



IN-VITRO ASSAYS TO INVESTIGATE ANTI- INFLAMMATORY ACTIVITY OF HERBAL EXTRACTS

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Abstract – The article reviews the different in-vitro assays can be carry out in the laboratory to check the anti-inflammatory activity for particular herbal extracts. These assays include protein denaturation assay, membrane stabilization assay, enzyme assays. Diclofenac, acetyl salicylic acid, indomethacin used as standard drugs in the following assays. all assays are possible to carry out inside the laboratory, so there is no animal involved and harmed and herbal drug shows less side effects than synthetic one with easy availability. The anti-inflammatory activity can be check for herbal drugs in less time, in-vitro by following methods. Protein denaturation assay includes heating of egg albumin it denature the protein and shows the anti-inflammatory activity. membrane lysis assay involves preparation of red blood cells suspension, then the heating of solution, centrifugation, and then absorbance is taken of the supernatant layer. hypotonicity induced haemolysis include hyposaline for anti-inflammatory activity. enzyme assays include collection of enzyme from animal cells or tissues. Enzymes such as lipoxxygenase, cyclooxygenase, hyaluronidase from animal tissues or cells. activities of enzymes takes place through reactions carried out under certain conditions are used for in-vitro study not the animals. Antiproteinase activity involves incubation, perchloric acid which terminate the reaction, centrifugation and absorbance. in lipoxxygenase enzyme assay linoleic acid used as substrate to start the reaction, absorbance and calculation gives inhibition of lipoxxygenase by using equations.

Keywords – anti-inflammatory, enzyme assays, membrane stabilization assay, protein denaturation assay.

1. INTRODUCTION

Injury to a living tissue causes inflammation. Inflammation can manifest itself in four ways. Pain, redness, heat or warmth, and swelling are examples. The arterioles dilate when there is an injury to any portion of the human body. The increased blood circulation towards the wounded tissue causes redness. [1] Inflammation is caused by a variety of events, one of which is arachidonic acid metabolism. The Cyclooxygenase (COX) pathway converts it to prostaglandins and thromboxane A₂, while the 5-lipoxygenase (5-LOX) pathway converts it to eicosanoids and leukotrienes (LT's), all of which are known to operate as chemical mediators in a range of inflammatory processes. [2] Anti-inflammatory medications on the market now block both enzyme activities and relieve symptoms, but they come with substantial side effects. [1] As a result, it's critical to use anti-inflammatory medications with fewer adverse effects. Studies using in vitro assays and in vivo models of inflammation have been employed in the endeavour to identify medicinal plants and their extracts with demonstrated anti-inflammatory potential. [3] Using animals in the early stages of medication development for inflammatory illnesses raises ethical concerns. [4] In vitro investigations aid in the investigation of cellular responses in a closed setting under controlled conditions. [5] These in vitro investigations are helpful in understanding the mechanism of herbal components' anti-inflammatory effect. [6] To assess the anti-inflammatory effect of herbs, different researchers have employed different in vitro techniques. The goal of this review is to compile a list of all the approaches that have been utilised to assess the anti-inflammatory properties of plant extracts in vitro tests.

2.PARAMETERS

2.1.Inhibition of protein denaturation assay

Protein denaturation causes protein molecules to lose their biological capabilities. Inflammatory diseases such as rheumatoid arthritis, diabetes, and cancer have all been linked to protein denaturation. As a result, a substance's ability to prevent protein denaturation may also aid in the prevention of inflammatory illnesses. [7] Proteins utilised in this experiment are either egg albumin[8,9] or bovine serum albumin (BSA)[10]. Protein denaturation is induced by incubating the reaction mixture in a water bath at 70°C for 10 minutes.[11] A reaction mixture comprises of 1000 litres of plant extract (100-500 g/ml), 200 litres of egg albumin, or 450 litres of 1400 L phosphate buffered saline, 5 percent bovine serum albumin w/v aqueous solution As a negative control, distilled water was used instead of extracts in the above mixture. After that, the mixture is heated at 70°C for 5 minutes after being incubated at 37°C for 15 minutes. Their absorbances are measured at 660 nm after cooling under running tap water. A positive control is acetyl salicylic acid[10,12], diclofenac sodium[8,9], ibuprofen[9], or indomethacin[13]. The experiment is repeated three times, and the percent inhibition of protein denaturation is computed using the equations below:

