Research Article

Preferential inhibition of bacterial elastase over human neutrophil elastase by leaf extracts of *Psidium guajava*: an in vitro study

Deena Mendez, AV Moideen Kutty, SR Prasad

Department of Biochemistry, Sri Devaraj Urs Medical College, Tamaka, Kolar, Karnataka, India.

Correspondence to: AV Moideen Kutty, E-mail: avmkutty511@gmail.com

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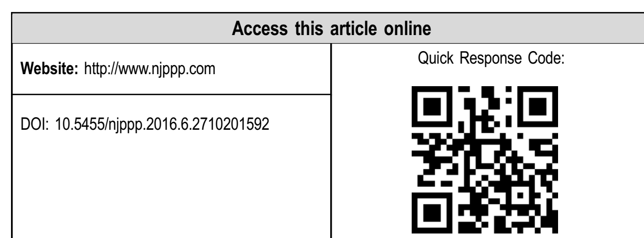
Abstract

Background: The guava (*Psidium guajava*), belonging to family Myrtaceae, is a small tree grown widely for its fruit in tropical and subtropical countries. Apart from its nutritional value, it is also known for its medicinal value and the antimicrobial activities of its leaves. **Aims and Objectives:** To demonstrate the inhibitory effect of *P. guajava* leaf extracts (aqueous and methanolic) on bacterial elastase from *Pseudomonas aeruginosa* (MTCC3541) and human neutrophil elastase (HNE); to explore the presence of a protease inhibitor of protein nature in the aqueous leaf extract. **Materials and Methods:** Purified bacterial elastase from *P. aeruginosa* (MTCC3541) and commercially purchased HNE were used as enzyme sources with succinyl trialanyl *p*-nitroanilide as the substrate. Varying concentrations of methanolic and aqueous extracts of the leaves were used as the source of the inhibitor. **Result:** The results of the study show that the methanolic and aqueous extract of the leaves inhibited both bacterial elastase and HNE. Calculation of K_i and IC_{50} showed that the bacterial elastase was inhibited by smaller amounts of the inhibitor suggesting that the inhibitor was more potent toward bacterial elastase. However, an aqueous extract prepared after removal of phenolic compounds did not show any inhibitory activity ruling out the presence of inhibitor of protein nature. **Conclusion:** This observation indicated that the inhibitory activity exhibited by the leaf extract is exclusively owing to the phenolic compounds.

KEY WORDS: *Psidium guajava*; Pseudomonas aeruginosa; Human Neutrophil Elastase; STANA

Introduction

Since the advent of civilization, plants have been used as a source of medicine. Recently, there has been a renewed interest in plant extracts that have medicinal properties, especially as these have been found useful in identifying molecules of importance in the pharmaceutical industry. *Psidium guajava* is cultivated commercially for its fruit, but its leaves, bark, and roots have a long history of medicinal use. The leaves of the guava plant are in use to treat wounds and ulcers, and an infusion of the leaves has been used to treat rheumatism, epilepsy, chorea, and diarrhea.[1,2] Phytochemical studies on *P. guajava* have shown the presence of tannins, triterpenes, flavonoids, and phenolic compounds.[3–5] Tannins present in the plant have been shown to act against bacteria by inactivating microbial adhesions and enzymes.[6] The growth of microorganisms can also be inhibited by essential oils in leaves.[7] Besides these, some plants also have antimicrobial proteins that play a role in defensive mechanism against bacteria, fungi, and viruses. Plants synthesize inhibitory proteins that suppress the extracellular enzyme activities released by pathogenic microorganisms.[8,9] These protease inhibitors (PIs) are of common occurrence and some serine PIs (serpins) involved in inhibiting proteases of plant pathogens have been isolated and used for



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medical treatments.[10–12] The observations on the antibacterial activity of *P. guajava* is nonspecific. Most studies have shown that there was a zone of inhibition when the leaf extract was applied on the microbial culture plate.[13] The mechanism as to whether its antimicrobial activity[14] is by inhibition of bacterial proteases of different origin is not clear. Elastase released by microorganisms is an important virulence factor in several types of infections,[15] and the invasive effect of elastase by *Pseudomonas aeruginosa* has been well documented.[16] Human neutrophil elastase (HNE) is involved in the degradation of bacteria and immune complexes phagocytosed by polymorphonuclear leucocytes.[17] As these leaf extracts are used to facilitate wound healing, it was felt appropriate to assess the inhibitory abilities of the *P. guajava* leaf extract on HNE and *P. aeruginosa* elastase to test the specificity of their inhibition on these enzymes. This study also explored the presence of PIs of protein nature present in the leaf extract.

