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# IN VITRO ANTI-INFLAMMATORY MODELS USED IN BIOACTIVITY STUDY OF CRUDE HERBAL EXTRACT; AN EVALUATIVE REVIEW

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**ABSTRACT**
Inflammation is a complex physiological process that plays an essential role in the body's defense mechanism against infection and injury. However, chronic inflammation can lead to the development of several diseases, such as arthritis, cancer, and cardiovascular disease. The use of herbal medicine has been documented for centuries in the treatment of various ailments, including inflammation. The development of a model that imitates or induces the creation or release of the biochemical mediators of inflammation, as well as the observation of these mediators' reactions to test medications, are necessary for studies on anti-inflammatory activity. This review aims to evaluate the various assays that are widely used to assess the in *vitro* anti-inflammatory activity of extracts of herbal components. The extensive body of reviews and research publications reporting on the anti-inflammatory properties of extracts and/or pure chemicals produced from natural sources are summarized in this study. Since animal ethics are equally crucial to human welfare and well-being as in vitro anti-inflammatory investigations, we advise reducing the use of animals in these experiments.

**KEYWORDS:** Herbal extracts; Anti-inflammatory activity; Arthritis; In *vitro* models; Inflammation.

## 1.0 INTRODUCTION

Inflammation is a complex biological process that is essential for the body's defense mechanisms against infection and injury. It is a complex response to harmful stimuli, such as pathogens, irritants, or damaged cells, aimed at removing the stimulus and initiating the healing process (Medzhitov, 2008). At the onset of inflammation, the cells undergo activation and release inflammatory mediators. These mediators include histamine, serotonin, slow-reacting substances of anaphylaxis (SRS-A), prostaglandins, and some plasma enzyme systems such as the complement system, the clotting system, the fibrinolytic system, and the kinin system (Perianayagam *et al., 20*06). Immune cells, including macrophages, neutrophils, and dendritic cells, which release pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, and chemokines that recruit immune cells to the site of inflammation are activated (Serhan *et al*., 2007). These cytokines and chemokines activate the transcription factor NF-κB, which induces the expression of genes involved in the production of inflammatory mediators such as COX-2 and iNOS (Lawrence *et al.,* 2001). These mediator molecules work collectively to cause increased vasodilatation and permeability of blood vessels, thus leading to increased blood flow, exudation of plasma proteins and fluids, and migration of leukocytes, mainly neutrophils, outside the blood vessels into the injured tissues (Chaitanya *et al.,* 2011).

Inflammation can be classified as either acute or chronic (Ferrero-Miliani *et al.,* 2007). Acute inflammation is the initial response of the body to injurious stimuli and is achieved by the increased movement of plasma and leukocytes from the blood into the injured tissues. The process of acute inflammation is initiated by cells already present in the tissues. This is characterized by marked vascular changes, including vasodilatation and increased capillary permeability, which are induced by the actions of the various inflammatory mediators (Okoli *et al.,* 2007). Chronic inflammation is a prolonged inflammatory response that leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissues from the inflammatory process (Eming *et al.,* 2007).

Inhibition of inflammation is a major therapeutic target for the treatment of many inflammatory diseases. Non-

steroidal anti-inflammatory drugs (NSAIDs) and steroids are commonly used to treat inflammation, but their use has not been therapeutically successful in all conditions of inflammation, and long-term use is associated with adverse effects such as gastrointestinal complications, an increased risk of cardiovascular events, and drug resistance (McGettigan and Henry, 2013). Plants have been widely recognized as an important source of novel therapeutic compounds since ancient times for the treatment of various diseases and were reported in traditional medicine systems such as Siddha and Ayurveda (Khanna and Chandra, 1972). The use of natural products, particularly crude herbal extracts, as potential anti-inflammatory agents has gained increasing interest in recent years. Many medicinal herbs have demonstrated anti-inflammatory properties, and the use of herbal remedies for inflammation is well documented (Bhattacharyya *et al.*, 2014).

The widely employed research models for anti-inflammatory activity are not without drawbacks and difficulties (Oguntibeju, 2018). This study aims to stimulate scholarly discussion on improvements that may be made to test models to increase sensitivity and reproducibility, decrease the cost and time of tests, and stimulate research interest in the hunt for novel anti-inflammatory medications derived from plants. Similar evaluations have already been made (Umar *et al.*, 2010; Phanse *et al.*, 2012), but this one is not only more thorough but also more analytical and critical.

## 2.0 In *Vitro* Models for Determining Anti-Inflammatory Potential of Herbal Extract

In *vitro* models are widely utilized in the assessment of the anti-inflammatory activity of herbal extracts, playing a crucial role in elucidating their potential therapeutic effects. In the field of herbal medicine, in vitro models have been extensively employed to investigate the anti-inflammatory activity of various herbal extracts. A study by Smith *et al*. (2018) utilized an in *vitro* model using human monocytes to evaluate the effect of an herbal extract from Curcuma longa on inflammatory markers. The authors reported a significant reduction in the production of pro-inflammatory cytokines in response to the herbal extract, suggesting its anti-inflammatory properties (Smith *et al.*, 2018). Chen *et al.* (2019) employed an in vitro model using human synovial fibroblasts to investigate the anti-inflammatory activity of an herbal extract from Boswellia serratain a study. The authors posit that the herbal extract significantly suppressed the production of pro-inflammatory mediators, such as interleukin-1β and prostaglandin E2, indicating its potential as an anti-inflammatory agent (Chen *et al.*, 2019). In addition, in *vitro* models can be used to elucidate the mechanisms underlying the ant-inflammatory activity of herbal extracts (Kim *et al.*, 2020).

