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Evaluation of phytochemical screening and *in vitro* anti-inflammatory activity of mangrove associate plant *Pemphis acidula* J.R. Forst and G. Forst (Lythraceae)

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Abstract--The study investigated the phytochemical screening and anti-inflammatory activity of *Pemphis acidula*. The phytochemical screening of methanol and ethanol extracts of *Pemphis acidula* revealed the presence of alkaloids, flavonoids, phenols, quinones, saponins, steroids, tannins, terpenoids, sugars, glycosides and xanthoproteins. The anti-inflammatory activity of ethanol extract of *Pemphis acidula* was evaluated using five *in vitro* based assays. Protein denaturation, proteinase inhibition, heat induced haemolysis, hypotonicity induced haemolysis and lipoxygenase inhibition at different concentration (100-1000 µg/ml). Aspirin, diclofenac sodium and indomethacin were used as standard drugs. The results obtained in the present study indicate that the ethanol extract of *Pemphis acidula* can be a potential source of anti-inflammatory agents.

Keywords---*Pemphis acidula*, phytochemical, anti-inflammatory, lipoxygenase.

Introduction

Inflammation is in general referred to as a complex biological response of vascular tissues to harmful stimuli. Inflammation is associated with pain, and it involves an increase of protein denaturation, an increase of vascular permeability, and membrane alteration, among others (Ferrero-Millani *et al.*, 2007). Inflammation is also said as the body response to inactivate or eliminate the invading stimuli or organisms, to eliminate the irritants and set the stage for tissue repair, and the process is associated by the release of chemical mediators from injured cells or tissues and migrating cells (Chandra *et al.*, 2012). The migration of leukocytes from the venous systems to the site of damage, and the release of cytokines are known to play a crucial role in the inflammatory response (Hollman, 2004). These work collectively to cause increased vasodilation and permeability of the capillaries. This leads to amplified blood flow to the injured site. Non-inflammatory drugs (NSAID) are commonly used for the management of inflammatory conditions. However, these drugs have several unpleasant side effects, especially gastric irritation, leading to the development of gastric ulcers. Therefore, the hunt for natural sources and phytochemicals with anti-inflammatory activity has greatly improved in recent years.

Pemphis acidula J.R. Forst & G. Forst belongs to the family Lythraceae, commonly known as bantigue or mentigi. It is a mangrove associate plant found throughout most of the tropical Indo-Pacific on rocky shores. An infusion of the sap plus a handful of bark has been consumed as an abortifacient. The bark contains 19-43% tannins. *In vitro* tests of the bark showed an increased activity on the amplitude and frequency of uterine contractions, which confirms the traditional use in Vanuata as an abortifacient (Bourdy and Walter, 1992). The bark is used to treat stomatitis. Bark extracts establish to have antioxidant, antibacterial and topoisomerase I inhibitor activities. Four galloyl flavonol glycosides with antioxidant activity have been isolated from leaf extracts (Ken 2022). Therefore, the present study was conducted to determine the qualitative phytochemical analysis and anti-inflammatory activity of *Pemphis acidula* whole plant using several *in vitro* bioassays such as inhibiting of albumin denaturation, antiproteinase activity, membrane stabilization anti-lipoxygenase activity.

Materials and Methods

Collection of plant samples

The whole plant of *Pemphis acidula* J.R. Forst & G. Forst was collected from Harbour estate. Thoothukudi coast, Gulf of Mannar Biosphere Reserve. The mangrove associate plant was identified with the help of local flora and authenticated by Botanical survey of India. The voucher specimen (EPH 364) was deposited in the Ethnopharmacology unit, P.G. & Research Department of Botany, V.O. Chidambaram College, Thoothukudi

Preparation of power samples

The whole plant of *P. acidula* was cut into small fragments and shade dried until the fracture was uniform and smooth. The dried plant samples were powdered using blender and sieved to get uniform particles by using sieve no 60. The final powder was used for extraction of phytochemicals.

