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EVALUATION OF *IN VITRO* ANTI-INFLAMMATORY ACTIVITY OF *PERGULARIA DAEMIA*

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ABSTRACT

Pergularia Daemai (PD) is an age-old traditional herb and is in use as folk medicine among the villagers even today. It had been screened for its bioactive compounds and various pharmacological activities. But its anti-inflammatory activity is not explored fully. In the present venture, we evaluated the anti-inflammatory effect of ethanolic extract of *PD* by *in vitro* method by using membrane stabilization test and protein denaturation test. Membrane stabilization test was done by using human red blood cells (HRBCs). Protein denaturation test was done by using bovine serum albumin (BSA). The results revealed that *PD* extract was capable of rendering membrane stabilization by inhibiting the hypotonically-induced hemolysis of HRBCs in dose-dependent manner (50, 100, 200, 300, 400, 500 and 1000 µg/mL). In lesser concentration (50 µg/mL), the % inhibition of hemolysis was less (26.80%) and in higher concentration (1000 µg/mL), the % inhibition of hemolysis was more (76.30%), which was comparable with that of standard anti-inflammatory drug viz. diclofenac sodium (200 µg/mL – 80.60%). The PD extract was also capable of inhibiting BSA denaturation in dose-dependent manner (50 µg/mL – 20.40%, 1000 µg/mL – 83.60%) which was comparable to that of diclofenac sodium (200 µg/mL – 86.60%). This finding confirms the potentiality of *PD* extract as an anti-inflammatory agent

and justifies the recommendation of *PD* extract for the treatment of painful inflammatory conditions.

KEYWORDS: Anti-inflammatory, *Pergularia daemia*, In vitro, Diclofenac sodium.

INTRODUCTION

Inflammation is the body's challenge in the form of immune response to produce self-protection against the damages like cell destruction, irritability, infection etc. and begin the healing process**.** The inflammatory response consists of several physiological processes, all of which are triggered by the release of pharmacologically active substances such as histamine and heparin and the chemical mediators released from injured tissues and migratory cells.[1] Overproduction of auto antigens by denaturation of tissue proteins as in case of arthritis may also be one of the main causes for inflammatory reactions. Whatever may be cause for inflammation, all are characterized by four cardinal signs viz. redness, swelling, heat and pain. So, the goal of treatment for inflammation is to reduce or prevent the production of inflammatory agents that triggers the signs and symptoms of inflammation.[2, 3] Fortunately there are anti-inflammatory agents like cortisol naturally produced in the body and also available commercially. But these substances are known to produce some adverse side effects like suppression of natural immune system in the body.[1] So, search is in progress to find out some natural herbs which may render anti-inflammatory action without producing any secondary problems. The present venture is one among them.

Non-steroidal anti-inflammatory drugs (NSAIDs) are commercially available and are commonly used for treating chronic health problems like rheumatoid arthritis, osteoarthritis etc. But long term use of NSAIDS is also associated with side effects like stomach bleeding, allergic reactions, kidney problems, heart problems etc.,[4] According to Leopold, “There are natural alternatives to NSAIDs that have a similar mechanism; some of them include turmeric, green tea, ginger, rosemary, cat's claw, devil's claw, and willow bark”.[5] Thus, herbal medicines are maintaining their popularity not only for their historical and cultural reasons but also for their safety with minimum or nil side effects.

PD is one of such traditional herb grown around the tropical and subtropical regions of India. It has widespread application in different folk medicine even in the Ayurveda and is believed to increase defense against various diseases. The whole plant is used as an antihelminthic, antiseptic, antivenin, emmenagogue, and in the treatment of infantile diarrhea and malarial

remittent fevers.[6, 7] Dried root is used as an abortifacient[8] and emetic and for bronchitis, cough, asthma and constipation.[9, 10] The juice of the leaves is applied to rheumatic swellings in rheumatism in combination with lime or ginger.[11] Its pharmacological potentiality like analgesic, anti-inflammatory, antirheumatic and antiarthritic[12], hepatoprotective[13], antidiabetic[14], antifertility[15], cardiovascular effect[16], antibacterial activity and wound healing capacity[17] have been already reported.

In our earlier study, we subjected the plant for phytochemical analysis and reported the presence of bioactive compounds like flavanoid, tannins, alkaloids, glycosides, terpenoids, steroids and carbohydrates.[18] In the present study, we explored the anti-inflammatory property activity of *PD* extract *by in-vitro* method.

