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Sandhia *et* al
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Original Article

In-Vitro Anti-inflammatory Activity Evaluation of the Latex Protease of *Holostemma Ada-Kodien* Schult

Dinta Daniel, S Subramaniyan, Sandhia G S*

Post GraduateDepartment of Botany and Research Centre, University College, Thiruvananthapuram – 695034, Kerala, India.

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ABSTRACT

Holostemma ada-kodien Schult. (family Asclepiadaceae), commonly known as adapathiyan is a laticiferous climber and a medicinally very important plant with therapeutic and pharmacological properties.This plant is beneficial for external use in various skin diseases, wounds and inflammation of the skin. The latex of Holostemma is applied on blisters and the plant as a whole is exploited for maintaining youthful vigour, strength and vitality. It is having antipyretic, antibacterial, anti-inflammatory, antioxidant, hypoglycemic and anti-diabetic properties. The present study was to evaluate the preliminary phytochemical screening of latex and the cyclooxygenase and lipoxygenase inhibitory activity of latex protease of *Holostemma ada-kodien*.The cyclooxygenase and 5-lipoxygenase inhibitory activities were evaluated to determine the promising mechanism of the anti-inflammatory activity of latex protease. The preliminary phytochemical screening of ethanolic extract of the latex showed positive result for reducing sugar, glycosides, flavanoids, alkaloids, terpenoids, steroids,saponins,iridoids,tannin and phlobatannin. The evaluation of anti-inflammatory activity by the inhibition of cyclooxygenase (79.20 % and 71.63 % at sample concentration of 100 µg/ml and 50µg/ml) and 5- lipoxygenase (92.93 % and 79.60 % at sample concentration of 100 µg/ml and 50µg/ml) showed that the latex protease possess good anti-inflammatory activity. Thus the use of latex in the treatment of wounds and blisters becomes effective use to the presence of the protease along with other secondary metabolites. Further isolation and purification of secondary constituents can lead to the identification of potent phytochemicals that may contribute towards the anti-inflammatory activity.

Keywords: 5- lipoxygenase, Anti-inflammatory,Cyclooxygenase, *Holostemma ada-kodien* Schult, Phytochemical screening.

1. INTRODUCTION

Many plants have long been recognized as important sources of therapeutically effective medicines [1].The commonly used drugs for management of inflammatory conditions are non-steroidal anti-inflammatory drugs. These drugs show an inhibitory action on the cyclooxygenase that catalyze the biosynthesis of prostaglandins and thromboxane from arachidonic acid, which have several adverse effects

Corresponding author *
Dr. Sandhia G.S
Assistant Professor
Post Graduate Department of Botany and Research Centre,
University College, Thiruvananthapuram, Kerala, India.
E-mail: sandhiags@rediffmail.com

especially gastric irritation leading to formation of gastric ulcers. Therefore, natural anti-inflammatory drugs with fewer side effects are used and are substitute to the chemical therapeutics.

Inflammation is a complex biological response of the body tissue to hurtful stimulus caused by injury, infection, environmental agents, malignancy and cellular changes. It is the protective response of the body to remove the injurious stimuli, inactivate or destroy the invading organisms as well as initiate the stage for tissue repair. This response is a complex process that includes activation of white blood cells, the release of immune system chemicals such as complements and cytokines, and the production and release of inflammatory mediators and prostaglandins [2].

The mechanisms of inflammation involve a series of events in which the metabolism of arachidonic acid plays an important role. Cyclooxygenase (COX) is the key enzyme that catalyzes the rate limiting step in prostaglandin synthesis, two cyclooxygenases (constitutive COX-1 and inducible COX-2) and lipoxygenase (5-LOX) enzymes are responsible for the transformation of arachidonic acid into the potent biologically active prostaglandin H2, which is further metabolized to prostaglandin E2(PGE2), prostaglandin F2(PGF2), prostaglandin D2(PGD2) and other eicosanoids, that are intimately involved in inflammation and cell growth[3]. Lipoxygenase (LOX) is an iron containing enzyme that hydrolyzethe dioxygenation of poly unsaturated fatty acids into lipid containing a *cis, cis*-1,4-pentadienes structure. LOX convert arachidonic acid into leukotrienes, which is further metabolized into leucotriene A4, leucotriene B4 and cysteinyl leucotrienes. These are called pro inflammatory mediators[4].