Preparation of plant extracts and phytochemical analysis

One hundred gram of the coarse powder of *P. acidula* was extracted successively with 250 ml of petroleum ether, benzene, ethyl acetate, methanol, ethanol and aqueous in a soxhlet apparatus for 24 hrs (Suvarchala et al. 2022). All the extracts were filtered through whatman no. 41 filter paper separately and all the extracts were concentrated in a rotary evaporator (Farzeen, 2022). All the concentrated extracts were subjected to qualitative phytochemical analysis for the identification of various phytochemical constituents as described by Brinda et al. (1981) and Trease and Evans (1986). Ethanol extracts were used for *in vitro* anti-inflammatory activity.

Methodology for *in vitro* antiinflammatory activity

Inhibition of protein denaturation

The antiinflammatory activity of ethanol extract of *P. acidula* was studied by using inhibition of protein denaturation technique which was studied according to Sakat et al. (2010) with some modifications as described in Gunathilake et al. (2018). The reaction mixture (5 ml) consisted of 0.2 ml of 1% bovine albumin, 4.78 ml of phosphate buffer saline (pH 6.4) and 0.02 ml of varying concentration of extracts, and the mixture was mixed and was incubated in a water bath (37 °C) for 15 min and then reaction mixture was heated at 51°C for 5 min. After cooling the turbidity was measured at 660 nm (using a UV/VIS spectrophotometer model SL 150 Elico, India Ltd). Phosphate buffer solution was used as the control. Aspirin (standard drug) was used as reference drug and treated as such for determination of absorbance. The percentage inhibition of protein denaturation was calculated by means of the subsequent formula

$$\% \text{ inhibition of denaturation} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Proteinase inhibitory activity

Proteinase inhibitory activity of the ethanol extract of *P. acidula* was performed according to the method of Sakat et al. (2010), which is modified by Gunathilake et al. (2018). Briefly, the reaction mixture (2 ml) consisted of 0.06 mg trypsin, 1 ml of 20 mM Tris – HCl buffer (pH 7.4) and 1 ml of varying concentration of extracts (100 – 1000 µg/ml). The mixture was incubated (37°C for 5 min) and then 1 ml of 0.8% (w/v) casein was added, and the mixture was further incubated for an additional 20 min. At the end of incubation, 2 ml of 70% perchloric acid was added to terminate the reaction. The mixture was centrifuged, and the absorbance of the supernatant was calculated at 210 nm against buffer as the blank. Phosphors buffer solution was used as control Aspirin was the standard

drug used. The experiment was performed in triplicate. The percentage inhibition of proteinase inhibitory activity was calculated using the following formula.

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Membrane stabilization

Preparation of Red Blood Cells (RBCs) suspension (Sadique *et al.*, 1989; Sakat *et al.*, 2010)

The blood was collected from healthy human volunteer who has not taken any NSAIDs (Non steroidal Antiinflammatory Drugs) for 2 weeks proceeding to the experiment and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 min and were washed three times with equal volume of normal saline (0.9 % NaCl). After the centrifugation, the blood volume was measured and reconstituted as a 10% (v/v) suspension with isotonic buffer solution (10 mM sodium phosphate buffer pH 7.4).

Heat induced haemolysis (Shinde *et al.*, 1999; Sakat *et al.*, 2010)

The 2ml reaction mixture is consisted of 1 ml of test extract at various concentrations (100 – 1000 µg/ml) and 1 ml of 10% RBCs suspension, instead of drug only saline was added to the control test tube. Diclofenac sodium was taken as a standard drug. All the centrifuged tubes containing reaction mixture were incubated in a water bath at 56°C for 30 min. At the end of the incubation, the tubes were chilled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatant was taken at 560 nm. The experiment was performed in triplicate. The percentage of inhibition of haemolysis was calculated using the equation given below.

$$.\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Hypotonicity induced haemolysis (Azeem *et al.*, 2010)

Varying concentration of the ethanol extract ranging from 100 to 1000 µg/ml along with the reference sample and control were separately mixed with 1 ml of phosphate buffer, 2ml, of hyposaline and 0.5 ml of HRBC suspension. Diclofenac sodium (100 – 1000 µg/ml) was used as a standard drug. The mixtures were then incubated for 30 min at 37°C. It was then centrifuged at 3000 rpm. The supernatant liquid was decanted and the haemoglobin content was estimated by spectrophotometers at 560 nm. The degree of haemolysis inhibition was calculated using the same formula as for the heat induced haemolysis activity.

