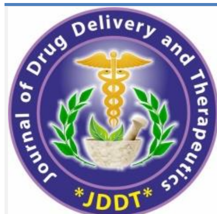

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Review Article

In-vitro approaches to evaluate the anti-inflammatory potential of phytochemicals: A Review

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Abstract

Phytochemicals, bioactive compounds derived from plants, have drawn considerable attention for their ability to modulate inflammatory pathways, presenting promising alternatives for the treatment of chronic inflammatory diseases. Inflammation, a complex biological response to injury or infection, involves a cascade of cellular and molecular events mediated by enzymes, cytokines, and reactive species. *In vitro* assays provide an essential platform for screening and investigating the anti-inflammatory potential of phytochemicals, offering valuable insights into their mechanisms of action. Commonly used techniques include the inhibition of protein denaturation and membrane stabilization, which evaluate the ability of compounds to prevent structural damage to proteins and cell membranes. Enzymatic assays, such as cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) inhibition tests, focus on the suppression of key enzymes involved in arachidonic acid metabolism, thereby reducing the production of pro-inflammatory mediators like prostaglandins and leukotrienes. Other assays, like proteinase and hyaluronidase inhibition tests, assess the ability of compounds to block enzymes contributing to tissue degradation and inflammation. These assays offer robust, reproducible frameworks for evaluating phytochemicals in preclinical research, helping to identify compounds with potential therapeutic value. However, their limitations, such as lack of *in vivo* context and inter-assay variability, necessitate their integration with complementary studies to validate findings and understand their translational significance.

Keywords: Phytochemicals, *in vitro* methods, anti-inflammatory activity, protein denaturation, membrane stabilization

Introduction

Inflammation is a natural defence mechanism for tissue damage brought on by harmful chemicals, microorganisms, or physical trauma. To eliminate the irritants and prepare the body for tissue healing, the body reacts by inactivating or destroying the invasive organisms. It is brought on by migrant cells and wounded tissue releasing chemical mediators. The symptoms of inflammation include heat, redness, discomfort, swelling, and disruption of physiological processes [1]. The inflammatory process involves a series of events in which the metabolism of arachidonic acid plays a critical role. The Cyclooxygenase (COX) pathway converts arachidonic acid into prostaglandins and thromboxane A2, whereas the 5-lipoxygenase (5-LOX) pathway converts it into leukotrienes (LTs) and eicosanoids. In a variety of inflammatory reactions, these metabolites play an important role as chemical mediators. Current anti-inflammatory medications successfully block both enzyme routes and reduce symptoms, but they often cause significant adverse effects [2]. There is a need to discover medicines that have fewer adverse effects as a result.

Plants are the source of bioactive substances known as phytochemicals. Multiple studies have been conducted on the anti-inflammatory properties of several plants. The anti-inflammatory qualities of phytochemicals have drawn attention because of their bioactive components, which include terpenoids, alkaloids, and flavonoids [3]. Phytochemicals are interesting options for the treatment of illnesses related to inflammation because of their capacity to modulate inflammation by blocking pro-inflammatory mediators and signalling pathways [4].

In vitro studies are a key investigation tool, particularly during the early phases of drug development and scientific investigations, because they have several advantages over *in vivo* tests. *In vitro* experiments are conducted in a controlled environment, such as cell cultures or biochemical assays, allowing researchers to isolate specific variables without interference from the complex interactions present in living organisms [5]. Studies conducted *in vitro* are less expensive than those conducted *in vivo*. They are a cost-effective option for early research since they do not require the costs of housing and caring for animal models or carrying out human trials. This review focuses on five widely used *in vitro* assays: inhibition of protein denaturation,

membrane stabilization, Cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) inhibition, proteinase inhibition, and hyaluronidase inhibition [6-10]. These assays are critical for elucidating the molecular mechanisms of phytochemicals and their potential therapeutic applications. This review aims to explore various *in vitro* methods used to evaluate the anti-inflammatory activity of phytochemicals, discussing their mechanisms of action and the challenges involved.

In-vitro methods to measure anti-inflammatory effects of phytochemicals

Inhibition of Protein Denaturation Assay

The Inhibition of Protein Denaturation Assay is a widely used in-vitro method to evaluate the anti-inflammatory potential of compounds, particularly phytochemicals. Whenever proteins undergo denaturation, they lose their tertiary structure because of external stress like heat, pH changes, or chemicals. This leads to the loss of their functional properties. Protein denaturation is a common result of inflammation and oxidative stress, which exacerbates tissue damage and progresses diseases [11]. This experiment involves applying heat or chemical stress to a protein solution, usually egg albumin or bovine serum albumin (BSA), to determine the ability of the compound to inhibit protein denaturation. The test compound is introduced to the protein solution, and the extent of denaturation is assessed by measuring changes in absorbance, turbidity, or the loss of enzymatic activity [12]. The compound's protective effect is determined by comparing the absorbance or activity levels in the presence of the test compound to a control.

