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ISOLATION AND SCREENING OF ANTIMICROBIAL PEPTIDES FROM KANTHARI MULAKU (*CAPSICUM FRUTESCENS*)

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ABSTRACT

Kanthari mulaku (*Capsicum frutescens*), as it is known in Kerala, India is one of the most commonly used pepper species in cooking as well as folk medicine. The work was carried out to isolate, purify and test the efficacy of the antimicrobial peptides (AMPs) from this species.The purified peptide fraction when tested against *E coli, Staphylococus aureus and Klebsiella pneumonia* by disk diffusion method showed inhibition zones of 3 to 12 mm range at 250 mg/ml concentration. The fungus *Alternaria* and *Colletotrichum* showed 100% inhibition of growth and Fusarium showed 66% inhibition of growth at 5 mg/ml concentration of fruit extract. The leaf extract however showed 75%, 12.5 % and 17.5% growth inhibition of *Alternaria, Colletotrichum and Fusarium* at 5 mg/ml concentration. On the basis of these findings, it can be concluded that the purified antimicrobial peptide from *C frutescens* could play strong roles in agriculture as a natural pesticide against various phytopathogens.

KEYWORDS: ***Antimicrobial peptides (AMPs), Kanthari mulaku, Capsicum frutescens***

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INTRODUCTION

The genus *Capsicum* belonging to the family Solanaceae comprises of more than 200 varieties. Among these, five main species commonly cited in literature includes *Capsicum annuum, Capsicum chinense, Capsicum frutescens, Capsicum baccatum,* and *Capsicum pubescens*. The present study focuses on "Kanthari mulaku" (*Capsicum frutescens*), a perennial crop commonly cultivated in Kerala, India. It is an errect, branched and half-woody plant, growing to a height of 0.5 to 1 meter. It is an introduced plant from America [1] and the greatest number of species is concentrated in Brazil [2]. Leaves are oblong-ovate to ovate-lanceolate, 3 to 10 centimeters long, and pointed at the tip, and 8 to 9 millimeters in diameter. Fruits are small, tapering capsules, often 2-3, at a node. The fruits of most varieties are white, dark green, pale green or purple getting red on ripening (Figure1). For more than 9000 years, this plant has been used as food, an additive in livestock feed and medicine in the pharmaceutical industries [3].

Figure I

Kanthari mulaku (C frutescens) a local capsicum species in South India

Fruit pungency is characteristic of the genus *Capsicum* due to some substances specific to peppers known as "capsaicinoids," - a group of compounds that includes more than 20 alkaloids including the principal compound, capsaicin [4]. It has very powerful pain-relieving properties and increases appetite by stimulating the gut**.** It is also a rich source of phenolics, particularly flavonoids such as quercetin and luteolin that are primary source of antioxidants. In humans these have demonstrated protective roles against coronary heart disease, stroke, and some forms of cancer [5]. There are also reports indicating other medicinal properties of "Kanthari mulaku" – reduces blood sugar, works against arthritis and rheumatism, prevents blood clots and knocks out cold and flu miseries. There have not been many reports on the use of Kanthari mulaku (*C. frutescens)* as an antmicrobial agent against phytopathogens. The potential of this crop due to the numerous biocompounds present has not been exploited towards the development of a natural antimicrobial agent. The present research work was henceforth carried out, with the objective of isolation and purification of antimicrobial peptides from leaf and fruit extracts of Kanthari mulaku and to evaluate its antibacterial and antifungal activity.

MATERIALS AND METHODS

I. Collection of Bacterial and Fungal culture

The bacterial cultures of *E coli, Staphylococcus aureus* and *Klebsiella pneumoniae* were obtained from Microbiology Laboratory of St Peter's College and the fungal cultures of *Fusarium, Phytophthora, Alternaria and Colletotrichum* were provided by Department of Plant Pathology, Kerala Agricultural University, Thrissur, Kerala. All the strains were confirmed by cultural and biochemical characteristics. The bacteria were cultured on Nutrient Agar (HiMedia) and the fungi were cultured on Potato Dextrose Agar (HiMedia) and maintained in slants till further use.