Lipoxygenase inhibition activity (Shinde *et al.*, 1999)

Lipoxygenase inhibition assay was studied using linoleic acid as substrate and lipoxygenase as enzyme. Test solution was dissolved in 0.25 ml of 2 M borate buffer

pH 9.0 and added 0.25 ml of lipoxidase enzyme solution (Final concentration 20,000 U/ml) and incubated for 5 min at 25°C. After which 1.0 ml of linoleic acid solution (0.6 mM) was added, mixed well and absorbance was measured at 234 nm using UV/Vis spectrophotometer. Indomethacin was used as reference standard. Phosphate buffer solution was used as the control. The percentage inhibition of lipoxygenase was calculated using the equation given below.

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Statistical analysis

Results are expressed of Mean $\pm$ SD. The difference between experiment groups were compared by one-way-analysis of variance (ANOVA) followed by Dunnett's Multiple comparison tests (Control Vs Test) using the soft ware graph pad instat.

Results and Discussion

Phytochemical analysis

The results of phytochemical screening of different solvent extracts of *P. acidula* are presented in Table 1. Among the solvents tested, methanol and ethanol extracts of *P. acidula* revealed the presence of alkaloids, flavonoids, phenols, quinones, saponins, steroids, tannins, terpenoids, sugars, glycosides and xanthoproteins. The presence of these phytochemicals in methanol and ethanol extracts of *P. acidula* possibly indicates their numerous medicinal properties such as anti-inflammatory, antiulcer, antioxidant, anticancer and antimicrobial properties, among others. The presence of these useful secondary metabolites could make these plants useful for treating different human ailments and thus providing a potential drug for human use. This is because; the pharmacological activity of any plant is usually traced to a particular phytochemical.

Table 1
Phytochemical screening of *P. acidula*

Bioactive components	Nature of extract					
	Petroleum ether	Chloroform	Ethyl acetate	Methanol	Ethanol	Aqueous
Alkaloids	–	–	–	+	+	–
Anthraquinone	–	–	–	–	–	–
Catachins	–	–	–	–	–	–
Coumarins	–	–	–	–	–	–
Flavonoids	+	+	+	+	+	+
Phenols	+	+	+	+	+	+
Quinones	–	–	+	+	+	–
Saponins	+	+	+	+	+	+
Steroids	–	–	+	+	+	–
Tannins	+	+	+	+	+	+

Terpenoids	+	+	+	+	+	+
Sugar	+	+	+	+	+	—
Glycosides	+	+	+	+	+	+
Xanthoprotein	+	+	+	+	+	+
Fixed oil	+	+	—	—	—	—

+ present - absent

***In vitro* anti-inflammatory activity** **Inhibition of protein (albumin) denaturation**

Many of the biological proteins lose their biological function when denatured. Denaturation of proteins is a well documented cause of inflammation. In the present study, the mechanism of the anti-inflammatory activity, ability of plant extract to inhibit protein denaturation was studied. Ethanol extract of *P. acidula* was able to inhibit protein denaturation in a concentration dependent manner. Maximum inhibition of 72.73% was observed at 1000 µg/ml concentration of *P. acidula* ethanol extract. Aspirin, a standard anti-inflammatory drug showed the maximum inhibition of 90.90% at the concentration of 1000 µg/ml compared with control (Fig. 1).

