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Invitro Anti-Inflammatory Activity of *Pachanamrutham Kashayam*

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Abstract: Inflammation is generally referred to as a complex biological response of vascular tissues to harmful stimuli such as pathogens damaged cells or irritants. *Pachanamrutham kashayam* , also known as *Amruthadi Kashayam* is a *kashaya yoga* used by traditional *ayurvedic* physicians since past due to its efficacy in the management of *jwaram,amam* and all other inflammatory conditions. The source of this *kashaya yoga* is *Sahasrayogam*..The present study compares anti-inflammatory potential of *Pachanamrutham kashayam with a standard anti-inflammatory drug* Diclofenac sodium and also with a LPS stimulated control group. The results were analysed statistically by ANOVA. Results revealed that *Pachanamrutham kashayam* significantly reduces inflammationby inhibiting the inflammatory processes($p < 0.0001$) comparing with control group . Diclofenac sodium has more inhibitory effects on inflammation in the same concentration of *kashayam* . IC 50 values of the *kashayam* and Diclofenac sodium was calculated which gives us a primary idea as to calculate on the dose and frequency of *Pachanamritam kashayam* to get comparable or better results to Diclofenac Sodium.

Keywords: Inflammation, *Aamam*, RAW264.5cells , Diclofenac sodium

1. Introduction

Inflammation is the immune system's response to harmful stimuli, such as pathogens, damaged cells, toxic compounds or irradiation[1]. At the tissue level, inflammation is characterized by redness, swelling, heat, pain, and loss of tissue function, which result from local immune, vascular and inflammatory cell responses to infection or injury[2]. It can be classified as either acute or chronic. During the mechanism of inflammation, arachidonic acid is metabolized by Cyclooxygnase pathway to prostaglandins and thromboxane A_2, whereas the 5-lipoxygenase pathway to eicosanoids and leukotriens (LT's), which are known to act as chemical mediators in a variety of inflammatory events. Accordingly *ayurveda* believes that *aama* is the root cause of metabolic and degenerative disease since it blocks *strotas* or micro channels that nourish tissues. NSAIDs are commonly used for the management of inflammatory conditions. Using higher doses of NSAIDs leads to an increased risk of upper gastrointestinal complications[3]. So the usage of pure herbal formulations has much more importance. Therefore a need arises for the development of newer anti-inflammatory agents and with lesser side effectsas substitutes for chemical therapy. *Pachanamrutham* kashayamis a *kashaya* yoga used by traditional *ayurvedic* physicians since past due to its efficacy in the management of *jwaram, aamam* and all other inflammatory conditions. The source of this *kashaya yogam* is *Sahasrayogam*[4].It is a polyherbal *ayurvedic* formulation and *samekashaya yoga* is also mentioned in the *Chikitsamanjari*[5],where instead of *vasa*, *pata* is mentioned. The main pharmacies that manufacture *Pachanamrutham kashayam* are AVS kottakkal, Vaidyaratnam oushadhasala and AVP Coimbatore.

1.1. Aim and Objectives

To determine the anti-inflammatory effect of *Pachanamrutham kashayam* in-vitro.

2. Materials and methods

2.1. Sample collection

The herbal raw materials were procured from authentic sources and authenticated by *Dravya guna*experts in the Govt. Ayurveda College,Thiruvananthapuram. Foreign matter in the drugs were removed manually, cut into small pieces and washed, cleaned and subjected to drying under shade.

2.2. Sample preparation

The drugs *useera (Vetiveria zizanioides Linn), vasa(Adhathoda vasica Nees) ,mustha(Cyperus rotundus Linn), viswa(Zingiber officinale Roscoe),bhoonimba(Andrographis paniculata Nees), valaka(Plectranthus vettiveroides(Jacob)) ,parpataka(Oldenlandia corymbosa Linn), dhanyaka*(*Coriandrum sativam Linn),* and *dhanvayasha(Tragia involucrata Linn*)are taken in dried form and *guduchI (Tinospora cordifolia(Wild) miers)*is taken in fresh form .All the drugs are crushed well and boiled in 16 times water in a mudpot and reduced to1/8. The resultant decoction was then filtered through clean white cloth (4layered) and the *kashaya* was kept in airtight container after cooling.

