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Research Article

Evaluation of *in-vitro* Anti-inflammatory Activity of Sinapic Acid

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ABSTRACT

Sinapic acid (SA) is a potent nutraceutical or natural bioactive compound which is commonly present in the human diet. It was evaluated for its anti-inflammatory activity by *in- vitro* methods like albumin denaturation assay, heat-induced and hypotonicity induced haemolysis, Cycloxygenase-2 (COX-2), and lipoxygenase (5-LOX) inhibition assay at different concentrations. Aspirin (200 µg/mL) and Indomethacin (100 µg/mL) were used as standard drugs. The results showed that Sinapic acid at a concentration range of 10-500 µg/mL significantly ($p < 0.001$) protected the heat-induced protein denaturation. Heat and hypotonicity induced haemolysis of erythrocyte was significantly ($p < 0.001$) inhibited at the concentration of 500 µg/mL. The (COX-2) and (5-LOX) enzymes were significantly ($p < 0.001$) inhibited by Sinapic acid. The results indicate that Sinapic acid may be a potential anti-inflammatory agent.

Introduction

Inflammation, a palliate response of the body to various offenses, is a double-edged sword. While it is typically recognized as a process for the remission of diseases, this process's persistence may lead to various diseases associated with chronic inflammation,[1,2] including arthritis, atherosclerosis, neuropathic pain and even cancer.[3-5]

Steroids are potent anti-inflammatory agents and have a wide range of adverse effects that increase blood sugar levels, retain fluid, impairs wound healing, increases appetite, and produces heart burn, irritability, and osteoporosis.[6] Non-steroidal anti-inflammatory agents (NSAIDs) are the most widely used over the counter drugs and have common unwanted effects like gastrointestinal irritability, gastric ulcers, nausea, vomiting, bloating and nephrotoxicity.[7] There is a pressing need for the development of novel anti-inflammatory drugs.

Sinapic acid is a phytochemical found in various edible plant spices such as, citrus and berry fruits, vegetables[8-10] cereals, and oilseed crops.[11,12] Sinapic acid is reported to be effective against infections,[13] oxidative stress,[14] cancer,[15] diabetes,[16] neurodegeneration,[17] and anxiety.[18] The present study is a preliminary work carried out to assess the anti-inflammatory activity of Sinapic acid.

Materials and Methods

Materials

Sinapic acid was purchased from Sigma Aldrich, USA. All the other chemicals used were of analytical grade.

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Assessment of *in - vitro* Anti-inflammatory Activity

Inhibition of Albumin Denaturation

The anti-inflammatory activity of SA was studied by using inhibition of albumin denaturation method according to Mizushima and Kobayashi M., 1968; Sakat et al. 2010[19,20] with minor modifications. The reaction mixture consisted of SA in different concentrations and 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted using 1N HCl. The samples were incubated at 37°C for 20 minutes and then heated to 51°C for 20 minutes, after cooling, the sample's turbidity was measured at 660 nm. The experiment was performed in triplicate. The percentage inhibition of protein denaturation was calculated using the following formula:

Percentage inhibition = (Absorbance of Control – Absorbance of Sample)/Absorbance of control × 100

Membrane stabilization

Preparation of Red Blood Cell (RBCs) Suspension

Sheep blood was collected from the slaughter house 1 hour before the experiment and transferred to centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 min and were washed three times with equal volume of normal saline. The volume of blood was reconstituted as 10 % v/v suspension with normal saline.[19]

Heat-induced Haemolysis

The reaction mixture (2 mL) consisted of 1 mL SA in different concentrations (10-500 µg/mL) and 1 mL of 10% RBCs suspension. Instead of SA, only saline was added to the control test tube. Aspirin (200 µg/mL) was used as a standard drug. Centrifuge tubes containing reaction mixture were incubated in a water bath at 56°C for 30 minutes. At the end of incubation, the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 minutes, and the absorbance of the supernatants was measured at 560 nm.[19,20] The experiment was performed in triplicate for all the test samples. The Percentage inhibition of hemolysis was calculated as follows:

Percentage inhibition = (Absorbance of Control – Absorbance of Sample)/Absorbance of Control × 100

Hypotonicity-induced Hemolysis

Different SA concentrations, standard sample and control were separately mixed with 1ml phosphate buffer, 2 mL of hypersaline, and 0.5 mL of HRBC suspension. Aspirin (200 µg/ml) was used as a standard drug. Assay mixtures were incubated at 37°C for 30 minutes and centrifuged at 3000 rpm. The supernatant liquid was decanted, and a Spectrophotometer estimated the hemoglobin content at 560 nm.[19,20] The percentage hemolysis was estimated by assuming the hemolysis produced in the control as 100%.

Percentage protection = 100 – (OD sample/OD control) × 100

Assay of Cyclooxygenase-2 and 5-Lipoxygenase Inhibition

Lymphocyte culture preparation

Human peripheral lymphocytes were cultured in RPMI 1640 [HIMEDIA] media, supplemented with 20% heat-inactivated fetal bovine serum, antibiotics (Penicillin and Streptomycin). Phyto haemagglutinin (HIMEDIA) was used as the stimulant for cell proliferation. The culture was filtered using 0.2 µm pore sized cellulose acetate filter (Sartorios) in completely aseptic conditions. Fresh plasma was aseptically added to the culture at a concentration of 1 x 106 cells/ml. The culture was then incubated for 72 hours. After 24 hours, culture is activated by adding 1 µL lipopolysaccharide and incubated for 24 hours. Ethyl acetate and aqueous extracts were added in a final concentration of 100, 500 µg/mL and incubated for 24 hours. Ibuprofen at a concentration of 100 µg/mL was added and incubated for 24 hours. After incubation, the cells were pelleted by centrifugation. The isolation was done by spinning at 6000 rpm for 10 minutes. The supernatant was discarded and 50 µL of cell lysis buffer was added and again centrifuged at 6000 rpm for 10 minutes. The supernatant was discarded and the anti-inflammatory assay was done using pellet suspended in required amount of supernatant.[21]

Assay of Cyclooxygenase-2

The assay mixture contained Tris- HCl buffer, glutathione, hemoglobin and enzyme. The assay was initiated by the addition of 200 µM of arachidonic acid incubated at 37°C and terminated after 20 min by the addition of 0.2 mL of 10% trichloroacetic acid (TCA) in 1N HCl, mixed and 0.2 mL of thiobarbiturate (TBA) was added and contents heated in a boiling water bath for 20 minutes, cooled and centrifuged at 1000 rpm for 3 minutes. The supernatant was measured at 632 nm for COX activity.[21].

Assay of 5-Lipoxygenase

70 mg of linoleic acid and equal weight of tween 20 was dissolved in 4 mL of oxygen-free water and mixed back and forth with a pipette avoiding air bubbles. A sufficient amount of 0.5 N sodium hydroxide was added to yield a clear solution and then made up to 25 mL using oxygen free water. This was divided into 0.5 mL portions and flushed with nitrogen gas before closing and kept frozen until needed. The reaction was carried out in a quartz cuvette at 25°C with 1-cm light path. The assay mixture had 2.75 mL Tris buffer of pH 7.4, 0.2 mL of sodium linoleate, and 50 µL of the enzyme. OD was measured in 234 nm.[21]

Statistical Analysis

Data were expressed as mean ± SEM (n = 3) and analyzed using one way analysis of variance (ANOVA) followed by Dunnet's T test using Graph pad prism 8.0. A value of $p < 0.05$ was considered to be statistically significant.

Results

Effect of Sinapic Acid on Albumin Denaturation:

Sinapic acid significantly ($p < 0.001$) inhibited the albumin denaturation in a dose-dependent manner and maximum inhibition (70 %) was observed at 500 µg/mL as compared with control. Aspirin, the standard drug used in this study showed maximum albumin denaturation inhibition (68.42 %) at 200 µg/mL when compared with control ($p < 0.001$) (Fig. 1).