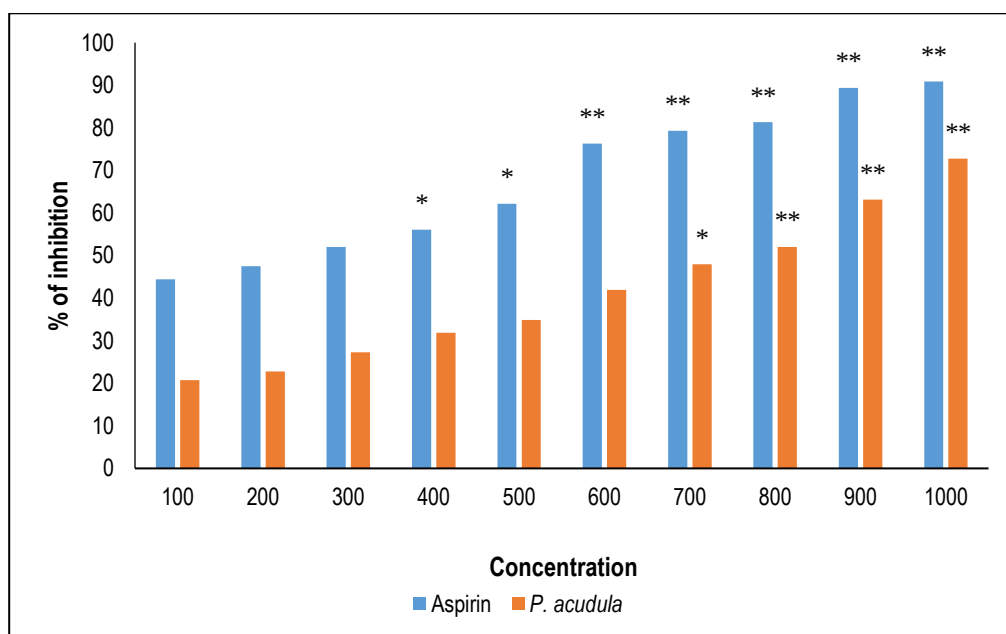


Figure 1. Effect of ethanol extract of *P. acidula* on heat induced proteinase denaturation

Data are presented as the means $\pm$ SD of three replicate determination. Experimental group were compared with control ** $P < 0.01$ considered extremely significant; * $P < 0.05$ considered significant.

Proteinase inhibitory action

During inflammation, as part of their defensive roles, leukocytes release their lysosomal enzymes, including proteases, causing further tissue damage and subsequent inflammation (Okali *et al.*, 2008). In the present investigation, ethanol extract of *P. acidula* exhibited significant antiproteinase activity at different concentrations as shown in Figure 2. It showed maximum inhibition of 94.02% at 1000 $\mu\text{g/ml}$ concentration of ethanol extract of *P. acidula*. Aspirin showed the maximum 92.39% at the same concentration.

