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Annona muricata Seed Trypsin Inhibitory Fraction as a Promising Antioxidant and Antibacterial Agent

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Abstract

Annona muricata is a source of many phytochemicals that serve as remedies for various illnesses. Although several studies have been conducted on *A. muricata*'s potential as a medicine, no potent trypsin inhibitory proteins identified so far, from any of its plant parts. The objective of this research was to examine the *in vitro* antioxidant and antibacterial properties of trypsin inhibitory fraction (AMTI) derived from the seed extract of *A. muricata*. AMTI was partially purified by ammonium sulphate fractionation. The fraction containing 0–30% ammonium sulphate was found to have the highest inhibitory activity and chosen for further analysis. Using the DPPH radical scavenging experiment, the antioxidant activity of partially purified AMTI was evaluated. It demonstrated significant radical scavenging action, with an IC_{50} value of 148.94 µg/mL. The antimicrobial activity of the AMTI against *Staphylococcus aureus* was done by agar well diffusion assay and Minimum inhibitory concentration (MIC) was determined. These findings suggest that AMTI possesses potent antioxidant and antimicrobial property and hence is a worthy candidate for the design of antioxidant and antimicrobial formulations.

1. Introduction

Proteases are a type of enzymes that catalyze the hydrolytic breakdown of peptide bonds in proteins (Barret *et al*., 2012). Proteases are classified as aspartic, cysteine, glutamic, serine, and threonine petidases based on functional amino acid residue that are present in their active site (Lopez Otin *et al*., 2008). These enzymes play an important role in many physiological and pathological processes, including the breakdown of proteins, cell division, tissue organization, developmental morphogenesis, inflammation, tumour growth and metastasis, blood coagulation, activation of zymogens, hormone release from precursor proteins, and secretory protein transport across membranes (Bond *et al*., 2019). Although proteolysis is essential for basic cellular response, an excessive proteolysis can be harmful to cellular functioning. Protease inhibitors (PIs) are examples of the control mechanism nature developed to manage this (Ehrman *et al*., 2004).

PIs are found in a wide range of organisms, including humans and viruses (Sabotic *et al*., 2012, Travis *et al*., 1983, Rachel *et al*., 2014). In plants, they are essential for defense against pathogens and insects. Although present in different plant parts, the largest quantities of these inhibitors

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are usually found in the seeds and tubers (Gahloth *et al*., 2010). PIs include trypsin inhibitors (TIs), of which there are three different kinds: Kunitz type (Machado *et al*., 2013), Bowman-Birk type (kumar *et al*., 2002) and Squash type. The molecular masses of the three varieties are around 20 kDa, 8 kDa, and 3 kDa respectively. Melons belonging to the Cucurbitaceae family has inhibitors of the squash type (Telang *et al*., 2003), while soybeans has inhibitors of both the Kunitz and Bowman-Birk types (Birk *et al*., 1996). The extraction and isolation of TIs from fruit seeds has been the subject of numerous studies (Lyu *et al*., 2015. Lijith *et al*., 2023, Chandran *et al*., 2022).

Annona muricata L., more commonly known by the names soursop, graviola, guanabana, paw-paw and sirsak, is a member of the Annonaceae family. In tropical and subtropical regions of the world, such as India, Malaysia, Nigeria, Australia and Africa, *A. muricata* is widely spread (Lebou *et al*., 1980, Adewole *et al*., 2006, Mishra *et al*., 2013). Numerous compounds and secondary metabolites are present in the plant. The primary components are carotenoids, amides, cyclopeptides, flavonoids, alkaloids, vitamins, and acetogenins (Vijayameena *et al*., 2013). Minerals including K, Ca, Na, Cu, Fe and Mg are also present in the plant (Gyamfi *et al*., 2011).

In this study we extracted TI fraction from *Annona muricata* L. mature seeds in order to investigate its antioxidant and antibacterial characteristics.

2. Materials and methods

N-benzoyl-DL-arginine-p-nitroanilide hydrochloride **(**BAPNA) were purchased from the Sigma Aldrich,St. Louis, USA. Rest of the chemicals used in this study were purchased from Sisco Research Laboratories Pvt. Ltd., India. All reagents used were of analytical grade. Himedia Pvt. Ltd. provided the dialysis membrane with a molecular weight cutoff of 12–14 kDa and Muller-Hinton agar.