MATERIALS AND METHODS

All chemicals used for the study were of analytical grade unless otherwise mentioned. Succinyl trialanyl *p*-nitroanilide (STANA) and HNE were obtained from Sigma, USA, and tannic acid used as standard was from Merck.

Cultivation and Growth of the Microorganism

The microorganism *P. aeruginosa* MTCC 3541 was purchased in a lyophilized form from Microbial Type Culture Collection and Gene Bank, Chandigarh, India. It was rehydrated and cultivated on nutrient agar plates for 24 h at 37°C. Protease release by the microorganism was confirmed by noting zone of clearance on caseinate agar plates, which were streaked and kept for incubation at 37°C for 48 h. The strain was then inoculated into a nutrient broth containing 1% glucose, 0.5% casein, 0.5% yeast extract, 0.2% K_2HPO_4, 0.2% K_2HPO_4, and 0.1% $MgSO_4$ $7H_2O$.[18] The broth was kept in a rotatory shaker at 120 rpm at 37°C for 24 h. It was then centrifuged at 10,000 rpm for 20 min at 4°C.

Purification of the Enzyme

The culture broth was subjected to 70% saturation with ammonium sulfate at 4°C in a cold room with constant stirring and allowed to stand overnight. It was then centrifuged at 10,000 rpm for 20 min. The precipitate was collected and dissolved in 200 mM Tris-HCl buffer, pH 8.0, and again centrifuged. The supernatant was collected and dialyzed against 200 mM Tris-HCl buffer, pH 8.0, with a change of buffers at every 12th h of dialysis. The dialysate (ammonium sulfate fraction) was collected and measured for protein by Lowry's method.[19] The elastase activity in this was determined using STANA as a substrate by the method of Bieth et al.[20] The fraction after ammonium sulfate precipitation was then subjected to further purification by ion exchange chromatography using DEAE cellulose column, which was equilibrated with 200 mM Tris HCl buffer. Elution was done in a stepwise manner using NaCl (0.05–0.5 M) in the same buffer. The eluted fraction that showed the highest proteolytic activity was then used as the source of the enzyme in this study. The protein content was estimated as per the method of Lowry[19] and elastase activity as per Bieth et al.[20]

Preparation of the Leaf Extract

Plant Materials: The fresh mature leave of *P. guajava* were collected in Kolar District, Karnataka, in the month of August . The leaves were washed thoroughly with distilled water and dried at room temperature. The dried leaves were then ground uniformly in a mechanical grinder to give a fine powder.

Five grams of the powder was then mixed with 30 mLof methanol in an Erlenmeyer flask. It was kept in a shaking incubator at 120 rpm for 24 h. The mixture was the filtered using Whatman filter paper No. 1, dried, and stored at 4°C. It was then dissolved when required for use.

The aqueous extract was prepared similarly using hot (60°C) deionized water. After filtration, one part of the aqueous extract was washed with an equal volume of 70% cold acetone and kept overnight at 4°C. It was centrifuged at 3000 rpm, and the precipitate formed was removed, dissolved in 0.2 M Tris HCl buffer, pH 8.0, and again centrifuged at 3000 rpm. The process was repeated several times until the precipitate was colorless. The supernatant obtained was used as the source of aqueous extract without phenolic compounds. Protein content in this extract was measured by Lowry's method.[19]

The total tannin content of the leaf extract was determined by the Folin–Ciocalteu reagent method[21] and compared with known standards prepared with tannic acid. The results obtained were expressed as micrograms of tannic acid per milliliter of the extract as per the method of Makkar.[21]

Enzyme Assay

Two sets of assay systems were run. In one set, the source of the enzyme elastase was that obtained from *P. aeruginosa* by the process mentioned earlier. Similarly, a fixed volume of HNE was then used as the enzyme source in a second system. The concentration of both enzymes, i.e., HNE and the bacterial elastase, was fixed at 10 U/L to get consistent enzyme activity so that it hydrolyzed the substrate to give an optical activity of 0.25. The assay system comprised 200 mM of STANA in 200 mM of Tris HCl buffer, pH 8.0, and the reaction was initiated by the addition of the enzyme. After 15 min of incubation at 37°C, the reaction was stopped by the addition of 1 mL of 30% acetic acid, and the optical density of *p*-nitroaniline liberated was measured at 410 nm. One enzyme unit was defined as the amount of enzyme required to release 1 μmol of *p*-nitroaniline per unit time (min).

