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IN-VITRO ANTI-INFLAMMATORY ASSESSMENT OF PLANT-DERIVED NATURAL PRODUCTS AS NOVEL DRUG LEADS

Prathitha Chathuri Tenne [1], Prasanna Galhena [2], Anura Dissanayake [3], Dinithi Peiris [4], Ajit Abeysekera [1] and Chayanika Padumadasa [*1]

Department of Chemistry [1], University of Sri Jayewardenepura, Sri Lanka.
Department of Biochemistry and Clinical Chemistry [2], University of Kelaniya, Sri Lanka.
Department of Obstetrics and Gynecology [3], University of Kelaniya, Sri Lanka.
Department of Zoology [4], University of Sri Jayewardenepura, Sri Lanka.

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Correspondence to Author:
Prof. Chayanika Padumadasa

Professor,
Department of Chemistry,
University of Sri Jayewardenepura,
Sri Lanka.

E-mail: chayanikapadumadasa@yahoo.com

ABSTRACT: Inflammation is considered as a biological response of the human body that counteracts harmful stimuli by activating different anti-inflammatory cellular signaling pathways to maintain good health and well-being. However, the persistence of inflammation in tissue or organ may lead to adverse chronic inflammatory responses resulting in diseases of chronic nature. Non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, and immune suppressants are widely used to minimize adverse responses mediated by inflammation. However, the development of detrimental side effects in long-term usage of these drugs has directed the scientific community towards identifying novel and safe plant-based anti-inflammatory agents. The plant-based novel drug discoveries involve in a wide range of preliminary *in-vitro* assessment methods. This article review the methods, principles, advantages and limitations of different assay procedures that have been widely used in evaluating anti-inflammatory properties. Aim of this review is to provide a guidance for identifying plant derived natural compounds with potential anti-inflammatory effects.

INTRODUCTION: Throughout human evolution, plants have played an enormous role in medicine and health. Plants have formed the basis of traditional medical practices that have existed since the commencement of human civilization [1, 2]. Chemical substances produced by living organisms found in nature are termed "natural products". Among the different sources, the plant kingdom is a rich source of such compounds. Until the 19th century, medicinal plants were used on an empirical basis, without any knowledge of natural products or their actions of mechanisms.

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Although, rational clinical investigation of medicinal plants commenced in the 18th century, it was the discovery of the analgesic and sleep-inducing agent morphine from opium by the German apothecary assistant Friedrich Sertürner in the beginning of the 19th century that led to coherent drug discovery from medicinal plants [3, 4].

Since then, many important natural products have been isolated from plants and other natural sources. Plant- based natural products have been reported to exhibit anti-oxidant, anti-bacterial, anti-fungal, anti-viral, anti-cancer and, more on anti-inflammatory activities. Detailed work has been published in literature on anti-inflammatory activities of medicinal plants and their derivatives [5-12]. Inflammation is a biological response of the immune system towards infection and tissue injury which enables the removal of harmful stimuli and the healing of damaged tissue which is essential to

maintain good health. Cardinal signs of inflammation are commonly characterized by redness, swelling, heat, pain, and loss of tissue function. The inflammatory response is mediated by the activation of nuclear factor kappa B (NF-κB), mitogen activated protein kinase (MAPK) and Cyclooxygenases (COXs) cellular signaling pathways. These signaling pathways consist of inducers, sensors, mediators, and target tissues. The inducers can initiate an inflammatory response and sensors can detect such initiation by expressing cell surface receptors on macrophages, dendritic cells, and mast cells in human tissues and organs [13]. These cells can trigger inflammatory mediators such as chemokines, cytokines, bioactive amines, and eicosanoids which may act on target tissues to elicit the inflammation and restore normal tissue function [14, 15].

During the acute inflammatory responses, the transition of the inflamed state of the body into the homeostatic state is regulated by molecular and cellular mechanisms and the entire process defines as a resolution of inflammation [16]. However, the persistence of the inflammatory response in inflamed tissue or organ may dysregulate the resolution phase of inflammation by overproduction of inflammatory mediators such as cytokines and chemokines. This may result in an uncontrolled tissue regeneration and remodeling at the site of inflammation leading to chronic inflammatory response [17, 18].

Most of chronic inflammatory responses can give rise to chronic diseases such as diabetes mellitus, neurodegenerative disease, chronic liver diseases, ischemic diseases, and malignancies. Currently available anti-inflammatory agents such as NSAIDs, glucocorticoids, immunosuppressant drugs are widely being used to treat adverse inflammatory responses. Despite their quick and effective action against inflammatory responses, development of detrimental side effects in long term usage appeared as the major drawback of these anti-inflammatory drugs [1, 3]. Plant based natural products have been identified as potential anti-inflammatory drug leads and currently a number of naturally derived pharmaceuticals are in clinical practice. Previous work clearly validates the use of naturally derived compounds as potential anti-inflammatory agents by using validated anti-inflammatory experimental models [19-23]. Despite the extensive studies on plant derived compounds over past decades, more studies would be urgently needed to discover novel potential anti-inflammatory drugs to treat adverse inflammatory effects by minimizing the uncontrolled activation of cellular inflammatory pathways of the human body. Therefore, investigations of potential anti-inflammatory agents derived from medicinal plants that are able to regulate key inflammatory signaling pathways have gained immense attention recently and more studies would be needed in future to combat with chronic inflammatory diseases.

