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Evaluation of in Vitro Anti-Inflammatory Potential of *Annona Squamosa* Linn Fruit Extract

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ABSTRACT:

Inflammation is a reaction of living tissues towards injury; it consists of bothsystemic and local responses. Inflammation initiates with vasodilatation, leads to the leakage of plasma, neutrophils and other cytokines at the injury site. The mediators of this phase are nitric oxide, neutrophils and macrophages. The migration of fluid into the wound site creates oedema, which contributes to the sensation of pain that characterizes inflammation. *Annonasquamosa* Linn is a deciduous plant used in traditional medicine. The intensified studies on *Annonaceae* species over the last decade showed that this family is a potent source of a wide variety of secondary metabolites. The *Annonasquamosa*Linn plants are traditionally used for the treatment of epilepsy, dysentery, cardiac problems, worm infestation, constipation, haemorrhage, antibacterial infection, dysuria, fever and ulcer. The bark and leaves possessed cytotoxicity,analgesic and anti-inflammatory activity. *Annona squamosa* Linn reported to contain alkaloids, aminoacids, glycosides, saponins, carbohydrates, flavonoids, tannins, proteins, phytosterols and phenolic compounds. The various chemical constituents isolated from leaves, stem and root of the plant including anonaine, norcorydine, aporpie, isocorydine, coryeline, and glaucine. Anti-inflammatory activity was confirmed by carrying out HRBC membrane stabilization assay and Protein denaturation test.

KEYWORDS: Anti-inflammatory, *Annonasquamosa*Linn, HRBC membrane stabilization, Proteindenaturation

INTRODUCTION

[1] Inflammation is a normal protective response to tissue injury that is caused by physical trauma, noxious chemicals or microbiological agents. [2] Inflammation is referred to as a complex biological response of vascular tissues to harmful stimuli. Inflammation involves in an increase of protein denaturation, an increase of vascular permeability and membrane alteration [2]. The cells undergo activation and release inflammatory mediators at the onset of inflammation. [3] These mediators include histamine, serotonin, slow-reacting substances of anaphylaxis, prostaglandins and some plasma enzyme such as the complement system, the fibrinolytic system and the kinin system. Initial phase of inflammatory process is marked by an acute phase where the cascade begins with primary response of immune and vascular systems right after damage to tissues and or infection. [4]This phase is rapid and with short duration. Acute inflammation also acts as a homeostatic mechanism.

The inflammatory responsesare designed to initiate and enhancehealing of body tissues. [5]The uncontrolled progression of such pro-inflammatory activities as part of the immune response has been associated with oxidative stress and other diseased conditions such as cancer, diabetes, hypertension, septic shock, asthma, arthritis, atherosclerosis, Parkinson's and Alzheimer's diseases. [6] Inflammatory diseases including different types of rheumatoiddiseases are very common throughout the world. The rheumatism, inflammation mediated disorder which is one of the oldest known disease which affects a large population of the world.

[7] Inflammation is usually treated by non-steroidal anti-inflammatory drugs (NSAIDs) and steroidalanti inflammatory drugs which are useful for reducing swelling and pain from inflammation. [8] NSAIDs represent a common class of anti-inflammatory and analgesic drugs for alleviating symptoms associated with inflammation by inhibiting the enzyme cyclooxygenase (COX). There are many side effectsassociated with the use of NSAIDs.The main mechanism of action of NSAIDs is the inhibition of prostaglandin (PG) synthesis or preferentialCOX -2 inhibition. [9]Due to prostaglandin synthesis inhibition, some toxic effects like bleeding, inhibition of platelet function, gastric mucosal damage, asthma and anaphylactic reactions occurs to some individuals. Aspirin, Celecoxib, Diclofenac, Etodolac, Flurbiprofen, Fenoprofen, Ibuprofen, Indomethacin, Ketoprofen, Ketorolac, Sulindac, Mefenamic

acid, Naproxen etc. are the examples of some FDA approved NSAIDs.The prolonged use of non-steroidal anti-inflammatory drugs (NSAIDs) such as Aspirin, and Coxibs includes increased risk of gastro-intestinal and cardiovascular complications.