Effect of Sinapic Acid on Heat-induced Hemolysis

Sinapc acid dose-dependently increased the percentage inhibition of heat-induced hemolysis. Sinapic acid significantly ($p < 0.001$) inhibited (57.14 %) the heat-induced hemolysis at 500 µg/mL when compared with control. Aspirin also significantly ($p < 0.001$) inhibited (70.12 %) the heat-induced hemolysis at 200 µg/mL when compared with control (Fig. 2).

Effect of Sinapic Acid on Hypotonicity induced Hemolysis

Sinapic acid significantly ($p < 0.001$) protected hypotonic solution induced lysis of the erythrocyte membrane in a dose-dependent manner and maximum inhibition of hemolysis (82.06 %) was observed at 500 µg/mL as compared with control. Aspirin at 200 µg/mL showed significantly ($p < 0.001$) protection (70.12%) against the damaging effect of a hypotonic solution when compared with control (Fig. 3).

Effect of Sinapic Acid on Cyclooxygenase-2

Sinapic acid significantly ($p < 0.001$) inhibited Cyclooxygenase-2 and maximum inhibition of Cyclooxygenase-2 (71.77 %) was observed at 500 µg/mL as compared with control. Ibuprofen at 100 µg/mL showed significant ($P<0.001$) inhibition of Cyclooxygenase-2 (87.88 %) (Fig. 4).

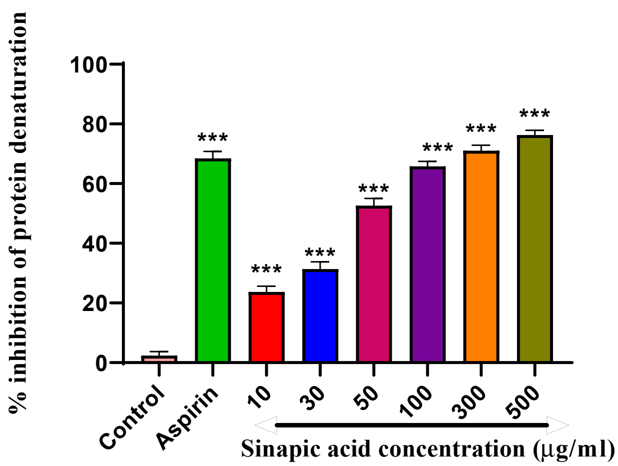

Fig 1: Effect of Sinapic acid on albumin denaturation
Values were expressed as mean ± SEM (n = 3); *** ($P < 0.001$) Vs Control group

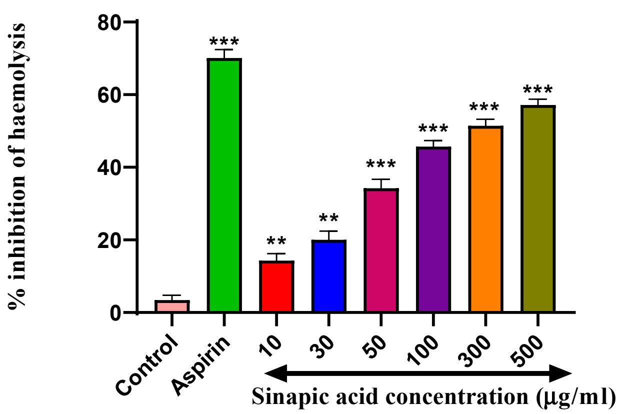

Fig 2: Effect of Sinapic acid on heat induced heamolysis
Values were expressed as mean ± SEM (n = 3); *** ($p<0.001$) Vs Control group

