**Review Article**

**Evaluation of *in vitro* anti-inflammatory activity: A comprehensive review of methods, advantages, and limitations**

**ABSTRACT**

Inflammation is a vital biological response that plays an important role during tissue repair and defense against infections. A range of transcription factors mediates this process (NF-KB and AP-1) and enzymes (Cycooxygenase-2 (COX-2) and 5-Lipoxygenase (5-LOX)). The dysregulation of inflammation can lead to chronic diseases such as atherosclerosis, cardiovascular disorders, and rheumatoid arthritis. Corticosteroids and Non-steroidal anti-inflammatory drugs (NSAIDs) are often used as anti-inflammatory drugs. Despite their effectiveness, the prolonged use of these drugs comes with various side effects. Therefore, there is a growing demand for safer but effective anti-inflammatory drugs. Plants and plant-based products have a rich history for their medicinal properties. Hence, medicinal plants can be a strong alternative for developing new anti-inflammatory drugs.

*In vitro* assays are frequently utilized for the initial screening of natural products for their pharmacological properties as they are cost-effective, direct, and time-efficient. Protein denaturation inhibition assays, membrane stabilization assays, enzyme inhibition (COX/LOX), and nitric oxide assays are commonly used to evaluate the anti-inflammatory properties of drugs. However, the inability of *in vitro* models to fully replicate the complexity of living organism responses remains a significant limitation. This review explores frequently utilized *in vitro* assays to evaluate anti-inflammatory activity, outlining their principles, methodologies, advantages, and limitations. It aims to help researchers choose appropriate techniques for drug discovery and the development of anti-inflammatory medications.

*Keywords: anti-inflammatory activity, protein denaturation, membrane stabilization, medicinal plants, in vitro assays*

**INTRODUCTION**

Inflammation is an important biological response to infection or injury. It activates the immune pathways to help in defense and healing processes (1). Activators of transcription and signal sensors are the key intracellular mediators of inflammation (2). Among the inflammatory transcription factors, nuclear factor NF-KB, nuclear factor of activated T cells, and activated protein (AP-1) are considered most significant. However, measuring these factors was found to be challenging (3). On the other hand, anti-inflammatory enzymes and proteins are recognized anti-inflammatory biomarkers that are easy to measure. They include cyclooxygenase‐2 (COX‐2), HAase, 5‐lipoxygenase (5‐LOX), interleukin‐6 (IL‐6), interleukin‐8 (IL‐8), tumor necrosis factor‐alpha (TNF‐α), and nitric oxide (NO) synthesized by inducible NO synthases (iNOS) (4).

In the events of inflammatory mechanisms, the metabolism of arachidonic acid (AA) is found to play an important role. It is used as a substrate by both COX-2 and 5-LOX, where COX-2 converts AA to prostaglandins while 5-LOX converts AA to leukotrienes (LTs). Prostaglandins are responsible for promoting most of the pain, fever, and other inflammatory symptoms, while LTs are accountable for enhancing inflammation and allergic reactions. NO plays its role in providing protection against infectious pathogens while modulating immunity. ILs and TNF-α are cytokines that are produced by inflammatory cells like macrophages. They are important pro-inflammatory mediators involved in apoptosis and cell differentiation (5).



Figure 1: Diagram of inflammatory pathway (5).

The presence of these factors and pro anti-inflammatory mediators can induce inflammatory related diseases including cardiovascular diseases, atherosclerosis, and rheumatoid arthritis. The most commonly prescribed drugs for treating acute inflammation are non-steroidal anti-inflammatory drugs (NSAIDs), but their efficacy in treating chronic inflammation has been found to be lower. For chronic inflammation, however, steroid anti-inflammatory medications work better than NSAIDs (6). Despite their effectiveness, prolonged use of these drugs comes with various side effects. Peptic ulceration, renal dysfunction, and mental confusion are some of them (1). Therefore, finding new anti-inflammatory drugs is an active and ongoing area of research in pharmaceuticals (7).

Since ancient times, plants have played a key role in human health care and wellbeing (8, 9, 10, 11, 12). As an adaptation to environmental stress and infections, plants hold a unique ability to produce small organic molecules through secondary metabolism. These molecules show various biological properties, and anti-inflammatory activity is found to be one of them (13, 14). According to estimates from the World Health Organization (WHO), nearly 65% of people around the world receives medical care through traditional medicine. The use of natural compounds derived from medicinal plants to treat a variety of illnesses has gained popularity in clinical research. As a result, plants are now the primary source of substances used for developing new medications, while a sizable portion of the medications prescribed worldwide are derived from them. In recent years, the anti-inflammatory properties of plants have been the subject of hundreds of research and review articles published (15).