[10] As the syntheticNSAIDs possessing several adverse side effects, including gastric irritation which finally leads to formation of gastric ulcers as well as serious cardiovascular adverse effects as mentioned above the search for safe natural sources and phytochemicals with anti-inflammatory potential has gained great interestin recent years.

[11,12] The use of natural products having therapeutic activities is an ancient practice for human civilization in which plants, animals and mineral products were the primary source of drugs belonging to this category [11].According to the WHO survey, 80% populations living in the developing countries following the practice to rely almost exclusively on traditional medicine for their primary health care need.

[13] The herbal natural products are the major sources of remedies for most human diseases. Natural products are usually the first point of call in the management of acute and chronic health complications owing to their predilection to availability, efficacy, and reported minimal side effects. Due to these factsthere is a growing demand for plant based medicines, health products, pharmaceuticals, food supplements, cosmetics etc.

GENERAL INFORMATION--*ANNONA SQUAMOSA* L.

*Annonasquamosa*Linn is a deciduous plant used in traditional medicine.*Annonasquamosa*Linn belonging to family Annonaceae is commonly found in India and cultivated in Thailand and originated from West Indiesand South America.

[14]The genus name ANNONA is from Latin word ‘ANON’, meaning ‘yearly produce’, referring to the production of fruits of the various species in this genus

Figure 1: *Annonasquamosa* Fruit

Synonyms

English- Custard apple, sugar apple, sweet apple.

Hindi- Sitaphal, Shareepha

Telugu-Sita-phalam

Taxonomy

Kingdom: Plantae

Order: Magnoliales

Family: Annonaceae

Genus: Annona

Species: Squamosa

[15]*Annonasquamosa* is known for its edible fruits, and the tree grows as a small sapling, rising from 3m and reaching up to 8m having brownish or light brownish bark with thin leaves. *Annonasquamosal* has been utilized as a natural medicine and in various other food applications. E.g.; its pulp as Flavoring agent in ice cream, and also for making juice.

[17,18]It contains appreciable vitamin C in the range of 35-42 mg per 100g, and dietary fiber, vitamin B1 and potassium contents also. Phytochemical analysis of *Annonasquamosal* revealed the presence of various phytochemicals such as proteins, carbohydrates, saponins, alkaloids, flavonoids, phenolic and glycosides..

[15]Extracts obtained from various sections of the *Annona squamosal* plant, such as its bark, roots, leaf, tem, fruit, peel and seeds have been utilised in traditional pharmacological applications in different countries to cure a diseases, such as epilepsy, haemorrhage, dysentery, fever and tumours.Manystudies on *Annona squamosal* have reported the biological activity of the plant as an anti-diabetic, anti-tumour, antimalarial and anthelminthic potential.

MATERIALS AND METHODS

The fruits of *Annonasquamosa* (500g) were collected from Pariyaram, Kannur, Kerala (India) in the month of October 2022 and authenticated by Dr. Harikrishnan.E, Department of Botany Payyanur College Kannur. A voucher specimen (No- 722001)wasdeposited in the Department of Pharmacology, College of Pharmaceutical Sciences, Government Medical College, Kannur.

Extraction

After authentication, the fruits were cleaned well and the pulp wasseparated from seeds and made a thick paste.The paste prepared is subjected for maceration with ethanol and water (hydro-ethanolic solution 95%) for 72 hours.The extract was shaken thoroughly, filtered through muslin cloth and marc was discarded. The filtrate obtained is then evaporated and stored for further use.

Preliminary Phytochemical Investigation

The extractof *Annonasquamosa* was subjected to phytochemical tests for carbohydrates, alkaloids, saponins, flavonoids,tannins, phenolic compounds, glycosides aminoacids, proteins, triterpenoids etc.

ESTIMATION OF *IN VITRO* ANTI-INFLAMMATORY ACTIVITY

The human red blood cell (HRBC) membrane stabilization assay and protein denaturation assay methods were employed for *in vitro* anti-inflammatory evaluation of *Annonasquamosa* extract.