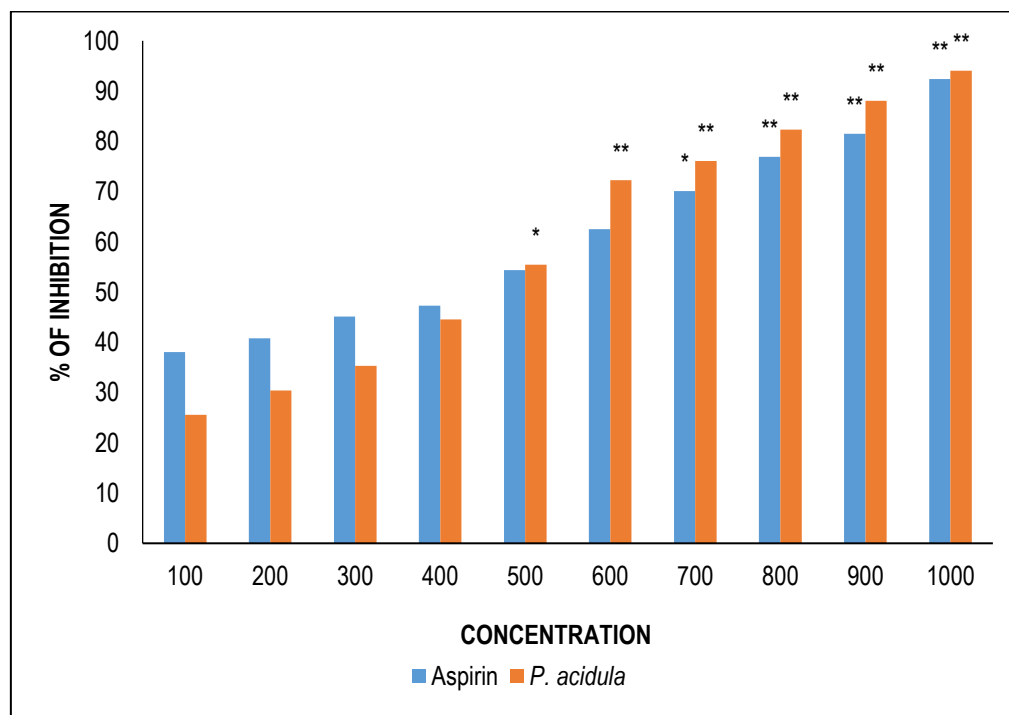


Figure 2. Effect of ethanol extract of *P. acidula* on proteinase inhibitory action

Data are presented as the means $\pm$ SD of three replicate determination. Experimental group were compared with control ** $P < 0.01$ considered extremely significant; * $P < 0.05$ considered significant.

Membrane stabilization

As the red blood cell membrane is similar to that of lysosomal membrane, inhibition of red blood cell haemolysis may provide insights into the inflammatory process (Umapathy *et al.*, 2016). Stabilization of these cell membrane may retard or inhibit the lysis and subsequent release of the cytoplasmic contents which, in turn, minimize the tissue damage and hence, the inflammatory response (Okoli *et al.*, 2008). Therefore, substance that contribute significant protection of cell membrane against injurious substances are important in the event of inhibiting the progression of inflammation.

Heat induced haemolysis

Ethanol extract of *P. acidula* was able to inhibit haemolysis in a concentration dependent manner. The results showed that *P. acidula* extract at concentration 1000 µg/ml protect significantly ($P < 0.01$; 75.30%) the erythrocyte membrane against lysis induced by heat (Fig. 3). Diclofenac sodium showed a significant ($P < 0.01$; 73.78%) protection against damaging effect of heat.

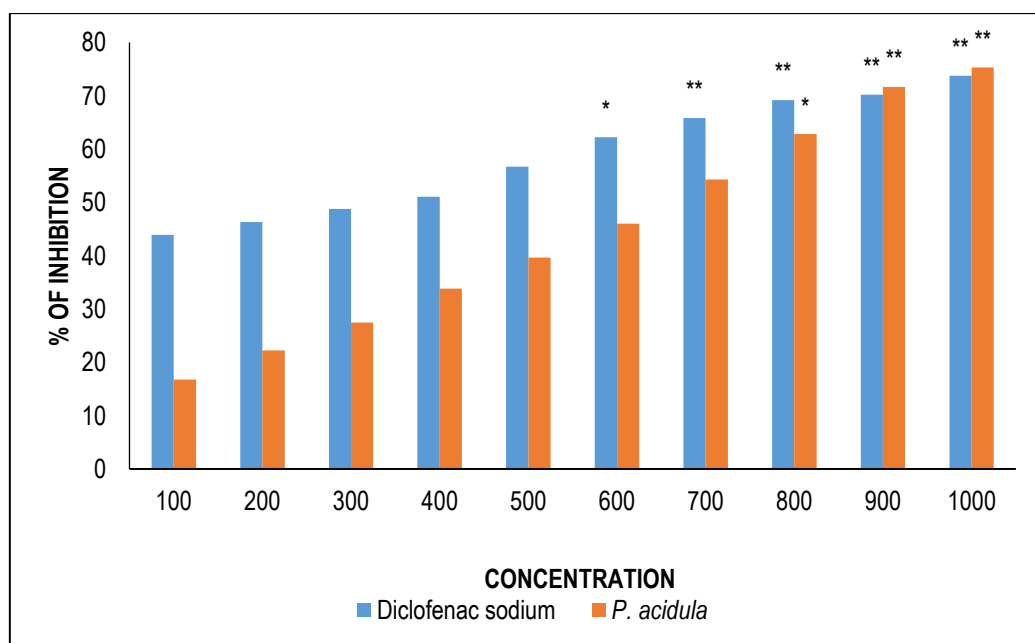


Figure 3. Effect of ethanol extract of *P. acidula* on heat induced haemolysis of erythrocyte

Data are presented as the means $\pm$ SD of three replicate determination. Experimental group were compared with control $**P < 0.01$ considered extremely significant; $*P < 0.05$ considered significant.