Elastase Inhibition assay

The inhibitor sources in each set were varied concentrations of the methanolic and aqueous extracts of the leaves and the aqueous fraction (without phenols). All of them were in the assay buffer, i.e., 200 mM Tris HCl buffer, pH 8.0. The leaf

extracts were preincubated for 10 min with a fixed volume of the enzyme. The reaction was initiated with a fixed volume of the substrate, i.e., 200 mM STANA. After 15 min incubation at 37°C, the reaction was stopped by the addition of 1 mL of 30% acetic acid, and the residual activity was determined by measuring optical density of *p*-nitroaniline liberated at 410 nm. Suitable controls were also run without the inhibitor. The inhibitory activity was determined by the difference between activity of the bacterial elastase without inhibitor and the residual activity of the same solution after adding the inhibitor. Similarly, the inhibitory activity on HNE was also determined. The percentage of inhibition was calculated using inhibition (%) = $[1 - (B/A)] \times 100$ where A is the activity of the enzymes without inhibitor and B is the activity in presence of inhibitor. One inhibitor unit was defined as the amount of inhibitor required to inhibit 50% of the corresponding enzyme activity per unit time. IC_{50} was determined for both elastase of *P. aeruginosa* and HNE with varying concentrations of the inhibitor in the assay systems.

Result

The methanolic extract of the leaves showed good inhibitory capacity, more than that of the aqueous extract, against both enzymes, elastase of *P. aeruginosa* and HNE [Figures 1 and 2]. However, IC_{50} (concentration in µg/mL at which the inhibition of the enzyme activity is 50%) for HNE was 40 and 44 µg/mL for the methanolic and aqueous extracts while, for bacterial elastase, 24 and 30 µg/mL, respectively. K_i values were calculated for methanolic and aqueous fractions and were 18.3 and 20.2 µg/mL for HNE and 10.4 and 13.0 µg/mL for bacterial elastase, respectively.

The aqueous extract after removal of phenolic substances with acetone did not exhibit any recordable inhibitory activity on both HNE and elastase of *P. aeruginosa.*

Discussion

The guava leaves known for its antibacterial properties has not been explored for the mechanisms as to how the leaf extract exert its action on the microorganisms. This study emphasizes on the inhibitory role of the extract on elastase as it is shown as a virulence factor and plays an important role in infection. The inhibitor effect of the methanolic and aqueous extracts here showed that both elastase of *P. aeruginosa* and HNE are inhibited. The data analysis showed that the amount of inhibitor required for inhibition of *P. aeruginosa* is about 40% lower than that of HNE. The lower K_i values for bacterial elastase in both methanolic and aqueous fractions suggest that it is more prone to the effect of the inhibitor in the leaves as the lower K_i indicates that less amounts of the inhibitor brings about greater degree of inhibition. The strain of *P. aeruginosa* MTCC 3541 used in this study had elastase activity. We had cultivated the organism and determined its proteolytic activity on casein substrate (results not shown in this study) and determined the purified elastase activity on STANA.

An earlier study had shown that porcine pancreatic elastase is inhibited by methanolic leaf extract of *P. guajava* and that the triterpene derivatives present in the leaf brought about the elastase inhibition.[22] There have been studies on elastase inhibition by extracts of plants such as *Areca catechu, Cornus kousa*, and *Achillea millefolium*. But the inhibition was brought about by phenolic substances, flavonoids and triterpenoids.[23–25]

So, in this study, we wanted to establish whether the wound healing capacity of the leaf extract is owing to a PI present in the leaves whose inhibition would be specific or if inhibition of elastase was nonspecific. These PIs are able to selectively abrogate protease actions by forming less active or fully inactive complexes with their cognate enzymes; so, they can be used as drugs by themselves or serve as a template for development of highly specific drugs.[26] Hence, an aqueous extract, a methanolic extract, and an aqueous extract after removal of phenolic compounds of the leaves were prepared as sources of inhibition. Elastase release