[16,19] Human red blood cell (HRBC) membrane stabilization assay

The HRBC method was used for the estimation of anti-inflammatory activity *in vitro*, as the erythrocyte membrane is analogous to the lysosomal membrane. Stabilization of lysosomal membrane is necessary for preventing the release of lysosomal constituents of activated neutrophils and proteases, thereby the inflammatory response.

Procedure

The collected blood from the blood bank, GMC, Kannur was mixed well with equal volume of Alsever's solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid, 0.42% sodium chloride) and centrifuged at 3000 rpm for 20 minutes. The packed cells were separated and washed with isosaline, and a 10% suspension was made. 37.5,75,150, and 300 µg /ml concentrations of extract was prepared using distilled water. To each concentration 1ml of phosphate buffer, 2ml hyposaline, 0.5% of HRBC suspension were added. It is incubated at 37 c for 3o minutes. Various concentrations of Diclofenac sodium (75mg/ml) 37.5, 75, 150 and 300 µg/ml were used as reference and control was prepared without standard or test. The hemoglobin content of the supernatant solution was estimated spectrometrically at 560nm.The percentage of HRBC membrane stabilization or protection was calculated using the following formula;

$$\% \text{ Protection} = \frac{\text{Ab(control)} - \text{Ab(test)}}{\text{Ab(control)}} \times 100$$

Ab(control) – absorbance of control.

Ab(test) – absorbance of test.

[16] **Protein denaturation assay**

In protein denaturation process, protein lose their tertiary and secondary structure by applying external stress or by use of compounds such as strong acid or strong base, organic solvent, a concentrated inorganic salt or heat. If denatured thebiological protein lose their biological function. This denaturationof protein is the cause of inflammation. In other words by preventing this protein denaturation, the inflammation can be prevented.

Procedure:

The hydro alcoholic extract of *Annonasquamosal* fruit pulp and a standard drug (diclofenac sodium-75mg/ml) were prepared in the concentration of 37.5,75,150,300, µg/ml. Then with 0.2ml of egg albumin (egg white), 2.8ml of phosphate buffered saline (pH 6.4) and 2ml of different concentration of *Annonasquamosal* and standard were mixed well. Similar volume of distilled water is taken as blank. This mixture is then incubated at 37°C for 15

minutes in the incubator and then heated at 70°C for 5 minutes. After cooling absorbance was measured at 660 nm using vehicle as blank.The percentage inhibition of denaturation was found out as follows

$$\% \text{ Protection} = \frac{\text{Ab(control)} - \text{Ab(test)}}{\text{Ab(control)}} \times 100$$

Ab(control) – absorbance of control.

Ab(test) – absorbance of test.

RESULTAND DISCUSSION

1. Phytochemical constituents

The results obtained from phytochemical screenings were given in the following table. (Table.1)

Table 1. Screening of Phytochemical

CONSTITUENTS	TESTS	OBSERVATION
Test for Carbohydrates	Fehling's test	+
Test for Alkaloids	Wagner's test	+
Test for Saponins	Foam or froth test	+
Tannins and phenolic compounds	Lead acetate test	+
Test for Glycosides	Legal's test	+
Test for Proteins and Amino acids	Million's test	+
Test for Triterpenoids	Salkowoski test	+
Flavonoids	Alkaline reagent test	+

The extract showed positive results for a wide range of constituents which elevated the therapeutic importance of these fruits. The valuable constituents include carbohydrates, alkaloids, saponins, tannins, phenolic compounds, glycosides amino acids, proteins andtriterpenoids. The tannins, phenolic constituents as well as flavonoids are well known free radical scavengers via possess promising therapeutic effects by controlling release of reactive oxygen species (ROS) which can be safely employed in the effective management of various complications of many acute and chronic disorders.