Holostemma ada-kodien Schult. is an important medicinal plant belonging to the family Asclepiadaceae. It is alaticiferous twining shrub with conspicuous flowers. The terpenoids, sugars and amino acids present in the *Holostemma* are responsible for its medicinal properties. The herb is beneficial for external use in various skin diseases, wounds and inflammation of the skin. Other uses include rejuvenative, aphrodisiac, expectorant, galactogogue, stimulant and in ophthalmic disorders.

Latex is a natural plant polymer secreted by highly specialized cells known as laticifers. More than 2500 species of plants produce latex[5]. Plant latex is a mixture of secondary metabolites and bioactive components[6], the composition of which vary from species to species. The milky latex of *Holostemma ada-kodien* is used for the treatment of wounds, blisters and heal ulcers[7].The present study was to investigate the anti-inflammatory activity of latex protease (HA protease) of *H. ada-kodien* and to evaluate the COX and LOX inhibitory prospective and primary phytochemical screening to understand their possible mechanism in the treatment of inflammatory disorders.

2. MATERIALS AND METHODS

Collection and authentification of plant material:

Holostemma ada-kodien was collected from Mannuthy, Thrissur District and was identified and deposited at Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, Thiruvananthapuram. The voucher specimen (number 84555) is preserved in the Botany Department, University College, Thiruvananthapuram and it has been deposited in herbarium of JNTBGRI for future reference.

Collection of Latex and Isolation of Crude Enzyme:

Latex samples were collected early in the morning from the plant species by superficial incision of the tender stem and dipping into the sterile glass vials containing 0.1 M citrate phosphate buffer (pH 6.5) with 0.05 mM ethylene diamine tetra acetic acid (EDTA).This was kept overnight in the refrigerator at 4˚C and was centrifuged at 12000 rpm for 20 minutes at 4^0C.The supernatant was used as crude enzyme and stored at 4˚Cuntil use [8].This crude enzyme was partially purified by ammonium sulphate precipitation. The fractions in the range of 10-70% was collected, dialyzed and used for anti-inflammation studies.

Preparation of Extracts

The latex was concentrated by lyophilization. 1 gm of the sample was mixed with 1 ml of phosphate buffer and 1ml of ethanol. This sample was concentrated and subjected to preliminary phytochemical screening studies.

Preliminary phytochemical screening:

Phytochemical examinations were carried out for all the extracts as per the standard methods.

Test for Reducing Sugars:

Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used for the presence of reducing sugars.

a) Fehling's Test:

Filtrates were hydrolyzed with dilute HCl, neutralized with alkali and heated with Fehling's A&B solutions. Formation of red precipitate indicates the presence of reducing sugars.

Test for Glycosides:

a) Killer- Killani Test:

0.5 ml extract was taken and it was diluted to 5 ml using distilled water. 2 ml glacial acetic acid containing one drop of ferric chloride solution was added to this. 1 ml of concentrated sulphuric acid was added along the side of the tube. Presence of brown ring at the interference indicates the presence of glycosides.

Test for Flavonoids:

a) Cyanidine Test:

0.5 g of the crude extract was dissolved in methanol and 2 ml of concentrated hydrochloric acid added. A spatula full of magnesium turnings was added and the mixture was observed for effervescence. A brick red colouration observed indicate the presence of flavonoids.

Test for Tannins:

a) Braymer's Test:

2 ml extract was taken and added 2 ml distilled water and 2-3 drops of 5% ferric chloride. Green precipitate indicates the presence of tannins.

Test for Terpenoids:

a) Liberman - Burchard's Test:

2 ml extract was taken, dried in air and dissolved in chloroform. Few drops of acetic anhydride and concentrated sulphuric acid was added to it. Pink colour developed after few minutes indicates the presence of terpenoids.

