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IN VITRO ASSAYS TO INVESTIGATE THE ANTI-INFLAMMATORY ACTIVITY OF HERBAL EXTRACTS: A REVIEW

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ABSTRACT

The article reviews recent studies on the anti-inflammatory effects of herbal extracts with emphasis on different assays that are frequently used to test the *in-vitro* anti-inflammatory activity of herbal constituents. Protein denaturation assays and membrane stabilization assays are frequently used to evaluate *in vitro* anti- inflammatory activities. Many researchers have used minimum of three *in vitro* assays to evaluate anti- inflammatory activity of herbal constituents. Diclofenac, acetyl salicylic acid and indomethacin are the drugs used as reference standard more frequently. We suggest to reduce the use of animals in conducting anti-inflammatory activity in vitro studies as animal ethics is important as human welfare.

KEYWORDS: Anti-inflammatory, herbal extract, *in vitro* assays, *in-vivo* assays.

INTRODUCTION

Inflammation is caused by injury to a living tissue. There can be four primary indicators of inflammation. i.e pain, redness, heat or warmness and swelling. When there is an injury to any part of the human body, the arterioles dilate. This produce redness by increased the blood circulation towards the injured tissue.[1]

The mechanisms of inflammation involve a series of events in which the metabolism of arachidonic acid plays an important role. It is metabolized by the Cyclooxygenase (COX) pathway to prostaglandins and thromboxane A2, whereas the 5-lipoxygenase (5-LOX)

pathway to eicosanoids and leukotrienes (LT's), which are known to act as chemical mediators in a variety of inflammatory events.[2] Currently available anti- inflammatory drugs block both enzyme activities and relief symptoms despite they have serious side effects.[1] Therefore it is essential to administer anti- inflammatory drugs with lesser side effects.

During the attempt of identification of medicinal plants and their extracts with proven anti-inflammatory activity, studies using *in vitro* assays and *in vivo* models of inflammation have used.[3] There are ethical issues of using the animals in the early stages of drug discovery for inflammatory diseases.[4] *In-vitro* studies help to study the cellular response in a closed system where the experimental conditions are maintained.[5] These *in vitro* studies are helpful in developing an understanding of the mechanism of anti- inflammatory activity of herbal constituents.[6]

Different workers have used different *in vitro* assays to evaluate the anti- inflammatory activity of herbs. This review aims to summarize all those methods that are repeatedly being used to evaluate the anti- inflammatory activity of plant extracts *in vitro* assays.

2. PARAMETERS

2.1. Inhibition of protein denaturation assay

Protein denaturation results loose of biological properties of protein molecules. Protein denaturation has been correlated with the formation of inflammatory disorders like rheumatoid arthritis, diabetes and cancer. Therefore ability of substance to prevent the protein denaturation may also help to prevent the inflammatory disorders.[7]

In this assay either egg albumin[8,9] or bovine serum albumin (BSA)[10] are used as protein. Denaturation of protein is induced by keeping the reaction mixture at 70°C in a water bath for 10 minutes.[11]

A reaction mixture consists of various concentrations of plant extract 1000 µL (100-500 µg/ml), 200 µL of egg albumin or 450 µL (5% w/v aqueous solution) bovine serum albumin, 1400 µL of phosphate buffered saline. Distilled water instead of extracts with above mixture is used as a negative control. Afterward, the mixtures is incubated at 37 °C for 15 min and then heated at 70°C for 5 min. After cooling under running tap water, their absorbances are measured at 660 nm. Acetyl salicylic acid[10,12] or diclofenac sodium[8,9] or ibuprofen[9] or

indomethacin[13] is taken as a positive control. The experiment is carried out in triplicates and percent inhibition for protein denaturation is calculated using following equations:

% Inhibition of denaturation = (1-D/C) X100.[9]

Where D is the absorbance of test sample and C is the absorbance of negative control (without the test sample or reference drug).