2.1 Plant collection and sample preparation

The mature seeds of *Annona muricata* was collected from Kollam district during the summer season. Dr. A. K Pradeep, an experienced taxonomist from the Department of Botany, University of Calicut, identified the plant and the plant specimen was deposited in the herbarium repository (Accession No. 178264).

The collected seeds were washed thoroughly with distilled water and dried in shade before being processed into powder using an electric blender. The powder was soaked in n-hexane at a ratio of 1:5 (W/v) and the fat was removed using a Whatman no 1 filter paper. Washed the sediment three times and filtered out any leftover oil from the grounded sample. The defatted sample was air dried and put to use for further studies.

2.2 Extraction and recovery of TI

Using standard protocols, the crude extract was prepared using 50 mM Sodium phosphate buffer by stirring continuously for 4 h using a magnetic stirrer. For 30 min, the crude extract was centrifuged at 8000 xg in a cooling centrifuge and the obtained supernatant was kept in 4^{o}C for further use. Using BAPNA as the substrate, the trypsin inhibitory

activity was measured following the method of Kakade *et al* (1974). The protein content was determined using Bradford method (1976) and specific activity was calculated.

2.3 Ammonium sulphate fractionation

The precipitation of TI proteins from the seeds was carried out according to a reported protocol (Saxena *et al*., 2007). Ammoniunm sulphate was added to the crude extract in three stages: 0-30-%, 30-60% and 60-90%. Protein content, specific activity and TI activity were measured for each fractions. To eliminate the ammonium sulphate in the fractions, those fractions were dialysed against a 50 mM Sodium phosphate buffer. The sample that had been dialysed were freeze-dried to preserve them using a lyophilizer (INLABCO). Lyophilized TI (henceforth referred to as AMTI) was dissolved in the appropriate solvents and utilized in further tests.

2.4 *In vitro* antioxidant assay by DPPH (2,2-diphenyl, 1-picrylhydrazyl) method

The antioxidant activity of the purified TI was determined using the 1, 1-diphenyl, 2-picryl hydrazyl (DPPH) free radical assay described by Salem *et al*., (2011). DPPH radical is scavenged by antioxidants through the donation of proton, forming the reduced DPPH. The colour changes from purple to yellow after reduction, which can be quantified by a decrease in absorbance at wavelength 517 nm. The percentage of inhibition was plotted against the samples' concentration. A logarithmic curve was established in order to calculate the IC_{50} which is the amount of sample necessary to decrease the absorbance of DPPH by 50%. The percentage of DPPH scavenging was calculated usingthefollowing formula.

$$percentage\ scavenging\ of\ DPPH = \left(\frac{Absorbance\ of\ control}{Absorbance\ of\ sample}\right) X\ 100$$

2.5 Antimicrobial activity of AMTI

2.5.1 Agar well diffusion method

Agar well diffusion method was used to assess the antibacterial activity of AMTI solution against *Staphylococcus aureus*(ATCC29213). Muller-Hinton agar wells were seeded with AMTI solution at 50 and 100 µg/mL concentrations, ampicillin (Positive control) at 0.3mg/mL, and DMSO (negative control). The swab culture method was used to inoculate the bacterial solution, and the zones of inhibition were determined after an overnight incubation at 37°C.

2.5.2 Minimum inhibitory concentration determination

A microplate was inoculated with *S. aureus* strain (10^5 CFU/mL) that had been prepared using 0.5 McFarland standards. A diluted culture of 100 µL was added to each well. After being loaded to the first well at 51 µg/mL, serially diluted to reach the 11^{th} well at 0.0498 µg/mL. 12^{th} well was used as control. For loading the positive control (ampicillin) the same protocol was used. And the plates were incubated overnight. After the incubation 5 µL of resazurin dye was added and after 20 min observed the colour. Pink showed live microbes and purple showed dead cells.

2.6 Statistical analysis

The Duncan's multiple range test was used to determine the differences between means after all the data were subjected to analysis of variance (ANOVA). The statistical package for social sciences (SPSS for

windows, version 21;Inc.) was used to conduct the statistical analysis.

3. Results and discussion

3.1 Extraction and partial purification of AMTI

Effective extraction and partial purification of a proteinaceous trypsin inhibitor from *A. muricata* seed extract was achieved. The Partial purification of AMTI involved extraction in a 50mM phosphate buffer (pH 7.6) and ammonium sulphate precipitation (0-30% saturation). After precipitation, it was dialyzed against a 50 mM phosphate buffer. Table 1 displays the amount of total protein, trypsin inhibitor activity and specific inhibitory activity of sample obtained from 10g seed powder.