Test for Coumarins:

1 ml extract was taken and dissolved in sodium hydroxide. **A** few drops of alcoholic sodium hydroxide added followed by the addition of concentrated hydrochloric acid through the sides of test tube. Appearance of yellow colour indicates the presence of coumarins.

Test for Alkaloids:

a). Mayer's Test:

A portion of the extract was dissolved in few drops of aqueous solution of hydrochloric acid and filtered. The filtrate was treated with 0.5 ml Mayer's reagent (Potassium Mercuric Iodide). Formation of white precipitate indicates the presence of alkaloids.

b)Dragendroff's Test:

A portion of the extract was dissolved in equimolar mixture of dilute HCl and Dragendroff's reagent. A brown colouration with precipitate indicates the presence of alkaloids.

Test for Saponins:

a) Froth Test: Extracts were diluted with distilled water to 20 ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

Test for Iridoids:

Extract was treated with 1 ml reagent. The mixture was heated over a flame. Development of a light blue colour indicated the presence of iridoids.

Test for Phlobatannins

Extract was boiled with 5 ml of 1% HCl. Red precipitate showed the presence of phlobatannins.

Test for Anthraquinones:

0.5 g of extract was taken and 10 ml of 1% HCl was added to this followed by boiling for 5 minutes. The sample was filtered and allowed to cool. Partitioned the cool filtrate against equal volume of chloroform. Carefully transferred the chloroform layer into clean test tube. Shaken well and added equal volume of 10% ammonia solution. The organic layer is separated to which ammonia is added slowly. Delicate pink colour indicated the presence of Anthraquinones.

Test for Steroids:

a) Liberman - Burchard's Test:

2 ml extractwas taken and dried in air and dissolved in chloroform. Added few drops of acetic anhydride and concentrated sulphuric acid to it. Kept it for few minutes. Green colour indicates the presence of terpenoids.

***In-vitro*anti-inflammatory activity:** *In vitro* anti-inflammatory activity of partially purified protease of *H. ada-kodien* was evaluated.

Cell lines:

RAW 264.7 cells were grown to 60% confluence followed by activation with 1 µL lipopolysaccharide (LPS) (1µg/mL). LPS stimulated RAW cells were exposed with different concentration (25,50, 100 µg/mL) of sample solution and Diclofenac sodium, a standard anti-inflammatory drug in varying concentration corresponding to the sample was added and incubated for 24 hours. After incubation the anti-inflammatory assays were performed using the cell lysate.

Cyclooxygenase (COX) activity

The COX activity was assayed by the method of Walker and Gierse (2010)[9]. The cell lysate was incubated in Tris-HCl buffer (pH 8), glutathione 5 mM/L, and hemoglobin 5 mM/L for 1 minute at 25°C. The reaction was initiated by the addition of arachidonic acid 200 mM/L and terminated after 20 minutes incubation at 37°C, by the addition of 10% trichloroacetic acid in 1 N hydrochloric acid. After the centrifugal separation and the addition of 1% thiobarbiturate, COX activity was determined by reading absorbance at 632 nm.

Percentage inhibition of the enzyme was calculated as:

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

Lipoxygenase (LOX) activity

The determination of LOX activity was as per Axelrod *et al*. (1981)[10]. Briefly, the reaction mixture (2 mL final volume) contained Tris-HCl buffer (pH 7.4), 50 µL of enzyme, and sodium linoleate (200 µL). The LOX activity was monitored as an increase of absorbance at 234 nm (Shimadzu), which reflects the formation of 5-hydroxyeicosatetraenoic acid.

Percentage inhibition of the enzyme was calculated using the formula:

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

3. RESULTS

Qualitative Analysis of Phytochemical Constituents:

The ethanolic extract of plant latex of *Holostemma ada-kodien* Schult. (Fig.1) was screened for the presence of various phytochemicals using standard procedures. This study has revealed the presence of phytochemicals considered as active medicinal constituents. Reducing sugars, tannins, alkaloids, iridoids, phlobatannins, terpenoids and steroids are present in ethanolic latex extract. These tests reveal the presence of various bioactive secondary metabolites which might be responsible for their medicinal attributes which could be the reason for high anti-inflammatory activity of the plant. Presence of terpenoid and steroids may be used in arthritis, in the treatment of joint

inflammation and as antiseptic and also reported to have anti-inflammatory, anti-viral, anti-malarial inhibition of cholesterol synthesis and anti-bacterial properties[11].The resultsare shown in Table.1.

