Introduction

Inflammation refers to the body’s normal physiological defense against tissue injury. The injury may be a result of physical or mechanical damage, trauma, autoimmune reaction, microbial attack, and burns. Inflammation can be acute or chronic [1-5]. During acute inflammatory reaction, cellular and molecular events reduce imminent injury or infection. This mitigation process contributes to the restoration of tissue homeostasis and resolution of the acute inflammation. Uncontrolled acute inflammation may, however, become chronic, contributing to a variety of chronic inflammatory diseases [6]. Inflammation and the immune system mechanism are closely connected. Mediators of inflammation (autacoids) include serotonin, histamine, prostaglandins, lipoxins, leukotrienes, bradykinin, platelet-activator factor, and lymphokines [7]. Histamine increases the porosity of the tissues, brings about contraction of the smooth muscles and causes bronchoconstriction [8].

Chronic inflammatory disorders, including arthritis and hemorrhoids, continue to torment humanity despite nature’s endowment of medicinal plant resources. A lot of plants employed in folkloric medicine for managing inflammation are yet to be evaluated scientifically. The currently used anti-inflammatory activity study models are not without storming limitations and challenges [9].

This review is aimed at arousing research interest toward the search for new anti-inflammatory drugs from plants as well as provoking scientific thought on positive modifications of the test models to improve sensitivity and reproducibility and reduce
cost and time of experiment. Similar reviews have been attempted in the past [10-12], but the current review is not only more comprehensive but also analytical and evaluative.

2 In vitro models for anti-inflammatory activity

2.1 Method involving phospholipase A2 enzyme activity

Phospholipase A2, designated as PLA2, is a fatty acid cleaving (lipolytic) enzyme that catalyzes the hydrolysis of the sn-2 ester bond into an array of different phospholipids. It is essential for the release of arachidonic acid (AA) which is an essential precursor in prostaglandin synthesis [13]. The anti-inflammatory activity assay involving this enzyme is based on the fact that drugs that inhibit the enzyme activity are capable of stalling the production of the inflammatory mediators.

The assay is carried out as previously reported [14]. The enzyme was prepared from bacteria, and the enzyme preparation incubated at 37°C with 0.5 mL aliquots of re-suspended erythrocytes in normal saline, 2 mM calcium chloride, and the test drugs for 1 h. Afterward, the reaction mixture was centrifuged at 3000 ×g for 10 min, and the absorbance of the supernatant determined spectrophotometrically against the blank at 418 nm. The enzyme creates leakage on erythrocyte membrane, causing hemoglobin to gush out into the medium. Hence, the enzyme activity correlates with the amount of hemoglobin in the medium. Hemoglobin has \( \lambda_{\text{max}} \) of 418 nm.

A known inhibitor of the enzyme, such as Prednisolone, is best suited for positive control [14].

2.2 Prostaglandin synthase enzyme assay

Prostaglandin synthase, also known as cyclooxygenase (COX), is the major enzyme implicated in the conversion of AA to prostaglandin H2, which is the intermediate in the synthesis of prostaglandins, prostacyclin, and thromboxanes.

According to previous reports [14], prostaglandin synthase was isolated from the ox-seminal vesicle. The assay was based on the formation of pregabalin (PGB) (\( \lambda_{\text{max}} \) 278 nm) from prostaglandin, PGE2, on treatment with concentrated alkali. The procedure involved incubation of the substrate (0.5 mL AA) with the reaction mixture (containing the enzyme) at 37°C for 2 min. The reaction mixture was composed of 1.5 mL cofactor solution (33 mM hydroquinone, 21 mM glutathione, 40 mM hemoglobin, 0.3 mL buffer, and 8 mg of the enzyme preparation). After 2 min. 1.5 ml of 0.2 M citric acid was added to quench the reaction. The mixture was extracted with 10 ml of ethyl acetate and centrifuged at 2500 ×g for 10 min. The supernatant was concentrated under a stream of nitrogen gas, and the dried extract dissolved in 2 ml methanol. The methanolic solution was then mixed with 0.5 mL of 3 M potassium hydroxide. Solution and the mixture allowed to stand for 15 min to produce PGB. The absorbance of tests against blank was read at 278 nm on a spectrophotometer. The enzyme activity was determined using the following relation [14]:

\[
\text{unit g}^{-1}\text{enzyme} = \frac{\text{Abs}_{278} \text{ min}^{-1} \times 10 \times 2.5 \times 1000}{25.6 \times 9 \times \text{mg}_{\text{enzyme test}}^{-1}}
\]