In-vitro experiments such as erythrocyte membrane stabilization assay, inhibition of protein denaturation assay, proteinase inhibitory assay, inhibition of 5-lipoxygenase enzyme action, inhibition of COX enzyme activity and inhibition of hyaluronidase enzyme activity are more common in searched literature due to their cost effectiveness, ethically favorable and rapid screening of anti-inflammatory properties over *in-vivo* and cell-based methods. Therefore, this review highlights the published literature on the most widely used anti-inflammatory assessment methods, explaining their background, assay principles, methodologies, and their respective pros and cons. Moreover, these insights may provide a useful platform for the development of novel plant derived anti-inflammatory compounds. Research papers and reviews published in literature that consist of *in-vitro* anti-inflammatory assay protocols used for the preliminary screening of plant derived chemical substances were reviewed and, a common assay procedure for each assay has been outlined.

In-vitro Anti-inflammatory Assays:

Erythrocyte Membrane Stabilization Assay:

Background and Principle: Lysosome is a membrane-bound cell organelle that contains hydrolytic enzymes. Degradation of macromolecules through a series of enzymatic reactions which are activated at low pH levels is the primary function of lysosomes. This process is needed to degrade macromolecules that are internalized by cells or to degrade endogenous molecules of damaged cells such as cell membranes, cytoskeleton and nucleic acids. When cells are exposed to stress conditions such as

starvation and exposure to toxic substances, this latter process is important to maintain normal homeostasis of cells. Lysosomes consist of different types of hydrolytic enzymes such as lipases, proteolytic enzymes, phospholipases, nucleases and glycosidases that exert their enzymatic activity at low pH levels. A complete or partial breakdown of the lysosomal membrane release higher amounts of hydrolytic enzymes in to the cytosol [24]. This may ultimately induce cytosolic acidification, which in turn can result an unregulated necrosis and apoptosis of the cell [24]. Release of these enzymes to the extracellular environment from the damaged cell may result degradation of connective tissue macromolecules and cause tissue injury at the site of inflammation leading to acute and chronic inflammatory diseases. Therefore, stabilization of lysosomal membrane is important during variety of stress conditions. NSAIDs prevent adverse inflammatory responses of these hydrolytic enzymes by stabilizing the lysosomal membrane [25-27].

Erythrocyte and lysosomal membranes share common properties. Therefore, stabilization of erythrocyte membrane by a test material upon the exposure to external stress stimuli is a measure for the stabilization of lysosomal membranes. Exposure to heat and hypotonic solutions may disrupt the erythrocyte membrane integrity and promote the release of their cytosolic content to the surrounding solvent; heamolysis [28-30]. Hypotonic solutions create a low osmotic pressure outside of the cell and facilitate the movement of solvent into the cell *via* the cell membrane. Swelling of erythrocytes disturb the membrane integrity and thereby release heamoglobinto the surrounding environment. Heating may increase kinetic energy of molecules inside the cell and this may trigger heamolysis of erythrocytes by disrupting the erythrocyte membrane integrity. Released heamoglobincontent can be determined using spectrophotometric methods. The hemiglobincyanide (HiCN) method is the standard method used to determine the total heamoglobin concentration in the heamolysate. In this method, all the heamoglobin derivatives including HbO_2 are converted in to HiCN and then HiCN concentration is determined at the wavelength of 540 nm at the pH range 7-7.4 [31]. However, HiCN method results an inaccurate estimation of the absorbance value at 540 nm due to the formation of a turbid appearance in the heamolysate. In addition, HiCN method does not distinguish hemoglobin derivatives which contain oxygen. This may overestimate the oxyheamoglobin (HbO_2) content in blood [32]. Direct measurement of HbO_2 content at 540 nm is the same as the measurement of HbO_2 which is converted in to HiCN at this wavelength. Thus, the measurement of the HbO_2 concentration is considered as one of the most applicable methods to quantify HbO_2 content in the heamolysate. Therefore, total oxyheamoglobin content in a heamolysate can be determined at the wavelength of 540 nm.

Experimental Procedure for Inhibition of Heat Induced Heamolysis: Brown and his collaborators in 1966 have reported a method to determine the inhibitory effect of a test material on heat induced heamolysis. This method has been followed with slight modifications up to now. The following method summarizes the methods reported for inhibition of heat induced hemolysis in literature [33-38].

- Prepare a concentration series of the test material (concentrations varying from 0.1 – 0.8 mg/mL) by dissolving the test material in a suitable solvent (dimethyl sulfoxide, dimethylformamide, methanol, normal saline or water) considering the solubility of the test material.

- Prepare 5.0 mL of the reaction mixture by adding 2.0 mL of normal saline (0.9% w/v NaCl), 1.5 mL phosphate buffer saline (PBS) at pH 7.4 and 1.0 mL test solution in a clean test tube.

- Add 0.5 mL of 10% erythrocyte suspension in to the tube containing the reaction mixture and mix well. Incubate the tube containing the reaction mixture at 56 °C in a water bath for 30 minutes and cool under running tap water at the end of the incubation period. Centrifuge the reaction mixture at 3000 rpm for 10 minutes. Measure the absorbance of the supernatant at 540 nm against PBS as the blank and carry out the experiment in triplicate. Repeat the above procedure for remaining test solutions.

- Prepare a concentration series of the standard drug (diclofenac sodium, indomethacin or asprin) by using PBS. Repeat the above-mentioned assay protocol for each of the prepared solutions of the standard drug. The standard drug will be the positive control in the experiment. Replace the test solution with 1.0 mL PBS in the reaction mixture to prepare the negative control and follow the above-mentioned assay protocol.

Experimental Procedure for Inhibition of Hypotonic Induced Heamolysis: The method described by Seeman and Weinstein in 1966 is used in order to determine the inhibition of hypotonic induced heamolysis of a particular test material in searched literature. This method has been followed to date with slight modifications. The following method summarizes the methods reported for inhibition of heat induced hemolysis during the past years [30, 33-35, 38].