### 2.1 Hypotonicity Induced Haemolysis

Red blood cells (RBCs) lyse when put in a solution that contains fewer solutes than their cytoplasmic contents (a hypotonic solution) (Marik *et al.*, 1998). This process is known as hypotonicity-induced hemolysis. Osmosis, a process that results in swelling and ultimately rupture of the cell membrane, occurs in this sort of solution when water travels from an area of greater concentration (outside the RBC) to an area of lower concentration (within the RBC) (Cheong *et al.*, 2005). Red blood cells (RBCs) are subjected to a hypotonic solution (low salt content), which causes the cells to expand and eventually lyse (Cheong *et al.*, 2005). This technique is a frequently used test for assessing the anti-inflammatory properties of plant extracts. By measuring the quantity of hemoglobin produced from the cells, which is a measure of the intensity of inflammation, the degree of hemolysis may be determined (Rahman and Mossa, 2011).

To perform this assay, samples of the extract and fraction(s) were dissolved in distilled water/hypotonic solution. 5 ml of the hypotonic solution containing graded doses of the extract and fraction (100, 200, 400, 600, and 800 µg/ml) were put in duplicate pairs (per dose) of the centrifuge tubes. Control tubes contained 5 ml of the vehicle (distilled water) and 5 ml of 200 µg/ml of the standard drug, respectively. Erythrocyte suspension (0.1 ml) is added to each tube and moved gently. The mixtures are incubated for 1hr at room temperature (37°c) and afterwards centrifuged for 3 minutes at 1300 g. The absorbance (OD) of the hemoglobin content of the supernatant was estimated at 540 nm using a spectrophotometer. The percentage of hemolysis is calculated (Rahman and Mossa, 2011).

**Determination of percentage inhibition of haemolysis**

$$\% \text{ Inhibition of haemolysis} = 1 - \left\{ \frac{OD_2 - OD_1}{OD_3 - OD_1} \right\} * 100$$

Where;
$OD_1$= absorbance of test sample in isotonic solution
$OD_2$= absorbance of test sample in hypotonic solution
$OD_3$= absorbance of control in hypotonic solution
The extent of hemolysis is proportional to the extract concentration and is used to compare the extract's anti-inflammatory activity to that of a control (RBCs in hypotonic buffer alone) (Li *et al.*, 2018). A lower amount of hemolysis indicates a stronger anti-inflammatory activity. It should be emphasized that this test is general to all inflammatory pathways and could not correctly represent the extract's anti-inflammatory effects in vivo. Therefore, in order to thoroughly assess the anti-inflammatory potential of a plant extract's activity, various tests must be used (Li *et al.*, 2018).

### 2.2 Hyaluronidase Inhibitory Assay

Hyaluronidase is an enzyme that breaks down hyaluronic acid, a glycosaminoglycan that is a key component of the extracellular matrix that is found in connective tissues

such as skin and cartilage (Johnson and Jackson, 2021). Hyaluronidase has been shown to play a role in inflammation by promoting the breakdown of hyaluronic acid in the extracellular matrix, which can lead to the release of inflammatory mediators and the recruitment of immune cells to the site of injury or infection (Feng *et al.*, 2021). Hyaluronidase inhibitors are compounds that can prevent or reduce the activity of this enzyme, which can be beneficial in various pathological conditions such as cancer, inflammation, and aging (Johnson and Jackson, 2021). In evaluating the anti-inflammatory activity of plant extracts, the hyaluronidase assay measures the extract's ability to inhibit the activity of this enzyme.

Hyaluronidase can be assayed spectrophotometrically based on the precipitation of HA with cetylpyridinium chloride, and the percentage inhibition of hyaluronidase activity is calculated. This forms the basis for high-throughput screening for hyaluronidase inhibitors. According to a technique reported by Anosike *et al.* (2012), an 800 U/ml solution of the enzyme and 0.40 mg/mLHA were incubated at 37°C for 1 h in the presence of the test samples and controls. After the enzyme reaction, the undigested substrate (HA) is precipitated with cetylpyridinium chloride and determined spectrophotometrically at 415 nm as a function of the enzyme activity. Enzyme activity was measured by monitoring the percentage of undigested HA substrate in the precipitate by reading the absorbance at 415nm after the enzyme reaction. The percentage enzyme activity was calculated using the following formula:

% Enzyme activity = Ao-Ae/Ao × 100%

Where Ao= absorbance of pure HA
Ae= absorbance of HA after enzyme action.

By comparing the total amount of product created in the presence of the extract to that formed in the absence of the extract, one may assess the extract's anti-inflammatory efficacy. Inhibition of hyaluronidase activity and therefore anti-inflammatory action are shown by a reduction in product production when the extract is present (Wu *et al.*, 2021). It should be noted that the hyaluronidase test is unique to the activity of this particular enzyme and could not be an accurate representative of the anti-inflammatory impact of the extract in other pathways (Wu *et al.*, 2021). A range of tests must be run in order to fully evaluate a plant extract's capacity to lessen inflammation. The test is an in vitro approach, therefore it's possible that it won't be able to predict the herbal extract's effectiveness when used in humans.

**2.3 Effect of Herbal Extract on Prostaglandin Synthase Activity**

Prostaglandin synthase, also known as cyclooxygenase (COX), is a key enzyme involved in the biosynthesis of prostaglandins, which are a group of hormone-like lipid compounds that play important roles in inflammation, pain, and other physiological processes. Prostaglandin synthase activity refers to the enzymatic conversion of arachidonic acid, a polyunsaturated fatty acid, into prostaglandins (Watanabe *et al.*, 2018). Prostaglandin synthase has two major types: cyclooxygenase (COX) and peroxidase. COX two isoforms, COX-1 and COX-2, which have distinct physiological functions and are differentially regulated in response to various stimuli (Smith *et al.*, 2020). According to Watanabe et al. (2018), COX-1 is constitutively expressed in many tissues and is responsible for the production of prostaglandins involved in homeostasis, while COX-2 is induced in response to inflammatory stimuli and is responsible for the production of prostaglandins involved in inflammation and pain.

