ISSN: 2287-6898
International
Journal of Bio-Pharma Research
Volume 8, Issue 5 (2019) pp. 2569-2574

Research Article Open Access

Assessment of *In vitro* anti-inflammatory activity of ethanol extract of *Petiveria alliacea* L. (Phytolaccaceae)

Rajesh A.[1], Doss A.[2], Tresina P.S.[2] and MohanV.R.*[3]

[1]Department of Medicinal Botany, Govt. Siddha Medical College, Tamilnadu, India.
[2]PG & Research Department of Botany, V.O. Chidambaram College, Tamilnadu, India.
[3]Department of Biomedical Science and Technology, Noorul Islam Center for Higher Education, Kumaracoil-629180, Tamilnadu, India.

Abstract: The aim of the present study, is to study the phytochemical and *In-vitro* anti-inflammatory activity of ethanol extract of *Petiveria alliacea* L. whole plant. Phytochemical screening of whole plant ethanol extract revealed the presence of alkaloid, flavonoid, saponin, steroid, phenol, tannin, glycoside and terpenoid. *In-vitro* anti-inflammatory activity was screened against inhibition of albumin denaturation, proteinase inhibitory activity, heat induced haemolysis, hypotonicity induced haemolysis and antilipoxygenase activity. Aspirin was used as standard drug. The results showed that *Petiveria alliacea* whole plant at a concentration of 500 µg/ml significantly ($P<0.01$) protect the heat induced protein denaturation, proteinase inhibitory action and heat induced haemolysis of erythrocyte. Hypotonicity induced haemolysis and lipoxygenase activity were significantly ($P<0.01$) inhibited at the concentration of 500 µg/ml. the results obtained in the present study indicate that ethanol extract of *Petiveria alliacea* whole plant can be a potential source of anti-inflammatory agents.

Key words: Phytochemical, anti-inflammatory, denaturation, HRBC, Lipoxygenase.

Introduction

Inflammation is a severe reaction by living tissue to any kind of injury. There are four primary indicators of inflammation pain, redness, heat or warmness and swelling. When there is an injury to any part of the human body, the arterioles in the encircling tissue dilate. This provides a raised blood circulation towards the area (redness) (1). Inflammation is either acute or chronic inflammation. Acute inflammation may be an initial response of the body to harmful stimuli. In chronic inflammation, the inflammatory response is one of the proportion resulting in damage to the body. Cyclooxugenase (COX) is the main enzyme in the synthesis of prostaglandins, prostacydins and thromboxanes which are absorbed in inflammation, pain and platelet aggregation (2).

Non-steriodal anti-inflammatory drugs (NSAIDS) represent one of the most common classes of medications used worldwide with an estimated usage of more than 30 million per day for inflammation and related disorders (3). Most of the NSAIDs are carboxylic acid containing drugs including salicylate derivatives (aspirin), carboxylic and heterocyclic acid derivatives (indomethacin) propionic acid containing drugs act at the active site, the enzyme preventing the access of arachiodonic acid (AA) to the enzyme and prevent the cyclooxygenase pathway (4). Unfortunately, besides the excellent anti-inflammatory potential of the NSAIDs the severe side effects such as gastrointestional (GI), perforation, obstruction, ulceration and bleeding has minimized the therapeutic usage of NSAIDs (5). The world population depend on traditional medicine for their healthcare. It is evident that several plants have been used in traditional ayurvedic medicine for treatment and management of distinct inflammatory disorder (6). Plants have the capacity to synthesize a wide variety of phytochemical compounds as secondary metabolites. Many of the phytochemicals have been used to effectively cure the various ailments for human race. *Petiveria alliacea* L. (Phytolaccaceae) is declared to have several medicinal properties. It is used in folk medicine to enhance memory and in the treatment of the common cold, flu, inflammation, diabetes

Corresponding Author:
Dr. V.R.Mohan,
Professor, Department of Biomedical Sciences and Technology,
Noorul Islam Center for Higher Education,
Kumaracoil, Tamilnadu, India.
E-mail: vrmohanvoc@gmail.com

and cancer, other viral or bacterial infections, (7, 8). Previous work on *P.alliacea* revealed the presence of triterpenoids, saponins, coumarins, benzaldehyde, polyphenols, benzoic acid, flavonoids, pimitol and allantonin varying their concentration in the root, stem and leaves (9). Taking into consideration the medicinal importance of the plant the ethanol extract of *P.alliacea* whole plant was analysed for the *In vitro* anti-inflamamtory activity using different models.

Materials and Methods

Collection of plant samples: Whole plant of *Petiveria alliacea* was collected from Kalakkad, South Western Ghats, Tamil Nadu, India. With the help of local flora, voucher specimen were identified and preserved in the Ethnopharmacology Unit, Research Dept. of Botany, V.O. Chidambaram College, Tuticorin–628008, Tamil Nadu, for further reference.