Fig. 1. Pictures of samples and assay: 1.Guduchi 2. Useera 3. Vasa 4. Mustha 5. Viswa 6. Bhoonimba 7.Valakam 8. Parpataka 9. Dhanyaka 10.Dhanvayasham

2.3 Experimental analysis - Anti inflammatory assays

Preparation of cell lines - RAW 264.7 cells were grown to 60% confluency. The cells were activated with 1µg/m Llipopolysaccharide (LPS). Exposed the LPS stimulated RAW cells with different concentration (25,50, 100µg/mL) of sample solution and Diclofenac sodium, astandard anti-inflammatory drug in varying concentration corresponding to the sample followed by incubation for 24 hours. After incubation the cell lysate was used for performing the anti-inflammatory assays. Inhibition of COX, LOX, and i NOS and nitrite levels were assessed spectrophotometrically to determine the anti-inflammatory effects of samples.

2.3.1 Assay of Cyclooxygenase (COX) activity

Method of Walker and Gierse was used for theassay of COX activity. The cell lysate was incubated inTris-HCl buffer (pH 8), glutathione 5 mM/L, andhaemoglobin 5 mM/L at 25°C for 60 seconds. Initiatedthe reaction by adding arachidonic acid 200 mM/L andterminated after incubation for 20 minutes at 37°C, bythe addition of 10% trichloroacetic acid in 1 Nhydrochloric acid. After the centrifugal separation,added 1%thiobarbiturate and determined the COXactivity[6] by measuring the absorbance at 632 nm. Percentage inhibition of the enzyme was calculated as,

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

2.3.2 Lipoxygenase (LOX) Activity

Method of Axelrod et al was used for LOX assay.The reaction mixture (2 mL final volume) containedTris-HCl buffer (pH 7.4), cell lysate (50 µL), and sodiumlinoleate (200 µL). The LOX activity was measured as anincrease of absorbance at 234 nm (Shimadzu), whichreflects the formation of 5-hydroxyeicosatetraenoic acid. Percentage inhibition of the enzyme was calculatedusing the formula[7].

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

2.3.3. Myeloperoxidase (MPO) Activity

In a solution containing 50 mM potassiumphosphate buffer and 0.57% hexadecyl trimethylammonium bromide (HTAB), the cell lysate was homogenised. Centrifuged the samples at 2000 g for 30minutes at 4°C, and assayed the supernatant for MPO activity. Added 50 mM phosphate buffer(pH-6)containing1.67mg/mL guaiacol and 0.0005% H2O2 to the sample to activate the MPO. The change inabsorbance at 460 nm was measured. MPO activity was presented as units per mL of cell lysate. One unit of MPO activity was defined as that degrading 1 µM of peroxide per minute at 25°C . Enzyme units for MPO was determined using the formula[8,9].

U= (OD x 4 x Vt x dilution factor)/ L x €460 x t x VsOD is optical density, Vt is total volume in ml, L is light path in cm, €460 ias extinction coefficient of tetraguaiacol, t is the time of measurement in minutes and Vs is sample volume in ml.

2.2.4 Inducible Nitric Oxide Synthase

Method used by Salter et.al was used to determine nitric oxide synthase. The cell lysate was homogenized in 2ml of HEPES buffer. The assay system contained substrate, 0.1ml L-Arginine, 0.1ml manganesechloride, 0.1ml dithiothreitol (DTT), 0.1ml NADPH,0.1ml tetrahydropterin, 0.1 ml oxygenated haemoglobinand 0.1ml enzyme (sample).Increase in absorbance was recorded at 401nm[10] . Percentage inhibition of the enzyme was calculated as,

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

2.3.5 Estimation Of Cellular Nitrite Levels

Method of lepoivre et al was used to estimatethe level of nitrite. 0.1 ml of sulphosalicylic acid wasadded to 0.5 mL of cell lysate and vortexed well for 30minutes. The samples were then centrifuged at 5,000rpm for 15 minutes. Nitrite level was estimated usingthe protein-free supernatant. Added 30 µL of 10%sodium hydroxide to 200 µL of the supernatantfollowed by 300 µL of Tris-HCl buffer and mixed well.Added 530 µL of Griess reagent to this mixture andincubated in the dark for 10–15 minutes. Theabsorbance was measured at 540 nm against a Griess reagent blank. Sodium nitrite solution was used as the standard. The amount of nitrite present in the samples `was estimated from the standard curves obtained[11].