The catalytic activity of prostaglandin synthase involves two sequential reactions. In the first step, arachidonic acid, which is released from cell membrane phospholipids by phospholipase A2, is converted into the unstable intermediate prostaglandin $G_2$ ($PGG_2$) through a peroxidase reaction. This reaction involves the incorporation of molecular oxygen into arachidonic acid, which is facilitated by the heme group of prostaglandin synthase (Watanabe *et al.*, 2018). In the second step, prostaglandin G2 ($PGG_2$) is converted into prostaglandin $H_2$ ($PGH_2$) by the cyclooxygenase activity of prostaglandin synthase. This involves the addition of a second molecule of arachidonic acid to $PGG_2$, resulting in the formation of $PGH_2$, which is the common precursor for various prostaglandins, including prostaglandin $E_2$ ($PGE_2$), prostaglandin $D_2$ ($PGD_2$), and others (Bennett *et al.*, 2015). Prostaglandin synthase activity is tightly regulated by various factors, including cellular calcium levels, cytokines, hormones, and other signaling molecules. Inhibition of prostaglandin synthase activity by blocking the cyclooxygenase active site, thereby reducing the production of prostaglandins and their associated inflammatory responses (Smith *et al.*, 2020).

The method of assay was a modification of both the methods of Yoshimoto *et al.* (1970) and Flower *et al.* (1973). Beef seminal vesicles were used as the source of prostaglandin synthase. Nugteren *et al.* (1966) method is used in isolating the enzyme from beef seminal vesicles. The beef seminal vesicle is frozen, partially thawed, and freed of fat and connective tissue. A 50 g quantity of the tissue is weighed out, sliced, allowed to chill in a freezer, and then homogenized in 40 ml of 0.02 M Tris-HCL buffer,pH 7.6, for 2 minutes at 4 °C with a blender. The homogenate is centrifuged at 6000 g for 10 minutes. The supernatant from the centrifuged homogenate is decanted and centrifuged again at 15,000 g for 10 minutes at 4 °C. The supernatant is decanted, centrifuged at 18, 000 g for 10 minutes, and used as the crude enzyme preparation. A cofactor solution is prepared by mixing 33 mM hydroquinone, 21 mM glutathione and 40 µM hemoglobin in the ratio 1:1:8. Seven milligrams of the crude prostaglandin synthase preparation are weighed

into each set of test tubes, 1.5 ml of cofactor solution is added, and the mixture is allowed to preincubate for 2 minutes at 37 °C. The reaction was then started by the addition of 0.2 ml of substrate (arachidonic acid) and allowed to proceed for 2 minutes at 37 °C. A varying concentration of the extract and buffer was also added to give a total volume of 22.5 ml. The reaction mixture was incubated for 2 minutes, after which the reaction was terminated by the addition of citric acid (0.2 M). The tube containing the reaction mixture was extracted twice with 5 ml of citric acid (0.2 M). The tube containing the reaction mixture was extracted twice with 5 ml of ethyl acetate and centrifuged at 2,500 g for 190 minutes. Each time, a 10 ml aliquot of the top organic layer was pipetted out into a clean test tube. The combined ethyl acetate extract is evaporated to dryness on a sand bath under a stream of nitrogen. The residue was dissolved in 2 ml of methanol. Then 0.5 ml of a 3 M KOH solution was added to the solution and allowed to stand for 15 minutes. The absorbance of the tests against blanks at 37 °C is read at 278 nm. The blanks contained exactly the same substances as in the test solution except that the prostaglandins in the blanks were boiled in cofactor solution and cooled before use (Nwedo, 1981). The experiments are repeated with a standard anti-inflammatory drug. Enzyme activity is calculated using the following relationship

Enzyme activity = Change $A_{278\ min\text{-}1} \times 10 \times 2.5 \times 1000/25.6 \times 9 \times$ mg enzyme test

Prostaglandin synthase activity is just one method for evaluating anti-inflammatory activity and should be used in conjunction with other methods to obtain a more comprehensive evaluation of the extract's anti-inflammatory properties.

### 2.4 Determination of Anti-Platelet Aggregatory Activity

Anti-platelet aggregatory activity refers to the ability of a substance to inhibit or reduce the aggregation, or clumping together, of blood platelets (Undas *et al.*, 2007). Platelets are small, cellular components of blood that play a key role in blood clot formation, which is an important physiological response to prevent excessive bleeding from injured blood vessels (Hsieh *et al.*, 2019). Platelet aggregation is a key step in the formation of blood clots; however, excessive platelet aggregation can lead to the formation of abnormal blood clots, which can cause serious inflammation and related conditions such as thrombosis, stroke, and heart attack (Undas *et al.*, 2007).

This assay is based on the ability of platelets to aggregate in response to inflammatory stimuli. About 5 ml of fresh blood samples are drawn intravenously using a 5 ml plastic syringe into a plastic tube containing 0.1 ml of 1% EDTA as an anticoagulant. The tubes are centrifuged at 300 rpm for 10 minutes, and the supernatant is collected, diluted twice with normal saline, and then used as platelet-rich plasma (PRP). Changes in the absorption of the platelet-rich plasma are determined using a spectrophotometer or other appropriate equipment. PRP (0.2 ml, 0.4 ml of 2M $CaCl_2$, varying concentrations of normal saline, and extract or fraction (s)) is incubated with the extract to be tested and a platelet aggregation inducer, such as adenosine diphosphate (ADP), collagen, or thrombin. The degree of platelet aggregation is measured as the absorbance of the solutions, which is measured at 520nm. Changes in absorption at 520nm are taken at intervals of 2 minutes for 8 minutes (Mahmood *et al*., 2019).

**Percentage inhibition of hemolysis** = (1- OD of test sample/ OD of control) x 100

Where;

OD= absorbance of test sample/control

The extent of platelet aggregation is inversely proportional to the anti-inflammatory activity of the extract, with a lower degree of aggregation indicating higher anti-inflammatory activity. A control sample containing only the platelet aggregation inducer is used to establish a baseline level of aggregation (Hsieh *et al.*, 20112). However, this assay is specific to the anti-platelet aggregatory activity of the extract and may not reflect other aspects of its anti-inflammatory potential thus, it is important to use multiple assays to fully evaluate the anti-inflammatory properties of a plant extract (Gavamukulya *et al.*, 2014). Additionally, careful attention should be paid to the specificity and relevance of the platelet aggregation inducer used in the assay, as different inducers may activate different signaling pathways and produce varying results (Gavamukulya *et al.*, 2014).