$$\% \text{ Inhibition of denaturation} = (1 - T/C) \times 100. [9]$$

Where T is the absorbance of test sample and C is the absorbance of negative control (without the test sample).

2.2.Membrane stabilization method

Lysosomal membrane lysis can occur during inflammation, releasing enzyme components that cause a range of problems. Nonsteroidal anti-inflammatory medications (NSAIDs) work by either inhibiting lysosomal enzyme release or stabilising lysosomal membranes. The breakdown of red blood cell membranes with hemolysis and the oxidation of haemoglobin might ensue from the exposure of red blood cells to harmful chemicals. [14] Hypotonic medium, heat, methyl salicylate, and phenyl hydrazine are all harmful chemicals. [15] Because the membranes of human red blood cells are comparable to those of lysosomes, the prevention of hypotonicity and heat-induced lysis of red blood cell membranes will be used to assess the mechanism of anti-inflammatory efficacy. Hypotonic solution causes the excessive accumulation of fluid within the red blood cells which resulting in the rupturing of its membrane. Finally the haemolysis of red blood cells take place. Injured red cell membrane produce the cell more susceptible to secondary damage through free radical induced lipid peroxidation. Bacterial enzymes and proteases can be found in active neutrophil lysosomes. Extracellular leakage of lysosomal contents causes further tissue inflammation and injury. As a result, lysosome membrane stability is critical for controlling the inflammatory response. As a result, there will be no leakage of its contents. [16] Heat induced haemolysis and hypotonic solution induced haemolysis utilising human erythrocytes[13,17], rat erythrocytes[18], or mouse erythrocytes can be used to test the extracts' membrane stabilising properties.[19] Because the erythrocyte membrane is similar to the lysosomal membrane, the effect of extracts on erythrocyte stabilisation can be extended to lysosomal membrane stabilisation. [20] The erythrocyte suspension was first prepared by all workers in order to test the methods mentioned above.

2.2.1.Hypotonic solution induced haemolysis

The hypotonic solution is used in this experiment. Hypotonic solutions can be made from a variety of substances, including hypo saline (50mM NaCl in a 10mM sodium phosphate buffer saline-pH7.4) and distilled water. [13] Erythrocyte suspension, plant extract, and hypotonic solutions are used in the reaction mixture. The plant extract is left out of the control. As a reference standard drug, acetyl salicylic acid[14], indomethacin[13], or diclofenac[7] can be used. This mixture is incubated for 30 minutes at 37°C and then centrifuged for 20 minutes at 3000rpm. Finally, the supernatant solution's haemoglobin content is determined using spectrophotometry at 560 nm. The following formulae are used to compute the percentage of red blood cell membrane stability or protection.

$$\% \text{ protection} = 100 - \text{OD of drug sample} \times 100 / \text{OD of control}$$