From a 10 g sample, 18 mLof crude extract in 50 mM phosphate buffer (pH 7.6) was obtained. Each gram of seed yields 1.9 mL extract. The total inhibitory activity and specific inhibitory activity of sample was 343.46 TIU and 313.53 TIU/mg respectively. The results indicate that *A. muricata* seeds also possess trypsin inhibitory activity. The 0-30% of the ammonium sulphate fraction was observed to have the greatest inhibitory activity (Figure 1) and hence was used for further studies.

3.2 Determination of Antioxidant Activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) test was used to spectrophotometrically measure the antioxidant activity of the trypsin inhibitor from *A. muricata* seed extract at 517 nm. It is a quick and inexpensive technique used for assessing the antioxidative capability of various natural sources. Following a half-hour incubation period, absorption measurements were performed to assess the response between the sample and DPPH, which acts as a free radical. The DPPH findings showed that AMTI has the ability to scavenge the free radicals. Figure 2 shows the results of the antioxidant activity of AMTI and standard.

The scavenging activities of the AMTI and standard (ascorbic acid) at 250 (µg/mL) are 60.43±0.03 and 66.81±0.06 respectively. Values for IC_{50} (concentration for 50% inhibition) a measure of antioxidant activity, were calculated. Radical scavenging assay exhibited IC_{50} of standard and AMTI as 111.7µg/mL and 148.9µg/mL respectively. According to previous reports, *Annona squamosa* trypsin inhibitors (IC_{50} 108µg/mL) have the potential to be antioxidants (Lijith *et al*. 2023). AMTI has a higher IC_{50} value compared to that of *Annona squamosa* trypsin inhibitors. An antioxidant substance is considered to be extremely strong if its IC_{50} value was less than 50µg/mL, strong if it was between 50 and 100 µg/mL, moderate if it was between 100 and 150 µg/mL, and weak if it was greater than 150 µg /mL (Molyenx., 2004) it is demonstrating that AMTI has a moderate level of antioxidant activity. The complex disorders including atherosclerosis, stroke, diabetes, Alzheimer's disease, and cancer are treated and prevented with a variety of antioxidant-based medication formulations (Harman D., 2000). These findings may lead to the production of powerful antioxidant agents from *A. muricata* seeds if the AMTI fraction is further purified.

3.3 Anti-microbial activity by agar well diffusion

Using the agar well diffusion technique, the antibacterial activity of AMTI extract with DMSO was evaluated *in vitro* against clinical isolates of *S. aureus* by measuring the diameter of growth inhibition zone. Ampicillin was used as the standard to compare the antibacterial effects of the test extracts. Antibacterial properties of seed extract from *A. muricata* are displayed in table 3.

Fig. 3 displays the microbial growth inhibition of AMTI extracts at different concentration. The zone of inhibition became apparent after 24 h of incubation. Measurement and recording of the zone of inhibition were made. In the DMSO-taken wells used as negative control, there is no zone of inhibition. A 30 mm diameter zone of inhibition is shown in ampicillin, which serves as a positive control. The results showed that AMTI extract had an inhibitory effect, with mean values of 8mm and 11 mm in 100µg and 150µg respectively. It has been reported that the leaves of *A. cherimola,* a member of the Annonacea family, have antibacterial action against *S. aureus*, with growth inhibition zone of 11 mm (Takahashi *et al*., 2006). The AMTI exhibited potent antimicrobial activity in comparison with control. It seems that inhibition rate increases in tandem with concentration. Higher concentrations of seed extract are demonstrated the greater antibacterial activity against clinical isolates of *Staphylococcus aureus* (Table 3). Numerous studies have confirmed that *A. muricata* leaf extract has antibacterial qualities against *S.aureus* (Bento *et al*., 2013, Abdulsalami *et al*., 2016). It is evident from the present study that AMTI has effect against the growth of *S. aureus.* Hence the research validates the use of *A. muricata* seeds in ethnomedicine to treat infections.

3.4 Anti-microbial study by assessing minimum Inhibition concentration

The lowest or minimum dilution that prevents an organism's apparent growth is known as the minimum inhibitory concentration or MIC. The minimum inhibitory concentration (MIC) of AMTI against *S.aureus* is displayed in Figure 4.