- Prepare a concentration series of the test material (concentrations varying from 0.1 – 0.8 mg/mL) by dissolving the test material in a suitable solvent (dimethyl sulfoxide, dimethylformamide, methanol, water or normal saline) considering the solubility of the test material.
- Prepare 5.0 mL of the reaction mixture by adding 2.5 mL hypo saline (0.25% v/v NaCl), 1.0 mL PBS at pH 7.4 and 1.0 mL test solution in a clean test tube.
- Add 0.5 mL of 10% erythrocyte suspension in to the tube containing the reaction mixture and mix well.
- Incubate the tube containing the reaction mixture at 37°C in a water bath for 30 minutes.
- After completing the incubation period, centrifuge the reaction mixtures at 3000 rpm for 10 min. Measure the absorbance of the supernatant at 540 nm against PBS as the blank. Carry out the experiment in triplicate. Repeat the above-mentioned procedure for remaining test solutions.
- Prepare a concentration series of the standard drug (diclofenac sodium, indomethacin or asprin) using PBS. Repeat the above-mentioned assay protocol for each of the prepared solutions of the standard drug. The standard drug will be the positive control in the experiment. Replace the test solution with 1.0 mL PBS in the reaction mixture to prepare the negative control and follow the same assay protocol.

Preparation of Erythrocyte Suspension: Collect fresh venous blood from a healthy volunteer donor without NSAID treatment two weeks prior to the experiment or from an anesthetized animal in to a sterile tube containing heparin, EDTA or Alsever solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid and 0.42% sodium chloride) as anticoagulants and centrifuge at 3000 rpm for 10 minutes. Discard supernatant and wash the resulting cell pellet three times with isotonic buffer (10mM sodium phosphate buffer at pH 7.4) or Alsevier solution. Reconstitute the cell pellet with fresh Alsevier solution, isotonic buffer or 1x PBS to prepare the erythrocyte suspension.

The percentage inhibition of heat and hypotonic induced hemolysis assays of test solution and the standard drug are calculated using the equation given below.

$$\text{Percentage Inhibition} = (A_1\text{-}A_2) / A_1 \times 100 \text{…..Equation (01)}$$

A_1- Absorbance of the negative control "A_2- Absorbance of the test solution/positive control".

Inhibition of Protein Denaturation Assay:

Background and Principal: Proteins are important macromolecules in all living organisms and, are important mediators in cell signaling pathways to maintain the homeostasis of the body during inflammation [39]. During the acute phase of inflammation, transition of inflamed state of the body in to the homeostatic state is regulated by the production of inflammatory cytokines which are proteins. Immune cells activated during the acute phase of inflammation such as macrophages, neutrophils and dendritic cells initiate the biosynthesis of pro-inflammatory cytokines to promote tissue repair and regeneration at the site of inflammation. This can be achieved through the activation of several cell signaling pathways. As an example, the activation of NF-Ƙb transcription factor proteins in immune cells are responsible for

the induction of the gene expression of pro-inflammatory cytokines. NF- ƙB proteins are composed of structurally related proteins namely, p^{50}, $p^{52,}$ p^{65}, Rel A, Rel B and C-Rel which are responsible to initiate the transcription of target genes by binding to the promoter region of the DNA upon their activation [40]. These proteins are naturally inhibited in the cytoplasm by inducible ƙB family member protein IƙBα. Binding of lipopolysaccharides, TNF-α and unknown ligands to its specific receptors present in the above-mentioned immune cells activate the phosphorylation of IƙBα by IƙB kinases (IKK). As a result, NF- ƙB proteins become activated and translocate to the nucleus from the cytoplasm to initiate the production of pro-inflammatory cytokines [40]. All above –mentioned processes are tightly regulated by proteins in its original molecular configuration. Disruption of protein structural integrity by external stress stimuli such as acids, bases, concentrated salts and thermal exposure reduce the biological potency of proteins leading to uncontrolled tissue damage and chronic inflammatory diseases.

Heat denaturation changes the thermodynamically stable native configuration of the protein by unfolding or altering their tertiary, quaternary or secondary structures. Protein denaturation may not affect the primary structure or amino acid sequence of the protein. However, the structural changes of protein can occur through the disruption of hydrogen and disulphide bonds, hydrophobic interactions and salt bridges of the protein. Deformities in functional properties of proteins may occur during heat denaturation of proteins such as changes in solubility, emulsifying capacity, gelation capacity, foaming properties, enzyme and biological activities. The temperature induced unfolding of protein structure promotes the protein aggregation via the formation of intermolecular cross-links between unfolded protein molecules[41].Bovine serum albumin (BSA) is commonly used as the protein source in inhibition of protein denaturation assay protocols. Temperature, pressure, pH and ionic strength of the surrounding solvent determine the stability of the protein structure. Heat exposure induces the unfolding of BSA in an aqueous solution leading to its thermal aggregation. Temperature, pH, protein concentration and salt concentration in the protein solution determine the rate of heat induced protein aggregation [42]. Several analytical methods have been designed to evaluate protein aggregation such as size exclusion chromatography, UV-VIS spectroscopy (turbidity/opalescence), analytical ultracentrifugation and static light scattering. Spectrophotometric determination of turbidity is used as a measure of protein aggregation in commonly reported assay protocols of inhibition of protein denaturation. Heat denaturation of proteins followed by protein aggregation show an optical property known as turbidity/opalescence in the protein solution [43]. Aggregated proteins span nanometers to micrometer particle size range and tend to scatter a considerable amount of light in the visible region of the spectrum. The intensity of the light beam may reduce when it passes through a turbid solution due to light scattering, reflection and absorption. Decreased intensity of light is measured in the determination of turbidity of a solution. In turbidity measurements, scattering light at 660 nm is measured using UV-VIS spectrophotometer [43]. This assay is designed to identify variety of test materials that can inhibit the heat-treated protein denaturation at a pathological pH (6.4) condition in early stages of drug developmental processes.