***In –vitro* anti-inflammatory activity**

Cyclooxygenase inhibitory assay:

The anti-inflammatory effects of *Holostemma ada-kodien* latex protease (HA protease) on the production of prostaglandins were estimated by inhibition of cyclooxygenase activity. The results are shown in the **Table. 2.** In this study, the HA protease has very good anti-inflammatory property as compared to control and standard.The percentage of inhibition of COX enzyme at various concentrations of standard Diclofenac sodium (25µg/ml, 50µg/ml, and 100µg/ml) were found to be 72.02%, 80.19% and 84.91%.In COX inhibition assay the percentage inhibition was found to be 45.26 %, 71.63%, and 78.20% for concentrations of HA Protease 10µg/ml, 50µg/ml and 100µg/ml, respectively.

5- Lipoxygenase Inhibitory Assay

The anti-inflammatory effects of *H. ada-kodien* latex protease (HA protease) on the production of leukotrienes were estimated by inhibition of lipoxygenase activity. Results are shown in the **Table.2.** The HA protease showed good 5-lipoxygenase inhibitory activity when compared to control and standard. These activities are due to the presence of flavonoids, steroids and terpenoids which act as free radical scavenger or acting possibly as primary oxidant thereby inhibiting inflammation.The results of the study indicated that inhibitory effect of each plant extract on enzymes were dose dependent.

Comparison of cyclooxygenase and lipoxygenase activity of latex extract are shown in the **Fig.2.**

Table 1: Preliminary Phytochemical Screening

No	Phytochemicals	Ethanolic latex extract
1	Reducing sugar	+
2	Glycosides	+
3	Flavonoids	+
4	Alkaloids	+
5	Tannins	+
6	Steroids	+
7	Terpenoids	+
8	Coumarins	_
9	Saponins	+
10	Anthraquinones	_
11	Phlobatannins	+
12	Iridoid	+

Table 2: Cyclooxygenase Inhibitory Assay

Sample Concentration (µg/ml)	Absorbance at 632 nm	Standard (diclofenac sodium % inhibition	% inhibition
Control	0.006± 0.119	-	-
25	0.004± 0.0641	72.021	45.26
50	0.0013± 0.0594	80.194	71.63
100	0.0072± 0.0504	84.9104	78.20

Table 3: Lipoxygenase Inhibitory Assay

Sample Concentration (µg/ml)	Absorbance at 234nm	Standard (Diclofenac sodium) % inhibition	% inhibition
control	0.0213±0.559	-	-
25	0.0176± 0.3384	71.93	38.05
50	0.016±0.3316	85.56	74.38
100	0.01006±0.2973	98.02	82.67