According to an alternative method described previously for COX-1 assay [15], 0.2 units of COX-1 were pre-incubated at 37°C with a mixture composed of sample solution (10 mL), of 0.1 M Tris (USB)-HCl (190 µl), L-adrenaline-D-hydrogen tartrate (18 mM), and hematine (10 µM). After 5 min of pre-incubation, 5 µM AA was added and the mixture was again incubated for 20 min. A 10% formic acid (10 µl) was then added to stop the incubation. The PGE2-concentration was determined measured using a PGE2 enzyme immunoassay. For COX-2 assay [16], the sample solution (10 µL) was pre-incubated with 0.2 units of COX-2 and a mixture composed of 190 µL of 0.1 M Tris (USB)-HCl, 18 µM of L-adrenaline-D-hydrogen tartrate, 10 µM Na₂ ethylenediaminetetraacetic acid (EDTA), and 10 µM of hematine for 5 min. This was followed by addition of 5 µM AA and further incubation at 37°C for 20 min. The incubation is terminated by addition of 10 ml of 10% formic acid. The PGE2 concentration was determined using a PGE2 enzyme immunoassay [15].

Prostaglandin is cytoprotective. Prostaglandin inhibitors are, therefore, likely to provoke erosion of gastric mucosa in the animals [14]. This model is versatile and the incubation conditions such as the reaction time and temperature can be varied. It, however, involves an expensive and high level
of technical expertise. Furthermore, the incubation procedure and analytical steps are very tedious and time consuming.

2.3 The mobility shift electrophoresis method

The electrophoretic mobility shift assay is probably the most sophisticated, most complicated, and the most expensive of all the anti-inflammatory activity study techniques. It evaluates the ability of the test drugs to inhibit NF-κB. It is based on electrophoretic separation of a protein–DNA or protein–RNA mixture.

According to the method of Aguilar [15], 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 extracts 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 re-constituted in 4 cell volumes of Totex buffer. 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) added to a reaction mixture composed as reported [15]. Inhibition of NF-κB activation, as a function of anti-inflammatory activity was indicated by the disappearance of this shift [15].

2.4 Hyaluronidase inhibitory assay

Hyaluronidases are a family of enzymes that catalyze the degradation of hyaluronic acid (HA). HA is a constituent of the extracellular matrix of connective tissues and advances the spread of inflammatory mediators throughout these tissues. It contributes to the pathogenesis of inflammatory disorders such as allergic reactions, migration of cancer cells, inflammation, and the increase in vascular permeability [17].

Hyaluronidase can be assayed spectrophotometrically based on precipitation of HA with cetylpyridinium chloride. This forms the basis for high throughput screening for hyaluronidase inhibitors. In a technique reported previously [17], 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:

\[
\text{% Enzyme activity} = \left(\frac{A_o - A_e}{A_o}\right) \times 100\%
\]

where \(A_o\) = absorbance of pure HA, \(A_e\) = absorbance of HA after enzyme action.

2.5 Stabilization of human red blood cell (HRBC) membrane test

Inhibition of hemolysis of the HRBC membrane induced by heat or hypotonicity has been related to anti-inflammatory activity. The assay procedure, according to previous reports [18], involves collecting fresh whole blood from healthy volunteers into heparinized tubes, centrifuging at 3000 rpm for 10 min and dissolving the red blood pellets into a volume of normal saline equivalent to that of the supernatant. The volume of the red blood pellets solution got was noted and reconstituted as a 40% v/v suspension in an isotonic buffer solution of pH 7.4 made of 10 mM sodium phosphate buffer which contained 0.2 g of NaH₂PO₄, 1.15 g of Na₂HPO₄, and 9 g of NaCl in 1 dm³ of distilled water. The re-suspended supernatant was used for the assay.

Solutions of the test samples were made in the isotonic phosphate buffer solution, and the red blood cell suspension (0.1 ml) added to each pair of the test tubes containing the test samples and controls and mixed gently. One of each pair was incubated for about 20 min at a temperature at 54°C. The other pair was maintained at −10°C in a freezer for 20 min. Later, the tubes were centrifuged at 1300 ×g for 3 min and the hemoglobin content of
the supernatant determined spectrophotometrically at 540 nm. The % inhibition of hemolysis by the test samples, as a function of anti-inflammatory activity, was calculated using the following formula:

\[
\text{% Inhibition of hemolysis} = 1 - \frac{A_2 - A_1}{A_3 - A_1} \times 100\%
\]

where \(A_1\) = absorbance of test drug (unheated), \(A_2\) = absorbance of test drug (heated), \(A_3\) = absorbance of the control (heated).

2.6 Lipopolysaccharide (LPS)-stimulated RAW 264.7 cells

RAW 264.7 cells are a macrophage-like cell line derived from BALB/c mice. It is usually the mouse macrophages model of choice for studying cellular reactions to microbes and their products.