3. Results and discussion

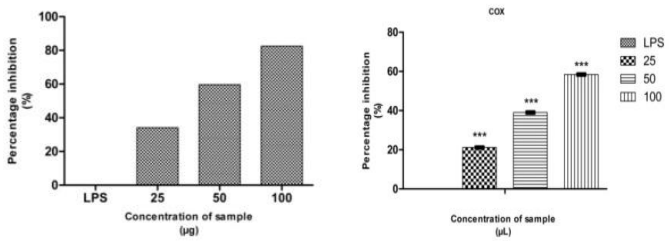
3.1.Results of anti inflammatory assays

3.1.1.Cyclooxygenase inhibitory assay

Pachanamrutham kashayam and Diclofenac sodium exhibited significant inhibition of cyclooxygenase activity in a dose dependent manner.The percentage of inhibition kashayam and Diclofenac sodium are expressed as Mean±SE(n = 3) and analyzed by one-way analysis of variance (ANOVA).

Table 1 : Cyclooxygenase inhibitory assay

Con(µg/ml)	% inhi- Diclofenac Sodium	% inhibition -Kashayam Mean±SE (Average)
LPS in all con	00	0
25	34.12	21.14333±0.313852997
50	59.73	39.05 ± 0.338427737
100	82.50	58.42± 0.344593158



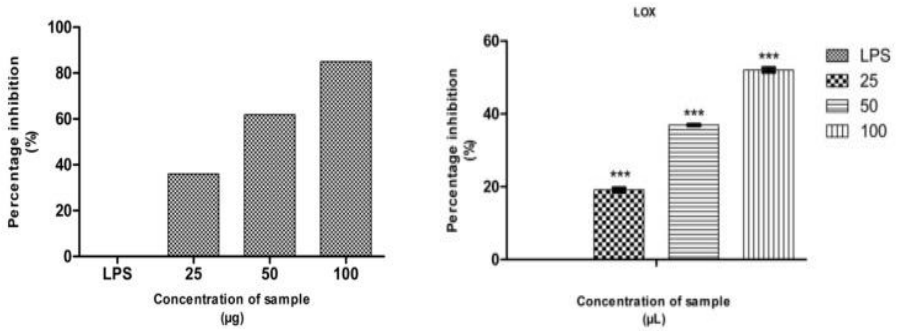
Graph number 1 - Cyclooxygenase inhibitory assay

3.1.2 Lipoxygenase inhibitory assay

Pachanamrutham kashayam and Diclofenac sodium exhibited significant inhibition of Lipoxygenase activity in a dose dependent manner. The percentage of inhibition *kashayam* and Diclofenac sodium are expressed as Mean±SE (n = 3) and analyzed by one-way analysis of variance (ANOVA).

Table 2 : Lipoxygenase inhibitory assay

Concentration(µg/ml)	**%inhibiion- Diclofenac**	**%inhibition -Kashayam Mean±SE (Average)**
LPSin all con		0
25	36.02	19.10667 ±0.323888412
50	61.88	36.93333 ±0.113153452
100	85.02	52.04667 ±0.366034809



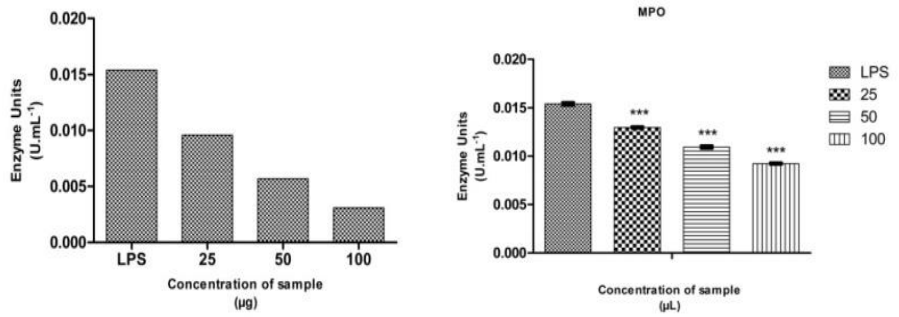
Graph number 2- Lipoxygenase inhibitory assay

3.1.3. Myeloperoxidase (MPO) estimation

Pachanamrutham kashayam and Diclofenac sodium exhibited significant inhibition of Myeloperoxidase enzyme activity in a dose dependent manner. The percentage of inhibition *kashayam* and Diclofenac sodium are expressed as Mean±SE (n = 3) and analyzed by one-way analysis of variance (ANOVA).