*In vitro* assays are routinely used for the preliminary screening of a poetical drug/chemical or compound (16). Compared to *in vivo* experiments, *in vitro* experiments are simpler, have shorter turnaround times, and are less expensive for quick screening for anti-inflammatory substances (17). Despite the widespread use*, in vitro* assays also come with certain limitations. The complex nature of whole-organism responses observed *in vivo* studies is sometimes not replicated in *in vitro* models is one of the limitations (18).

Researchers use different methodologies to evaluate the anti-inflammatory activity of plant materials. This review aims to provide a comprehensive review of all the frequently used in vitro anti-inflammatory assays, including their principles, methods, and applicability to drug discovery. Moreover, it aims to highlight the advantages and disadvantages of *in vitro* assays.

**1. *IN VITRO* ASSAYS TO EVALUATE THE ANTI-INFLAMMATORY ACTIVITY**

**1.1. Protein denaturation inhibition assays**

Egg albumin or bovine serum albumin (BSA) is used as the protein in this assay (19).

**1.1.1 Principle of the assay**

The process by which proteins lose their secondary and tertiary structures as a result of external pressures or chemicals like heat, an organic solvent, a concentrated inorganic salt, or a strong acid or base is known as protein denaturation. Proteins lose their biological functions when they become denatured. Protein denaturation is a key feature of inflammation. Therefore, the ability of a drug or a pant product to stop or reduce protein denaturation may have the ability to stop or control inflammation. Heat-induced egg albumin /bovine serum albumin denaturation inhibition assays are frequently used as protein denaturation inhibition assays. When the reaction mixture is exposed to heat, protein denatures and increases the turbidity of the mixture. This leads to an increase in the absorbance of the mixture. If a potential drug/pant product has anti-inflammatory properties, it will inhibit protein denaturation, lowering the progressive increase in absorbance due to protein denaturation (1, 19).

**1.1.2 Method of heat-induced egg albumin denaturation inhibition assay**

Making a 1% egg albumin solution: 1% egg albumin solution can be prepared with fresh hen eggs or easily found egg albumin powder from stores.
To correctly make an egg-albumin solution with a fresh hen's egg, crack the egg carefully. Then add 1 mL of the translucent part to 100 mL of w/V distilled water, and stir until well combined. Egg albumin is the transparent part of the egg. When creating the solution, the water should be cold. When water is brought to a boil, it will coagulate.

For the test mixture, mix 2.8mL of phosphate-buffered saline (PBS) with a pH adjusted to 6.4, 0.2mL of egg albumin solution, and 2mL of plant extract (concentration 15.625, 31.25, 62.5, 125, 250, 500, 1000, and 2000 μg/ml) to obtain a volume of 5mL.

For the standard mixture, mix 2.8mL of PBS (pH =6.4), 0.2mL of egg albumin solution, and 2mL of the reference drug (e.g.: Diclofenac Sodium, acetylsalicylic acid, indomethacin) (concentration 15.625, 31.25, 62.5, 125, 250, 500, 1000, and 2000 μg/ml (prepared from Diclofenac Sodium crystalline powder) to obtain a volume of 5mL.

For positive control, mix 2.8mL of PBS (pH =6.4), 0.2mL of egg albumin solution, and 2mL of distilled water to obtain a volume of 5mL.

As the negative control, 5mL of distilled water can be used.

Incubate all the mixtures (tests, standards, controls) at 37 ± 2 ºC for 15 minutes. Then, increase the temperature gradually to 70 ºC. Once it reaches 70 ºC, keep the mixtures at 70 ºC for another 5 minutes. Take out the mixtures from the water bath and allow them to cool down for 15-20 minutes. After cooling, measure the absorbance of each mixture at a wavelength of 660 nm using a spectrophotometer. Carry out the test in triplicates

Finally, calculate the percentage of protein denaturation inhibition using the formula below (1, 20, 21).

% Inhibition of egg albumin denaturation = $\frac{(Vc- Vt)}{Vc}$ × 100

(Vc = absorbance of the positive control, Vt = absorbance of the test)

*1.1.2.1 Ensuring a standard egg albumin concentration*

The reliability of this assay depends on maintaining a consistent egg albumin concentration. Hen’s breed, weight, diet, and egg size are some factors that can affect the albumin concentration of eggs. Few studies have outlined strategies for standardizing albumin concentration in eggs for this assay.