Where OD.- optical density

2.2.2. Heat induced haemolysis

This approach involves producing heat in order to aliquot through incubation. The reaction mixture (2 ml) consists of 1 ml test sample with concentrations ranging from 100 to 600 g/ml[21] or 5 ml isotonic buffer with 2.0 mg/ml extractives and 10% erythrocyte suspension. Instead of test sample, 1 ml[21] or 30 L[14] of vehicle is put to the control test tube. 50 g/ml - 400 g/ml A usual medicine is diclofenac sodium[22] or acetyl salicylic acid (aspirin)[10]. Inversion is used to gently mix this reaction mixture. [18] All centrifuge tubes containing reaction mixture are incubated in a water bath for 30 minutes[21] or 20 minutes at 60°C[21], 56°C[23], or 54°C[18]. Some workers made a duplicate of the above reaction mixture, and the other pair is kept in an ice bath at 0-5°C. [24] The tubes are cooled under running tap water at the end of the incubation. The reaction mixture was centrifuged for 5 minutes at 3000 rpm or 10 minutes at 2500 rpm[26] or 3 minutes at 1300 rpm[18], and the absorbance of the supernatants was measured at 560 nm. For each test sample, the procedure is repeated three times. The following formula is used to compute the percentage inhibition of haemolysis:

$$\text{Percentage inhibition of haemolysis} = \frac{\text{Abs control} - \text{Abs test} \times 100}{\text{Abs control}}$$

$$\% \text{ Inhibition of haemolysis} = 100 \times [1 - (\text{OD1} - \text{OD2}) / (\text{OD3} - \text{OD1})]. [24,25]$$

Where OD1 = Optical density of unheated test sample OD2 = Optical density of heated test sample OD3 = Optical density of heated control sample

2.3. Assay of cyclooxygenase and 5-lipoxygenase inhibition

The enzymes Cyclooxygenase and 5-LOX are involved in the metabolism of arachidonic acid (AA) in the body. Prostaglandins and thromboxane are produced by the COX route, whereas eicosanoids and leukotrienes are produced by the 5-LOX system. [26] It has been claimed that blocking both pathways prevents the formation of prostaglandins and leukotrienes, resulting in synergistic effects and optimal anti-inflammatory action. As a result, inhibiting both the COX and 5-LOX pathways simultaneously could have a broader range of anti-inflammatory effects. [2]

2.3.1. Anti-cyclooxygenase activity

The colorimetric COX (ovine) inhibitor screening assay kit is used to determine this enzymatic assay. [28,29] The chromogenic assay uses a spectrophotometer to evaluate the change in colour caused by the oxidation of TMPD during the conversion of PG-G2 (prostaglandin-G2) to PG-H2. [27] In a nutshell, the assay mix consists of test substances in various concentrations, a reference medication such as aspirin[29] or indomethacin[27], and other chemicals added according to the manufacturer's recommendations. The plate is shaken for few seconds and incubated for 5 minutes at 25°C. The reaction is initiated by the addition of arachidonic acid (20 µl) and TMPD (20 µL) to all the wells. The plate is shaken for few seconds and incubated for 5 minutes at 25°C. The absorbance is measured at 590 nm using micro plate reader. All the reactions are carried out in triplicates.[29]

2.3.2. Anti-lipoxygenase activity

Using linoleic acid as a substrate and lipoxidase as an enzyme, anti-lipoxygenase activity was investigated. [23,28] As an enzyme, you can employ soybean lipoxygenase[28] or human recombinant lipoxygenase[27]. The procedure of assessing this assay is summarised below. The reaction mixture is made up of 160 l of sodium phosphate buffer (pH 8.0), 10 l of plant extract in various concentrations (10, 25, 50, 100, 200 g/mL), and 20 l of soybean lipoxygenase solution (167 U/ml) that is mixed and incubated at 25°C for 10 minutes. The addition of initiates the reaction. 10 litres of sodium linoleic acid solution as a substrate. Using a UV-vis spectrophotometer, the absorbance is measured at 234 nm every minute for 3 minutes. [27,30] NDGA (nordihydroguaiaretic acid) or indomethacin or quercetin[29] is used as positive reference drug. Control is prepared by omitting the plant extract/ drug to the above mixture. All the reactions are performed in triplicates. The percentage of inhibition is calculated as:

$$\% \text{ inhibition} = \frac{\text{Abs control} - \text{Abs extract} \times 100}{\text{Abs cl}}$$