MATERIALS AND METHODS

Collection of the plant material

The whole plant was collected from Karuppur, Salem district, Tamil Nadu in India, and the voucher specimen was deposited in the Herbarium and authenticated in CAS Botany, Department, University of Madras. (Authentication No. MUCASB - H106).

Preparation of the extract

The aerial part of the plant with leaves, stem and flowers was separated from the whole plant. It was cleaned in water and dried in shade at room temperature. Then it was powdered in an electric blender and sieved to get a fine powder. From the powder, the ethanolic extract was prepared by treating it with 95% ethanol in a soxhlet apparatus by continuous heat exposure for 72 hours. The extract was then concentrated in rotary vacuum evaporator at 40°C and reconstituted in minimum amount of dimethyl sulphoxide (DMSO). The dried extract was stored in refrigerator at 40°C for future use.[18]

Determination of anti-inflammatory activity by membrane stabilization activity

The membrane stabilizing activity of *PD* extract was assessed by the method developed by Shinde et al.[19], and designed and employed by Sikder et al.,[20, 21] and Md Reyad-Ul-Foerdous et al with a slight modification, ie., mice RBCs were used in their studies whereas healthy human volunteer's RBCs (HRBCs) were used in our study.

HRBCs preparation: The venous blood was collected from the anticubital vein by using a syringe that contained EDTA as anticoagulant. After centrifuging, the RBCS were separated

and was washed three times in isotonic buffered saline at (154 mM Nacl) in 10 mM sodium phosphate buffer at a pH 7.4 through centrifugation for 10 min at 3000 rpm.

The test sample consisting of 0.5 ml of RBC suspension from the HRBC stock and mixing it with 5 ml of hypotonic solution (50 mM Nacl) in 10 mM sodium phosphate buffered saline with pH 7.4. To this, the PD extract in different concentrations of 50, 100, 200, 300, 400, 500, 1000 µg/mL was added in test sample tubes and diclofenac sodium (200 µg/mL) was added to the standard tube. The control sample tube containing 0.5 ml of HRBCs was mixed with only hypotonic buffered saline. The tubes with these mixtures were incubated for 10 min at 56ºC and cooled under running tap water for 20 min. Then the tubes were centrifuged for 10 min at 3000 rpm and the supernatant was collected to measure the absorbance at 540 nm.

The membrane stabilization was calculated by using the formula.

% inhibition of hemolysis = $(OD_1 – OD_2/OD_1)$ X 100

Where, OD_1 = optical density of hypotonic-buffered saline solution alone (control)

OD_2 = Optical density of test sample in hypotonic solution.

Protein denaturation method

The ability of *PD* extract to inhibit protein denaturation was studied by the method of Shravan kumar et al[22] to evaluate the anti-inflammation activity of *PD* extract.

Procedure: The tubes were taken as for control, product control, standard tube (with Diclofenac sodium at 200 µg/ml) and test (with *PD* extracts).The reagents were added in the following order, 0.45 ml of 5% aqueous solution of Bovine serum albumin (BSA), 0.050 ml of *PD* extract in different concentrations as 50,100,200,300,400,500,1000 µg/ml. In control, distilled water was used instead of *PD* extract .In product control, all reagents were added except BSA. Then, the pH of the solution in all the tubes was adjusted to 6.3 using 1N Hcl. The tubes with the samples were incubated at 37 ºC for 20 mins and then heated at 54 ºC for 3 mins in water bath. All the tubes were later cooled in running tap water and then 2.5 ml of phosphate buffer solution in each tube. A turbidity was produced in the tubes, which was measured spectrophotometrically at 600 nm. Control represents 100 % protein denaturation. The results were compared to the standard diclofenac sodium.

Percentage inhibition = 100 - OD of test – OD of control/OD control X 100

RESULTS

Membrane stabilization effect.

PD extract effectively inhibited the membrane lysis of HRBCs which was expressed as the percentage inhibition (%inhibition) of hemolysis. It was dose-dependent: in lesser concentration (50µg/ml), the %inhibition was much less (26.80%) with more hemolysis and as the concentration increased (100, 200, 300, 400, 500 and 1000 µg/mL), the membrane stabilization effect was also increased accordingly with more %inhibition of hemolysis (35.20%, 50.40%, 56.20%, 59.50%, 72.40%, 76.30%): this was in comparison with the anti-inflammatory effect of standard drug viz. Diclofenac sodium (200 µg/ml, 80.60%) (Table 1).