Solvent extraction

Ethanol was used as solvent to prepare the plant extracts. The whole plant was directly soaked for 12 hrs in 500 ml ethanol and then subjected to extraction by reflexing for 6 to 8 hrs below the boiling point of the solvent. The ethanol extracts were concentrated by evaporating at a reduced pressure using rotary evaporator. In order to facilitate complete evaporation of the solvents, the concentrated extracts were further dried at 37^{0}C for 3 to 4 days.The concentrated extracts were used for qualitative analysis of phytochemicals with standard protocols.

Assessment of *In vitro* anti-inflammatory activity

Inhibition of albumin denaturation

The anti-inflammatory activity of *P.alliacea* was intended by using inhibition of albumin denaturation technique.This was calculated according to Mizushima *et al*. (10) and Sakat *et al*. (11) followed with negligible modifications. The reaction mixture had test extracts and 1% aqueous solution of bovine albumin fraction. The pH of the reaction mixture was changed using small amount of 1N HCl. The sample extracts were shielded at 37 ^{o}C for 20 min. It is then heated to 51^o C for 20 min. After cooling the samples the turbidity was computed at 660nm. (UV Visible Spectrophotometer Model 371, Elico India Ltd) The experiment was repeated thrice. Using the following formula the Percentage inhibition of protein denaturation was calculated:

Percentage inhibition = (Abs Control –Abs Sample) X 100/ Abs control.

Antiproteinase action

The test was performed using the modified method of Oyedepo and Femurewa, (12) and Sakat *et al*. (11). The reaction mixture (2 ml) contains 1 ml 20 mMTrisHCl buffer (pH 7.4), 0.06 mg trypsin, and 1 ml test sample of different concentrations (100 - 500 µg/ml). The mixture was set aside warm at 37^{o}C for 5 min. 1 ml of 0.8% (w/v) casein was added to this. The mixture was kept warm for an additional 20 min. Inorder to arrest the reaction 2 ml of 70% perchloric acid was added to it. Then the cloudy suspension was centrifuged. Further the absorbance of the supernatant was read at 210 nm beside buffer as blank. The experiment was duplicated thrice. Using the following formula the percentage inhibition of proteinase inhibitory activity was calculated.

Percentage inhibition = (Abs control –Abs sample) X 100/ Abs control

Membrane stabilization

Preparation of Red Blood cells (RBCs) suspension (11, 13)

Blood was collected from healthy human helper who has not used any NSAIDs (Non-Steroidal Anti-Inflammatory Drugs) for 2 weeks prior to the experiment. It is then transferred to the centrifuge tubes. At 3000 rpm for 10min the tubes were centrifuged. They were washed using normal saline three times with equal volume. The volume of blood was found out.It was re-constituted as 10% v/v suspension with normal saline.

Heat induced haemolysis (11, 14)

The reaction mixture (2ml) consisted of 1 ml test sample of dissimilar concentrations (100 - 500 µg/ml) and 1 ml of 10% RBCs suspension. In its place of test sample, only saline was connected to the control test tube. As a standard drug Aspirin can be used. All the centrifuge tubes containing the reaction mixture were incubated in water bath at 56 ^{o}C for 30min. The tubes were cooled using running tap water at the end of the incubation. The absorbance of the supernatants was taken at 2500 rpm for 5 min. At 560 nm the reaction mixture was centrifuged. The experiment was repeated thrice for all the test samples. Using the following formula. The Percentage inhibition of Haemolysis was calculated:

Percentage inhibition = (Abs control –Abs sample) X 100/ Abs control

Hypotonicity-induced haemolysis (15)

The different concentration of extract, different reference sample, (100-500µg/ml), were mixed individually with 1ml of phosphate buffer, 2ml of hyposaline, and 0.5ml of HRBC suspension. Diclofenac sodium (100µg/ml) was utilized as a normal drug. All the assay mixtures were maintained warm at 37°c for 30minutes.It is then centrifuged at 3000rpm. Followed by this the supernatant liquid was poured and the haemoglobin content was approximated using a spectrophotometer at 560nm. The percentage hemolysis was calculated approximately by assuming the haemolysis produced in the control as 100%.

Percentage protection = 100- (OD sample/OD control) x 100

Anti-lipoxygenase activity (14)

Using linoleic acid as substrate and lipoxidase as enzyme Anti-Lipoxygenase activity was considered. Test samples were suspended in 0.25ml of 2M borate buffer pH 9.0. An additional 0.25ml of lipoxidase enzyme solution (20,000U/ml) is added.This is maintained warm for 5 min at 250°c. After which, 1.0ml of lenoleic acid solution (0.6mM) was added and mixed well. Then the absorbance was measured at 234nm. Indomethacin was used as indication standard. Using the following formula the percent inhibition was calculated:

% inhibition= [{Abs control- Abs sample}/Abs control] x 100

To establish the IC_{50} values, a dose response curve was plotted. IC_{50} is termed as the concentration sufficient to obtain 50% of a maximum scavenging capacity. All tests and analyses were done three times and averaged.