The assay procedure, according to a previous report [19], 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 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 [19]. The reaction of NO with oxygen yields nitrite.

3 In vivo models for anti-inflammatory activity

3.1 Model involving carrageenan as phlogistic agent

Carrageenan is a sulfated, jell-like polysaccharide extracted from certain algae such as the red seaweeds. The Carrageenan-induced rat paw edema is a typical systemic model for evaluating acute inflammation. Carrageenan is the preferred phlogistic agent for evaluating anti-inflammatory activity as it is non-antigenic and is without noticeable adverse reactions [20]. Inflammation induced by carrageenan is known to occur in two phases. The first phase is ascribed to the release of histamine, serotonin, and kinins in the 1st, while the second phase involves the release of prostaglandins, protease, and lysosome enzymes in the 3rd–4th h [21,22].

In this model, groups of experimental animals are orally or intraperitoneally treated, respectively, with the test drugs and controls. After 30 min 1 h of drug administration (for intraperitoneal or oral route, respectively), 1% carrageenan suspension (0.1 mL) is carefully injected into the subplantar region of the hind paw of each rat [23]. The paw volumes are determined before and then at 1-h intervals for 6 h after carrageenan challenge using plethysmometer, Vernier caliper, liquid displacement method, or with the aid of cotton thread [24]. The % inhibition of edema is quantified using the following formula [21].

\[
\text{% Inhibition of edema} = \frac{EV_0 - EV_t}{EV_0} \times 100
\]

where \(EV_0\) = edema volume of negative control group, \(EV_t\) = edema volume of test group at a particular time. \(EV_0\) and \(EV_t\) are obtained by taking the differences between the paw volumes before and after the challenge.

3.2 Egg albumin edema model in animals

The egg-albumin edema model is similar to the carrageenan model except that the phlogistic agent is raw, undiluted egg albumin. The experiment is used to assess sub-acute inflammation and is carried out as previously described [21,25].
Edema is provoked in the animals by subplantar administration of fresh egg albumin (0.1 mL) into the hind paw of each animal 30 min (or 1 h for oral route) after drug administration. Paw volume or thickness is determined appropriately.

Edema induced by egg albumin is long-lasting (sub-acute inflammation). It is attributed to the discharge of histamine and 5-hydroxytryptamine [21]. Egg albumin stimulates mast cell degranulation resulting in the release of histamine. Histamine has been linked to increased dilation and enhanced permeability of blood vessels [26]. An advantage of this method over the carrageenan edema is the ready availability and low cost of egg-albumin, which can easily be withdrawn from the raw egg with syringe and needle.

3.3 Model involving kaolin as phlogistic substance

The kaolin-induced edema model is suitable for sub-acute inflammation. The experiment is performed as described [27]. Edema was induced in the animals by the introduction of 5% suspension of Kaolin in normal saline (0.1 mL) into the plantar surface of the animal paw after 30 min of drug treatment. Alternatively, a blend of kaolin and carrageenan may be used as the phlogistic agent [28]. A mixture comprising 20% kaolin suspension and 1% carrageenan (0.2 ml) was injected into the rat footpad, and the test samples administered 3 times (every 4 h) beginning 18 h after the phlogistic challenge.

In a method described by Sur et al. [28], inflammation was produced by injecting a 3% suspension of carrageenan/kaolin mixture in normal saline into the left joint. Knee thickness, knee flexion test, and weight distribution ratio were the parameters measured as related to inflammation. These parameters were observed for 6 days. Daily administration of the test samples and controls began 24 h after the injection and maintained until day 5.

Kaolin is preferred to carrageenan when a longer period of inflammation is required. Furthermore, it is not likely to have antigenicity or cause hypersensitivity reactions since it is a clay mineral. It is suggested that kaolin-induced edema is mediated through prostaglandin biosynthesis [28].

3.4 Dextran-induced paw edema in rats

Dextran is a complex polysaccharide derived from glucose molecules. It is synthesized from sucrose by certain lactic acid bacteria. Edema induced by dextran results from enhanced vascular permeability, kinins trigger, and the discharge of histamine and 5-hydroxytryptamine from mast cells, resulting in osmotic edema containing poor quantities of protein and neutrophils. The dextran model, like the carrageenan model, is used to assess the effect of test drugs on systemic acute inflammation. Inflammation is provoked by injecting 2% dextran solution in normal saline (0.1 mL) into the subplantar region of the animal paws 1 h after drug treatment with test samples and controls [29].