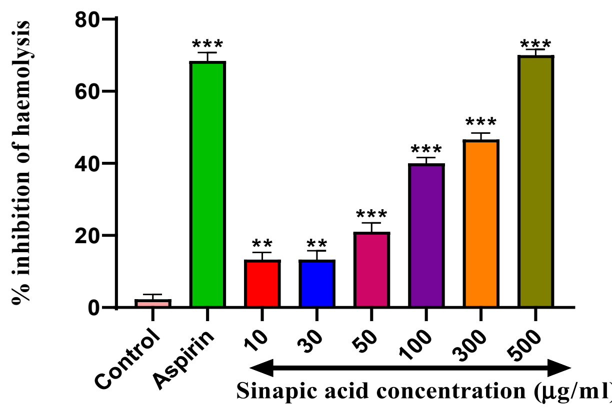

Fig 3: Effect of Sinapic acid on hypotonicity induced heamolysis
Values were expressed as mean ± SEM (n = 3); ***($p < 0.001$) Vs Control group

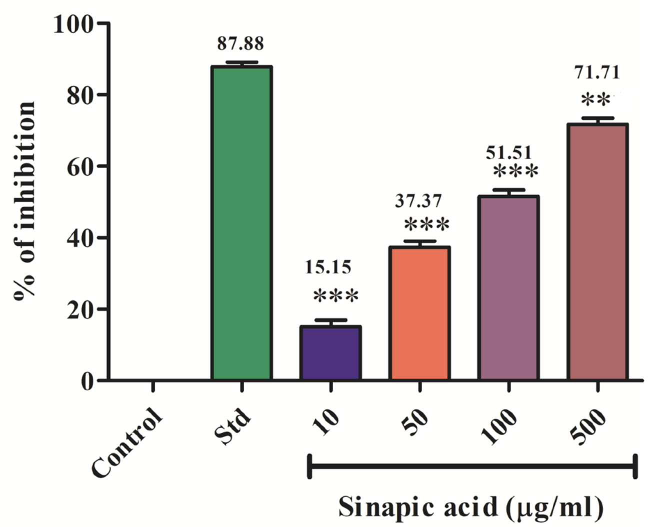

Fig 4: Effect of Sinapic acid on Cyclooxygenase-2
Values were expressed as Mean ± SEM (n = 3); **($p < 0.01$), ***($p < 0.001$) Vs Std group

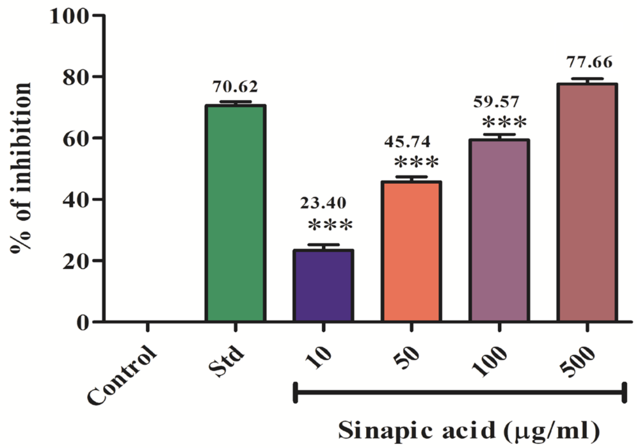

Fig 5: Effect of Sinapic acid on 5-lipoxygenase
Values were expressed as mean ± SEM (n = 3); ***(p < 0.001) Vs Standard group

Effect of Sinapic Acid on 5-lipoxygenase

Sinapic acid significantly ($p < 0.001$) inhibited lipoxygenase and maximum inhibition of 5-lipoxygenase (77.66 %) was observed at 500 μg/mL as compared with control. Ibuprofen at 100 μg/mL showed significant ($p < 0.001$) inhibition of lipoxygenase (70.62 %) (Fig. 5).