Fig 1: .*Holostemma ada-kodien* Shult

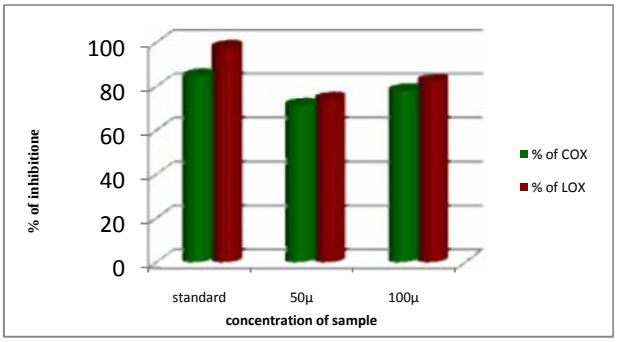

Fig 2: Comparison of COX 2 and 5- LOX Inhibition Activity

4. DISCUSSION

Medicinal plants are rich source of secondary metabolites that could attribute to its medicinal property.These secondary metabolites are used as food additives, in pharmaceuticals industry and as herbicides[12, 13, 14]. Most of the secondary metabolites are present in the latex which helps its medicinal property. The presence of flavonoids may have an effect on anti-inflammatory mechanisms by means of their ability to inhibit reactive oxygen or nitrogen compounds. They have also been recommended to inhibit the pro-inflammatory activity of enzymes involved in free radical production, such as cyclooxygenase, lipoxygenase or inducible nitric oxide synthase, and to modify intracellular signaling pathways in immune cells, or in brain cells after a stroke. The presence of terpenoids, coumarins and phlobatannins have been reported for its wound healing properties, and also have anti-inflammatory and analgesic [15] and antitoxidant properties[16]. Plants having alkaloids are used in medicines for reducing headache and fever. These are attributed for antibacterial and analgesic properties[17].These phytochemicals are known to promote anti-inflammatory activity in some plants such as *Syzygium*

zeylanicum, Solanum nigrum, Abroma augusta and *Desmodium gangeticum.*

Inflammation may be acute or chronic. Acute inflammation is characterized by heat, erythema, pain, swelling and loss of function. Pain is a common feature of many diseases and analgesics relieve pain by the action of central nervous system. Chronic inflammation is the result of progressive shift in inflammatory cells characterized by immediate damage and healing of the injured tissue. Inflammation is a complex physiopathological response to different stimuli and involves the activity of mediators such as reactive oxygen species, neutrophil derived free radicals, nitric oxides, prostaglandins and cytokines[18]. COX and LOX play an important role in the regulation of inflammatory response.

The anti-inflammatory activity of latex protease of *Holostemma ada-kodien* has been attributed to the inhibition of the synthesis of prostaglandins (PGs) and leukotrienes. Cyclooxygenase enzymes are most important and responsible for the conversion of arachidonic acid in to prostaglandins. At the same time it's overexpression may leads to serious problem especially inflammation, malignant tumors of the colon and rectum. So its inhibition is very necessary for the treatment of inflammation and cancer. Likewise,LOX enzyme metabolites also produce severe problems related to inflammation, augment metastasis of tumor cells and stimulate tumor cell adhesion [19, 20.]

In the present study, HAprotease was capable of inhibiting COX and LOX enzyme of the arachidonic acid cascade in human cellular system. Anti-inflammatory activity of latex protease has already been reported in other medicinal plants. Anti-inflammatory activity of fruit stem latex protease extracted from the plant *Artocarpus heterophyllus* Lam against carrageenan induced rat paw oedemabas been reported[21].Anti-inflammatory activity of the latex has been observed in*Calotropis procera* against carrageenan and formalin-induced rat paw oedema model[22]. The ethanolic extract of leaves of *H. ada-kodien*possess good anti-inflammatory activity against albino rats[23]. The present study confirmed that the anti-inflammatory activity of *H. ada-kodien* latex protease that showed high percentage of inhibition in COX and LOX activity.

In this study, HA protease showed good anti-inflammatory activity in both cyclooxygenase inhibition assay (COX) and 5-lipoxygenase inhibition assay (LOX). The anti-inflammatory activity may also be due to the presence of secondary metabolites in the latex. So, further isolation and purification of secondary constituents might lead to the identification of potent phytochemicals that contributes towards the anti-inflammatory activity of *H. ada-kodien* latex.