3.5 Serotonin paw edema model

Serotonin, or 5-hydroxytryptamine, is a neurotransmitter chemical linked to mood and well-being. It is one of the messengers of inflammation and can be used as a phlogistic agent. In this model, groups of experimental rats are first treated orally with the test drugs and controls, followed by injection of 0.1 mL serotonin into the animal paws, 1 h after drug administration [30,31]. The paw volume or circumference is measured accordingly at 0 h and thereafter at hourly intervals. The degree of edema inhibition is determined as usual (Equation 1). This method is serotonin-specific and can be used as a confirmatory test for serotonin involvement. It is, however, tasking to obtain pure serotonin.

3.6 Method involving histamine as phlogistic agent

Histamine, a vasoactive amine, is a chemical mediator that is implicated in both allergic and inflammatory processes. It is a vasodilator drug. It is involved in both acute and sub-acute hypersensitivity responses [31]. In chemically induced inflammation, paw edema is believed to occur in earlier stage chiefly due to histamine release. Histamine release results from degranulation of the mast cells by some other autacoids.

The histamine model involves the injection of about 0.1 ml of 1% solution of histamine (in 0.9% saline solution) into the subplantar region of the left hind paw of the animals 1 h after oral treatment. The
edema volume is determined before the histamine challenge, 30 min later and at hourly interval for 6 h [31]. The paw edema volume is measured as a function of inflammation. This method is histamine-specific and can be used to confirm the inhibition of histamine by the test sample.

### 3.7 The Formalin-induced Edema Model

This method involving formalin as the phlogistic agent is appropriate for testing drugs against chronic inflammation. The nociceptive action of formalin occurs in two phases. The early phase involves a neurogenic component, while the later phase involves tissue-mediated reaction. The early phase is ascribed to the discharge of histamine, 5-HT, and kinin, while prostaglandin is implicated in the second phase [32].

In this model, edema is induced by subplantar injection of freshly prepared 2% formalin (about 20 μL) into the rat paw. Drug administration is repeated daily for about 6 consecutive days, and paw volume determined each time 1 h after treatment. Percentage edema inhibitions are calculated as described previously [32]. The advantage of this model is that it offers a method for testing drug activity on long-lasting inflammation. It is, however, time consuming.

### 3.8 Agar-induced inflammation

Agar is composed of a blend of two complex carbohydrates, namely, agarose and agarpectin. It has wide application in microbiological and biochemical research and is implicated in vascular inflammation. The agar model is suited for acute inflammatory experiments. In this model, the phlogistic substance is 2% agar suspension (0.1 mL) [33]. The paw edema volume is determined appropriately. The safety nature and ready availability of agar are obvious advantages of this method. Agar is, however, injected as a suspension since the appropriate sterile, non-irritant solvent is not readily available.

### 3.9 Zymosan-induced edema in rat

Zymosan reagent is a glycoside produced from yeast cell walls and used widely in immunology and chronic inflammatory experiments. It activates complement by the alternative pathway and induces arthritis within 48 h of injection in animals. Arthritis subsides within 14 days. The mechanism entails neutrophils infiltration followed by synovial hyperplasia and then infiltration of macrophage, with the formation of pannus [34]. The zymosan model is well suited for both topical and systemic inflammation. It is used for ear-skin inflammation locally (ear swelling) as well as systemically (rat paw edema). Administration of zymosan is known to activate the complement system, promote increased activity of COX-2 and prostaglandin production, largely the PGE2 type, and increase the production of NO [34]. Systemic edema is induced in animals using 1% suspension of zymosan in normal saline (0.1 mL) injected into the paws. To test local activity, it is injected into one ear of the animals and edema measured relative to the other ear [34].

### 3.10 The xylene model of topical edema

The xylene ear edema model is used to evaluate topical acute anti-inflammatory reactions. Xylene is known to provoke remarkable dilation of blood vessels leading to edematous changes in the skin [35,36]. It is suited for the assessment of anti-inflammatory properties of both steroids and nonsteroidal drugs, particularly those that inhibit phospholipase A2 enzyme. It is, however, less sensitive nonsteroidal drugs. Hence, steroidal drugs are more suited for positive control [35,36].

Edema is induced in each animal by dropping about 0.1 mL of pure xylene into the inner surface of one of the mouse ear 1 h after oral administration of test drugs. After 3 h of phlogistic challenge, the animals are anaesthetized, and both ears carefully chopped off to approximately equal size and weighed carefully. The difference in weight between the challenged and non-challenged ear is determined as a measure of inflammation [35,36].