The second well with AMTI shows the minimum inhibitory concentration at 25.5µg/mL, while the MIC of positive control (ampicillin) is 3.188µg/mL. The presence of dead microorganism is indicated by purple colour, which is due to the microbes got inhibited by AMTI. The pink colour indicates the presence of live microbes, it means microbes are not inhibited by AMTI (Sarker *et al*., 2007). The indication of inhibition is done by comparing the AMTI and positive control wells. Minimum Inhibitory Concentrations (MIC) of 50 µg/mL have been recorded for the aqueous and methanolic seed extracts of *A. squamosa*'s efficacy against *S.aureus* (Nasser *et al*., 2017). According to the current study findings, AMTI generates inhibitory activity on the tested strains of bacteria at a concentration of 25.5 µg/mL as compared to the positive control. The research indicates that AMTI exhibits effectiveness against *S. aureus.*

4. Conclusion

In conclusion, studies on the trypsin inhibitory fraction of *A. muricata* seed extract (AMTI) has shown potential as a source of antibacterial and antioxidant activity.

It demonstrated moderate antioxidant potential compared to ascorbic acid. Its antibacterial properties were demonstrated against *S. aureus*, in a concentration-dependent manner. These findings suggest the AMTI's potential for therapeutic use in infections and diseases, advancing natural medicine and drug discovery.

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Table 1: Quantification of trypsin inhibitor activity

SAMPLE (Crude extract)	**Total Protein (mg)**	**Trypsin Inhibitory activity (TIU)**	**Specific inhibitory activity (TIU/mg protein)**
A.muricata	1.09523	343.46	313.5391

Table 2: Zone diameter of inhibition obtained at different conditions

DMSO	**-**
AMPICILLIN	**++**
AMTI 50µg	**+**
AMTI 100µg	**+**

(-) No zone of inhibition
(+) Inhibition of zone diameter 0-20mm
(++) Inhibition of zone diameter > 20mm

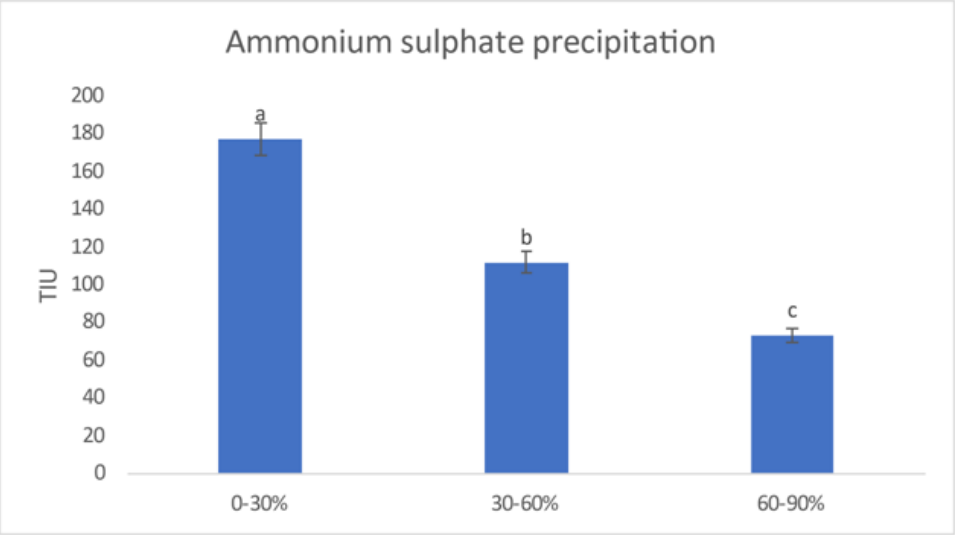

Fig. 1: Trypsin inhibitory activity in different ammonium sulphate fraction. Values are mean of three estimations. Error bars indicate Standard Deviation (SD). Different alphabets indicate significant difference at P< 0.01. F value 645.53.