Experimental Procedure: Mizushima and kobaysh in 1968 described a method to determine the inhibition of protein denaturation of test materials. This method with slight modifications has been followed till present day. The following method is a summary of the methods reported for protein inhibitory assay over the past years [44-46].

- Prepare a concentration series of the test material (concentrations varying from 0.1 – 0.8 mg/mL) by dissolving the test material in a suitable solvent (dimethyl sulfoxide, dimethylformamide, methanol or water) considering the solubility of the test material

- Prepare 3.0 mL of the reaction mixture by adding 0.8 mL bovine serum albumin (BSA) or egg albumin, 1.2 mL PBS at pH 6.4 and 1.0 mL test solution in a clean test tube. Incubate the tube containing the reaction mixture at 37 °C in a water bath for 20 minutes. After completing the incubation period, heat the reaction mixture at 70°C for 5 minutes and cool under running

tap water. Measure the absorbance of the reaction mixture at 660 nm against PBS as the blank. Carry out the experiment in triplicate. Repeat the above procedure for remaining test solutions.

- Prepare a concentration series of the standard drug (diclofenac sodium, indomethacin or asprin) using PBS. Repeat the above-mentioned assay protocol for each of the prepared solutions of the standard drug. The standard drug will be the positive control in the experiment. Replace the test solution with 1.0 mL PBS in the reaction mixture to prepare the negative control and follow the above-mentioned assay protocol.

The percentage inhibition of protein denaturation of the test solution and the standard drug can be calculated using the equation 01.

Proteinase Inhibitory Assay:

Background and Principal: Proteolytic enzymes are capable of hydrolyzing peptide bonds in proteins and are called peptidases, proteases or proteinases. Proteolytic enzymes are classified based on their origin: microbial, plant, animal and human. They are also classified as exopeptidases and endopeptidases depending on the site of enzyme action.

Exopeptidases catalyze the hydrolysis of terminal or penultimate peptide bonds of the polypeptide chain in the substrate protein at N or C terminals to release amino acids or dipeptides whereas endopeptidases cleave internal peptide bonds of the polypeptide chain in the substrate protein to release small peptides [43].

Proteolytic enzymes stored in azurophilic granules of activated neutrophils are released upon the activation of neutrophils during the acute phase of inflammation. Neutrophils are the earliest inflammatory cells that migrate towards the inflammatory site in order to act against invading pathogens *via* activating the short-term phagocytosis during the acute phase of inflammation. Recruitment of neutrophils during inflammation is important for host defense, however, their excessive accumulation may trigger prolonged and uncontrolled release of proteolytic enzymes. As a result, degradation of extracellular matrix components at the inflammatory site may occur leading to excessive tissue damage and chronic inflammatory diseases [47, 48]. These enzymes are capable of hydrolyzing the substrate protein at an optimum temperature of 37° C to 60 °C and pH between 6 and 10. This optimum temperature and pH is important for protein hydrolysis to take place by maintaining the correct conformational state of the substrate protein towards the active site of the proteolytic enzyme. This may facilitate the hydrolysis of the peptide chain of the substrate protein and release amino acids and small peptides to the surrounding reaction mixture. During protein hydrolysis, H^+ ions are released to the surrounding reaction mixture decreasing the optimum pH at which protein hydrolysis occurs. These pH changes can be eliminated by dispersing the substrate protein in a buffer solution which has been adjusted to the optimum pH of the proteolytic enzyme [49].

Commonly, tris HCl buffer at pH 7.4 has been used to maintain the optimum pH throughout the assay. The optimum temperature is maintained by incubating the reaction mixture at 37 °C. Upon completion of the protein hydrolysis in a given time period, the enzyme-protein reaction can be terminated by using different methods; keep the reaction mixture at a temperature level that will denature the proteolytic enzyme and stop the reaction (usually above 80 °C) or keeping the reaction mixture at cold temperatures and adjust the pH level where the enzyme is inactive by adding a weak acid: TCA [49]. Addition of TCA terminates the enzyme-protein reaction by decreasing the optimum pH of the reaction mixture [50].

At this point, the reaction mixture consists of products formed during protein hydrolysis such as small peptides, free amino acids and partially-hydrolyzed or non-hydrolyzed substrate proteins. This mixture is collectively known as the protein hydrolysate. Addition of TCA also induce the precipitation of partially hydrolyzed or non-hydrolyzed substrate proteins by increasing the solubility of small peptides and free amino acids formed after the protein hydrolysis. Therefore, TCA is used to separate partially and non-hydrolyzed proteins in the protein hydrolysate [49, 51]. The TCA induced protein precipitation is independent with size of the protein and it appears to be less effective in precipitating proteins in its

disordered states suggesting that TCA has a high selectivity towards the precipitation of proteins with its own structure. Detection of TCA soluble small peptides dissolved in the protein hydrolysate can be achieved using spectrophotometric methods. Universally applicable wavelength for measuring absorbance of the peptide bond of proteins and small peptides is 205 nm. The peptide bond in proteins and small peptides is the major chromophore which gives a strong absorbance band at 190 nm and a weak absorbance band at 220 nm.

Absorbance band of oxygen interferes with the strong absorbance band of the peptide bond at 190 nm and this gives an erroneous absorbance value for the peptide bond at this wavelength. Therefore, 190 nm wavelength is shifted towards the 205 nm where the molecular absorbance coefficient of the peptide bond is about half of that at 190 nm. Protein hydrolysate containing proteins and small peptides with aromatic side chains such as tryptophan, phenylalanine, tyrosine, and histidine absorb UV radiation at 280 nm and these proteins and small peptides also absorb maximum UV radiation at 205 nm.