2.2. Membrane stabilization method

During inflammation, lysis of lysosomal membrane may occur which release their enzyme components that produce a variety of disorders. Non-steroidal anti-inflammatory drugs (NSAIDs) produce their effects by either inhibit the release of lysosomal enzymes or by stabilizing the lysosomal membranes. The lysis of the red blood cell membranes with hemolysis and oxidation of haemoglobin may occur by the results of injurious substances exposure to red blood cells.[14] Injurious substances are hypotonic medium, heat, methyl salicylate and phenyl hydrazine.[15] Since human red blood cell membranes are similar to lysosomal membrane, the inhibition of hypotonicity and heat induced lysis of red blood cell membrane will be taken as a measure of the mechanism of anti-inflammatory activity. 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 are exist in lysosomes of activated neutrophils. Leakage of lysosomal constituents cause the further tissue inflammation and damage upon extra cellular release. Therefore membrane stabilization of lysosomes are important to control the inflammatory response. This will leads to prevention of leakage of its constituents.[16]

The membrane stabilizing activity of the extracts can be determined through heat induced haemolysis and hypotonic solution induced haemolysis using human erythrocytes[13,17], rats erythrocytes[18] or mice erythrocytes.[19] The erythrocyte membrane analogues to lysosomal membrane therefore the effect of extracts on the stabilization of erythrocyte applied to the stabilization of lysosomal membrane.[20] All workers have initially prepared the erythrocyte suspension for assessing the above mentioned methods.

2.2.1. Hypotonic solution induced haemolysis

This experiment carries out with hypotonic solution. A number of different agents can be used as hypotonic solutions, including hypo saline (50mM NaCl in 10mM sodium phosphate

buffer saline-pH7.4)[14,18] and distilled water.[13] Reaction mixture contain erythrocyte suspension, plant extract and hypotonic solutions. Control is prepared by omitting the plant extract. Acetyl salicylic acid[14] or indomethacin[13] or diclofenac[7] can be used as reference standard drug. This mixture is incubated at 37^0C for 30 minutes and centrifuged at 3000rpm for 20 minutes. Finally the hemoglobin content of the supernatant solution is estimated by spectrophotometrically at 560 nm. The percentage of Red blood cell membrane stabilization or protection is calculated by the following equations.

$$\% \text{ protection} = 100 - \frac{\text{Optical density of drug treated sample}}{\text{Optical density of control}} \times 100$$

2.2.2. Heat induced haemolysis

This method carries out with produce the heat to aliquot by incubation. The reaction mixture (2ml) consist of 1 ml test sample of different concentrations 100 - 600µg/ml[21] or 5ml of isotonic buffer containing 2.0mg/ ml with different extractives and 10% erythrocyte suspension 1 ml[21] or 30 µL[14], instead of test sample only vehicle is added to the control test tube. 50 -400µg/ml Diclofenac sodium[22] or acetyl salicylic acid (aspirin)[10] is used as a standard drug. This reaction mixture is mixed gently by inversion.[18] All the centrifuge tubes containing reaction mixture is incubated in water bath at 60ºC[21] or 56ºC[23] or 54ºC[18] for 30 minutes[21] or 20 minutes.[18] Some workers have prepared the duplicate of above reaction mixture and the other pair is maintained at 0-5ºC in an ice bath.[24] At the end of the incubation the tubes are cooled under running tap water. The reaction mixture was centrifuged at 3000 rpm for 5 min or 2500 rpm for 10 minutes[26] or 1300rpm for 3 minutes[18] and the absorbance of the supernatants is taken at 560 nm. The experiment is performed in triplicates for all the test samples. The percentage inhibition of haemolysis is calculated as follows:

$$\text{Percentage inhibition of haemolysis} = \frac{\text{Absorbance control} - \text{Absorbance test}}{\text{Absorbance control}} \times 100$$ [21]

$$\% \text{ Inhibition of haemolysis} = 100 \times [1-(OD_1-OD_2)/ (OD_3-OD_1)].$$ [24,25]

Where OD_1 = Optical density of unheated test sample

OD_2 = Optical density of heated test sample

OD_3 = Optical density of heated control sample

2.3. Assay of cyclooxygenase and 5-lipooxygenase inhibition

Arachidonic acid (AA) is metabolized in the body through two main metabolic pathways with the enzymes: Cyclooxygenase and 5-LOX. The COX pathway produce prostaglandins and thromboxane whereas 5-LOX pathway produce eicosanoids and leukotrienes.[26] It has been suggested that the inhibition of both pathways prevent the production of prostaglandins and leukotriene thereby it might produce the synergistic effects and achieve optimal anti-inflammatory activity. Hence the dual inhibition of the COX and 5-LOX pathway could have a wider spectrum of anti-inflammatory effects.[2]

2.3.1. Anti- cyclooxygenase activity

This enzymatic assay is determined by the using colorimetric COX (ovine) inhibitor screening assay kit.[28,29] The chromagenic assay is based on the oxidation of TMPD during reduction of PG-G2 (prostaglandin-G2) to PG-H2 and the change in colour is measured using a spectrophotometer.[27]

