

Original Article

IN VITRO ANTI-INFLAMMATORY ACTIVITY OF SYRINGIC ACID

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ABSTRACT

Objective: The present study was carried out to investigate the *in vitro* anti-inflammatory activity of syringic acid (SA).

Methods: SA was tested for its *in vitro* anti-inflammatory activity at different concentrations in protein denaturation, proteinase inhibition and human red blood cell (HRBC) membrane stabilization assay. The reference drugs used were aspirin and diclofenac sodium.

Results: SA showed concentration-dependent inhibition of protein denaturation and proteinase activity with a half-maximal inhibitory concentration (IC₅₀) value of 49.38±0.56 µg/ml and 53.73±0.27 µg/ml respectively. Heat-induced haemolysis was inhibited by SA with an IC₅₀ value of 57.13±0.24 µg/ml. SA also inhibited the hypotonicity-induced haemolysis (IC₅₀ value of 53.87±0.72 µg/ml).

Conclusion: From the present study, we can conclude that SA possesses appreciable anti-inflammatory effect against denaturation of proteins, proteinase activity, and human red blood membrane stabilization assays. Further studies are required for determining the possible mechanisms behind its anti-inflammatory action.

Keywords: Syringic acid, Antioxidant, Anti-inflammatory, Protein denaturation, Proteinase inhibition, Membrane stabilization

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INTRODUCTION

Inflammation is the protective mechanism present in the body against injury or damage. In case of an injury to a particular tissue, the adjacent tissues take part in the process of inflammation, to heal the affected tissue. There are a number of agents that may damage or kill the cells, such as trauma, heat, cold, radiation, certain chemicals like acids or organic poisons such as paraquat. Inflammation may be caused by the invasion of immunological agents and microbes such as bacteria, viruses, and parasites [1].

The release of chemical mediators like prostaglandins, histamine, serotonin, kinins, and cytokines present at the site of injury is responsible for triggering the inflammatory response. These mediators are known as biological messengers that act on the inflammatory cells and contribute to the development of the inflammatory response. The complex process of inflammation involves an increase in the vascular permeability, denaturation of proteins and alteration in the membrane stability of the injured cells [2].

Inflammation can be grouped as acute or chronic, depending on the severity of the condition. Acute inflammation is one that involves only the initial stages of the process marked by the exudation of plasma and white blood cells from the blood to the site of injury. In cases, where the causative agent of inflammation is not successfully removed, the acute inflammation is then followed by chronic inflammation [2].

The current regime for the management of inflammation includes the administration of the non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids and opioids. These therapies suffer from the drawback of long-term toxicity and variation in clinical efficacy [3]. Syringic acid (SA) (4-hydroxy-3,5-dimethoxybenzoic acid) is a polyphenolic compound (fig. 1) found in abundance in olives, dates, spices, pumpkin, grapes, acai palm, honey, and red wine. SA is also present in large quantities in plants namely, *Annona squamosa*, *Catunaregam*, *Raphanus sativus*, *Hemidesmus indicus* and *Tagetes erecta* Linn flower [4]. SA has previously shown to possess strong antioxidant properties along with neuroprotective, hepatoprotective, anti-angiogenic, anti-steatotic, anticancer and anti-osteoporotic activities [5-12].

Extracts of a number of plants containing SA have demonstrated anti-inflammatory activity [4]. A survey of the literature showed that no systematic approach was undertaken to evaluate the anti-inflammatory potential of SA *in vitro*. The aim of the present study was to evaluate the anti-inflammatory potential of SA by assessing its effect on protein denaturation, proteinase activity, and membrane stabilization assays.

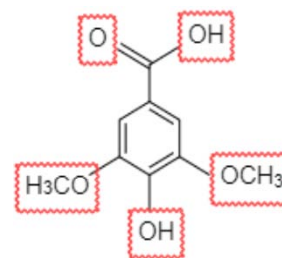


Fig. 1: Structure of syringic acid

MATERIALS AND METHODS

Drugs and chemicals

Syringic acid was purchased from Tokyo Chemical Industry Ltd. (Mumbai, India). Diclofenac sodium and aspirin were procured from SD Fine Chemicals Ltd. (Mumbai, India). The other chemicals were obtained from commercial sources and were of analytical grade.