2.1. Assay of proteinase inhibition

It is demonstrated that proteinase implicate the tissue damage during the inflammatory reactions. Proteinases abundantly exist in lysosomal granules of neutrophils. Therefore proteinase inhibitors provide the significant level of production.[32] Different enzymes and proteins can be employed in this experiment; enzymes include proteinase[21] and trypsin[32,33], while proteins include casein and bovine serum albumin[34]. 0.06 mg proteinase[21] or trypsin, 1

ml 20 mM Tris HCl buffer (pH 7.4), and 1 ml test sample/standard drug, Diclofenac sodium, of varied concentrations 100-600 g/ml are included in the reaction mixture (2 ml). After 5 minutes of incubation at 37°C, 1 ml of 0.8 percent (w/v) casein or 4 percent (w/v) bovine serum albumin[34] is added to the mixture. After that, the mixture is incubated for another 20 minutes. To stop the process, add 2 ml of 70 percent perchloric acid or 5 percent trichloroacetic acid (TCA)[35]. Cloudy suspension is centrifuged at 3000 rpm for 10 minutes[36] or 2500 rpm for 5 minutes[37] and the absorbance of the supernatant is read at 210 nm[21] or 217 nm[38] against buffer as blank. The experiment is performed in triplicate. The percentage inhibition of proteinase inhibitory activity is calculated using the following equation.

$$\text{Percentage inhibition} = (\text{Abs control} - \text{Abs sample}) \times 100 / \text{Abs control.}[21]$$

2.4. Hyaluronidase inhibition assay

One of the enzymes involved in tissue remodelling during inflammation is hyaluronidase. In both human and animal tissues, it destroys hyaluronic acid. Hyaluronic acid is a key component of connective tissue's extracellular matrix. [38] The enzyme is known to be implicated in allergic reactions, inflammation, and enhancing vascular membrane permeability by lowering hyaluronic acid viscosity. [37]The substrate in this assay is hyaluronic acid, and the assay begins after the substrate is added.[39] Plant extract samples (5 mg) are dissolved in 250 litres of dimethylsulphoxide. The samples are produced in sodium phosphate buffer at varied concentrations (100, 200, 300, 400, and 500 g/mL) (200 mM, pH 7). Hyaluronidase (4U/mL, 100L) is mixed with sample solution (25L) and incubated at 37°C for 30 minutes.10min.[39] Some researchers have incorporated calcium chloride 2.5mM, 1.2 µL[41] or 12.5Mm, 50 µL[42] to activate the enzyme and the mixture is again incubated at 37oC for 20 minutes. Some researchers have omitted the addition of CaCl2.[39,40] The reaction is then started by adding the substrate, hyaluronic acid solution (0.03 percent in 300mM sodium phosphate, pH 5.4, 100L), and incubated for 45 minutes at 37°C. Acid albumin solution (0.1 percent bovine serum albumin in 24 mM sodium acetate, pH 3.8, 1 mL) is used to precipitate the undigested hyaluronic acid. At 600nm, absorbance is measured after a 10-minute incubation period at room temperature. In the absence of enzyme, the absorbance measurement is utilised as a control value for maximum inhibition. The assay's performance is checked using either quercetin[40] or indomethacin[38] as a positive control. The test is carried out three times.The percentage of inhibition is determined using the following formula: hyaluronidase

$$\text{Percentage inhibition} = \text{Abs sample} / \text{Abs control} \times 100.[40]$$

Conclusion

The review gives an information about the in-vitro assays to check the anti-inflammatory activity of herbal extracts. As compare to in-vivo studies in-vitro require less use of animals and less time. NSAID that is synthetic drugs shows more side effects as compare to herbal drugs. Above anti-inflammatory assays that is protein denaturation require heating and absorbance, membrane lysis assay needs some drops of human blood and then absorbance, and enzyme assays can be carried out in laboratory no need of animals. Thus we can check the anti-inflammatory activity of herbs in easy way.

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