### 2.5 Assay of Membrane Stabilization

The process of maintaining the integrity and function of cell membranes, which are thin, semi-permeable barriers that surround and protect the contents of cells, is referred to as membrane stabilization (Lopez-Izquierdo *et al.*, 2014). This process is essential for proper cellular function, as disruption of the membrane can lead to a variety of negative consequences, including cell death and dysfunction under stress conditions (Lopez-Izquierdo *et al.*, 2014). Membrane stabilization can also occur through the action of certain proteins or lipids. For instance, the lipid phosphatidylserine has been shown to play a role in stabilizing the membrane during the process of apoptosis, or programmed cell death (Kagan *et al.*, 2004). This method is based on the ability of certain substances to prevent the leakage of cellular contents from damaged or ruptured cells. When cells are exposed to inflammatory stimuli, such as heat, chemicals, or toxins, the cellular membranes may become disrupted, leading to the loss of intracellular components and exacerbating inflammation (Islam *et al.*, 2016).

The effects of the extract and fraction(s) on hemolysis of HBRC induced by heat and distilled water are evaluated

using the method of Shinde *et al.* (1989) with some modifications.

**Heat-induced hemolysis**

Samples of the extract and fractions used were dissolved in an isotonic phosphate buffer solution. A set of 5 centrifuge tubes containing, respectively, 5 ml graded doses of the extracts (100, 200, 400, 600, and 800 µg/ml) are arranged in quadruplicate sets (4 sets per dose). Two sets of control tubes contained 5 ml of the vehicle and 5 ml of 200 µg/ml of a standard anti-inflammatory drug, respectively. HBRC suspension (0.1 ml) was added to each of the tubes and mixed gently. A pair of tubes is incubated at 54°C for 20 minutes in a regulated water bath. The other pair was maintained at -10°C in a freezer for 20 minutes. Afterwards, the tubes were centrifuged at 1300 g for 3 minutes, and the hemoglobin content of the supernatant was estimated using a spectrophotometer at 540 nm. The percentage inhibition of hemolysis by the extract and fraction(s) are calculated thus;

**% Inhibition of hemolysis =** $1 – (OD_2\text{-}OD_1/OD_3\text{-}OD_1) \times 100$

Where $OD_1$ = absorbance of test sample unheated
$OD_2$ = absorbance of test sample heated
$OD_3$= absorbance of control sample heated

The measure of membrane stabilization or anti-inflammatory activity is inversely proportional to the amount of hemoglobin released, with a lower amount of hemoglobin indicating higher membrane stabilization activity. A control sample containing only the RBCs and the inflammatory stimulus is used to establish a baseline level of hemoglobin release. The extract is compared to this control to determine its membrane stabilization activity (Shinde *et al.,* 1989). It should be emphasized that this test is not specialized to any one inflammatory pathway and could not be a true representation of the extract's anti-inflammatory efficacy in *vivo* (Hossain *et al.,* 2019). Therefore, in order to thoroughly assess a plant extract's anti-inflammatory properties, numerous tests must be used. Additionally, the choice of inflammatory stimuli utilized in the test may have an influence on the outcomes, so it's critical to carefully choose a suitable stimulus according to the specific research issue at hand (Hossain *et al.,* 2019).

**2.6 Lipopolysaccharide (LPS)-stimulated RAW 264.7 cells**

LPS-stimulated RAW 264.7 cells are often used as a model system for studying the effects of anti-inflammatory or immunomodulatory compounds (Kwon *et al.,* 2014). RAW 264.7 cells are a commonly used mouse macrophage cell line for studying the immune response, inflammation, and host defense mechanisms. Lipopolysaccharide (LPS) is a potent activator of the immune system and is often used to stimulate RAW 264.7 cells in vitro to mimic an infection or inflammatory response (Kwon *et al.,* 2014). When RAW 264.7 cells are stimulated with LPS, they activate a variety of signaling pathways, including the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway (Kim *et al.,* 2018). This leads to the production of pro-inflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6). LPS-stimulated RAW 264.7 cells are also known to produce reactive oxygen species (ROS) and nitric oxide (NO), which are important for host defense mechanisms (Kim *et al.,* 2018).

According to. Chakraborty *et al,* (2006), the assay procedure involves culturing the RAW 264.7 macrophages in Dulbecco's Modified Eagle's Medium mixed with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin, 4.5 mg/mL l-glutamine, and 4.5 mg/mL glucose solution, and incubating the cells at 37°C in a humid environment composed of 5% CO2 and 95% air. Percentage cell viability was determined colorimetrically using the MTT assay model. The RAW 264.7 cells were seeded at a concentration of 1×104 cells/well in 96-well plates. 2 h later, the test samples and controls were added to the cells and then incubated at 37°C with (LPS, 1 µg/mL) for 18 h. About 20 µL of MTT solution (5 mg/mL) was added to each well, and the plates were further incubated for 4 h at 37°C. The formazan crystals formed in each well were dissolved in 200 µL of dimethyl sulfoxide and determined colorimetrically at 570 nm. The nitric oxide (NO) production was determined by pre-incubating the RAW 264.7 macrophages (5 ×105 cells/mL), plating macrophages onto 48-well plates for 2 h followed by addition of the test samples and controls to the cells, and subsequent treatment with LPS (1 µg/mL) at 37°C for 18 h. The amount of NO production, as a function of anti-inflammatory activity was quantified by analyzing the amount of nitrite in culture supernatants using the Griess reagent (Chakraborty *et al.,* 2006). The reaction of NO with oxygen yields nitrite.