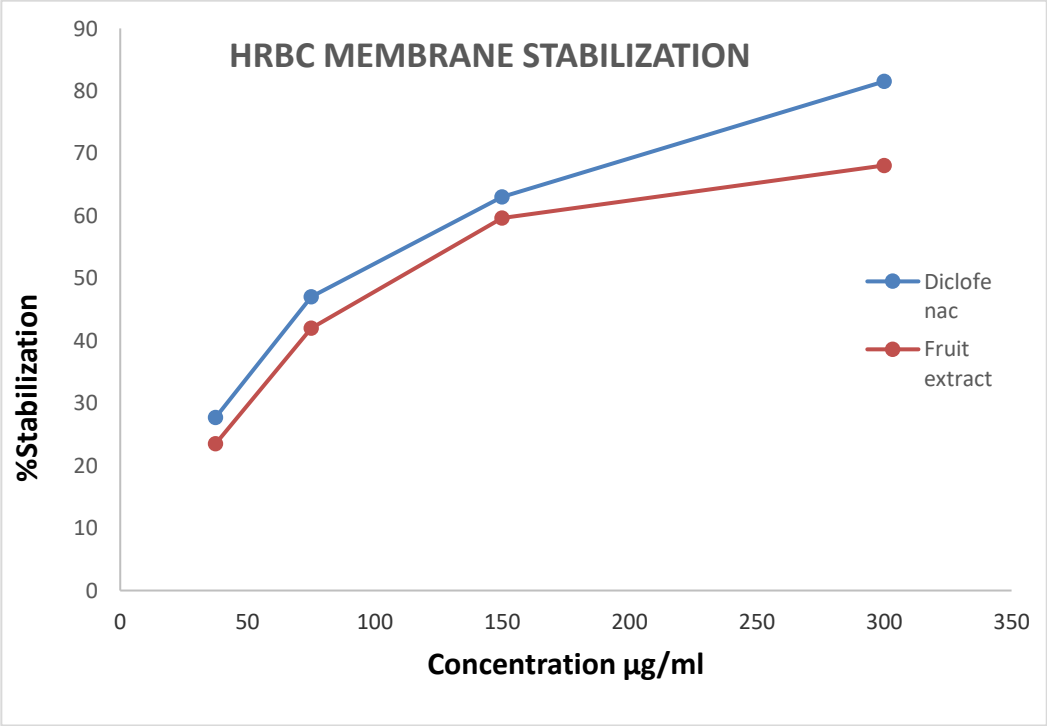
2. *In vitro* anti-inflammatory activity

2.1.Hrbc membrane stabilization assay

The results of membrane stabilisation of test, standard were stated in the following table (Table.2)

Table2. Effect of ethanolic extract of *Annona squamosal* on HRBC membrane stabilization assay

SI NO	GROUP	CONCENTRATION (µg/ml)	ABSORBANCE	PERCENTAGE OF STABILIZATION (%)	IC_{50} VALUE
1	Control		0.119± 0.0171	-	-
2	Standard (Diclofenac sodium)	37.5	0.086± 0.0171	27.73	100
		75	0.063±0.0132	47.05	
		150	0.044±0.0126	63.02	
		300	0.022±0.01149	81.51	
3	Ethanolic extract of Extract	37.5	0.091±0.0127	23.52	125
		75	0.069±0.0163	42.01	
		150	0.048±0.152	59.66	
		300	0.038±0.154	68.06	