Proteins and small peptides lack of aromatic side chains do not absorb UV radiation at 280 nm but strongly absorb at 205 nm [52]. Peptide bonds in the polypeptide chain of proteins and small peptides have extinction coefficient values in the range of 30-35 at 205 nm.

The position and intensity of the main л- л* transition of the peptide bond can vary with the polarity of the solvent and therefore maximum absorbance of the peptide bond can be slightly shifted towards 210 nm where the extinction coefficient value is in the range of 20-24.

Since there is no much difference between these two values and therefore, absorbance of proteins and small peptides dissolved in protein hydrolysate also measured at 210 nm in reported methods [53].

Experimental Procedure: In 1965, Samy and co-workers reported a method to determine the proteinase inhibitory activity of a test material. This method has been followed up to date with slight modifications [2, 54, 55]. The following method is a summary of the methods reported for proteinase inhibitory assay in literature [35, 56].

- Prepare a concentration series of the test material (concentrations varying from 0.1– 0.8 mg/mL) by dissolving the test material in a suitable solvent (dimethyl sulfoxide, dimethylformamide, methanol, water or tris HCL buffer) considering the solubility of the test material.
- Prepare 2.0 mL of the reaction mixture containing 0.06 mg proteolytic enzyme (protease or trypsin), 1.0 mL 20 mM tris HCl buffer at pH 7.4 and 1.0 mL test solution in a clean test tube.
- Incubate the tube containing the reaction mixture at 37 °C in an incubator or a water bath for 5 minutes.
- After completing the incubation period, add 1.0 mL substrate protein (4% w/v bovine serum albumin or 0.8 % w/v casein) dissolved in 20 mM tris HCl buffer in to the reaction mixture, mix well and incubate for another 20 minutes at 37 ° C.
- Add 2.0 mL 70% (w/v) trichloroacetic acid (TCA) to terminate the enzyme-protein reaction. Centrifuge the cloudy suspension at 3000 rpm for 10 minutes and measure the absorbance of the supernatant at 210 nm against tris HCl buffer as the blank. Carry out the experiment in triplicate. Repeat the above-mentioned procedure for remaining test solutions.
- Prepare a concentration series of the standard drug (diclofenac sodium, indomethacin or asprin) using 20 mM tris HCl buffer. Repeat the above-mentioned assay protocol for each of the prepared solutions of the standard drug. The standard drug will be the positive control in the experiment. Replace the test solution with 1.0 mL 20 mM tris HCl buffer in the reaction mixture to prepare the negative control and follow the same assay protocol.

The percentage inhibition of proteinase activity of the test solution is calculated using the equation mentioned under the section 3.1.2. The same equation can be used to calculate percentage inhibition of proteinase activity of the standard drug.

Inhibition of COX Enzyme Activity:

Background and Principal: Arachidonic acid (AA) is a polyunsaturated fatty acid distributed throughout membranes of most of the cells in the human body. They are precursor molecules of far-ranging cellular effects [57]. Phospholipase A and phospholipase C enzymes in the cytosol catalyze the release of membrane bound AA to the cytosol upon irritation or injury to the cell. Released AA is metabolized through the major anti-inflammatory pathways; cyclooxygenase (COX), lipoxygenase and cytochrome P-450 monooxygenase pathways. However, COX pathway is the most extensively studied pathway among all inflammatory pathways and its activation may produce prostaglandins (PGs). PGs are important lipid mediators involved in a variety of biological functions such as regulation of immune responses, blood pressure, gastrointestinal integrity, fertility and acute inflammatory responses leading to characteristic inflammatory signs at the site of inflammation [58].

COX enzymes are monotopic integral membrane proteins which are located in membranes of endoplasmic reticulum and nuclear membranes of cells. They catalyze the conversion of AA in to an unstable prostaglandin known as prostaglandin G_2 (PGG_2). This PGG2 is then converted in to prostaglandin H_2 (PGH_2) which is the precursor for the formation of structurally related bioactive types of PGs including prostaglandin E_2 (PGE_2), prostacyclin (PGI_2), prostaglandin D_2 (PGD_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) via the action of cell specific prostaglandin synthases. PGs exert their autocrine and paracrine actions upon binding with specific G-protein coupled receptors (GPCR) located in the same cell or nearby cell membranes to activate their downstream signaling pathways at the site of inflammation [59]. Activation of these GPCR, activate MAPK and NF-kB activator proteins. NSAIDs mainly target COX-2 enzymes by competing with the AA binding site of the COX enzyme. This may ultimately reduce the production of prostaglandins and thereby, reduce adverse inflammatory responses at the site of inflammation [60].

COX enzymes contain Fe^{3+} protoporphyrin IX as a co-factor and its dissociation may affect for the COX enzyme activity. Therefore, hemin (hematin) is added to the reaction mixture in order to maintain the maximum COX enzyme activity by stabilizing the Fe^{3+}-protoporphyrin IX co-factor throughout the COX enzyme reaction [61]. The peroxidase activity of COX enzymes (conversion of PGG_2 in to PGH_2) can be determined using co-substrates such as 5-phenyl-4-pentenyl hydroperoxide (PPHP), 10-acetyl-3,7-dihydroxy-phenoxazine (ADHP), luminol and N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) to generate colored products during reduction of PGG_2 into PGH_2. The peroxidase activity of COXs is commonly measured using TMPD, which is an artificial electron donor oxidized in to a blue color product with a maximum absorbance at 590 nm. AA is used as the substrate for the COX enzyme reaction. This method is an indirect method of measuring COX enzyme activity. The oxidation of TMPD accurately reflects the rate of conversion of AA in to PGH_2. Therefore, this assay has been widely used to determine potential COX inhibitors. Commercially available colorimetric inhibitor screening assay kits also use this peroxidase mediated TMPD co-oxidation to identify variety of COX (COX-1 and COX-2) enzyme inhibitors [61].