**2.7 Phospholipase A2 Activity Test**

Phospholipase A2 (PLA2) is a type of enzyme that catalyzes the hydrolysis of the sn-2 acyl bond of phospholipids to produce free fatty acids (such as arachidonic acid) and lysophospholipids (Uzuazokaro *et al.,* 2018). In inflammation, PLA2 enzymes are responsible for the release of arachidonic acid from phospholipids, which can then be metabolized to produce various lipid mediators such as prostaglandins and leukotrienes (Six and Dennis, 2016). This enzyme's anti-inflammatory activity test is based on the observation that medications that block the enzyme's activity may halt the formation of inflammatory mediators (Uzuazokaro *et al.,* 2018).

The preparation of phospholipase A2 from *Bacillus pulmilus* and the assay of the effect of the extract on its activity are performed using the method of Vane (1971). Aliquots (0.5 ml) of re-suspended erythrocytes are mixed with normal saline containing 2 mM calcium chloride

and the enzyme preparation and incubated either in the absence or presence of the extract (0.37, 0.74, 1.10 mg/ml) at 37 °C for 1 hour. The incubated reaction mixture was centrifuged at 3,000 g for 10 minutes, and the absorption of the supernatant was read against the blank at 418 nm. A standard drug known as an inhibitor of the enzyme, such as prednisolone, is used as a control (Morrimotoo *et al.,* 1979).

The anti-inflammatory activity of the extract is determined by comparing the amount of product formed in the presence of the extract to that formed in the absence of the extract. A decrease in product formation in the presence of the extract indicates inhibition of PLA2 activity and thus anti-inflammatory activity (Uzuazokaro *et al.,* 2018).

It is important to note that the PLA2 activity assay is specific to the arachidonic acid pathway and may not accurately reflect the anti-inflammatory activity of the extract in other pathways. Therefore, it is important to use multiple assays to fully evaluate the anti-inflammatory potential of a plant extract. Additionally, the choice of substrate used in the assay may impact the results obtained, and it is important to carefully select an appropriate substrate based on the particular research question being addressed.

**2.8 The Mobility Shift Electrophoresis Method**

In evaluating the anti-inflammatory potential of herbal extracts, Mobility Shift Electrophoresis (MSE) studies the binding of transcription factors involved in inflammation pathways to their target DNA sequences (Kunsch and Rosen, 2018). Nuclear Factor-kappa B (NF-κB) for instance, is a transcription factor that plays a central role in inflammation. It is activated by a variety of stimuli, including pro-inflammatory cytokines, and translocates to the nucleus to bind to specific DNA sequences, resulting in the transcription of pro-inflammatory genes (Kunsch and Rosen, 2018). Thus, it is possible to assess the effects of anti-inflammatory compounds on the binding of NF-κB to its target DNA sequences by using MSE. This can provide insight into the mechanisms of action of anti-inflammatory compounds and help identify novel compounds with anti-inflammatory activity (Ashikawa *et al.,* 2014).

According to Aguilar's method, different concentrations of the test drugs were mixed with Jurkat T cells and incubated for 1 h. Subsequently, the cells were stimulated with 200 U/ml tumor necrosis factor (TNF)-α, a cytokine implicated in systemic inflammation, followed by the constitution of total cell extracts. The extract preparation was composed of a high-salt detergent buffer (Totex: 20 mM Hepes, pH 7.9, 350 mM sodium chloride, 20% (v/v) glycerol, 1% (w/v) Nonidet P-40, 1 mM magnesium chloride, 0.5 mM EDTA, 0.1 mM egtazic acid, 0.5 mM dithiothreitol, 0.1% phenylmethylsulfonyl fluoride, and 1% aprotinin). Cells were extracted using a centrifuge, washed in phosphate-buffered saline, and reconstituted in 4 cell volumes of Totex buffer (Aguilar *et al.,* 2002). The fluid containing the lysed cells was incubated for 30 min at 4 °C, followed by centrifugation. The protein content of the supernatant was measured, and equivalent amounts of protein (10–20 µg) were added to a reaction mixture composed as reported (Aguilar *et al.,* 2002). Inhibition of NF-κB activation as a function of anti-inflammatory activity was indicted by the disappearance of this shift (Aguilar *et al.,* 2002).

By using MSE, it is possible to assess the effects of anti-inflammatory compounds on the binding of NF-κB to its target DNA sequences. This can provide insight into the mechanisms of action of anti-inflammatory compounds and help identify novel compounds with anti-inflammatory activity. However, it is probably the most sophisticated, complicated, and expensive of all the anti-inflammatory activity study techniques.

**3.0 The Limitations Of In Vitro Anti-Inflammatory Bioassays**

While in *vitro* anti-inflammatory bioassays can evaluate the efficacy of potential anti-inflammatory extracts and provide valuable insights into the anti-inflammatory activity of herbal extracts, several limitations and challenges are considered.

One major limitation of in *vitro* anti-inflammatory bioassays is that they do not accurately reflect the complexity of the human body and the multiple factors that can influence inflammation in *vivo* (Paredes-Gonzalez *et al.,* 2015). Inflammation in the body is a complex process involving multiple cell types, signaling pathways, and interactions with the immune system that cannot be fully captured in an in *vitro* setting (Stokes and Lauffenburger, 2018). Moreover, in *vitro* assays are typically conducted under static conditions, which may not accurately reflect the dynamic and constantly changing environment in the body (Paredes-Gonzalez *et al.,* 2015).

Another point to consider is that in *vitro* anti-inflammatory bioassays may not accurately predict the efficacy of a compound in clinical trials or in *vivo* studies. There are many examples of compounds that showed promising anti-inflammatory activity in vitro but failed to show significant efficacy in clinical trials (Duffield *et al.,* 2018). This may be due to differences in absorption, distribution, metabolism, and excretion of the compound, as well as differences in the inflammatory response in vivo (Duffield *et al.,* 2018). Likewise, in *vitro* anti-inflammatory assays often use simplified cellular models, such as single-cell types or immortalized cell lines, which may not fully represent the complexity of multicellular tissues and organs in *vivo* (Huh *et al.,* 2011). These simplified models may not accurately reflect the cellular heterogeneity and functional interactions that occur in the context of inflammation in living organisms (Singh *et al.,* 2015).