A lower absorbance or higher enzyme activity in the presence of the compound indicates its potential to inhibit protein denaturation. In this test, phytochemicals like flavonoids, polyphenols, and alkaloids showed promising results, exhibiting their protective and anti-inflammatory qualities by stabilizing proteins and preventing their denaturation [13]. The protein model that is frequently utilized is bovine serum albumin (BSA). The protein solution is heated at 70°C for 10 minutes to cause denaturation.

A reaction mixture contains 1000 µL (100–500 µg/ml) of plant extract, 200 µL of egg albumin or 450 µL (5% w/v aqueous solution) of bovine serum albumin, and 1400 µL of phosphate buffered saline in different concentrations. As a negative control, distilled water is used in place of the extracts in the mixture above. Afterward, the mixtures are incubated at 37 °C for 15 min and then heated at 70°C for 5 min. Once the protein solution has cooled to room temperature, a spectrophotometer is used to measure the degree of denaturation at 660 nm [14]. Standard anti-inflammatory drugs, such as ibuprofen or diclofenac sodium, are utilized for comparison. Percentage inhibition of protein denaturation is calculated using following formula.

$$\text{Inhibition (\%)} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} \times 100$$

Despite being an easy and economical technique, this assay fails to give any direct insights into cellular pathways or *in vivo* relevance. It does, however, function as an initial screening method for substances with anti-inflammatory qualities.

Membrane Stabilization Method

The membrane stabilization method is a widely used technique for evaluating the anti-inflammatory properties of herbal extracts, synthetic compounds, and pharmaceutical preparations. The theory behind this technique is that certain substances can prevent biological membranes from lysing when they are subjected to osmotic, chemical, or physical stress. Red blood cell membranes are a suitable model for this assay because of their structural similarities to lysosomal membranes [15-16].

Immune cell activation and the production of inflammatory mediators are two characteristics of inflammation, a complex biological reaction to tissue damage or infection. Intracellular organelles called lysosomes are essential to this process because they release hydrolytic enzymes that cause tissue destruction. Inflammatory reactions can be reduced by stabilizing lysosomal membranes during inflammation, which stops these enzymes from leaking out. Therefore, a compound's capacity to stabilize erythrocyte membranes is an alternative for its anti-inflammatory properties [17].

In the membrane stabilization assay, human erythrocytes are usually exposed to heat or hypotonic solutions, which cause haemolysis by rupturing the erythrocyte membrane. The membrane stabilization method has the following benefits: it is easy to use, economical, and repeatable. Furthermore, it is an ethical substitute for the initial screening of anti-inflammatory medications because it does not require the use of complex animal models. But it has limitations, like not being able to take into consideration the full inflammatory cascade or the test compound's systemic effects [18].

I. Hypotonic solution induced haemolysis

This technique exposes human erythrocytes to hypotonic solutions, which cause water to enter the cells and cause an osmotic imbalance. This leads to swelling, disruption of the membrane, and subsequent release of haemoglobin, a process termed haemolysis. Anti-inflammatory compounds can prevent or reduce haemolysis by stabilizing the erythrocyte membrane and maintaining its structural integrity.

After that, an erythrocyte suspension is made and incubated with the test chemical while a hypotonic solution is present. To separate intact cells from the supernatant, the reaction mixture is centrifuged at 3000 rpm following incubation at a particular temperature and time (usually 37°C for 30 minutes). A spectrophotometer is used to measure the absorbance of the released haemoglobin in the supernatant at 540 nm in order to quantify the degree of haemolysis. The haemolysis percentage is computed in relation to a control sample that does not contain the test drug [19]. The following formula is used to calculate the percentage inhibition of haemolysis, which is a sign of membrane stabilization:

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

II. *Heat induced haemolysis*

Inflammatory responses often involve cellular damage and membrane destabilization caused by physical or biochemical stress. During inflammation, lysosomal enzymes are released due to membrane lysis, which exacerbates tissue damage. Compounds that stabilize membranes can potentially reduce this damage by preventing lysosomal enzyme release. In the heat-induced haemolysis assay, the ability of a compound to protect erythrocyte membranes from heat-induced lysis serves as an indirect indicator of its anti-inflammatory potential [20].