Hypotonicity induced haemolysis

The results showed that ethanol extract of *P. acidula* at concentration of 1000 µg/ml protect significantly ($P < 0.01$; 79.37%) the erythrocyte membrane against lysis induced by hypotonic solution (Fig. 4). Diclofenac sodium (1000 µg/ml) exhibited a significant ($P < 0.01$; 88.25%) protection against damaging effect of hypotonic solution.

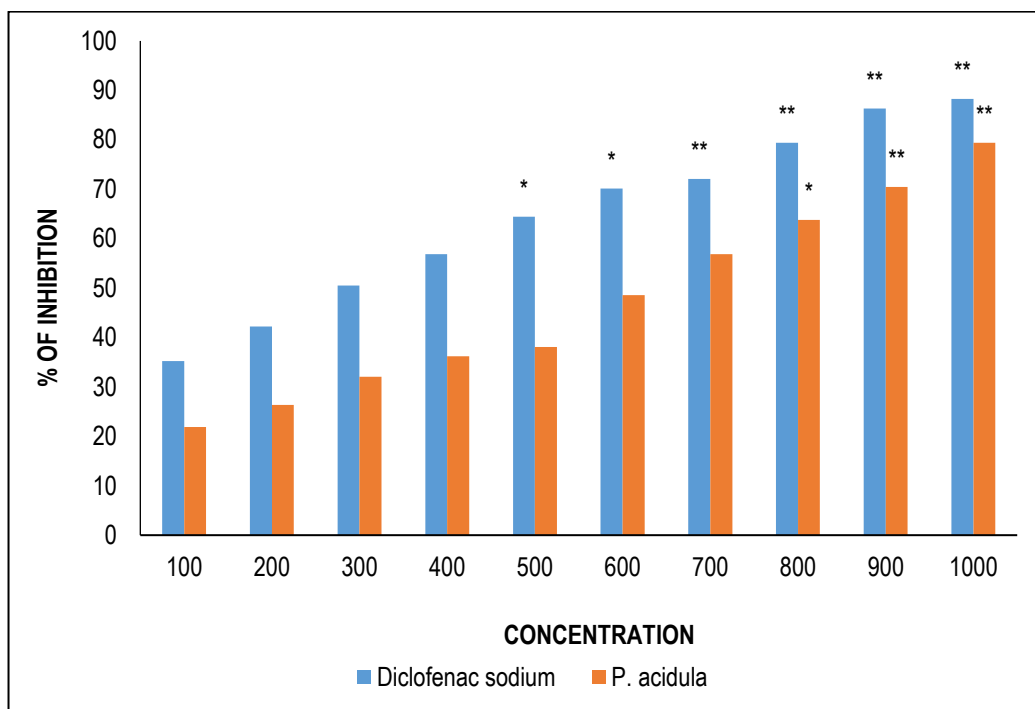


Figure 4. Effect of ethanol extract of *P. acidula* on hypotonicity induced haemolysis of erythrocyte

Data are presented as the means $\pm$ SD of three replicate determination. Experimental group were compared with control ** $P < 0.01$ considered extremely significant; * $P < 0.05$ considered significant.