In vitro anti-inflammatory activity

Protein denaturation assay

The assay was performed according to the procedure described previously with slight modifications [13]. The reaction mixture prepared consisted of 1 ml of SA at different concentrations (table 1), to which 1 ml of 1% bovine serum albumin was added. The pH of the reaction mixture was adjusted to 7.4, and the mixture was incubated at room temperature for 20 min. This was followed by

heating at 56 °C for 20 min. The samples were left to cool to room temperature and the turbidity developed was measured spectrophotometrically at 660 nm. The samples were prepared in triplicates.

Percentage inhibition of protein denaturation was calculated by: $[(Ac - At)/(Ac)] \times 100$

Where Ac denotes the absorbance of the control and At denotes the absorbance of the test sample.

Proteinase inhibition assay

The test was performed according to the method provided in the literature with minor modifications [13, 14]. To 100 µl of the test sample prepared at varying concentrations (table 1), was added 100 µl of 1% bovine serum albumin and the reaction mixture was incubated at room temperature for 5 min. 250 µl of trypsin was added, followed by centrifugation. The absorbance of the supernatant was noted at 210 nm using a UV-visible spectrophotometer. The samples were prepared in triplicates.

Percentage inhibition of proteinase activity was calculated by: $[(Ac - At)/(Ac)] \times 100$

Where Ac denotes the absorbance of the control and At denotes the absorbance of the test sample.

Membrane stabilization assay

Preparation of human red blood cell (HRBC) suspension

Blood was collected (approval number ICT/IAEC/2017/P59) and taken into heparinized centrifugation tubes. The blood was mixed with sterile Alsever's solution in equal proportion. The resulting mixture was then centrifuged at 3000 rpm for 10 min. The supernatant plasma was removed, and the erythrocyte sediment was washed thrice with normal saline. The blood was reconstituted with isotonic phosphate buffer, whose pH was adjusted to 7.4, and a 10% v/v human red blood cell (HRBC) suspension was prepared [15].

Heat-induced haemolysis

Different concentrations of SA (10, 20, 40, 60, 80 and 100 µg/ml) were prepared in isotonic phosphate buffered solution. The reaction mixture consisted of 5 ml of the test drug solution and 0.1 ml of HRBC suspension. The control tube composed of 5 ml of the vehicle (isotonic phosphate buffer) and the standard tube consisted of 5 ml of diclofenac sodium (prepared in isotonic phosphate buffer at concentrations of 100 and 200 µg/ml). To each of the control and standard tube were added 0.1 ml of HRBC suspension. All of the tubes were then incubated at 56 °C for a duration of 30 min. After the incubation period, the tubes were allowed to cool to room temperature, followed by centrifugation at 3000 rpm for 5 min. The spectrophotometer was used to measure the absorbance of the supernatant at 540 nm. The assay was performed in triplicates [15, 16].

Percentage inhibition of heat-induced haemolysis was calculated by:

$$[(Control\ OD - Test\ OD) / (Control\ OD)] \times 100$$

Where Control OD is the optical density of heated control and Test OD is the optical density of the heated test sample.

Hypotonicity-induced haemolysis

SA was dissolved in distilled water to prepare the different concentration of the test sample (10, 20, 40, 60, 80 and 100 µg/ml). The reaction mixture consisted of 5 ml of the test sample, to which was added 0.1 ml of HRBC suspension. The control tubes contained 5 ml of distilled water, and the standard tube contained diclofenac sodium dissolved in distilled water to obtain the concentrations of 100 µg/ml and 200 µg/ml. To each of the control and standard tube were added 0.1 ml of HRBC suspension. The reaction mixture was incubated at 37 °C for 1 h, followed by centrifugation at 3000 rpm for 5 min. The spectrophotometer was used to measure the absorbance of the supernatant at 540 nm. The assay was performed in triplicates [15].

Percentage inhibition of hypotonicity-induced haemolysis was calculated by:

$$[(Control\ OD - Test\ OD) / (Control\ OD)] \times 100$$

Where Control OD is the optical density of hypotonic control and Test OD is the optical density of the hypotonic test sample.

Statistical analysis

Data obtained are expressed as the mean±standard deviation (SD) (n=3). The half-maximal inhibitory concentration (IC₅₀) value was calculated using the linear regression analysis.