Prepare a standardized Albumin Solution: It is recommended to make a consistent albumin solution when working with fresh eggs. For this, carefully separate the egg whites from a fresh hen’s egg and mix 1mL of egg whites with 100 mL of cold distilled water. This technique aids in reaching a steady concentration for use in experiments (21).

Measurement and Adjustment: Determine the protein concentration of the albumin solution after it has been prepared using spectrophotometric techniques or a protein assay kit. Adjust the concentration as necessary to ensure that your essays meet the required standard. For this, a Nanodrop spectrometer or applying Beer Lambart’s Law can be involved (21).

* Using a Nanodrop for albumin measurement (22)

Prepare the albumin solution (e.g., 1% v/w). Aliquot the solution and measure the absorbance at 280nm.

* Applying Beer Lambert’s Law

A = ɛ.c.l

A – Absorbance at 280nm

ɛ - Molar extinction coefficient of egg albumin (known or determined experimentally)

(The extinction coefficient of egg albumin at 280nm is, ~30,590cm-1M-1, or 7.16 of a 1% solution) (23)

c- Concentration of albumin of the solution

l- Path length ((usually 1 cm for standard cuvettes, but if Nanodrop spectrophotometry is used, it uses a shorter pathlength, typically ~0.05 mm, which is auto-calibrated)

Therefore, c = $\frac{A}{ɛ. l}$

Using commercial egg albumin powder: utilizing commercially available egg albumin powder can be a consistent and standard albumin source (21).

**1.1.3 Method of heat-induced Bovine Serum Albumin (BSA) Denaturation inhibition assay**

0.5% Bovine serum albumin (BSA)- dissolve 500 mg of BSA in 100 mL of distilled water.

Phosphate Buffered Saline (PBS), pH = 6.3 - to 800 mL of distilled water add 8 g of sodium chloride (NaCl), 0.2 g of potassium chloride (KCl), 1.44 g of disodium hydrogen phosphate (Na2HPO4), and 0.24 g of potassium dihydrogen phosphate (KH2PO4). Adjust the pH to 6.3 using 1M HCl and top up the final volume to 1000 mL with distilled water.

Test solution (0.5 mL) - mix 0.45 mL of 0.5% BSA with 0.05 mL of the test solution in various concentrations.

Standard solution (0.5 mL) - mix 0.45 mL of 0.5% BSA with 0.05 mL of Diclofenac Sodium in various concentrations.

Positive control - mix 0.45 mL of 0.5% BSA with 0.05 mL of distilled water. The control represents a 100% protein denaturation.

After the preparation of all sample materials, incubate at 37 0C for 20 minutes gradually increase the temperature to 57 0C and keep for another 3 minutes. Then, take out all the samples and let them cool for 15-20 minutes. After cooling add 2.5 mL of PBS (pH = 6.3) to all samples and measure the absorbance at 255 nm using a spectrophotometer. Carry out the test in triplicates

The % of inhibition of protein denaturation can be calculated using the following equation.

The results are then compared with the reference drug, Diclofenac Sodium (24).

% inhibition of denaturation = $ 100-[\frac{(Optical density of the test solution - Optical density of the control)}{Optical density of the test}x 100] $

**1.2 Membrane stabilization assays**

**1.2.1. Principle of the Membrane stabilization assays**

During an inflammatory condition, lysosomal membranes can lyse, releasing enzymes that can further enhance the inflammation. The action of NSAIDs is to inhibit the release of lysosomal enzymes or stabilize the lysosome membranes. Human red blood cell (HRBC) membranes are considered to be similar to lysosomal membranes. When RBCs are exposed to harmful substances like heat, hypotonic mediums, phenylhydrazine, and methyl salicylate, it can lyse the RBC membranes.

The anti-inflammatory properties of a plant material can be evaluated with membrane stabilization assays. In this assay, heat-induced hemolysis or hypotonic solution-induced hemolysis is observed using humans, rats, or mice RBCs. As the lysosomal membrane is considered similar to the erythrocyte membrane, the effects of a potential plant extract on the membrane of RBCs under harmful environments prove its ability to stabilize the lysosomal membranes and thereby show its anti-inflammatory properties (1, 19, 25).

**1.2.2. Hypo tonicity induced Human Red Blood Cell Membrane Stabilization assay**

Preparation of Alsever's solution: Alserver’s solution consists of 0.8 g sodium citrate, 2 g dextrose, 0.05 g citric acid, and 0.42 g sodium chloride dissolved in distilled water in a final volume of 100 mL.