Lipoxygenase inhibitory action

Lipoxygenase are the key enzyme in the biosynthesis of leukotrienes. Leukotrienes play an important role in several inflammatory diseases, such as arthritis, asthma, cancer, and allergic diseases (Rackova *et al.*, 2007). The mechanism of antiinflammation may involve series of events in which the metabolism of arachidonic acid plays an important role (Akinwunmi and Oyedapo, 2015). In this process, arachidonic acid is cleaved from the membrane phospholipids upon suitable stimulation of neutrophils and can be changed to leukotrienes and prostaglandins through lipoxygenase and cyclooxygenase pathways respectively (Akinwunmi and Oyedapo, 2015). Lipoxygenase catalyzes deoxygenation of polyunsaturated fatty acids to produce *cis*, *trans* conjugated diene hydroperoxides, such as leukotrienes, events (Khasawneh *et al.*, 2011). In the present study, results for lipoxygenase inhibitory activity of *P. acidula* are shown in figure 5. Inhibition levels were within the range of 18.69 – 76.89% within the concentration of 100 – 1000 $\mu\text{g/ml}$. The standard drug indomethacin showed an 81.07% inhibition at a concentration of 1000 $\mu\text{g/ml}$. previous studies have shown that some herbs also have high lipoxygenase inhibitory activity, such as *Leptadenia pyrotechnica* (Khasawneh *et al.*, 2011). *Mahonia aquifolium* (Rackova *et al.*, 2007) and selected green leafy vegetables (Gunathilake *et al.*, 2018).

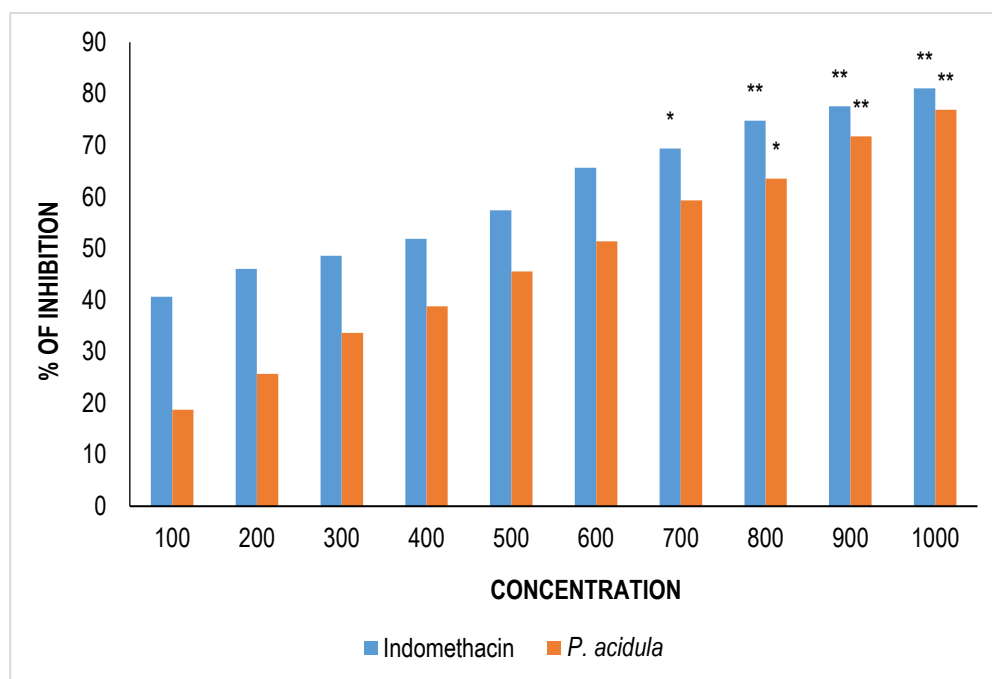


Figure 5. Effect of ethanol extract of *P. acidula* on lipoxygenase inhibitory action

Data are presented as the means $\pm$ SD of three replicate determination. Experimental group were compared with control $**P < 0.01$ considered extremely significant; $*P < 0.05$ considered significant.

The results obtained from this studies on *P. acidula* have shown a potential anti-inflammatory activity.

Conclusion

In the present study, results indicate that the ethanol extracts of *P. acidula* possess anti-inflammatory properties. These activities may be due to the strong occurrence of polyphenolic compounds, alkaloids, flavonoids, tannins and steroids. This study gives on idea that the compound of the plant *P. acidula* can be used as lead compound for designing a potent anti-inflammatory drug which can be used for treatment of various diseases such as cancer, neurological disorder, aging and inflammation.

Conflict of interest

The authors have no conflict of interest to declare.

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