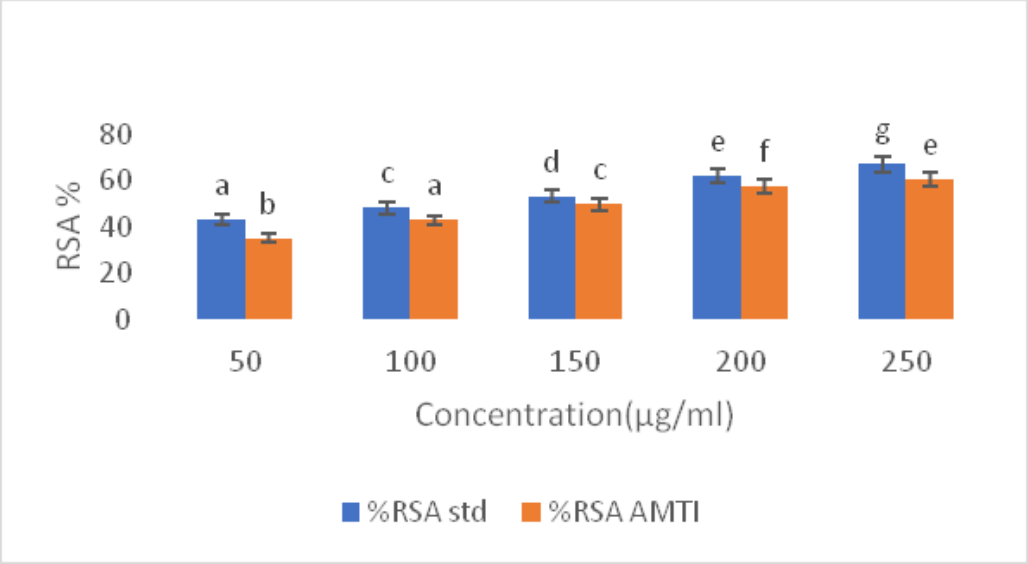

Fig. 2: DPPH radical scavenging activity (RSA) of ascorbic acid and *A. muricata.* Values are mean of three estimations. Error bars indicate Standard Deviation (SD). Different alphabets indicate significant difference at P< 0.01. F value 292.201.

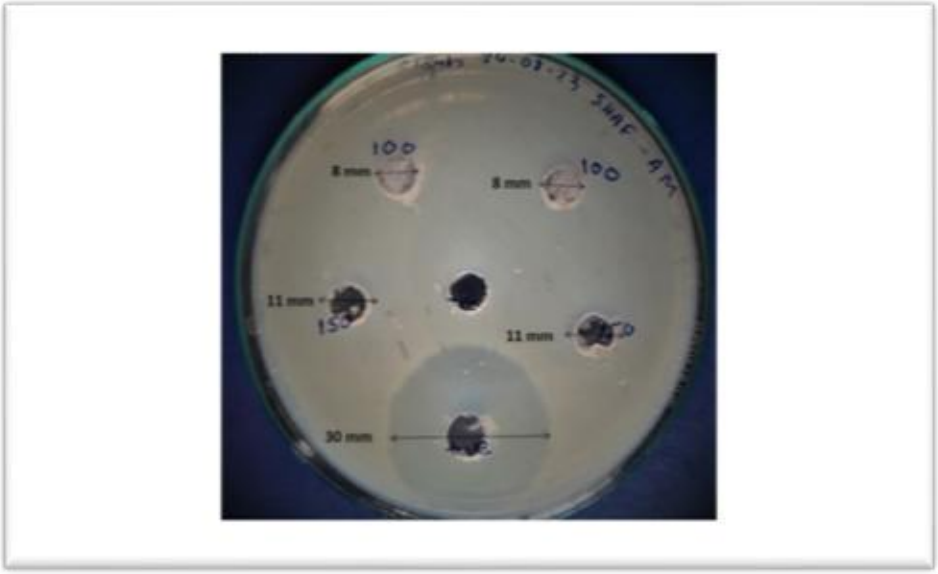

Fig. 3: Agar well diffusion

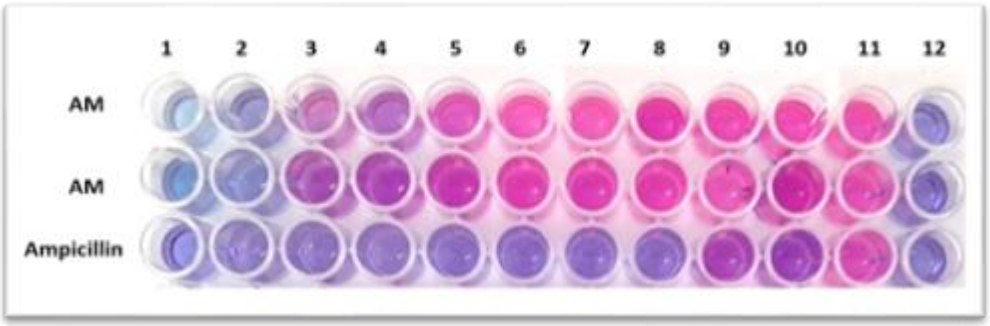

Fig. 4: Minimum inhibition concentration test of AMTI against Gram-positive *Staphylococcus aureus.*