Experimental Procedure: Three basic assay protocols have been used to measure COX enzyme (both COX -1 and COX-2) activity. These assay protocols measure the final product (PGE2) of the enzyme reaction using enzyme-linked immunosorbent assay (ELISA), uptake of oxygen during the conversion of AA into PGG2 and the peroxidase function of the COX enzyme in conversion of PGG2 into PGH2 using N, N, N', N'tetramethyl-p-phenylenediamine (TMPD) [60]. The latter assay protocol has been reported in literature to determine COX enzyme activity *in-vitro*. Richard and Kulmacz in 1987 described a method to determine peroxidase function of COX enzyme activity using TMPD. This method with slight modifications has been used over past three decades in order to determine inhibitory effects of test materials on COX -2 enzyme action [57, 60, 61].

The following method summarizes the determine peroxidase function of COX enzyme activity using TMPD protocol that has been used to determine the inhibition of the COX enzyme activity by a particular test material. The same assay protocol with slight modifications has been implemented in commercially available colorimetric COX (ovine)

inhibitor screening assay kits which should be used according to the manufacturers' instructions, and therefore, inhibitory effects of a test material on COX enzyme activity can be determined [57].

- Prepare a concentration series of the test material (concentrations varying from 0.1 – 0.8 mg/mL) by dissolving the test material in a suitable solvent (dimethyl sulfoxide, dimethylformamide, methanol or water) considering the solubility of the test material.
- Prepare 0.04 mL of the reaction mixture containing 0.02 mL of 10 U/mL of COX enzyme solution and 0.02 mL test solution in a 96-well plate.
- Prepare endpoint assay mixture consisting of 100 µM bovine hemin chloride, 10 mM of arachidonic acid, 17 mM of TMPD and 100 mM Tris-HCl buffer at pH 8.0.
- Add 0.16 mL of endpoint assay mixture in to each well containing the reaction mixture and mix well. Incubate the mixture for 5 minutes at room temperature and measure absorbance at 590 nm. Carry out the experiment in triplicate. Repeat the above-mentioned procedure for remaining test solutions
- Prepare a concentration series of the standard drug (diclofenac sodium, indomethacin or asprin) using Tris-HCl buffer. Repeat the above-mentioned assay protocol for each of the prepared solutions of the standard drug. The standard drug will be the positive control in the experiment. Replace the test solution with 0.02 mL of Tris-HCl buffer in the reaction mixture to prepare the negative control and follow the same assay protocol.

The percentage inhibition of COX enzyme activity of the test solution and the standard drug can be calculated using the equation 01.

Inhibition of 5-lipoxygenase Enzyme Action:

Background and Principal: Leukotrienes (LTs) are biologically active compounds derived from the arachinodic acid (AA) metabolism through 5-lipoxygenase (5-LOX) pathway. In contrast to the production of PGs in most of the cells in the human body, LTs are predominantly produced by inflammatory cells such as polymorphonuclear leukocytes, activated neutrophils, dendritic cells and mast cells. 5- LOXs are key enzymes involved in the production of LTs. These enzymes catalyze the hydroperoxidation of polyunsaturated fatty acids. Binding of AA with 5- lipoxygenase activating protein (FLAP) in the cytosol facilitates the 5-LOX mediated oxygenation of AA. This yields an unstable 5*S*-hydroxy-peroxyeicosatetraenoic acid (5S-HPETE), which in turn, dehydrated in to an unstable inflammatory lipid mediator leukoterine A_4 (LTA_4). These LTAs are hydrolyzed in to LTB4 and LTC4 via specific LTA4 hydrolases. All these LTA4, LTB4 and LTC4 are collectively known as LTs. They exert their inflammatory activity upon the binding of cell specific G protein couple receptors present in the same cell or nearby inflammatory cells. Synthesis of LTs is important to maintain normal physiological functions of the body during inflammation but their overproduction may cause adverse inflammatory responses leading to chronic inflammatory disease [62, 63].

NSAIDs mainly target 5-LOX enzymes by competing with the AA binding site and this may ultimately reduce the production of LTs and thereby, reduce adverse inflammatory responses at the site of inflammation.

Lipoxygenases catalyze the oxygenation of polyunsaturated fatty acids yielding a hydroperoxy fatty acids that contain a penta-l,4-diene moiety. Linoleic acid is the polyunsaturated fatty acid which has been used as the substrate for the soybean lipoxygenase enzyme action, however, its poor water solubility may result difficulties when maintaining the optimum pH for the lipoxygenase action in the reaction mixture. Therefore, homogeneous and clear aqueous solution of sodium linoleic acid can be obtained by dissolving them in borate buffer solution at pH 9 [64-68]. A clear aqueous solution of linoleic acid can also be obtained by adding an emulsifier in to the reaction mixture. Polysorbate 20 emulsifier is one of the most commonly emulsifier that can be used to maintain optimum pH of the reaction mixture by increasing the solubility of linoleic acid. Different theories have been established to explain the inhibitory effects of test materials on LOX enzyme activity [69]. One hypothetical theory describes this enzyme

reaction as a free radical mediated process that can occur due to interactions between substrate, enzyme and oxygen. Therefore, it is believed that test materials can inhibit the enzyme activity by scavenging the free radicals generated during the oxygenation of polyunsaturated fatty acids and thereby limiting the available hydroperoxide fatty acid substrate for the LOX enzyme action [64]. The LOX enzyme activity can also be determined by measuring the molecular oxygen uptake by the substrate using manometric and polarographic techniques and spectrophotometric techniques. However, direct spectrophotometric analysis of the formation of hydroperoxide fatty acid intermediate which contains a conjugated diene with a maximum absorbance at 234 nm is widely used to determine the LOX enzyme activity.