Table 3 : Myeloperoxidase (MPO) estimation

Con(µg/ml)	Enzyme Activity (U/ml) Diclofenac	Enzyme Activity (U/ml) Kashayam Mean±SE (Aver)
LPS	0.0154	0.0154±0
25	0.0096	0.012967±0
50	0.0057	0.01094 ±0
100	0.0031	0.009233±0



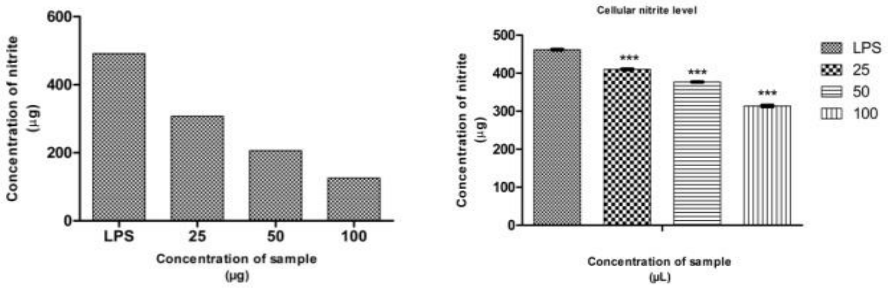
Graph number3Myeloperoxidase (MPO) estimation

3.1.4. Estimation of Cellular Nitrite Levels

Pachanamrutham kashayam and Diclofenac sodium exhibited significant inhibition of Nitrite Levels in a dose dependent manner. The percentage of inhibition *kashayam* and Diclofenac sodium are expressed as Mean±SE (n = 3) and analyzed by one-way analysis of variance (ANOVA).

Table 4 : Cellular Nitrite Levels Estimation

Con(µg/ml)	**% inhibition Diclofenac**	**Cellular Nitrite Levels Mean±SE (Average)**
LPS	491.535	462±0.779759578
25	307.89	410.355±0.99
50	206.91	377.19±0.59491596
100	125.73	313.995±1.533109585



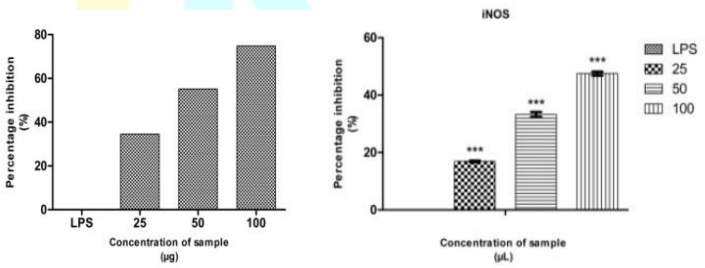
Graph numbe4Cellular Nitrite Levels Estimation

3.1.5. Inducible Nitric Oxide Synthase determination

Pachanamrutham kashayam and Diclofenac sodium exhibited significantreduction in the inducible nitric oxide synthase level in a dose dependent manner. The percentage of inhibition kashayam and Diclofenac sodium are expressed as Mean±SE (n = 3) and analyzed by one-way analysis of variance (ANOVA).

Table 5 : Nitric Oxide Synthase determination

Con(µg/ml)	% inhibition-Diclofenac	% inhibition -Kashayam Mean±SE (Average)
LPS	0	0
25	34.55	16.90667±0.153526448
50	55.14	33.28333±0.491870956
100	74.81	47.52±0.388344063



Graph number 5 Nitric Oxide Synthase determination

3.2. IC50 calculation (Comparing invitro anti-inflammatory activity of Diclofenac Sodium and *Pachanamrutham kashayam*)

IC 50 values of *Pachanamrutham Kashayam* and Diclofenac sodium were evaluated using graphical method.

Table 6: IC50 Calculation

IC50 Values	Diclofenac sodium	*Pachanamritam Kasayam*	IC 50 (Kashayam : Diclofenac sodium)
COX	42.50	78.75	1.85 :1
LOX	38.75	93.75	2.42 : 1
i NOS	43.75	110	2.51 : 1