The xylene method, and other ear-edema models, offers a method of testing topical anti-inflammatory activity, eliminating the pharmacokinetic problems that may be associated with the drugs. Furthermore, it can be performed with smaller animals like mice, which are cheaper. An obvious shortcoming of this method, like other topical edema models, is the assumption that the two ears are of equal weight before induction of inflammation. It also requires that the animals be sacrificed before measurement with its cost and cultural implications.
3.11 Croton oil-induced topical ear edema in mice

Croton oil, like xylene, is a phlogistic agent for topical inflammation studies. It is an irritant oil obtained from the seeds of *Croton tiglium* and contains mainly the phorbol esters, notably 12-o-tetracanoilphorbol-13-acetate (TPA). TPA induces diarrhea systemically and provokes topical inflammation and irritation by activating protein kinase, which in turn triggers the activities of other enzymes such as phospholipase A2, resulting in the release of platelet activation factor. This flow of events releases histamine and serotonin, and promotes vascular permeability, vasodilation, and polymorphonuclear leukocytes migration. It also stimulates a reasonable production of inflammatory eicosanoids by COX and 5-lipoxygenase [37].

In this topical model, the test drugs are applied on the inner and outer surfaces of one of the mouse ear while the other ear is treated with the vehicle only. After about 15 min, edema is induced on both ears by applying 5% (v/v) Croton oil in acetone topically [37]. The animals are humanely sacrificed 6 h after Croton oil application, and a 7 mm diameter disc removed from each ear with a steel punch. The edema volume is calculated in comparison with the weight of the untreated ear.

Apart from xylene, croton oil, or TPA, other appropriate phlogistic agents often used for topical anti-inflammatory activity study include ethyl phenylpropionate and tissue plasminogen activator.

3.12 Heat blister-induced inflammation

This model involves inflicting injury on the animal by applying heat. Edema can be induced in the rat paw by a hot blister. As described previously [38], the right hind paws of the animals were drenched in hot water maintained at 53 ± 0.5°C, with cutoff time of 14 s, 30 min after administration of test samples. The paw edema volume was determined at hourly intervals for 6 h and then on the 24th h after hot scald. In the method of Kuhns et al. [39], blisters were raised on healthy, normal human volunteers, and the study carried out as follows. The volar surface of the forearm was first disinfected with alcohol and sterile 8-well skin suction chamber secured on the arm and a vacuum of 350 mmHg applied with a vacuum pump. The vacuum was maintained for 1–2 h. The swelling was facilitated by warming the skin suction chamber with a 60-W tungsten bulb held 15 cm from the chamber. This condition was maintained until sufficient blisters were achieved. The mobilized neat blister fluid was carefully aspirated using a tuberculin syringe and stored for investigation. The cell pellet was re-suspended with 0.1 ml Hank’s Balanced Salt Solution and the leukocytes count carried out using a hemocytometer. The amount of cytokines and inflammatory mediators in the various biological fluids was determined using standard kits. Neutrophils’ migration into the chamber fluid was detectable within 3 h and appeared to stabilize at 16–24 h.

3.13 Collagen-induced arthritis

This technique is carried out, as described in Chondrex [40]. In this model, BB rats aged 7–8 weeks, with a mature immune system are most appropriate. Type II collagen is prepared and purified to remove minor impurities, such as pepsin, which are likely to produce false-positive results in a T-cell stimulation test. A 2–4 mg/ml solution of the collagen is made in 0.05M acetic acid with constant stirring at 4°C for about 6 h. Various methods can be employed in emulsion preparation. The sonication method, however, yield emulsions that are not appreciably stable for inducing arthritis. Furthermore, sonication fragments collagen, denaturing them at physiological temperatures. The emulsion is best prepared using an electric homogenizer. Arthritis is induced by subcutaneous injection of 0.2 ml (200 mg collagen) of the emulsion at the base of the tail of each rat.

This method is suitable for chronic inflammation study. Noticeable arthritis with swollen joints usually develops within 14–21 days after the first collagen challenge. BB rats are the most suitable species because they show the quickest onset of arthritis, within 12–14 days. The extent of arthritis development can be determined using appropriate methods [40].

3.14 Adjuvant-induced arthritis

This technique, like that of collagen, offers a good method for determining chronic inflammation. The phlogistic agent is Freund’s complete adjuvant (FCA), which constitutes *Mycobacterium*
tuberculosis killed by heat, suspended in liquid paraffin. About 0.1 ml of 0.4% FCA is injected intradermally into the animal paw 24 h after administration of test drugs. Afterward, drug treatment is continued daily for 13 days. The foot size and body weights of the rats are measured appropriately [41].

Alternatively, the percentage inhibition of arthritis can be determined on days 1, 3, 5, 9, 13, and 21. The arthritic parameters to be measured include the primary and secondary lesions. Primary lesions imply the edema formation in the injected paw that reaches climax 3–5 days after phlogistic challenge. It is usually measured on the 5th day. Secondary lesions involve the inflammation of the non-injected sites and a decrease in animal weight that occurs after 11–12 days due to immune response. The arthritic index was determined as the sum of the scores [41].

