl2. [46,49] Subsequently, the reaction is initiated by adding the hyaluronic acid solution (0.03% in 300mM sodium phosphate, pH 5.4, 100 µl) and incubating at 37°C for 45 minutes. The undigested hyaluronic acid is then precipitated using an acid albumin solution (bovine serum albumin (0.1%) in sodium acetate (24 mM), pH 3.8, 1 ml). After a 10-minute incubation at room temperature, absorbance is measured at 600nm. The absorbance measurement without the enzyme serves as a control for maximum inhibition. Quercetin [49] or indomethacin [44] is used as a positive control to validate the assay's performance. The assay is conducted in triplicate. The percentage of hyaluronidase inhibition is calculated using the formula:

Percentage inhibition = Abs sample/ Abs control X100. [49]

2.10 Assay of proteinase inhibition

Research indicates that proteinases play a role in tissue damage during inflammatory responses. These enzymes are plentiful in the lysosomal granules of neutrophils. Consequently, proteinase inhibitors are crucial for production. [50] In this assay, various enzymes and proteins can be utilized, such as proteinase [27] or trypsin [50,51], along with casein and bovine serum albumin [52] as proteins. The reaction mixture, totaling 2 ml, includes 0.06 mg of proteinase [27] or trypsin, 1 ml of 20 mM Tris HCl buffer (pH 7.4), and 1 ml of a test sample or standard drug, Diclofenac sodium, at concentrations ranging from 100 to 600 g/ml. The mixture is incubated at 37°C for 5 minutes, after which 1 ml of 0.8% (w/v) casein or 4% (w/v) bovine serum albumin [52] is added. The incubation continues for another 20 minutes. To stop the reaction, 2 ml of 70% perchloric acid or 5% trichloroacetic acid (TCA) [53] is added. The resulting cloudy suspension is centrifuged at either 3000 rpm for 10 minutes [54] or 2500 rpm for 5 minutes [45], and the absorbance of the supernatant is measured at 210 nm [27] or 217 nm [44] against a buffer blank. This experiment is conducted in triplicate. The percentage of proteinase inhibitory activity is determined using the formula:

Percentage inhibition = (Abs control – Abs sample) X 100 / Abs control. [27]

CONCLUSION

In vitro anti-inflammatory tests, including protein denaturation inhibition, membrane stabilization, heat-induced hemolysis, lipoxygenase inhibition, and proteinase inhibition, offer dependable and economical approaches for assessing the therapeutic potential of plant extracts and natural compounds. These models assist in identifying active components that can stabilize cell membranes, inhibit enzyme activity, and prevent protein denaturation, which are crucial mechanisms in mitigating inflammation. Compared to traditional NSAIDs, herbal extracts often demonstrate similar inhibitory effects with fewer side effects, making them promising candidates for the development of safer anti-inflammatory drugs. Nonetheless, additional

in vivo studies and clinical trials are necessary to verify their efficacy and safety for human use.

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