3.3 Discussion

Inflammation is a natural response of immune and non-immune cells against infection caused by exogenous pathogens, leading to alteration in tissues and organs. Vasodilation, increased vascular permeability, cellularinfiltration, changes in the biosynthetic, metabolic andcatabolic profiles of many organs and activation of cells of immune system and enzyme systems of blood plasma are the main inflammatory responses. The metabolism of arachidonic acid has a significant role in the inflammation mechanism. NSAIDs although structurally heterogeneous, possess a common mode of action which is to inhibit the cyclo-oxygenase(COX) enzyme in the arachinodonic acid cascade to prevent the synthesis of prostanoids(PGs,PG12,TXA2). Arachidonic acid can be metabolised to prostaglandins and thromboxane A2 by cyclooxygenase (COX) pathway and to hydroperoxy-eicosatetraenoic acids (HPETE's)and leukotrienes (LT's) by 5lipoxygenase (5-LOX) pathway. Inhibition of 5-LOX and COX decreases the production of these biologically active mediators of inflammation. So agents that inhibit these enzymes can act as anti – inflammatory can act as anti-inflammatory agents. NSAID s are generally used for the symptomatic relief of many inflammatory conditions.Inflammation is implicated in the pathogeneses of arthritis, cancer, stroke, neurodegenerative and cardiovascular disease. Long term use of NSAIDs can lead to various harmful effects. So it is necessary to explore plants to obtain traditional herbal medicines.*Pachanamrutham Kashayam* is a polyherbal ayurvedic formulation.The ingredients of this *kashayam* are *amrutha, useera, vasa, abda, viswa, bhoonimba, valaka,parpataka, dhanyaka and dhanvayasha*. In *ayurvedic* view , the pathogenesis of inflammation includes the involvement of *ama and tridoshas.*

The aim of the present study is to do invitro anti inflammatory activity of *Pachanamrutham kashayam* using five anti inflammatory assays and compared each other. It is compared with standard drug Diclofenac sodium in three different concentrations (25 µg/mL, 50 µg/mL, and 100 µg/mL).LPS stimulated cells were taken as control group.In all the five assays maximum percentage of inhibition of enzyme activity was obtained at the concentration 100µg/mL.As per the results obtained from the above five assays, in both the cells treated with Dicofenac Sodium as well as *kashayam*, as the concentration increased from 25 µg/ml to 100 µg/ml, the percentage of inhibition also showed corresponding increase and the maximum percentage of inhibition was obtained in the highest concentration of 100 µg/ml while the control group shows zero percentage of inhibition. Statistical analysis was done and results represented as Mean+/- SE. One-way ANOVA Dunnets test were performed to analyse data. From the table ANOVA followed by LSD test analysed that the average percentage inhibition of *Pachanamrutham kashayam* and Dicofenac Sodium in all the five assays . From the results, it may be concluded that the polyherbal decoction *Pachanamrutham kashayam* significantly reduced the inflammation by inhibiting the inflammatory processes ($p<0.001$) . But Diclofenac sodium had shown more efficient anti-inflammatory effect than the *Pachanamrutham kashayam in comparable concenteration.*. *Pachanamrutham kashayam* at higher concentration can achieve comparable results to Diclofenac sodium. As the study was not done in higher concenterations, with the available data, IC 50 are calculated with percentage of inhibition values COX , LOX and iNOS of Diclofenac sodium and *Pachanamrutam Kashayam* using graphical presentation. From the IC50 result of COX It was found that *Pachanamrutam kashayam* need to be given in a dose equal to 1.85 times of Diclofenac Sodium to get comparable results. From the IC50 result of LOX It was found that *Pachanamrutam kahayam* need to be given in a dose equal to 2.42 times of Diclofenac Sodium to get comparable results. From the IC50 result of iNOS, it was found that *Pachanamutam kasayam* need to be given in a dose equal to 2.51 times of Diclofenac Sodium to get comparable results.

4. Conclusion

The present study "*Invitro anti-inflammatory activity of Pachanamrutham kashayam*" can be concluded as *Pachanamrutham kashayam* showed significant dose dependant anti-inflammatory activity in all the five anti-inflammatory assays by inhibiting the inflammatory processes ($p<0.001$) . But, the standard drug Diclofenac Sodium has showing higher anti-inflammatory activity than the *Pachanamrutham kashayam since* the Diclofenac Sodium shows high percentage of inhibition in all the five anti inflammatory assays , in all the three concentrations ie25 µg/mL, 50 µg/mL,100 µg/mL.. So *Pachanamrutham kashayam* at higher concentration can achieve comparable results to Diclofenac Sodium . As the study was not done in higher concenterations ,with the available data , IC 50 values are calculated with percentage of inhibition in COX , LOX and iNOS assays of Diclofenac sodium and *Pachanamrutam kashayam* were using graphical presentation.This knowledge gives us a primary idea as to calculate on the dose and frequency of *Pachanamrutam Kashayam* to get comparable or better results to DiclofenacSodium. The formulation under study being a herbal formulation with all its ingredients being potent anti inflammatory drugs and safe to use on prolong as well as increased intake , can be judiciously adviced to all the inflammatory conditions in all range of patients with adequate *anupana* as per the condition of the patients. Further the drug being mentioned in *amahara* and *jwaraharaprakarana,* is aimed at resolving the basic metabolic imbalance and thereby resolving the inflammatory pathogenesis.

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