3.15 The cotton pellet-induced granuloma model

This model is generally employed in assessing the composition of transudative and proliferative substances associated with chronic inflammation. It is based on the release of granuloma induced by implanting cotton pellets in the groins of the animals subcutaneously. The wet weight of the pellets associates with transudate, while the dehydrated has a direct relationship with the size of granulomatous tissues. Chronic inflammation results from the development of proliferating cells which can be spread or appear in granuloma form. The nonsteroidal anti-inflammatory drugs diminish the size of granuloma which results from cellular reaction by restraining granulocyte penetration, averting the production of collagen fibers and repressing mucopolysaccharides [23].

The test procedure involves careful implantation of about 30 mg of sterile cotton pellet in the groins of the animals under anesthesia, followed by drug treatment for about 4 days. On day 5, the animals are humanely sacrificed and the pellets with the attached granuloma cautiously detached, dried to a constant mass at 60°C, and weighed. The granuloma mass formed is quantified as a measure of inflammation [23]. This technique is suited for chronic inflammation. The major disadvantages of this method include that it is tedious, and it requires expertise and involves loss of many animals.

3.16 Carrageenan (or zymosan) air-pouch model

Air pouch can be induced in animals with carrageenan or zymosan resulting in tissue degradation and fibrosis. This model evaluates the activity of substances against the proliferative stage of inflammation [42,43].

According to reports [42,43], air cavities were produced in the animals under anesthesia by subcutaneous injection of 20 mL of sterile air into the interscapular area of the back. An extra 10 mL of air was injected into the cavity every 3 days to keep the space open. On day 7, the animals were administered orally with the test drugs and controls. Two hours later, 2 mL of 1% solution of carrageenan (or zymosan) dissolved in normal saline was introduced directly into the pouch to trigger an inflammatory reaction. The drug treatment was repeated 24 h after the first treatment. After 48 h of injection of carrageenan or zymosan, the animals were anesthetized, the pouch opened, and the volume of exudates collected and determined. The leukocyte concentration was quantified appropriately [42,43]. This technique also offers a good method of monitoring the immune system response. It is, however, not only tedious and time consuming but also requires sophistication and expertise.

3.17 Prostaglandin E2 (PGE$_2$)-induced paw edema in rats

PGE$_2$, or dinoprostone, is a naturally occurring prostaglandin often used as medicine. It is a strong mediator of an inflammatory response and can be used to provoke edema in animals. The procedure involves subplantar injection of 100 μl of 0.01 μg/ml solution of PGE$_2$ into the animal paws 1 h after oral administration of the test drugs and controls. Paw edema volume is determined at an hourly interval for 6 h as a function of inflammation [44].

3.18 AA-induced paw edema

AA is an intermediate in the synthesis of various prostaglandins, thromboxanes and leukotrienes, respectively, which are essential mediators in the regulation of signal transduction involved in pain and inflammatory reactions. In this model, the inhibition of these substances is evaluated [45].
The procedure involves subplantar injection of 0.1 mL of 0.5 % AA in 0.2 M carbonate buffer into the animal paws. The edema volume is measured appropriately at hourly intervals after AA challenge. Arachidonic is an intermediate in prostaglandin synthesis and is likely to induce the synthesis [45].

3.19 Vascular permeability test

The increased flow of cells in and out of the blood vessels results from the contraction and separation of endothelial cells at their borders, exposing the basement membrane and becomes freely porous to plasma proteins and fluid [46]. Exudation is a major characteristic of inflammation and is a result of increased vascular permeability. Drugs that give a positive result in this model are likely to block the production of inflammatory-mediating cytokine and myeloperoxidase activity mediated by neutrophils. They are also likely to inhibit interleukin -1β and TNF-α and prevent leukocyte accumulation. Acetic acid provokes instant vascular permeability that persists over 24-h period [47].

The practice involves an intravenous injection of 0.2 ml of 0.25% Evans blue dye in normal saline through the tail vein to each mouse 1 h after treatment with test samples. This is followed by intraperitoneal injection of each animal with 1 ml/100 g of acetic acid (0.6%, v/v) after 30 min. After an additional 30 min, the treated animals are humanely sacrificed. The peritoneal cavity of each animal is washed with a given volume of normal saline into a test tube and centrifuged. The concentration of the dye in the supernatant (as a function of vascular permeability) is measured spectrophotometrically at a wavelength of 610 nm [48,49].