Preparation of hypotonic saline and isotonic saline: To make hypotonic saline, dissolve 0.36 g of sodium chloride in 100 mL of distilled water. In the meantime, isotonic saline can be made by dissolving 0.85 g of sodium chloride in 100 mL of distilled water.

Preparation of Phosphate Buffered Saline (pH 7.4, 0.15M): Dissolve 0.19 g of potassium dihydrogen phosphate, 2.38 g of disodium hydrogen phosphate, and 8 g of sodium chloride in 100 mL of distilled water.

Preparation of the blood sample: Collect a blood sample from a volunteer who had not taken any NSAIDs for at least two weeks before the experiment. This should be done by a trained nurse/ phlebotomist. Then mix the sample with an equal volume of sterilized Alsever's solution. Then, centrifuge the blood solution at 3000 rpm to separate the packed cells. Then, separated packed cells should be washed with an iso-saline solution. Finally, prepare a 10% v/v HRBC suspension with isosaline for later use.

Test mixture: Mix 2 mL of hypotonic saline, 1 mL of PBS (pH 7.4), 0.5 mL of test extract at various concentrations (15.625, 31.25, 62.5, 125, 250, 500, 1000, and 2000 μg/ml) , and 0.5 mL of 10% v/v HRBC to get a final volume of 4 mL.

Standard solution: Mix 2 mL hypotonic saline, 1 mL PBS (pH 7.4), 0.5 mL standard drug solution (Diclofenac Sodium) of varying concentrations (15.625, 31.25, 62.5, 125, 250, 500, 1000, and 2000 μg/ml), and 0.5 mL 10% v/v HRBC suspension.

Control: Mix 2 mL of distilled water with 1 mL of PBS (pH 7.4), and 0.5 mL of 10% v/v HRBC suspension. Use distilled water for the negative control.

Incubate all mixtures at 37 0C for 30 minutes. Then, take them out of the water bath and centrifuge them at 3000 rpm for 5 minutes. Separate the supernatant and measure the hemoglobin content of the supernatant at 560 nm. Carry out the test in triplicates.

Use the following formula to calculate the protection against HRBC membrane lysis (1, 26).

% protection = $100-[\frac{(Optical density of the sample )}{(Optical density of the control)}x 100]$

**1.2.3. Heat-induced Human Red Blood Cell Membrane Stabilization Assay**

Preparation of isotonic buffered solution (10 mM sodium phosphate buffer, pH 7.4): Dissolve 0.2 g of NaH2PO4, 1.15 g of Na2HPO4, and 9 g of NaCl in 1L of distilled water.

Preparation of red blood cell suspension: collect fresh whole blood (3 mL) into heparinized tubes and centrifuge at 3000 rpm for 10 minutes. Discard the supernatant and add normal saline with a volume equal to the discarded supernatant to dissolve the RBC pellet. Measure the volume of dissolved RBC and prepare a 40% (v/v) suspension with the isotonic buffered solution.

Dissolve the test sample (e.g., plant extract) in an isotonic buffered solution to obtain a concentration series of 100, 200, 400, 600, and 800 μg/mL. Arrange the series in quadruplicate sets (4 sets per concentration) containing 5 mL of the sample in each tube.

For the controls, use 5 mL of 200 μg/mL of indomethacin and 5 mL of vehicle (the solvent used to dissolve the drug) in 2 sets.

Add 0.1 mL of the HRBC suspension to each tube and mix gently.

Incubate a pair of tubes (2 sets of test sample series, and a set of controls) at 54 0C for 20 minutes in a regulated water bath. The other pair should be maintained at -10 0C for 20 minutes. Afterward, centrifuge the tubes at 1300 g for 3 minutes.

Then, measure the hemoglobin content of the supernatant using a spectrophotometer at 540 nm.

The % inhibition of hemolysis can be calculated using the following formula (27).

% Inhibition of hemolysis =$ 1-[\frac{(OD2 - OD1)}{( OD3- OD1)}] x 100 $

OD1 = Absorbance of the test sample (unheated)

OD2 = Absorbance of the test sample (heated)

OD3= Absorbance of the control (heated)

**1.3 COX inhibition test**

COX is a rate-limiting enzyme in prostaglandin (PG) synthesis from Arachidonic acid (AA). It has both catalase and oxygenase activities. COX is identified to have two isoenzymes with different biological effects, which are structural COX-1 and inducible COX-2 (28).