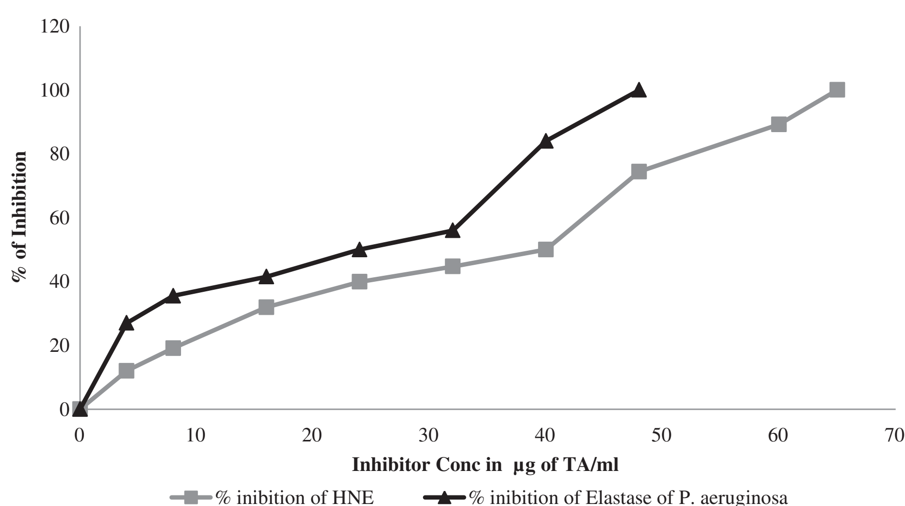

Figure 1: Percentage of inhibition of HNE and elastase of *P. aeruginosa* by methanolic extract of *P. guajava*.

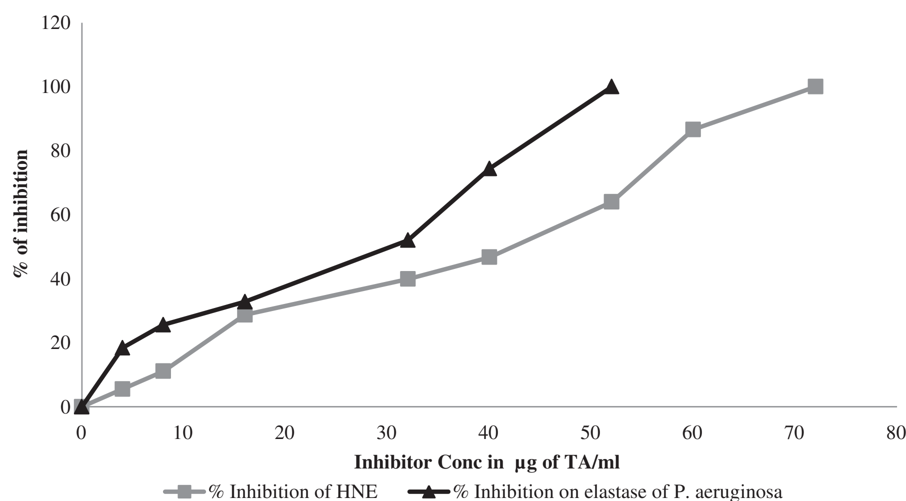

Figure 2: Percentage of inhibition of HNE and elastase of *P. aeruginosa* by aqueous extract of *P. guajava*.

by microorganisms is an important invasive factor. So, it was thought appropriate to use an extracellular enzyme released by an invasive microorganism as only porcine pancreatic elastase had been used in earlier elastase inhibition studies. Inhibition of HNE has been shown by some medicinal plants in Yemen in an earlier study.[27] In our study, the methanolic extract of the leaves showed good inhibitory capacity, more than that of the aqueous extract, against both elastase of P. *aeruginosa* and HNE. This was probably because methanol as a solvent had extracted many of the active compounds present in the leaf more than that of water; so, percentage of inhibition brought about was more in the methanolic fraction.

During wound healing, in the initial stages, some amount of proteolytic activity is required to break down the extracellular matrix and promote wound healing. This role is usually provided by HNE.[28] As per the results of this study, application of the leaf extract appears to preferably inhibit bacterial elastase over that of HNE. So, there does appear to be a scientific basis for the application of these leaves on a wound. Attempts to isolate an inhibitor of protein nature, which would have been more specific in its inhibition, however, failed as the aqueous extract, which was treated with acetone and had the major part of the bioactive compounds washed out, showed negligible inhibitory activity. Moreover, when protein content was measured in this fraction, it was present only in trace amounts, indicating that not much water-soluble proteins were present under the given set of conditions.

Conclusion

The results of the study clearly demonstrate more specific inhibition of elastase of *P.aeruginosa* in comparison with HNE by the leaf extract of *P. guajava.* It provides a scientific basis that this leaf extract facilitates the wound-healing process by curtailing progress of invasion by microorganisms. So, there is scope for further isolation and chemical characterization of the active molecules present, which inhibit elastase of *P. aeruginosa* and exhibit potential use for topical application. However, its action on other vital enzymes and physiological processes in host systems need to be evaluated.

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