Statistical analysis

Results are expressed as Mean ± SD. Using One Way Analysis of Variance (ANOVA) the difference between experimental groups are compared. Making use of the soft ware Graph Pad Instat, it is followed by Dunnet Multiple comparison test (control Vs test).

Results and Discussion

Inhibition of albumin denaturation

A well documented cause of inflammation is denaturation of protein. The inflammatory drugs have shown dose dependent capability to thermally induced protein denaturation (10). Protein denaturation denotes loses of biological properties of protein molecules. Denaturation of proteins is accountable for the cause of inflammation in conditions like rheumatoid arthritis, diabetes, cancer etc. Therefore, prevention of protein denaturation may also aid in preventing inflammatory conditions. The current study demonstrated the *In-vitro* anti-inflammatory activity of ethanol extract of *P.alliacea* whole plant on inhibitory denaturation of proteins and is shown in table 1. Maximum inhibition of 69% was seen in the whole plant extract of *P.alliacea* at the concentration of 500 µg/ml. Aspirin a normal anti-inflamamtory drug showed the maximum inhibition of 66% at the concentration of 100 µg/ml in comparison with control.

Table 1: Effect of *Petiveria alliacea* extract on heat induced haemolysis of erythrocyte

Treatment	Concentration Ug/ml	Absorbance at 560 nm	% of inhibitionof heat induced haemolysis of erythrocyte
Control	-	0.33±0.02	-
PA Extract	100	0.29±0.01	12
PA Extract	200	0.24±0.03	27
PA Extract	300	0.18±0.07**	45
PA Extract	400	0.14±0.03**	58
PA Extract	500	0.10±0.07**	70
Aspirin	100	0.07±0.01**	78

Each value represents the mean ± SD. N=3, Experimental group were compared with control
**p<0.01, considered extremely significant

Table 2: Effect of *Petiveria alliacea* extract on heat induced protein denaturation

Treatment	Concentration Ug/ml	Absorbance at 660 nm	% of inhibition of protein denaturation
Control	-	0.36±0.03	-
PA Extract	100	0.24±0.02	33
PA Extract	200	0.21±0.07**	41

PA Extract	300	0.17±0.04**	52
PA Extract	400	0.14±0.03**	61
PA Extract	500	0.11±0.02**	69
Aspirin	100	0.12±0.05**	66

Each value represents the mean ± SD. N=3, Experimental group were compared with control
**p<0.01, considered extremely significant

Table 3: Effect of *Petiveria alliacea* extract on hypotonicity induced haemolysis of erythrocyte

Treatment	Concentration Ug/ml	Absorbance at 560 nm	% of inhibitionof hypotonicity inducedhaemolysis of erythrocyte
Control	-	0.32±0.04	
PA Extract	100	0.22±0.02	31
PA Extract	200	0.17±0.01**	47
PA Extract	300	0.14±0.02**	56
PA Extract	400	0.11±0.03**	66
PA Extract	500	0.08±0.001**	75
Aspirin	100	0.10±0.03**	68

Each value represents the mean ± SD. N=3, Experimental group were compared with control
**p<0.01, considered extremely significant

Table 4: Effect of *Petiveria alliacea* extract on lipoxygenase inhibitory action

Treatment	Concentration Ug/ml	Absorbance at 234 nm	% of inhibition of lipoxygenase action
Control	-	0.40±0.02	
PA Extract	100	0.33±0.01	17
PA Extract	200	0.28±0.03	30
PA Extract	300	0.24±0.03**	40
PA Extract	400	0.16±0.04**	60
PA Extract	500	0.14±0.04**	65
Aspirin	100	0.07±0.01**	82

Each value represents the mean ± SD. N=3, Experimental group were compared with control
**p<0.01, considered extremely significant

Table 5: Effect of *Petiveria alliacea* extract on proteinase inhibitory action

Treatment	Concentration Ug/ml	Absorbance at 210 nm	% of inhibition of proteinase action
Control	-	0.37±0.06	-
PA Extract	100	0.32±0.03	14
PA Extract	200	0.27±0.02	27
PA Extract	300	0.22±0.01**	46
PA Extract	400	0.17±0.04**	54
PA Extract	500	0.13±0.02**	65
Aspirin	100	0.14±0.01**	62

Each value represents the mean ± SD. N=3, Experimental group were compared with control
**p<0.01, considered extremely significant

Proteinase Inhibitory action

Neutrophils are identified to be a rich source of serine proteinase and are contained at lysosomes. Serine protease inhibitors (serpins) are a superfamily of proteins involved in many important biological processes, including inflammation. It was previously reported that leukocytes proteinase play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors (16). Ethanol extract of *P.alliacea* whole plant exhibited significant antiprotienase activity at different concentrations as shown in table 2. It shows maximum inhibition of 65% at 500 µg/ml. Aspirinshowed the maximum inhibition 62% at 100µg/ml.