3.20 Castor oil-induced diarrhea model

Prostaglandins are implicated in both diarrhea and inflammation, and this forms the basis for correlating diarrhea with inflammation. It is assumed that inhibition of diarrhea results from obstruction of prostaglandin biosynthesis which in turn translates to anti-inflammatory activity.

According to the previously reported procedure, 20 ml/kg castor oil is administered to each animal 1 h after administration of test samples and standard drug. The clean white paper is placed at the cage floor, and the animals monitored for diarrheal droppings at hourly intervals for about 4 h. The parameters measured include the onset of diarrhea, the number of wet stools, semi-solid, and solid stools. Nonappearance of diarrheal stool or delay in commencement of diarrhea is taken as an indicator of possible inhibition of prostaglandin biosynthesis and hence anti-inflammatory activity [49]. This method is obviously not sensitive as it is difficult to correctly quantify diarrheal droppings. It is very tedious to monitor the animals separately as a lot of precautions are required since urine can also wet the stool. Furthermore, it cannot be used in isolation since prostaglandin is not the only mediator of inflammation.

3.21 Carrageenan-induced pleurisy in rats

This method tests a reduction in the pleural exudates as a function of the inhibitory effect on leukocyte migration. It is carried out as previously reported [50]. The practice entails intrapleural injection of 0.25 ml of 1% solution carrageenan in 0.9% saline on the right side of the thorax 1 h after test drug administration. After 3 h, the animals are humanely bled to death through the portal vein under anesthesia. The pleural exudate is recovered and the pleural cavity rinsed with 1.0 ml saline containing heparin (10 IU/ml). Leukocytes count in the exudates follows appropriately as a measure of inflammation.

4 Challenges and prospects of anti-inflammatory bioassays

4.1 In vitro models

In vitro, anti-inflammatory activity study models involve cell culture techniques in which the enzymes and the inflammatory mediator chemicals are directly exposed to the test drugs. They have numerous advantages. Such studies are very helpful in elucidating the mechanism of anti-inflammatory activity of the test drugs. They usually involve limited number of experimental variables and make collection of significant data simpler than animal models. They take less time to accomplish [51]. They require smaller quantities of the test samples and hence, best suited for pure compounds isolated from plants. Sometimes, however, it is difficult to reproduce the same in vitro results in vivo due to pharmacokinetic and pharmacodynamic
parameters. *In vitro* models are more sophisticated and more complicated in terms of instrumentation and procedures, but once the reagents are prepared and materials assembled, the tests can be quite simple to run, and numerous subsequent tests can be run with the same materials and with ease. Cell subculturing and maintenance are, however, required with their associated cost and material implications. Consequently, many developing countries resort mainly to animal models for economic reasons.

### 4.2 In vivo models

The advantages and challenges of some *in vivo* anti-inflammatory assay protocols are shown in Table 1. *In vivo* anti-inflammatory activity experiments have numerous challenges. Cultural restrictions on animal use and rigorous procedures for obtaining ethical approval are major drawbacks to animal experiments. Despite these factors, the aggressiveness of the animals poses risk to the researchers and restricts the study only to researchers courageous enough to handle animals. Furthermore, poor compliance from the animals makes drug administration and measurement of edema volumes difficult. Paw edema models require larger animals, like rats, which are expensive. In some cases, the animals die or are deliberately sacrificed with its associated consequences on animal rights and conservation. Animal studies generally require large quantities of test samples. Accurate measurement of paw edema volume is associated with several problems. Detection of