RESULTS AND DISCUSSION

Protein denaturation is a well-known cause of inflammation. Denaturation of a protein involves the disruption of the tertiary and secondary structure of the proteins caused by the application of external stress, leading to loss of its biological function [14, 17]. The transformation of protein from soluble to an insoluble form is known as denaturation [18]. The mechanism of denaturation involves the change in disulphide and hydrogen bonds, along with an alteration in the hydrophobic and electrostatic forces of attraction [19]. As part of the investigation on the mechanism of the anti-inflammation activity, the ability of SA to inhibit protein denaturation was studied. The inhibition of protein denaturation by SA was produced in a dose-dependent manner. The IC₅₀ value of SA was found to be 49.38±0.56 µg/ml. SA at 100 µg/ml exhibited comparable protein denaturation inhibition with that of the standard drug, aspirin (table 1).

Neutrophils play an important role in the pathogenesis of inflammation that release mediators such as serine proteinases localized in the lysosomes. These leukocyte proteinases act as key participants in the development of tissue damage. Inhibition of these proteinases has suggested the anti-inflammatory action of drugs [20, 21]. SA showed inhibition of proteinase activity in a concentration-dependent manner. The IC₅₀ value of SA was calculated to be 53.73±0.27 µg/ml. SA at a concentration of 100 µg/ml showed inhibition of proteinase activity similar to aspirin (table 1).

Table 1: Effect of syringic acid on protein denaturation, proteinase inhibition, and membrane stabilization assay

	Mean % inhibition				
	Concentration (µg/ml)	Protein denaturation	Proteinase Inhibition	Heat-induced haemolysis	Hypotonicity induced haemolysis
Syringic acid	10	31.12±1.23	9.020±0.25	35.53±0.98	17.91±0.94
	20	35.91±0.78	18.39±0.81	35.71±1.55	44.17±1.43
	40	45.50±0.29	37.13±0.89	46.69±1.87	48.65±1.76
	60	55.09±1.14	55.87±0.97	66.42±0.67	57.61±0.69
	80	64.68±0.97	74.61±1.18	86.24±0.82	88.76±0.38
100	74.26±0.87	93.35±0.52	117.92±0.56	118.44±1.34	
Aspirin	100	87.46±0.40	92.44±0.31	----	----
Diclofenac Sodium	200	----	----	69.47±0.76	77.57±0.31
IC ₅₀ (µg/ml)	----	49.38±0.56	53.73±0.27	57.13±0.24	53.87±0.72
Correlation coefficient (r)	----	0.978	0.998	0.997	0.967

Values are expressed as mean±SD (n=3). Linear regression analysis was used to calculate the IC₅₀ values.

The HRBC membrane stabilization assay is used as a method to study the *in vitro* anti-inflammatory activity of potential compounds because the red blood cell membrane is analogous to the lysosomal membrane [22-24]. One of the limiting steps in the process of inflammatory response is the stabilization of the lysosomal membrane, which would prevent the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases, leading to further tissue inflammation and damage upon extracellular release [25, 26]. The haemolysis of red blood cell can be induced by heat as well as hypotonicity. In hypotonicity-induced haemolysis, thereby occurs shrinkage of cells, characterized by the release of intracellular electrolytes and fluid components that takes place as a result of osmotic loss [27]. SA has shown to inhibit the haemolysis triggered by both heat and hypotonicity in a dose-dependent manner probably owing to the ability of SA to stabilize the membranes. This action may be attributed to the anti-inflammatory activity of SA. The heat-induced haemolysis was inhibited by SA with an IC₅₀ value of 57.13±0.24 µg/ml. On the other hand, SA showed inhibition of hypotonicity-induced with an IC₅₀ value of 53.87±0.72 µg/ml. SA exhibited comparable anti-inflammatory activity as that of the standard drug, diclofenac sodium (table 1).

Our results indicated that SA possesses anti-inflammatory properties *in vitro*. The results of the present study are consistent with the findings of previous studies that have reported similar observations with regards to the anti-inflammatory activity of the plant extracts containing SA [28, 29].

CONCLUSION

In conclusion, the present preliminary study using *in vitro* anti-inflammatory assays suggested that SA has potential anti-inflammatory and anti-arthritis effects. Further work can be done on testing the anti-inflammatory activity of SA using *in vivo* models and studying its underlying mechanism of action.

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AUTHORS CONTRIBUTIONS

Shilpee Chanda as a first author was involved in the designing of the work, performing the experiments, data analysis, data interpretation and drafting of the manuscript. Archana R. Juvekar as the corresponding author was involved in the critical revision of the article and approval of the final manuscript.

CONFLICT OF INTERESTS

The authors have no conflict of interest to declare

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