In brief, the assay mix consist of test compounds in different concentrations, either asprin[29] or indomethacin[27] is used as a reference drug and other chemicals are added according to the manufacturer's instructions. The plate is shaken for few seconds and incubated for 5 minutes at 25^{O}C. The reaction is initiated by the addition of arachidonic acid (20µl) and TMPD (20 µL) to all the wells. The plate is shaken for few seconds and incubated for 5 minutes at 25^{O}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

Anti-lipoxygenase activity has studied using linoleic acid as substrate and lipoxidase as enzyme.[23,28] Soybean lipoxygenase[28] or human recombinant lipoxygenase[27] can be used as enzyme.

Here we summarize the method of analyzing this assay. Reaction mixture consist of sodium phosphate buffer 160 µl of 100 mM (pH 8.0), 10 µl of plant extract with various concentrations (10, 25, 50, 100, 200 µg/mL) and 20 µl of soybean lipoxygenase solution (167 U/ml) are mixed and incubated at 25°C for 10 min. The reaction is initiated by the addition of 10 µl of the substrate in the form of sodium linoleic acid solution. The absorbance is measured at 234 nm over a period of 3 minutes in every minute using UV-vis spectrophotometer.[27,30] Nordihydroguaiaretic acid (NDGA)[31] or indomethacin[23] 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:

$$\% \text{ inhibition} = \frac{\text{Abs control} - \text{Abs extract}}{\text{Abs control}} \times 100$$[30]

2.4. 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]

In this assay different enzymes and different protein can be used, enzymes are proteinase[21] or trypsin[32,33] and casein and bovine serum albumin[34] are used as protein. The reaction mixture (2 ml) contain 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 different concentration 100-600 g/ml. The mixture is incubated at 37°C for 5 min and then 1 ml of 0.8% (w/v) casein or 4% (w/v) bovine serum albumin[34] is added. The mixture is incubated for an additional 20 min. 2 ml of 70% perchloric acid or 5% trichloroacetic acid (TCA)[35] is added to terminate the reaction. 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.

Percentage inhibition = (Abs control –Abs sample) X100/ Abs control.[21]

2.5. Hyaluronidase inhibition assay

Hyaluronidase is also one of the enzyme which involves in tissue remodelling during inflammation. It degrades hyaluronic acid, in human and animal tissues. Hyaluronic acid is important constituents of the extracellular matrix of connective tissues.[38] The enzyme is known to be involved in allergic reactions, inflammation and increasing permeability of vascular membrane by lowers the viscosity of hyaluronic acid.[37]

In this assay, hyaluronic acid is used as the substrate and the assay is initiated after adding the substrate.[39] Plant extract samples (5mg) are dissolved in dimethylsulphoxide (250 µL). The samples are prepared at various concentrations (100, 200, 300, 400 and 500 µg/mL) by dissolving in sodium phosphate buffer (200 mM, pH 7). Hyaluronidase (4U/mL, 100 µL) is mixed with sample solution (25 µL) incubated at 37^{o}C for 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 37°C for 20 minutes. Some researchers have omitted the addition of $CaCl_2$.[39,40]

After that, the reaction is initiated with the addition of substrate, hyaluronic acid solution (0.03% in 300mM sodium phosphate, pH 5.4, 100 µL) and incubated at 37°C for 45min. The undigested hyaluronic acid is then precipitated with acid albumin solution (bovine serum albumin (0.1%) in sodium acetate (24 mM), pH 3.8, 1 mL). After 10min incubation at room temperature, absorbance are measured at 600nm. The absorbance measurement in the absence of enzyme is used as a control value for maximum inhibition. Either quercetin[40] or indomethacin[38] is used as the positive control to verify the performance of the assay. The assay is performed in triplicate. The percentage of hyaluronidase inhibition is determined using the following formula:

Percentage inhibition = Abs sample/ Abs control X100.[40]

3. CONCLUSION

This review gives an insight of the frequently used *in-vitro* assays to test anti-inflammatory activity of herbal extracts. Although some workers have relied only one assay to evaluate the *in-vitro* anti-inflammatory properties of herbal extracts, most of the workers have preferred to use more than one assay at the same time. Although many researchers have used human whole blood to evaluate membrane stabilisation assays where as some workers have used animal blood for this purpose. We suggest to reduce the animal use *in vitro* assays as animal ethical issue is important as human welfare. Most of the workers have used either non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin, diclofenac and acetyl salicylic acid as positive reference.

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