Discussion

Inflammation may be a pathophysiological state related to pain. The free nerve endings of peripheral nerve fibers in general tissues respond to inflammatory factors, like pH, bradykinin, histamine or prostaglandins, by generating electrical activity which is perceived as pain,[22] this useful pain state helps to safeguard us from adverse environments and might be managed by over-the-counter medications.[23] Arachidonic acid undergoes 2 metabolic pathways: the cyclooxygenase (COX) pathway involving cyclooxgenase-1 (COX-1) and cyclooxgenase-2 (COX-2) to synthesise the prostaglandins and thromboxanes[24] and the lipoxygenase (LOX) pathway, involving 5-lipoxygenase (5-LOX), 12-lipoxygenase (12-LOX) and 15-lipoxygenase (15-LOX), to produce the leukotrienes and hydroperoxy fatty acids.[25,26] COX and LOX pathways participate in the induction of numerous pathologies, particularly inflammatory diseases. These include arthritis,[27] fever, chronic pain,[28] sepsis, burn injury,[29] inflammatory bowel disease,[30] and malignant neoplastic disease processes in large intestine cancer.[31] In recent years, the search for phytochemicals possessing anti-inflammatory properties is on rise because of their potential use within the medical care of assorted chronic and infectious diseases. In the present study, we investigated the anti-inflammatory potential of SA by *in-vitro* strategies.

Protein Denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compound, such as strong acid or base, a concentrated inorganic salt, an organic solvent, or heat. Most biological proteins lose their biological function when denatured.[32] Protein denaturation is well correlated with the inflammatory response occurrence and leads to various inflammatory diseases.[33] Tissue injury during life might be referred to as denaturation of cells' protein constituents or intercellular substances.[34] Hence, a substance's ability to inhibit the denaturation of protein signifies apparent potential for anti-inflammatory activity. Sinapic acid effectively inhibited heat-induced albumin denaturation, indicating its anti-inflammatory property.

Membrane stabilization is used as a method to study the *in-vitro* anti-inflammatory activity, the erythrocyte membrane is analogous to the lysosomal membrane[35,36] and its stabilization implies that the SA may well stabilize lysosomal membranes. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophils, such as bacterial enzymes and proteases, further aggravating tissue inflammation and damage.[37] The lysosomal enzymes released during inflammation produce various disorders. The extracellular activity of these enzymes is related to acute or chronic inflammation. The non-steroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane.[38] SA significantly inhibited the heat and hypotonicity induced hemolysis.

Medications that inhibit both COX-1/2 and 5-LOX pathways are believed to be superior to conventional NSAIDs, they produce a synergistic effect and achieve optimal anti-inflammatory activity by blocking the production of both leukotrienes and prostaglandins.[39,40] This is supported by a significant reduction of collagen-induced arthritis in animal models upon concomitant administration of NSAID and leukotriene synthesis inhibitors.[40,41] 5-LOX is the first and the key enzyme involved in the arachidonic acid pathway to produce leukotrienes.[42] The 5-LOX pathway is associated with various diseases, including asthma,[43] inflammatory bowel disease,[44] cancers (prostate, pancreatic, and breast);[45,46] and cardiovascular diseases including atherosclerosis, heart attack and stroke.[47,48] COX and LOX inhibitors are considered promising agents for the treatment of inflammatory diseases on the basis of the important roles of LOX pathways.[49] In this study, SA significantly inhibited both COX and LOX, indicating it's anti-inflammatory activity.

Steroidal drugs are more potent anti-inflammatory agents but have adverse metabolic effects; NSAIDS have a dose ceiling effect and are not devoid of numerous adverse effects. 5LOX and CoX1/2 inhibitors are more potent anti-inflammatory agents. Sinapic Acid was screened in-vitro for anti-inflammatory activity using standard protocols. The results are encouraging as it blocks LOX COX and protein denaturation. Our results indicate that sinapic acid possesses good anti-inflammatory activity by inhibiting protein denaturation, hemolysis, Cyclooxygenase-2 and lypoxygenase, indicating its potential as novel lead molecule for inflammation. Hence, further *in -vivo* studies are required to explore Sinapic acid's mechanism as anti-inflammatory agent.

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