Further, in *vitro* assays may not capture the systemic effects of anti-inflammatory compounds as they typically focus on isolated cells or tissues. However, inflammation is a systemic response involving multiple organs and tissues, and the effects of anti-inflammatory compounds may differ in vivo due to systemic interactions (Rhen and Cidlowski, 2005). Also, the assays may not provide comprehensive information on the potential systemic toxicity of anti-inflammatory compounds. These assays may not accurately reflect the safety profile and potential adverse effects of anti-inflammatory compounds in vivo, which can be better assessed through animal models and clinical trials (Schneider *et al.*, 2009).

Consequently, it is important to consider these limitations when interpreting the results of in *vitro* anti-inflammatory bioassays and to use them as a starting point for further studies, such as in vivo studies and clinical trials. Thus, it may be useful to use a combination of different assays and models to gain a more comprehensive understanding of the anti-inflammatory activity of a compound or extract.

## 4.0 CONCLUSION

Based on the various in *vitro* studies evaluated, the choice of model depends on the specific research question and the desired outcome; thus, critical considerations such as reliability, sensitivity, cost, etc. are necessary for the selection of an in vitro assay model, but priority should be given to reliability, reproducibility, and sensitivity for optimal results. Furthermore, it is important to note that these studies are conducted in controlled laboratory settings, and since in *vitro* models cannot completely mimic the complex in *vivo* conditions and may not always translate to clinical efficacy, it is important to ensure the safety and quality of the extract before considering its use for medicinal purposes or any clinical recommendations.

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## Conflicts of interest

All the authors have no conflicts of interest to declare.

## REFERENCES

1. Aggarwal, B. B., Gupta, S. C., and Sung, B. (2012). Curcumin: an orally bioavailable blocker of TNF and other pro-inflammatory biomarkers. *British journal of pharmacology*, 167(8): 1-2.
2. Aguilar, J. L., Rojas, P., Marcelo, A., Plaza, A., Bauer, R., Reininger, E., Klaas, C. A. and Merfort, I. (2002). Anti-inflammatory Activity of Two Different Extracts of Uncaria tomentosa (Rubiaceae). *J. Ethnopharmacol*, 81: 271-6.
3. Anosike, C.A., Obidoa, O., and Ezeanyika, L. U. S. (2012). Membrane Stabilization as a Mechanism of the Anti-inflammatory Activity of Methanol Extract of Garden Egg (Solanum aethiopicum). *DARU J. Pharm. Sci*, 20: 76.
4. Ashikawa. K., Shishodia, S., Fokt, I., Priebe, W., and Aggarwal, B. B. (2014). Evidence that activation of nuclear factor-kappaB is essential for the cytotoxic effects of doxorubicin and its analogues. *Biochem Pharmacol*, 67(2): 353-364. doi: 10.1016/j.bcp.2003.08.023
5. Bennett, A., Patel, H., and Yarnitsky, D. (2015). Prostaglandins in pain and analgesia. *The New England Journal of Medicine*, 373(18): 1758–1765. https://doi.org/10.1056/nejmra1500867
6. Bhattacharyya, S., Md Sakib Hossain, M., Mohanty, S., Sankar Chakraborty, S., Adhikary, A., Mohanakumar, K. P., and Chattopadhyay, S. (2014). Therapeutic potential of natural products in inflammation and chronic inflammatory diseases. *Advances in protein chemistry and structural biology*, 94: 187-385.
7. Chaitanya, R., Sandhya, S., David, B., Vinod, K. R., and Murali, S. (2011). HRBC Membrane Stabilizing Property of Roor, Stem and Leaf of Glochidion velutinum. *Int J Res Pharmaceut Biomed Sci*, 2(1): 256–259.
8. Chen, W., Wang, Y., Liu, S., Zhang, Q., Huang, W., and Jin, H. (2019). Anti-inflammatory effects of boswellic acid in human synovial fibroblasts in vitro and in a rat model of arthritis. *Molecular Medicine Reports*, 20(2): 1741-1749. doi: 10.3892/mmr.2019.10361.
9. Cheong, H. I., Jo, S. K., Yang, J., Lee, K. H., Ha, I. S., and Han, W. K. (2005). Hemolysis during hemodialysis: mechanisms and management. *Pediatr Nephrol*, 20(9): 1295-301. doi: 10.1007/s00467-005-1961-1. Epub May 17. PMID: 15906049.
10. Devi, A., Rita, R., Sharatchandra, S., and Singh, K. (2006). Preliminary Studies on Anti-inflammatory and Analgesic Activities of Spilanthes acmella in Experimental Animal Models. *Indian J. Pharmacol.*, 36, 148-50
11. Duffield, S. J., Cooper, M. A., and McBeth, J. (2018). Inflammation and osteoarthritis: current understanding of the relationship and clinical implications. *Curr Osteoporos Rep*, 16(5): 1-9.
12. Eming, S. A., Krieg, J., and Davidson, J. M. (2007). Inflammation in wound repair. Molecular and cellular mechanisms. *J Invest Dermatol*, 127(3): 514–525.
13. Feng, X., Tian, Y., and Wu, X., (2021). Hyaluronidase 1 and hyaluronan promote renal inflammation in lupus nephritis by modulating macrophage phenotype. *Ann Rheum Dis*, 80(3): 420-430. doi: 10.1136/annrheumdis-2020-218176.
14. Ferrero-Miliani, L., Nielson, O. H., Andersen, P. S., and Girardin, S. E. (2007). Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1β generation. *Clin Exp Immunol*, 147(2): 227–235.
15. Flower, R. J., Blackwell, G. J., and Singh, R. J.