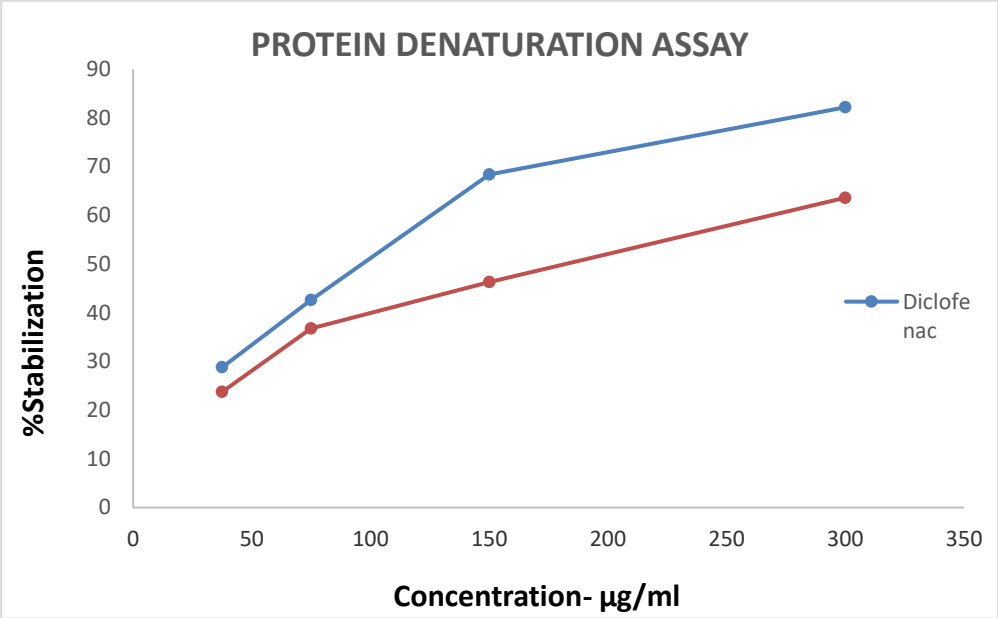
As of the above results it was clear that the extract possess membrane stabilization potential as that of the standard drug Diclofenac sodium. A dose dependent increase in the effect was also evident from the results. These facts indicate the possibility of an effective anti-inflammatory property for the extract.The IC_{50}values of standard drug as well as extract were 100 and 125 respectively.

2.2 Protein denaturation assay

The results of inhibition of protein denaturation by test and standard were given in the following table (Table.3)

Table 3. Effect of ethanolic extract of *Annona squamosal* on protein denaturation assay

SI NO.	GROUP	CONCENTRATON(µg/ml)	ABSORBANCE	% INHIBITION	IC 50 VALUE
1	Control		2.817± 0.0153		-
2	Standard (Diclofenac sodium)	37.5	2.006±0.0142	28.78	100
		75	1.617±0.0137	42.59	
		150	0.891±0.0175	68.37	
		300	0.502±0.0132	82.17	
3	Ethanolic extract of Annonasquamosa	37.5	2.148±0.0158	23.74	185
		75	1.782±0.0129	36.74	
		150	1.513±0.0125	46.29	
		300	1.025±0.0172	63.61	



The demonstrated results above stated that the extract exhibited promising ability to prevent denaturation of proteinscompared to that of the standard Diclofenac sodium. The extract also showed dose dependent increase in the effect from 75 to 300µg/ml. The fruit extract and standard found to have an IC50 value as 185 and 100 respectively.

CONCLUSION

The herbal source based research is getting a great boom in these decades to their safety and therapeutic potency. The Phytochemical investigations of fruit extract showed positive results for various valuable constituents such ascarbohydrates, alkaloids, saponins, tannins, phenolic compounds, glycosides, amino acids, proteins and triterpenoid. These phytoconstituents can ensure a wide and varying therapeutic potential as well as safety of the extract. The extract exhibited a prominent human RBC membrane protection with an IC_{50} value which was comparable to that of aneffective NSAID drug Diclofenac. The protein egg albumin denaturation was also effectively prevented by various doses of the extract. The effect was dose-dependent as that of the standard. The RBC stabilization and inhibition of albumin denaturation might be due to the regulation of release of free radicals, inflammatory mediators, cytokines, leukotrienes and so on by the valuable constituents present in the extract like tannins, flavonoids, phenolic constituents etc. A detailed *in vivo* pharmacological and clinical examination are yet to be conducted to exploit the whole pharmacological and therapeutic potentials of the extract. The individual constituent elucidation and path way analysis is also needed to understand the real ongoing mechanism of the extract completely. As a base this work could enrich the awareness regarding varying chemical constituents and their therapeutic potentials exhibited by this extract which can be applied in future research for the betterment of public health.

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