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6. REFERENCES

1. Newman DJ, Cragg GM, Snader KM. Natural products as sources of new drugs over the period 1981-2002. J Nat Prod2003; 66 (7): 1022-37.
2. Cotran RS, Kumar V, Collins T. Robbins pathological basis of disease. 6th ed. WB Saunder's company, 2001. 51 -55.
3. Mariana D, Anna S, Elizabeth H, Carlos LC. Effects of extracts, flavanoids and irioids from *Penstemon gentianoides* (Plantaginaceae) on inhibition of inducible nitric oxide synthase (iNOS) cyclooxygense-2 (COX-2) in LPS-activated RAW 264.7 macrophage cells and their antioxidant activity. J. Boletin Latino americanoydei Caribe de Plantas Medicinalesy Aromaticas 2010; 9 (5): 397-413.
4. Iranshahi M, Askari M, Sahebkar A, Hadjipavlou Litina D. Evaluation of antioxidant, anti-inflammatory and lipoxygenase inhibitory activities of the prenylated coumarin umbelliprenin. J. tums.ac.ir.Daru 2009; 17(2): 99-103.
5. Agrawal AA, Kotaro Konno. Latex: A Model for Understanding Mechanisms, Ecology, and Evolution of Plant Defense against Herbivory. Annual Review of Ecology, Evolution, and Systematics 2009; 40: 311-331.
6. Upadhyay RK. Plant latex: a natural source of pharmaceuticals and pesticides. Int. J Green Pharm 2011; 5:169.
7. Subhasini R, Jeyam M. Traditional medicinal plants used in the healing of skin related problems in Coimbatore district: A Review. World Journal of Pharmaceutical Research 2013; 2(6): 2111-2124.
8. Liggieri C, Obregon W, Trejo S, Priolo N. Biochemical analysis of a papain like protease isolated from the latex of *Asclepias curassavica* L. Acta Biochim Biophys Sin 2009;41 (2): 154-162.
9. Walker MC, Gierse JK. *In vitro* assays for cyclooxygenase activity and inhibitor characterization. Methods Mol Biol 2010;644:131-44.
10. Axelrod B, Cheesebrough TM, Laakso S. Lipoxygenase from soyabean. Methods Enzymol 1981; 71:441-5.
11. Mahato SB, Sen S. Advancede in triterpenoid research, 1990-1994. Phytochemistry 1997; 44(7): 1185- 1236.
12. Okwu DE. Phytochemicals, vitamins and mineral contents of two Nigeria medicinal plants. In: Int. J. Mol. Med. Adv. Sci 2005; 1(4): 375-381.
13. Ramawat K.G, Dass S. Herbal Drugs: Ethnomedicine to Modern Medicine. Springer-Verlag, Berlin; Heidelberg, 2009.
14. Ramu G, Mohan, GK. Preliminary phytochemical and antioxidant study of hydroalcoholic extracts from

selected genera of Indian Lamiaceae. Asian Pacific J.Trop. Biomedicine.2012; 685-688.
15. Ayinde F. A, Bolaji O.T, Abdus-Salaam, R. B, Osidipe O. Functional properties and quality evaluation of "kokoro" blended with beniseed cake *Sesame indicum.* African Journal of Food Science. 2012;6(5):117-123.
16. Okwu D E. Phytochemicals and vitamin content of indigenous species of South Eastern Nigeria. J. Sustain Agric. Environ 2004; 6: 30-34.
17. Pietta PG. Flavonoids as antioxidants. J Nat Prod 2000; 63: 1035-1042.
18. Udegbunam RI, Obinna KN, Udegbunam SO, Chinaka ON, Gregory EO. Evaluation of anti-inflammatory activities of root extracts of *Stephania dinklagei* (Engl.) Diels. African Journal of Pharmacy and Pharmacology 2010; 6: 834-839.
19. Chi YS, Jong HG, Son KH, Chang HW, Kang SS, Kim HP. Effects of naturally occurring prenylated flavonoids on enzyme metabolizing arachidonic acid: cyclooxygenase and lipoxygenase. Biochem. Pharmacol 2001;62:1185-1191.
20. Funk CD. Prostaglandins and leukotrienes: advance in eicosanoid biology.Science 2001; 298: 1871-1875.
21. Indranil Chanda, Smriti Rekha Chanda, Sadhan KR Dutta.Anti-inflammatory Activity of a Protease Extracted from the Fruit Stem Latex of the Plant A*rtocarpus heterophyllus* Lam. Research Journal of Pharmacology and Pharmacodynamics.2009;1(2). 70-72.
22. Kumar VL, Babu N. Anti- inflammatory activity of latex of *Calotropis procera.* Journal of Ethnopharmacology 1994;44(2):123-125.
23. Yasodha Krishna J, Kumar Ponnusamy, Kahsay Getu. Anti-inflammatory activity of alcoholic extract of leaves of *Holostemma ada-kodien* Shult. in albino rats. International Journal of Pharmacy education and research 2016; 3(2): 13-16.

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