Heat induced haemolysis

Stabilization of lysosomal membrane is important in limiting the inflammatory response by inhibition the release of lysosomal constituted of activated neutrophil such as bactericidal enzymes and proteases which cause further tissue inflammation and damage upon extracellular release or by stability the lysosomal membrane. HRBC or erythrocyte membrane is analogous to the

lysosomal membrane (17, 18) and its stabilization implies that the extract may well stabilize lysosomal membrane. The lysomal enzymes released during inflammation produce a various disorders. The extracellular activity of these enzymes are said to be related to acute or chronic inflammation. The non steroidal drugs act either by inhibitory these lysosomal enzymes or by stabilizing the lysosomal membrane (19). Ethanol extract of *P.alliacea* was effective in inhibitory the heat induces haemolysis at different concentrations. The results showed that ethanol extract of *P.alliacea* at concentration 500 µg/ml protect significantly ($p<0.01$) the erythrocyte membrane against lysis induced by heat (Table 3). Aspirin at concentration 100 mg/ml exhibited a significant ($p<0.01$) protection against damaging effect of heat solution.

Hypotonicity induced haemolysis

Exposure of red blood cells (RBCs) to injurious substances such as hypotonic medium results in the lysis of the membranes accompanied by haemolysis and oxidation of haemoglobin (20). Since human red blood cells (HRBC) membranes are similar to lysosomal membrane components the inhibition of hypotonicity induced red blood cell membrane lysis was taken as a measure of the mechanism of anti-inflammatory activity of *P.alliacea* whole plant extract. The haemolytic effect of hypotonic solution is related to excessive accumulation of fluid within the cell resulting in the rupturing of its membrane. Injury to red cell membrane will be the cell more susceptible secondary damage through free radical induced lipid peroxidation (21). Membrane stabilization leads to the prevention of leakage of serum protein and fluids into the tissues during a period of increased permeability caused by inflammatory mediators. The present study the results showed the ethanol extract of *P.alliacea* whole plant at concentration range of 200 to 500 µg/ml protect significantly ($p<0.01$) the erythrocyte membrane against lysis induced by hypotonic solution (table 4). Aspirin (100 µg/ml) offered a significant ($p<0.01$) protection against the damaging effect of hypotonic solution. At the concentration of 500 µg/ml, ethanol extract of *P.alliacea* whole plant showed maximum of 75% protection whereas aspirin (100 µg/ml) showed 68% inhibition of RBC haemolysis when compared with control.

Antilipoxygenase activity

Lipoxygenase (LOXs) enzymes are reported to convert the arachidonic, linolic and other polyunsaturated fatty acid into biologically active metabolites that are involved in the inflammatory and immune responses (22). LOXs are the key enzymes in the biosynthesis of leukotriens (LTs), that play an important role in several inflammations related diseases such as arthritis, asthma, cancer and allergic diseases (23). High levels of LTs could be observed in the case of asthma, psoriasis, allergic rhimitis, rheumatidarhtiritis and colitis ulcerosa. Therefore, it is of the view that the production of LTs can be prevented via inhibition of the LOX pathway and targeting LOX with inhibitors is of a promising therapeutic target for treating a wide spectrum of human diseases. The present study, the strongest inhibition was observed at concentration 500 µg/ml ethanol extract of *P.alliacea* whole plant. The standard aspirin showed an 82% inhibition at a concentration of 100 µg/ml.

In conclusion, the present study revealed the *In-vitro* anti-inflammatory activity of ethanol extract of *Petiveria alliacea* whole plant. The presence of flavonoids and polyphenolic compounds may be responsible for the *in-vitro* anti-inflammatory activity. Further investigations are required to find active component of the extract and to confirm the mechanism of action.

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Cite this article as:

Assessment of *In-vitro* anti-inflammatory activity of ethanol extract of *Petiveria alliacea* L. (Phytolaccaceae), Rajesh A., Doss A., Tresina P.S. and V.R., Mohan. *International Journal of Bio-Pharma Research*, Volume 8, Issue 5 (2019) pp. 2569-2574.

http://dx.doi.org/10.21746/ijbpr.2019.8.5.3

Source of support: Nil; **Conflict of interest:** Nil.