A 10% erythrocyte suspension is prepared and incubated with the test compound in an isotonic buffer. Control samples (without the test compound) are included for comparison. The test samples are subjected to heat stress by incubating them at elevated temperatures, typically 54°C, for a fixed duration (usually 30 minutes). Following incubation, the samples are cooled to room temperature and centrifuged to separate intact cells from the supernatant. The extent of haemolysis is determined by measuring the absorbance of released haemoglobin in the supernatant at 540 nm using a spectrophotometer.

The percentage inhibition of haemolysis, which reflects the membrane-stabilizing activity of the test compound, is calculated using the formula [21]:

$$\%\ \text{Inhibition of haemolysis} = \frac{(\text{Absorbance control} - \text{Absorbance test})}{\text{Absorbance control}} \times 100$$

% Inhibition of haemolysis = 100 X [1- (OD_1-OD_2) / (OD_3-OD_1)]

Where OD_1 =Optical density of unheated test sample

OD_2 = Optical density of heated test sample

OD_3 = Optical density of heated control sample

Assay of Proteinase Inhibition

Proteinases and other proteolytic enzymes contribute to tissue damage, facilitate immune cell migration to inflammatory areas, and break down extracellular matrix components, all of which are important aspects of inflammatory processes. Compounds that inhibit proteinase activity can mitigate these effects, making proteinase inhibition assays valuable tools in pharmacological and biochemical research. The assay is based on the ability of a test compound to inhibit the activity of proteinases, such as trypsin or chymotrypsin, on a specific protein substrate. Proteinase activity is commonly measured by detecting the cleavage products of the substrate, either spectrophotometrically, fluorometrically, or by other analytical methods. The reduction in enzymatic activity in the presence of the test compound indicates its inhibitory potential [22].

In this experiment, commercially available proteolytic enzymes such papain, chymotrypsin, or trypsin are frequently used. To maintain its optimum enzymatic activity, the enzyme is dissolved in an appropriate buffer, such as Tris-HCl buffer or phosphate-buffered saline (PBS). Casein, bovine serum albumin (BSA), and synthetic chromogenic substrates such as Nα-Benzoyl-DL-arginine-p-nitroanilide (BAPNA) are examples of common substrates. A buffer is used to dissolve the substrate at a concentration high enough to allow for enzymatic cleavage. To enable binding and possible inhibition, the test substance is incubated with the enzyme for a predetermined amount of time at 37°C. The substrate is added to start the reaction after pre-incubation. In parallel, a control response is conducted without the test chemical [23-24].

To ensure sufficient enzymatic cleavage of the substrate, the reaction mixture is incubated at 37°C for a predetermined amount of time, such as 30 minutes. By adding a stop solution, like trichloroacetic acid (TCA), which precipitates undigested proteins or inhibits enzyme activity, the reaction is stopped. A particular wavelength, such as 410 nm, is used to monitor the release of chromogenic compounds. Following the precipitation of undigested proteins, the turbidity decreases for casein or BSA as substrates is evaluated spectrophotometrically at 280 nm. The percentage inhibition of proteinase inhibitory activity is calculated using the following equation [25-26].

$$\text{Inhibition}\ (\%) = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} \times 100$$

Assay of Hyaluronidase Inhibition

The enzyme hyaluronidase catalyzes the breakdown of hyaluronic acid, an essential part of the extracellular matrix that keeps tissues hydrated, elastic, and intact [27]. Excessive activity of hyaluronidase is linked to a wide range of clinical conditions such as arthritis and skin aging, as well as inflammation and tissue damage [28]. Hyaluronidase inhibition can lessen these effects, which makes this assay a valuable tool for study in pharmacology and cosmetics. The assay measures a compound's capacity to prevent hyaluronic acid from being broken down by hyaluronidase. The breakdown of hyaluronic acid yields smaller oligosaccharides that can be identified by precipitation processes, colorimetry, or spectrophotometry. The test compound's inhibitory impact on hyaluronidase is indicated by a decrease in degradation in its presence [29].

Hyaluronic acid serves as the substrate in this test, and the assay is started once the substrate has been added. Samples of plant extract (5 mg) are dissolved in 250 µL of dimethylsulfoxide. The samples are produced by dissolving them in sodium phosphate buffer (200 mM, pH 7) at different concentrations (100, 200, 300, 400, and 500 µg/mL). Sample solution (25 µL) is combined with hyaluronidase (4U/mL, 100 µL) and incubated for 10 minutes at 37ºC [30-31]. To activate the enzyme, some researchers have added calcium chloride at 2.5 mM, 1.2 µL [32], or 12.5 m, 50 µL [33]. The mixture is then incubated for another 20 minutes at 37 °C. The inclusion of $CaCl_2$ has been left out by some researchers.