COX-1 is present in various cells and tissues, primarily regulating the synthesis of PGs, which regulates cell functions and different physiological processes (29). On the other hand, COX-2 is under-expressed in normal cells and tissues but becomes overexpressed in almost all inflamed tissues and tumor cells. Therefore, evaluating the selectivity of an anti-inflammatory compound towards COX-1 and COX-2 is important. This is crucial because while inhibiting COX-2 can reduce inflammation, inhibiting COX-1 can lead to other complications since COX-1 plays an important role in the body (30).

COX-2 measurement is used to evaluate the anti-inflammatory activity of a potential compound. The inhibition of COX in a sample is evaluated using radio autography (14C labeling). The COX activity is evaluated using commercially available kits.

**1.3.1 14C labeling method (measurement of the conversion of radioactive Arachidonic acid)**

The inhibition of COX is evaluated using radio autography, which measures the conversion of radioactive AA using 14C labeling. The method of radio autography provides precise identification and distribution mapping of radioactive materials. However, it requires long exposure times in low concentration situations, and this method can only be involved in relative quantification measurements (31).

***1.3.1.1. Experimental procedure:***

Mix 5 μL epinephrine (1.3 mg/mL), 10 μL glutathione (3 mg/mL), and 127.5 μL tris buffer (0.1 mol/L) in a test tube and place in an ice bath for 5 minutes. Then, add 10 μL of COX-1 or COX-2 and 10 μL of the test sample to the mixture and pre-incubate on ice for another 10 minutes.

After the pre-incubation, add 10 μL of 14C labeled AA and incubate at 37 0C for 20 minutes. Then add 10 μL HCl 2 mol/L to stop the reaction and add 800 μL of Ether to facilitate the extraction. Mix the mixture for 30 seconds.

Centrifuge for 1 minute at 2500 rpm. After centrifugation, collect 800 μL of the supernatant and keep at 37 0C. Then, dissolve 30 μL of the supernatant in acetone to obtain a resulting solution which has 14C labelled prostaglandins.

Use Thin Layer Chromatography (TLC) to develop the sample. The solvent system should contain chloroform: methanol: acetic acid 18: 1: 1.

After TLC development, the 14C labelled prostaglandins are detected using electron autoradiography.

The amount of prostaglandins produced will indicate the level of COX enzyme activity and, consequently, the test sample's potential anti-inflammatory effect.

Calculate the inhibition rate using the following formula (31).

Inhibition (%) = =$ \left[\frac{\left(CB - CA\right)}{\left( CB\right)}\right]x 100\% $

CB = the concentration of 14C labelled AA

CA, the concentration of 14C labelled prostaglandins

**1.4 LOX inhibition test**

LOXs are non-heme, iron-containing enzymes that are commonly found in animals and plants (32). While they bio-transform AA in mammals, LOXs mainly metabolize linoleic acid (LA) in plants (33). LOXs are mostly present in leukocytes, platelets, and the lungs of mammals. They are classified into four primary types according to their capacity to introduce oxygen atoms into AA: 5-LOX, 8-LOX, 12-LOX, and 15-LOX, with 5-LOX directly associated with human diseases (34).

AA can undergo oxidation at five sites to 5‐hydroperoxide 20‐carbo4‐enoic acid (5‐HPete) when LOX is present. Following oxidation, it is converted to 5, 6-epoxide (LTA4) (35). LTA4 is then converted to 5, 12-dihydroxy-20-carbo4-enoic acid (LTB4) through hydrolysis by hydrolases. LTA4 is transformed into leukotriene C4 (LTC4) by glutathione S-transferase, which then loses glutamate to form leukotriene D4 (LTD4) through γ-glutamyltransferase. Glycine is removed by LTD4 to create LTE4, which can then attach to glucosyltransferase to create LTF4. Leukotrienes linked to peptides, known as peptide leukotrienes (pLTs), include LTC4, LTD4, LTE4, and LTF4 (36).

Leukotrienes and their products trigger lysosomes to release enzymes, amplifying inflammation. They contribute significantly to allergy-related inflammations by inhibiting 5‐LOX activity and regulating LT biosynthesis (31).

**1.4.1 Manometry (Warburg breathometer method)**

LOXs are enzymes that can add O2 to unsaturated fatty acids to help create specific hydroperoxides. These fatty acids have certain double bonds, and LOXs can specifically target double bonds with a cis-cis pentadiene structure (37).