Experimental Procedure: Soybean lipoxygenases have widely been used to determine the inhibitory effects of test materials on enzyme action in published literature. Linoleic acid is used as the specific substrate for soybean lipoxygenase enzyme action. Cemal and co-workers in 1981, reported a method to determine inhibitory effects of a particular test material on lipoxygenase enzyme activity. This method has been followed with slight modifications up to date. The following method is a summary of the methods reported for inhibition of lipoxygenase activity [64-67].

- Prepare a concentration series of the test material (concentrations varying from 0.1 – 0.8 mg/mL) by dissolving the test material in a suitable solvent (dimethyl sulfoxide, dimethylformamide, ethanol, water or normal saline) considering the solubility of the test material. 1
- Prepare 0.2 mL of the reaction mixture containing 0.02 mL of 100U/mL lipoxygenase enzyme, 0.16 mL of 0.2 M borate buffer at pH 9 and 0.01 mL test solution in a clean test tube.
- Incubate the tube containing the reaction mixture at 25℃ for 10 minutes. After completing the incubation period , add 0.01 mL substrate linoleic acid in the form of sodium lineloic acid solution in to the reaction mixture , mix well and incubate for 5 minutes at 25℃. Measure the absorbance of the reaction mixture at 234 nm and carry out the experiment in triplicate. Repeat the above-mentioned procedure for remaining test solutions.
- Prepare a concentration series of the standard drug (indomethacin, asprin or nordihydroguaiaretic acid) using 0.2 M borate buffer. Repeat the above-mentioned assay protocol for each of the prepared solutions of the standard drug. The standard drug will be the positive control in the experiment. Replace the test solution with 0.01 mL 0.2 M borate buffer in the reaction mixture to prepare the negative control and follow the same assay protocol.

The equation 01 can be used to calculate the percentage inhibition of lipoxygenase activity of the test solution and the standard drug.

Inhibition of Hyaluronidase Enzyme Activity:

Background and Principal: Hyaluronic acid (HA) is an unbranched polymer belonging to a group of glycosaminoglycan heteropolysaccharides and an important component in the extra cellular matrix (ECM) of tissues and organs in the human body. HA regulates key functions in the human body such as cell signaling, cell differentiation, cell proliferation, tissue regeneration, morphogenesis and maintain the ECM structure. HAs exert their biological activities upon binding with cell specific surface HA receptors located on the same cell or nearby cells in the ECM [70]. Hyaluronidases (HYALs) are involved in tissue remodeling processes during inflammation. HYALs are present in different organs in the human body such as testes, spleen, skin, eyes, liver, kidneys, uterus and placenta, as well as different body fluids such as tears, blood and semen. They are the key enzymes involved in the degradation of HA. Five types of HYALs are present in the human body: HYAL1, HYAL2, HYAL3, HYAL4 and HYAL5, however, HYAL1 and HYAL2 are predominantly found in most of the human tissues and organs. HYALs mediated degradation of HA forms a mixture of HA fragments varied in their molecular weights and are classified as high-molecular weight HA fragments (HMWHA) and low molecular weight HA fragments with contrasting biological functions. HMWHA possesses anti-inflammatory and immunosuppressive properties whereas low molecular weight HA (LMWHA) fragments are

potent pro-inflammatory mediators. These LMWHA fragments interact with cell-specific surface receptors to activate their downstream signaling pathways to produce cytokines [71]. Unregulated production of these cytokines may occur as a result of uncontrolled HYALs mediated degradation of HA at the site of inflammation leading to chronic inflammatory diseases. Therefore, it is important to maintain basal HA levels by minimizing the HAYLs enzyme action. HAYALs inhibitors such as proteins, fatty acids, plant derived bioactive compounds and NSAIDs are used as potent regulatory to maintain the basal levels of HA during inflammation.

A variety of methods have been used to estimate HYAL enzyme activity such as measure the spreading effect of HYALs, reduction of viscosity, prevention of mucin clot formation and measure the liberated N-acetylglucosamine or D-glucuronic acid during HYALs enzyme action. However, all the above-mentioned methods have less sensitivity [72]. HA can form a HA-protein complex with acidified proteins which may develop a turbidity in the reaction mixture. Development of this turbidity has a positive correlation with the HYALs enzyme concentration. Therefore, this development of turbidity has been identified as an accurate and convenient method to determine the HYAL enzyme activity.

HYAL enzymes are capable of hydrolyzing the substrate HA at their optimum temperature (37 °C) pH. Optimum pH can be varied between 3-8 depending on the source of the enzyme. This optimum temperature and pH of the enzyme is important to maintain the correct conformational state of the substrate. HA is a polymer of disaccharides which are composed of D-glucuronic acid and N-acetylglucosamine linked via alternating β-1,3 and β-1,4 glycosidic bonds. HYALs cleave HA at β-1,4 and β-1,4 glycosidic bonds during the enzyme action. The inhibition of enzyme activity is determined by measuring the turbidity produced by undigested HA with acidified albumin solution at the end of the reaction time. HA-acid albumin complex has been formed upon addition of acidified albumin solution to develop a turbid appearance in the reaction mixture and this has been measured immediately at 600 nm. The pH and ionic strength of the reaction mixture may affect for the formation of this complex. A maximum turbidity is produced when acidic albumin solution is at pH 3.8 and the ionic strength of reaction mixture should not be below 0.15 Mm [72].