<table>
<thead>
<tr>
<th>Assay model</th>
<th>Advantages</th>
<th>Challenges</th>
</tr>
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<tbody>
<tr>
<td>Carrageenan-induced inflammation</td>
<td>Sensitive, highly reproducible, non-immune suited for systemic, acute inflammation, orally active drugs [52,53]</td>
<td>Involves animal stress; difficulty with edema measurement; needs extra attention</td>
</tr>
<tr>
<td>Egg-albumin-induced edema</td>
<td>Egg-albumin is cheap and readily available; specifies histamine/serotonin involvement [53,54]</td>
<td>Involves animals; does not test lipid mediators; edema measurement problems</td>
</tr>
<tr>
<td>Kaolin-induced edema</td>
<td>Suited for sub-chronic inflammation; not likely to have antigenicity or cause hypersensitivity reactions [28]</td>
<td>Involves animals; difficulty associated with edema measurement</td>
</tr>
<tr>
<td>Dextran-induced edema</td>
<td>Suited for systemic acute inflammation; leads to the rapid development of edema</td>
<td>Involves animals; not suitable for lipid mediators</td>
</tr>
<tr>
<td>Histamine and Serotonin edema models</td>
<td>Confirm histamine/serotonin involvement (acute)</td>
<td>Not readily available; produce minimal edema</td>
</tr>
<tr>
<td>Formalin-induced edema model</td>
<td>Suitable for both chronic anti-inflammatory and analgesic activities</td>
<td>Time consuming; severe animal stress and pain</td>
</tr>
<tr>
<td>Agar-induced inflammation</td>
<td>Safe; tests acute systemic effect</td>
<td>Poor agar solubility</td>
</tr>
<tr>
<td>Xylene, croton oil, or TPA-induced edema</td>
<td>Suitable for topical inflammation; requires mice</td>
<td>Animals are sacrificed; measurement is relative</td>
</tr>
<tr>
<td>Adjuvant-induced arthritis</td>
<td>Ideal for chronic inflammation and arthritic alterations</td>
<td>Sophisticated; tedious; severe animal pain and stress; animals are sacrificed</td>
</tr>
<tr>
<td>Cotton-pellet granuloma test</td>
<td>Ideal for chronic inflammation; gives information about the proliferative changes</td>
<td>Tedium; requires expertise; sepsis may occur; involves loss of many animals</td>
</tr>
<tr>
<td>Air pouch model [53]</td>
<td>Good for sub-acute inflammation; direct contact of test drugs with the target cells; monitors immune system response</td>
<td>Tedium; requires expertise; severe animal stress; involves loss of many animals</td>
</tr>
<tr>
<td>Carrageenan-induced pleurisy test</td>
<td>Suitable for acute anti-inflammatory activity; assess fluid extravasation, leukocyte migration and biochemical parameters in the exudates [53]</td>
<td>Tedium; severe animal stress; animals are sacrificed; may result in infection</td>
</tr>
</tbody>
</table>
small increments in paw volume precisely using the liquid displacement method is difficult. Due to surface tension, water or mercury does not overflow immediately until a certain volume of object is immersed. Some volume of water is also lost due to wetting of the paws. Furthermore, aggressiveness and poor compliance of the animals aggravate the problem, causing liquid splashes, and agitation. Vernier caliper, on its own, measures paw diameter and not volume. It is only an approximation to correlate paw diameter with paw volume since the rat paw is not perfectly cylindrical, and inflammation could be non-uniform across the paw surface. The reading also depends on the extent of tightening of the Vernier caliper knob. Since the rat paw surface is not rigid, variations in the degree of tightening are possible. Paw volume is also measured by winding a piece of cotton thread around the paw circumference. The sensitivity of this technique can be increased by winding the thread several turns around a specified paw length. The more the number of turns, the more sensitive the measurement. This is because the minor increment in diameter is multiplied by the number of turns, hence, magnifying the volume differences.

5 Perspective

The in vitro models involving direct monitoring of inhibition of inflammatory mediators or enzyme activities are no doubt more preferable in terms of sensitivity, reproducibility, and reliability. They offer better insight into the mechanism of action and the inflammatory mediators involved. However, where the resources and expertise are lacking, the carrageenan-induced edema model is most recommended. Among all the animal models, it is the most preferred and the most commonly used method for systemic acute inflammation. It evaluates both phases of inflammation. It is very sensitive and reproducible, provided the carrageenan injection, and the edema volume measurements are accurate. Plethysmometer remains the best option for edema volume measurement. The egg albumin model is also commonly used because of the ready availability of the egg albumin. To improve accuracy and sensitivity as regard edema volume, it is recommended to employ up to three different methods to determine edema volume and use the mean values. While using the liquid displacement methods, it is recommended to immerse the animal paw in a narrow calibrated vessel not filled to the brim and measure the change in liquid level instead of volume of water displaced from overflowing vessel. This reduces error due to liquid surface tension. Subplantal injection of the phlogistic agent has to be done carefully and by an expert too to avoid severe injury on the paw tissues. Otherwise, it would be difficult to tell if the edema is due to carrageenan or tissue injury.

Researchers from most developing countries resort to the crude paw edema models, not because they are not aware of the merits of the in vitro models, but because of the economic realities. Where cost and expertise are barriers to sound research, researchers are encouraged to engage in collaboration with other more privileged ones instead of resorting to improvised techniques, which may lead to false results. There is a wide gap between the rate at which numerous plants have been reported with good anti-inflammatory activity in recent times and the rate at which potent new anti-inflammatory drugs are being discovered.

6 Conclusion

Numerous techniques are at the disposal of researchers for assessing the anti-inflammatory activities of test drugs, each of which is associated with some peculiar advantages and limitations. Critical considerations such as sensitivity, reliability, and cost are, therefore, necessary in the choice of assay model. However, priority should be given to reliability, reproducibility, and sensitivity for optimal results.

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Conflicts of interest

All the authors have no conflicts of interest to declare.

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