In this assay, the activity of the LOX enzyme is measured by maintaining the concentration of the substrate (polyunsaturated fatty acids) at a constant level. LOX utilizes the dissolved O2 in the solution while it oxidizes the substrate, producing hydrogen peroxides that contain double bonds. Essentially, while LOX catalyzes the reaction, the O2 level in the solution decreases. Therefore, the rate at which the O2 level in the solution decreases is directly proportional to the activity of the LOX enzyme. The decrease in the O2 level can be measured using an O2 electrode. This assay gives a clear and reliable measurement of the LOX activity based on O2 consumption as the setup is straightforward and easy to operate (31).

***1.4.1.1 Preparation of the sample***

Collect fresh plant samples and carefully remove surface contamination. Finely chop the samples and weigh them 0.5g precisely to place them in a motor. Add 5 mL of Tris-hydrochloric acid buffer (25mmoL/L, pH=7.5) to the motor and grind the mixture in an ice bath, followed by homogenization. Then, centrifuge the mixture in a refrigerated centrifuge at 0-4 0C at 4000 rpm for 5 minutes. Consider the resulting supernatant as the crude enzyme extract (31).

***1.4.1.2. Experimental procedure***

Prepare a 1:10 dilution of enzyme solution with distilled water and add 0.4 mL of the diluted solution to the side arm of the reaction bottle. To the main chamber of the reaction bottle, add 2 mL of phosphoric acid buffer and 1 mL of the substrate emulsion (a mix of 15 mL of distilled water, 5 mL of linoleic acid, and 2 mL of Tween-20 stirred thoroughly). Following this, allow the reaction bottle to equilibrate by vibrating at a constant temperature in a water bath set at 30 0C for 5 minutes. After reaching equilibrium, seal the piston of the manometer and adjust the liquid level of the closed end to match the reference point. Start the reaction by adding the enzyme solution in the side arm to the main chamber and start timing. After 3 minutes, bring back the liquid column of the closed end of the barometer to its original reference point. Immediately measure the column height at the open end and correct it according to temperature and barometric readings. The enzyme activity can be expressed as microliters of O2 consumed in 1 minute (31).

**1.4.2. Colorimetric method (determination of the product hydrogen peroxide)**

LOX enzyme catalyzes the production of H2O2 which has a characteristic absorption peak at 234 nm. Readily available substrates such as linoleic acid, linolenic, and arachidonic acids can be utilized for this assay. Hence, this assay is also considered easy to perform (31).

***1.4.2.1 Experimental procedure.***

Prepare the substrate solution by carefully dispensing 0.4 mL of linoleic acid and mixing it with 0.5 mL of 10% NaOH to ensure complete dissolution. Then, adjust the total volume to 100 mL by adding distilled water to result in a clear and colorless solution. Mix 20 mL of this prepared mixture with 0.1 mL of Tween-60 (emulsifier), and bring the total volume to 100 mL by adding 0.2 mol/L boric acid (pH =9.0). Before using, dilute the solution 40 times to achieve a linoleic acid concentration of 2.57 mmol/L.

Mix 0.5 mL of the sample solution with Tri buffer (containing 0.015 mol/L CaCl2 and 13% sucrose at pH 8.2). Stir this mixture at 40C for 10 minutes and centrifuge at 4000 rpm for 5 minutes to yield the crude extract (this extract can be refrigerated for subsequent use).

During the determination step, mix 2.8 mL of the substrate solution with 0.2 mL of the enzyme extract. Observe and record the changes in optical density (OD) at 234 nm wavelength in 15-second intervals (31).

The enzyme activity can be calculated using the following formula:

A= =$ 4 \left[\frac{\left(OD30s-OD 15s\right)}{\left( 0.01\right)}\right]$; where,

A - Activity of the enzyme (per minute)

OD30s - OD15s - OD values at 30s and 15s, respectively

The constant 0.01 - activity required to increase the absorbance by 0.01 per minute

**1.5 Proteinase inhibitory assay**

The release of proteinases during inflammation can contribute to tissue damage as they can degrade structural proteins in tissues. Proteinases are present abundantly in lysosomal granules and are released by lysosomes as a part of the inflammatory responses. The body produces proteinase inhibitors to protect the tissues from damage by proteinases and therefore, these inhibitors are considered crucial during inflammation (38).

**1.5.1 Experimental procedure**

Prepare the reaction mixture by mixing 0.06 mg of trypsin, 0.02 mL of cooked plant extract, 0.980 mL of methanol, and 1 mL of Tri-HCl buffer (pH= 7.4) and incubate at 37 0C for 5 minutes. Following incubation, add 1 mL of 0.8% (w/v) of casein and incubate the mixture for another 20 minutes. Then, add 2 mL of perchloric acid (70%) to stop the reaction. Centrifuge the mixture and measure the absorbance of the supernatant at 210 nm. The buffer can be used as the blank while PBS without the sample can be used as the control (39, 40).