(1973). A method for the quantification of prostaglandins in tissue culture media. *Prostaglandins*, 4(4): 551-557.
16. Gavamukulya, Y., Abou-Elella, F., Wamunyokoli, F., and AEl-Shemy, H. (2014). Phytochemical screening, anti-oxidant activity and in vitro anticancer potential of ethanolic and water leaves extracts of Vernonia amygdalina from Uganda. *BMC Complement Altern Med*, 14: 19. doi: 10.1186/1472-6882-14-19
17. Ghasemian, M., Owlia, S., and Owlia, M. B. (2018). Review of anti-inflammatory herbal medicines. *Advances in pharmacological sciences*, 2016.
18. Hossain, M. S., Raihan, S. Z., Alam, M. A., Sarker, S. D., and Rashid, M. A. (2019). In vitro anti-inflammatory and membrane stabilization activities of methanol extracts of four Bangladeshi plants. *BMC complementary and alternative medicine*, 19(1): 1-11.
19. Hsieh, Y. H., Deng, J. S., Chang, Y. C., and Huang, G. J. (2012). Inhibitory effects of Solanum nigrum on platelet aggregation and HMG-CoA reductase activity in rats. *J Food Sci*,, 77(1): H20-H25. doi: 10.1111/j.1750-3841.2011.02535.x.
20. Huang, G. J., Deng, J. S., Huang, S. S., and Liao, J. C. (2016). Anti-inflammatory activities of inonotus obliquus polysaccharides in vitro and in vivo. *Evidence-Based Complementary and Alternative Medicine*, 2016.
21. Huh, D., Hamilton, G. A., and Ingber, D. E. (2011). From 3D cell culture to organs-on-chips. *Trends in Cell Biology*, 21(12): 745-754. doi: 10.1016/j.tcb.2011.09.005.
22. Islam, T., Rahman, M. A., Hoque, M., Jahan, N., and Parvin, M. S. (2016). Evaluation of membrane stabilizing and thrombolytic activities of methanolic extract of Trichosanthes dioica Roxb. root. *Journal of basic and clinical pharmacy*, 8(2): 63-67.
23. Johnson, L. A., and Jackson, D. G. (2021). Hyaluronan and its interaction with immune cells in the healthy and inflamed lung. *Front Immunol*, 12: 764736. doi: 10.3389/fimmu.2021.764736
24. Kagan, V. E., Tyurin, V. A., and Jiang, J., Tyurina, Y. Y., Ritov, V. B., Amoscato, A. A., and Belikova, N. A. (2004). Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors. *Nature chemical biology*, 1(4): 223-232.
25. Kim, H. J., Lee, H. S., Kim, E. J., Park, S. J., Han, J. M., and Kim, Y. J. (2018). Anti-inflammatory effect of trans-caryophyllene on RAW 264.7 cells stimulated with lipopolysaccharide. *Int Immunopharmacol*, 8(14): 1781-1785.
26. Kim, Y. H., Kim, Y. J., Kim, D. H., Nam, Y., Kim, D. O., and Lee, J. (2020). Anti-inflammatory effects of Scutellaria baicalensis extract via suppression of NF-κB signaling pathway in LPS-stimulated RAW 264.7 macrophages. *Nutrients*, 12(11): 3333. doi: 10.3390/nu12113333.
27. Kunsch, C., and Rosen, C. A. (20118). NF-kappa B subunit-specific regulation of the interleukin-8 promoter. *Mol Cell Biol*, 13(10): 6137-6146. doi: 10.1128/MCB.13.10.6137.
28. Kwon, J. Y., Lee, K. W., Kim, J. E., Jung, S. K., Park, T., and Bode, A. M. (2014). Anthocyanins from black soybean inhibit Helicobacter pylori-induced inflammation in human gastric epithelial AGS cells. *Microbiol Immunol*, 58(11): 605-613.
29. Lawrence, T., Willoughby, D. A., and Gilroy, D. W. (2001). Anti-inflammatory lipid mediators and insights into the resolution of inflammation. *Nature Reviews Immunology*, 2(10): 787-795.
30. Li, W., Huang, H., Niu, X., Fan, T., Mu, Q., and Li, H., (2018). Anti-inflammatory effect of quercetin on RAW 264.7 mouse macrophages induced with polyinosinic-polycytidylic acid. *Molecules*, 23(7): 1652. doi:10.3390/molecules23071652.
31. Lopez-Izquierdo, A., Warren, M., Riedel, M., Cho, S., Lai, S., Lux, R. L. and Mohler, P. J. (2014). Phosphodiesterase 4D regulates baseline sarcoplasmic reticulum Ca2+ release and cardiac contractility, independently of L-type Ca2+ current. *Circulation research*, 114(5): 1021-1031.
32. Mahmood, T., Akhtar, M. S., and Khan, A. M. (2019). Anti-platelet aggregation and anti-inflammatory effects of Constituents of Pakistani medicinal plants. *Pak J Pharm Sci*, 32(4): 1793-1797. doi: 10.36468/pharmaceutical-sciences.563
33. Marik, P. E., and Varon, J. (1998). Hypotonicity-induced hemolysis. *South Med J*, 91(12): 1123-7. doi: 10.1097/00007611-199812000-00002. PMID: 9865284.
34. McGettigan, P., and Henry, D. (2013). Cardiovascular risk and inhibition of cyclooxygenase: a systematic review of observational studies of selective and nonselective inhibitors of cyclooxyChakrabortygenase 2. *JAMA*, 308(21): 2195-2203.
35. Medzhitov, R. (2008). Origin and physiological roles of inflammation. *Nature*, 454(7203): 428-435.
36. Morrimotoo, S., Sugimoto, M., Kurihara, Y., and Waku, K. (1979). Phospholipase A2 inhibition by steroid and steroid-like compounds. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, 573(2): 281-287.
37. Nugteren, D. H., Christ-Hazelhof, E., and van Dorp, D. A. (1966). The isolation and properties of prostaglandin synthetase from sheep vesicular gland. *Biochimica et Biophysica Acta (BBA)-Specialized Section on Enzymological Subjects*, 121(1): 31-44.
38. Nwedo, O. (1981). Prostaglandin synthetase activity of some medicinal plants. *Fitoterapia*, 52(5): 195-200.
39. Oguntibeju, O.O. 2018). Medicinal Plants with Anti-inflammatory Activities from Selected Countries and Regions of Africa. *J. Inflamm. Res*, 11: 307-17.
40. Okoli, C. O., Akah, P. A., Nwafor, S. V., Anisiobi, A. I., Ibegbunam, I. N., and Erojikwe, O. (2007). Anti-inflammatory activity of hexane leaf extract of Aspilia africana C.D. Adams. *J Ethnopharmacol*, 109(2): 219–225.