Experimental Procedure: Dorfman and co-workers in 1947 described a method to determine the HYALs enzyme action using a turbidimetric method. This method with slight modifications has been followed over the past six decades to determine the inhibitory effects of test materials on HYALs enzyme activity. The following method is a summary of the reported methods in searched literature [72-74].

- Prepare a concentration series of the test material (concentrations varying from 0.1– 0.8 mg/mL) by dissolving the test material in a suitable solvent (dimethyl sulfoxide, dimethylformamide, methanol, water or Tris-HCl buffer) considering the solubility of the test material.
- Prepare 0.125 mL of the reaction mixture containing 0.1 ml 4U/mL HYAL dissolved in 200 mM sodium phosphate buffer at pH 6, and 0.025 mL test solution in a clean test tube.
- Incubate the tube containing the reaction mixture at 37 °C in an incubator or a water bath for 10 minutes.
- After completing the incubation period, add 0.1 mL hyaluronic acid (0.03% w/v) dissolved in 200 mM sodium phosphate buffer at pH 5.4 in to the reaction mixture, mix well and incubate for 45 minutes at 37 ° C.
- Add 1.0 mL 0.1% w/v bovine serum albumin solution prepared in 24 mM sodium acetate buffer at pH 3.8 and incubate for 10 minutes at room temperature. Measure the absorbance of the reaction mixture at 600 nm against sodium phosphate buffer as the blank and carry out the experiment in triplicate.
- Repeat the above-mentioned procedure for remaining test solutions. Prepare a concentration series of the standard drug (diclofenac sodium, indomethacin or asprin) using 200 mM sodium phosphate buffer. Repeat the above-mentioned assay protocol for each of the prepared solutions of the standard

drug. The standard drug will be the positive control in the experiment. Replace the test solution with 0.1 mL 200 mM sodium phosphate buffer in the reaction mixture to prepare the negative control and follow the same assay protocol.

The percentage inhibition of HYAL enzyme activity of the test solution and the standard drug are calculated using the equation 01 mentioned above. The half maximal inhibitory concentration (IC_{50}) values of test materials and the standard drugs in each *in-vitro* anti-inflammatory assay can be calculated using a statistical software (Graphpad Prism for Windows or Origin) in non-linear regression analysis. The significant differences between IC_{50} values obtained for test material and the standard drugs are analyzed where the level of significance has been set at $p<0.05$.

DISCUSSION: Plant based natural products have been used in traditional medicine practices including Ayurveda since 2600 B.C [2, 24]. Studying of *in-vitro* anti-inflammatory properties of plants would be needed when defining novel and safe potential anti-inflammatory compounds as alternatives to synthetic anti-inflammatory drugs. Moreover, evaluating the anti-inflammatory properties of different plant-based compounds prior to their clinical application is essential to minimize potential adverse effects on humans and animals [75]. *In-vitro* cell-based and *in-vivo* assay procedures provide more accurate results for specific test materials; however, these methods are more expensive and time consuming compared to preliminary assessment methods. **Table 1** summarizes the advantages and disadvantages of these assays. The erythrocyte membrane stabilization assay, inhibition of protein denaturation assay and proteinase inhibitory assay procedures have been widely used over the other assay protocols discussed in this manuscript by different scientific communities due to their simplicity, rapid use and cost effectiveness [76]. However, different external factors such as temperature, pH, concentration of the reaction mixture constitutes can directly affect for the final outcome. These methods would not measure specific inflammatory mediators or the cellular mechanisms involved in inflammation directly. Consequently, results of these assay procedures would not correlate with *in-vivo* potency of a particular test material. However, accuracy and reliability of the possible outcomes can be achieved by maintaining the optimal physical and chemical conditions within each assay procedure. Eventhogh inhibition of COX enzyme activity, inhibition of 5-lipoxygenase activity and inhibition of hyaluronidase enzyme activity assay procedures are closely related to human inflammatory pathways, and provide more accurate results resembling NSAID activity, they require expensive assay kits with highly purified enzymes. Therefore, application of these methods is less common than the other *in-vitro* methods described above.

TABLE 1: ADVANTAGES AND DISADVANTAGES OF *IN-VITRO* ASSAYS

***In-vitro* Assay**	**Advantages**	**Disadvantages**
"Erythrocyte membrane stabilization assay"	Simple Inexpensive and easy to perform No require purified enzymes	Indirect method Colored compound can interfere with the final outcome Not correlate with *in-vivo* studies
"Inhibition of protein denaturation assay"	Simple and rapid Needed for initial screening Broad application against many compounds	Indirect method Temperature can affect for the final outcome
"Proteinase inhibitory assay"	Easy to perform and adopt Applicable to wide range of proteinases	Not compatible with *in-vivo* studies Assay conditions can affect for the final outcome
Inhibition of COX-2 activity	Specific to key inflammatory pathway Clinical relevance Simple and Rapid	Commercial kits are expensive Plant compounds can interfere with assay reagents and give false results
Inhibition of 5-lipoxygenase activity	Target Leukotrienes Simple and cost effective	Difficulty to work with enzyme substrates (Arachidonic acids) 5-Lox is unstable and need microsomal fractions
Inhibition of Hyaluronidase enzyme activity	Simple and Rapid Cost effective	Less specific Source of enzyme can affect the result

CONCLUSION: This review provides comprehensive explanation of widely used *in-vitro* anti-inflammatory assay protocols such as erythrocyte membrane stabilization assay, inhibition of protein denaturation assay, proteinase inhibitory assay, inhibition of COX enzyme activity, inhibition of 5-lipoxygenase activity and inhibition of hyaluronidase enzyme activity in literature giving relevant justifications, advantages and their limitations where necessary. These assay procedures can be used to assess preliminary *in-vitro* anti-inflammatory properties of plant derived compounds prior to their cell based and clinical applications.

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