Subsequently, the substrate, a hyaluronic acid solution (0.03% in 300 mM sodium phosphate, pH 5.4, 100 µL), is added to start the reaction, and it is then incubated for 45 minutes at 37ºC. Acid albumin solution (0.1%) in sodium acetate (24 mM), pH 3.8, 1 mL) is then used to precipitate the undigested hyaluronic acid. Absorbance is measured at 600 nm following a 10-minute incubation period at room temperature. For maximum inhibition, the

absorbance measurement without the enzyme is utilized as a control value. The assay's performance is confirmed by using either quercetin or indomethacin as the positive control [31].

The percentage inhibition of hyaluronidase activity is calculated as:

$$\text{Inhibition (\%)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Assay of Cyclooxygenase (COX) and 5-Lipoxygenase (5-LOX) Inhibition

Cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) enzyme inhibition is a commonly used technique to assess a compound's anti-inflammatory potential. These two enzymes are essential for the metabolism of arachidonic acid because they generate the important inflammatory mediators prostaglandins and leukotrienes, respectively [34]. These assays are crucial in pharmacological and drug development research because drugs can have strong anti-inflammatory effects by blocking these enzymes. The tests for COX and 5-LOX inhibition are essential for finding new anti-inflammatory medications, especially dual inhibitors that target both enzymes. The potential for dual inhibitors to lessen gastrointestinal adverse effects linked to selective COX inhibition while offering more extensive anti-inflammatory effects makes them desirable [35-36].

I. COX Inhibition Assay:

There are two primary isoforms of cyclooxygenase: constitutively produced COX-1, which maintains physiological processes such protecting the stomach mucosa, and inducible COX-2, which is primarily engaged in inflammation and pain. The COX inhibition assay quantifies a compound's capacity to prevent these enzymes from converting arachidonic acid to prostaglandins [37]. Enzyme extracts from biological tissues or commercially available pure COX enzymes (COX-1 and COX-2) are used. In the assay, the enzyme is incubated with arachidonic acid (substrate) either with or without the test substance present. To speed up the process, hematin or other cofactors are introduced [38-39]. Prostaglandin synthesis is measured by enzyme immunoassay, colorimetric, or fluorometric techniques. The reaction product is usually combined with a chromogenic substrate, like N,N-dimethyl-p-phenylenediamine (DMPD), to create a detectable color shift for colorimetric detection. In comparison to a control response in which the test substance is absent, the percentage inhibition of prostaglandin synthesis is computed [40].

II. 5-LOX Inhibition Assay:

5-LOX is an enzyme that converts arachidonic acid into pro-inflammatory mediators called leukotrienes, which have been associated in allergic responses, asthma, and arthritis. The 5-LOX inhibition test assesses a compound's capacity to prevent the formation of leukotrienes [41]. Purified 5-LOX or leukocyte enzyme extracts (such as human polymorphonuclear leukocytes or rat basophilic leukemia cells) are frequently utilized. In the presence of the test substance, the enzyme is incubated with either linoleic acid or arachidonic acid (substrate). Leukotriene biosynthesis intermediates, hydroperoxyeicosatetraenoic acids (HPETEs), are produced and quantified using high-performance liquid chromatography (HPLC) or spectrophotometry at 234 nm (UV absorbance). Leukotriene levels can also be found using particular enzyme-linked immunosorbent tests (ELISAs). In comparison to the control, the percentage suppression of leukotriene or HPETE generation is computed [42-44].

Conclusion

In vitro assays are indispensable for evaluating the anti-inflammatory potential of phytochemicals, offering valuable insights into their mechanisms of action. The methods discussed in this review, including inhibition of protein denaturation, membrane stabilization, COX and 5-LOX inhibition, proteinase inhibition, and hyaluronidase inhibition, provide a comprehensive framework for preclinical studies. While *in vitro* models offer valuable insights, several challenges need to be addressed: Many *in vitro* models use immortalized cell lines or animal-derived cells, which may not fully replicate the complexity of human inflammation. The lack of a complete immune system and tissue-specific responses is a limitation. Phytochemicals often have low bioavailability, and their effects can vary depending on their absorption, distribution, metabolism, and excretion. *In vitro* studies may not always account for these factors. The concentration of phytochemicals used in *in vitro* studies is often much higher than what is achievable in humans. This raises concerns about the translation of *in vitro* findings to clinical settings.

Future research in this area should focus on development of more physiologically relevant models: Integrating co-culture systems, organ-on-a-chip technology, and 3D cell cultures can provide more accurate models for studying phytochemical effects on inflammation. Although *in vitro* results are promising, clinical studies are needed to confirm the therapeutic potential of phytochemicals. Clinical trials that assess the bioavailability, efficacy, and safety of phytochemicals in humans are essential for translating *in vitro* findings into real-world applications.

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