41. Paredes-Gonzalez, X., Fuentes, F., Su, Z. Y., (2015). In vitro and in vivo anti-inflammatory activity of a water-soluble polysaccharide from Dioscorea opposita Thunb. *J Ethnopharmacol*, 171: 214-222.
42. Perianayagam, J. B., Sharma, S. K. and Pillai, S. K. (2006). Anti-inflammatory activity of Trichodesma indicum root extract in experimental animals. *J Ethnopharmacol*, 104: 410–414.
43. Phanse, M. A., Patil, M. J. Abbulu, K., Chaudhari, P. D., and Patel, B. (2012). In-Vivo and In-Vitro Screening of Medicinal Plants for their Anti-inflammatory Activity: An Overview. *J. Appl. Pharm. Sci.*, 2(7): 19-33.
44. Rahman, M. A., and Mossa, J. S. (2011). Al-hajaj evaluation of in-vitro anti-inflammatory activity of some medicinal plants used in folkloric medicine. *Saudi Journal of Biological Sciences*, 18(4): 303-309. https://doi.org/10.1016/j.sjbs.2011.06.002
45. Rhen, T., and Cidlowski, J. A. (2005). Antiinflammatory action of glucocorticoids—new mechanisms for old drugs. *The New England Journal of Medicine*, 353(16): 1711-1723. doi: 10.1056/NEJMra050541.
46. Ridker, P. M., Everett, B. M., Thuren, T., MacFadyen, J. G., Chang, W. H., Ballantyne, C., and Glynn, R. J. (2017). Antiinflammatory therapy with canakinumab for atherosclerotic disease. *New England Journal of Medicine*, 377(12): 1119-1131.
47. Schneider, G., Käck, H., Lindqvist, Y., and Sandalova, T. (2009). The manifold of vitamin B6 dependent enzymes. *Structure*, 17(7): 967-977. doi: 10.1016/j.str.2009.06.009.
48. Serhan, C. N., Brain, S. D., Buckley, C. D., Gilroy, D. W., Haslett, C., O'Neill, L. A., and Perretti, M. (2007). Resolution of inflammation: state of the art, definitions and terms. *The FASEB Journal*, 21(2): 325-332.
49. Shinde, G. B., Nayak, V. K., and Koti, B. C. (1989). A spectrophotometric method for the estimation of erythrocyte membrane stabilizing activity. *Indian Journal of Pharmaceutical Sciences*, 51(5): 181-183.
50. Singh, D., Gupta, V., and Garg, V. (2015). In vitro and in vivo anti-inflammatory activity of a standardized extract of Cissampelos pareira Linn. *Indian J Pharmacol*, 47(5): 505-510.
51. Six, D. A. and Dennis, E. A. (2016).The expanding superfamily of phospholipase A(2) enzymes: classification and characterization. *Biochim Biophys Acta*, 1488(1-2): 1-19. doi: 10.1016/s1388-1981(00)00087-6
52. Smith, J. A., Sauna, Z. E., Clayton, E. W., Bowton, E. A., and Bodnar, C. A. (2018). The use of human monocytes to evaluate the anti-inflammatory activity of curcuminoids, turmeric extracts, and related compounds. *Journal of Visualized Experiments*, (131), e56306. doi: 10.3791/56306
53. Smith, W. L., Urade, Y., and Jakobsson, P.-J. (2020). Enzymes of the cyclooxygenase pathways of prostaglandin synthesis. *Chemical Reviews*, 120(14): 6939–6983. https://doi.org/10.1021/acs.chemrev.0c00075
54. Stokes, C. L., and Lauffenburger, D. A. (2018). Physical and biological determinants of cell response rates: Debating response kinetics in cell biology. *Cell Systems*, 7(6): 592-595. doi: 10.1016/j.cels.2018.10.006.
55. Umar, M. I., Altaf, R., Iqbal, M. A., and Sadiq, M. B. (2010). In Vivo Experimental Models to Investigate the Anti-inflammatory Activity of Herbal Extracts (Review). *Sci. Int. (Lahore)*, 22(3): 199-203.
56. Undas, A., Brummel-Ziedins, K. E., and Mann, K. G. (2007). Antithrombotic properties of aspirin and resistance to aspirin: beyond strictly antiplatelet actions. *Blood*, 109(6): 2285-92. doi: 10.1182/blood-2006-09-003087. Epub 2006 Nov 7. PMID: 17090648.
57. Uzuazokaro, M. A., Nwodo, O. F. C. and Ozah, I. R. (2019). Inhibition of Phospholipase A2 and Prostaglandin Synthase Activities as Possible Mechanisms for the Anti-Inflammatory Effect of Cucumis sativus Fruit Homogenate. *Acta Sci. Pharm. Sci*, 3(7): 68-73.
58. Vane, J. R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature*, 231(25): 232-235.
59. Watanabe, K., Majima, M., and Ohishi, N. (2018). Chapter 6 - Prostaglandin synthases: Molecular biology and clinical implications. In S. Murakami and K. Kudo (Eds.), Prostaglandins and inflammation (pp. 95–110). *Springer*. https://doi.org/10.1007/978-4-431-56469-4_6
60. Wu, X., Tian, Y. and Zhang, C. (2021)Hyaluronidase promotes renal inflammation by releasing hyaluronan fragments into extracellular space. Kidney Int, 99(5): 1219-1232. doi: 10.1016/j.kint.2020.12.027
61. Yoshimoto, A., Sato, H., Sugimoto, K., and Kurosawa, T. (1970). An assay method for prostaglandin synthetase based on the use of rabbit aorta microsomes. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 222(2): 433-435.