Calculate the percentage inhibition of proteinase activity using the following formula;

% inhibition = $\frac{(1- A2)}{A1}$ × 100

A1- absorption of the control

A2- absorption of the test sample

**1.6 Hyaluronidase inhibition assay**

Hyaluronidase is an enzyme that plays a role in tissue remodeling during inflammation. It is an important constituent present in the extracellular matrix of connective tissues. Hyaluronidase enzymes are involved in inflammation and allergic reactions by increasing vascular membrane permeability and decreasing hyaluronic acid viscosity (41).

**1.6.1. Experimental procedure:**

Prepare the sample at various concentrations (100, 200, 300, 400, and 500 μg/mL) by dissolving it in sodium phosphate buffer (200 mM, pH=7.0). Dissolve 5mg of the sample (e.g. plant extract) in 250 μL of dimethyl sulfoxide (DMSO). Mix 25 μL of the sample solution with 100μL of Hyaluronidase (4U/mL) and incubate at 37 0C for 10 minutes (39). (In some research, the investigators add 1.2 μL, 2.5mM or 50μL, 12.5 mM CaCl2 and incubate at 37 0C for 20 minutes to activate the enzyme (42).

Following incubation, add 100 μL of the substrate (Hyaluronic acid, 0.03% in 300mM sodium phosphate, pH 5.4) to initiate the reaction and incubate at 37 0C for 45 minutes. Precipitate the undigested hyaluronic acid with 1 mL of acid albumin solution (bovine serum albumin (0.1%) in sodium acetate (24 mM), pH 3.8). Incubate at room temperature for 10 minutes and measure the absorbance at 600 nm (43,44).

Quercetin (45) or indomethacin (41) can be used as the positive control.

For the control value for maximum inhibition, the absorbance measurement in the absence of enzymes can be used.

Perform the assay in triplicates.

Calculate the percentage of hyaluronidase inhibition using the following formula (45);

% inhibition = $\frac{(Absorbance of the sample)}{Absorbance of the control}$ × 100

**1.7 Determination of NO**

NO is linked to a variety of physiological and pathological conditions. It plays a role in inflammation by causing vasodilation, activating prostaglandin synthase, oedema, increased exudation, local erythema, and sepsis. NO can promote inflammatory toxicity by involving in the development of rheumatoid arthritis (46). There are three forms of NO synthases (NOS). Both endothelial NOS (eNOS) and neuronal NOS (nNOS) are constitutively expressed and produce low levels of NO, which maintains tissue homeostasis. However, inducible nitric oxide synthase (iNOS) produces high levels of NO and is associated with various pathological states (47), including chronic inflammatory diseases such as asthma and rheumatoid arthritis. Hence, regulating the level of iNOS is an important factor in managing inflammatory diseases (48).

NOS can catalyze the transformation of L-Arginine to NO inside the human body. However, in *in vivo* or aqueous solutions, NO is readily oxidized to nitrites (NO2 2- ) and nitrates (NO2 3-) end products of the NO oxidation pathway. The concentration of these end products is evaluated to measure the NO production in cell cultures and biological liquids.

Figure 2: The production of NO catalyzed by iNOS in the human body (31).

A few methods are developed to evaluate the NO2 2- activity. They are chemiluminescence, gas chromatography, Griess colorimetry, fluorescence, and hemoglobin capture. Among these methods, the Griess method has been identified as the most convenient method to measure the NO2 2- level in aqueous solutions (49).

**1.7.1 Griess colorimetric method**

In this method, NO2 2- reacts with sulphonamides in alkaline conditions resulting in a diazo compound. This compound further undergoes a coupling reaction with N-(1-Naphthyl) ethylene diamine dihydrochloride to produce a colored compound. The absorbance is then measured at 546 nm with a spectrophotometer and a standard curve is generated to determine the concentration of NO in the sample (31).

The Griess method has a low detection limit of 1 µM for NO2 2-. The limited sensitivity makes it unsuitable for measuring micromolar levels of nitrite and nitrate in biological samples. However, this method is still widely used for detecting nitrites due to its simplicity, ease of use, and practical benefits (31).

**1.8 Advantages and disadvantages of *in vitro* assays.**

"*In vitro*" comes from Latin and means "in the glass." It is a study model in which a procedure is carried out in laboratories. Interventions can be performed on a specific cell culture to grow a virus, bacteria, or fungus, or to test a new drug. Because an "*in vitro*" procedure examines specific cells rather than the entire organism, these experiments are better suited for research and analysis.