Table 1. Effect of *PD* Extract on membrane stabilization activity.

Extract/ Drug	Concentration	% inhibition of hemolysis
		***By PD* extract**
PD extract	50 µg/mL	26.80
	100 µg/ml	35.20
	200 µg/ml	50.40
	300 µg/ml	56.20
	400 µg/ml	59.50
	500 µg/mL	72.40
	1000 µg/mL	76.30
Diclofenac sodium	200 µg/mL	80 .60

Protein denaturation study

Protein denaturation was effectively inhibited by PD extract in dose-dependent manner (50 µg - 20.40%, 100 µg - 34.60%, 200 µg - 49.20%, 300 µg - 56.30%, 400 µg - 62.30%, 500 µg - 68.20% and 1000 µg – 83.40). When compared to diclofenac sodium (200 µg/mL, 86.60%), PD extract was almost equally effective in inhibiting the protein denaturation in higher concentration (Table 2).

Table 2- Effect of *PD* Extract on protein denaturation assay

Extract/ Drug	Concentration	% inhibition of protein denaturation)
		PD extract (6)
PD extract	50 µg/ml	20.40
	100 µg/ml	34.60
	200 µg/ml	49.20
	300 µg/ml	56.30
	400 µg/ml	62.30
	500 µg/ml	68.20
	1000 µg/m	83.40
Diclofenac Sodium	200 µg/mL	86.60

DISCUSSION

Inflammation is a positive sign shown by the living tissue to raise an alarm regarding some irritation or/and infection or/and injury that is taking place inside or outside the body. The inflammatory process starts by activating the neutrophils and releasing the enzymes like bactericidal enzymes and proteases. These enzymes induce lipid peroxidation and release of lysosomal enzymes which will damage the macromolecules. This chain of reactions may ultimately cause a lot of pathophysiological changes ending up with inflammatory diseases like rheumatoid arthritis, septic shock, heart attack etc.,[1, 21, 22] So, stablization of lysosomal membrane is important to prevent the inflammatory processes.

We have chosen the hypotonically induced erythrocyte lysis to prove the membrane stabilizing effect because HRBC membrane is considered to be similar to the lysosomal membrane.[23]

Reduction in protein denaturation is another method of showing the anti-inflammatory capacity. Protein denaturation is a pathological process by which the proteins lose their configuration and become functionless.[2] This usually happens when the proteins are exposed to external stress like heat, strong acid or base. These external forces make the organic or inorganic solvents in the proteins to lose their tertiary and secondary structure and make them lose their functional capacity.[24] Protein denaturation may take place even by autoimmune inflammatory process in which production of auto-antigens is increased as in case of rheumatoid arthritis.[2, 24] Thus, any product that prevents protein denaturation can be an effective anti-inflammatory agent.[25] Apart from changing the configuration, the mechanism of denaturation also involves alteration in electrostatic hydrogen, hydrophobic and disulphide

bonding.[26, 27] Its capacity in reducing protein denaturation was proved by comparing the similar action by the standard drug viz. diclofenac sodium.

The effective anti-inflammatory activity of *PD* extract may be attributed to the presence of bioactive compounds like saponins, alkaloids, steriods and flavanoids present in it as reported in our earlier study.[18]

Though there are many commercial steroidal and non-steroidal anti-inflammatory drugs availabe in the market, the surge for finding some natural herbal products as an alternative for these is not quenched yet, mainly because of the minimum or nil secondary effects by these herbal products. The present study is one among them and the results encourage us to say that *PD* extract can be an effective herbal anti-inflammatory agent.

CONCLUSION

PD extract is an effective anti-inflammatory herbal product as revealed by the significant inhibition against hypotonically-induced membrane lysis and the inhibition of protein denaturation by *in vitro* study; these effects are comparable to those of standard anti-inflammatory drug viz. diclofenac sodium. Thus, we conclude that PD extract can be an effective anti-inflammatory and analgesic herbal product. However, for determining the pattern of dosage regimens, further evaluation is necessary on bioavailability of *PD* compounds and their pharmacokinetic properties. And it gives hopes that new herbal medical products can be derived from *PD* extract with anti-inflammatory, anti-analgesic and anti-bacterial effects which may be useful in places where conservative medication fails.

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