*In vivo* assays are research models for a process or procedure performed "on" a living being rather than in cell samples. The Latin phrase "*in vivo*" means "within the living, and *in vivo* methods mimic the biological conditions found in a living subject. *In vivo* studies can be carried out on both animals and humans.

In research, "*in vivo*" and "*in vitro*" models are used in clinical trials, scientific studies, and new medical procedures. Scientists use both of these models to understand how a drug (or intervention) affects the body (pharmacodynamics profile) and how the body affects/metabolizes the drug (pharmacokinetic profile). Both of these studies aim to develop a safer and more effective drug profile before it is approved for use by the general public (50).

*In vitro* assays are the frequently used methods by pharmaceutical companies to produce drugs in large-scale settings due to the ease of culture for production when compared to the use of animals, as well as cost considerations. Since there won't be any involvement of animals, in *in vitro* assays, avoid the need to submit animal protocols to the Institutional Animal Care and Use Committee (IACUC). Although mice can be used as a source of feeder cells once antibody generation is underway, *in vitro* techniques minimize the use of mice during the antibody-production stage. *In vitro* techniques reduce or eliminate the need for specially trained staff to handle animals (18).

*In vitro* methods fail to model the complex interactions and feedback responses that happen in cells, tissues, and organs in living organisms (51). The complex interactions and feedback systems that take place within living things are frequently not replicated by *in vitro* assays. For instance, the way proteins are denatured in these tests might not accurately replicate inflammatory processes that occur *in vivo*. Rather than specifically inhibiting inflammatory mediators or pathways, these assays measure a general anti-denaturation effect, which may limit their applicability to biological inflammation mechanisms (52).

One important factor affecting reproducibility and reliability in *in vitro* assays is the impact of experimental conditions. Inconsistent cell culture practices, such as variations in handling protocols, culture conditions, and cell line authentication, can lead to variability. These differences could have a major impact on assay results, making it difficult to replicate findings within as well as among labs. To reduce such variability, Standard Operating Procedures (SOPs) must be followed and Good Cell Culture Practice (GCCP) principles must be followed. To guarantee consistency, variables like pH, temperature, and protein concentration need to be carefully managed and recorded. By taking care of these factors, scientists can improve the precision and repeatability of *in vitro* tests, which will ultimately increase their applicability to clinical settings and biological processes (53).

It is widely acknowledged that plant extracts can cause interference in spectrophotometric tests, especially because of their complex composition and inherent color. Interference of this kind can result in inaccurate absorbance measurements, which can jeopardize the validity of tests. (e.g: DPPH (2,2-diphenyl-1-picrylhydrazyl) antioxidant activity test) (54). Researchers have investigated several strategies that could mitigate these problems. Using activated charcoal to remove interfering substances from leaf extracts is one efficient method. By adsorbing pigments and other substances that cause background interference, this technique has been demonstrated to improve the precision of spectrophotometric measurements (55). Derivative spectrophotometry has also been used to address the background interference that is frequently observed in plant extracts. This method enhances assay specificity and sensitivity by distinguishing the analyte signal from the background absorbance, which improves the detection of particular analytes, such as nitrate in plant tissues (56).

There are ethical and scientific issues with using fetal bovine serum (FBS) in cell culture. FBS is ethically extracted from bovine fetuses during slaughtering, frequently by cardiac puncture without anesthesia, which raises questions regarding animal welfare because the fetuses may be in discomfort or pain (57). Given its unclear composition, FBS introduces variability into experimental results, which can impact data interpretation and reproducibility from a scientific standpoint. To improve the scientific accuracy and ethical standards of in vitro research, attempts are being made to decrease or substitute FBS with synthetic substitutes or serum-free media (57, 58). Furthermore, the use of fetal blood for the production of FBS has been examined for its effects on animal welfare, which has led to debates about the need for the biotechnology sector to adopt more ethical and humane procedures (59).

*In vitro* assays are typically more affordable for large-scale drug production. However, they can be very expensive for small- and medium-scale operations, particularly if specialized equipment or reagents are needed. These expenses can be decreased by creating strong procedures that maximize reagent use and minimize waste (60).

**CONCLUSION**

*In vitro* assays have become invaluable tools in the preliminary screening of anti-inflammatory agents, offering benefits such as cost-effectiveness and rapid results. However, researchers must remain mindful of their limitations, particularly the inability to fully replicate the complexities of *in vivo* responses.

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