II. Collection of Plant materials

The fruit and leaf samples were collected from the Botanical garden of St Peters College, Kochi, India.

III. Extraction of Antimicrobial Proteins

Antimicrobial peptides were extracted from fruits and leaves of the plant using protein extraction buffer (50mM phosphate buffer pH 7.0, 50mM NaCl, 2mM EDTA). This extract was further centrifuged at 10,000 rpm at 4^{o}C for 15 minutes. The crude extract obtained was saturated with 80% ammonium sulphate. This was further purified using Sephadex G50 gel filtration column and protein estimation was carried out.

IV. ASSAY METHODS

a. Assay by Disk Diffusion Method

The antibacterial activity screening of the purified extract was carried out by Disk diffusion method [6]. Briefly, 100 µl of the test bacteria (*E. coli, K. pneumonia,* and *S.aureus)* were grown in 10 ml of fresh media until they reached a count of approximately 10^8 of microbial suspension. A sterile swab dipped in this culture was used for inoculating the dried surface of the Mueller Hinton Agar (HiMedia) plate. The petriplate was rotated approximately 60 degrees and the streaking motion was repeated three times over the entire agar surface. Small sterile disks (5 mm) were impregnated with 50µL of extract (50-250 mg/ml concentration) and placed on the agar plate. Filter paper disk impregnated with 50 µl of distilled water served as the control. The Petri plates inoculated with each of the test organisms were incubated at 37 ^{0}C for 24 hours and the diameters

of the inhibition zones were measured. Each antimicrobial assay was performed in three replicates and mean values taken.

b. Assay by Agar Incorporation Method

The antifungal screening activity of the extract was examined by Agar incorporation method [7]. This method involves the mixing of the plant extract with (PDA) agar and then transferring the fungi to determine the inhibitory activity of the extract. The strains were cultivated on potato dextrose agar (PDA) in petri-dishes. Twenty ml of PDA was aseptically mixed with the extract in varying concentration (1mg/ml, 3 mg/ml and 5 mg/ml). After solidification, the seeding was carried out by inoculating the media with the fungal isolates separately in the middle of the petri dishes. The petri dishes were then incubated at room temperature for 5 days. Each concentration is replicated three times. PDA media without extract is used as the control. Percentage inhibition caused by extract was calculated as follows: % inhibition = (diameter of growth in the control – diameter of growth in the treatment sample)*100/diameter of growth in the control[8].

c. Broth Dilution Method

The MIC of the extracts was examined by broth dilution method using spectrophotometer. For this extract was serially diluted to concentrations of 1 to 6 mg/ml of extract in broth medium in a series of test tubes. The test organism (fungi) was aseptically inoculated to the tubes containing extracts and a tube without extract serves as positive control. Uninoculated broth served as blank. Incubated the tubes in a shaker for 120hrs and turbidity was read at 600 OD using spectrophotometer[9].

RESULTS

The organisms used and zone of inhibition to the corresponding extracts of *C frutescens* and *C annuum* are shown in Table 1. Antibacterial activity at different doses was done for both the purified leaf and fruit extract by disk diffusion method. Concentration was in the range of 50 to 250 mg/ml per disk. Activity was dependent on the dose of the test material. As the concentration increased the inhibition zone was also increased. Maximum inhibition was obtained for 250 mg/ml per disk of purified fruit and leaf extract. *E coli, S aureus* and *K pneumonia* showed inhibition zone of 7, 9 and 11mm respectively when *C frutescens* fruit extract was used. In the case of *C frutescens* leaf extract, *E coli ,S aureus* and *K pneumonia* showed inhibition zone of 3, 12 and 8 mm respectively (Figure 2). The fruit and leaf extract of *C annuum* showed inhibitory action only against *S aureus* (8mm and 5 mm respectively)

Table I

Antibacterial activity of leaf and fruit extracts of C frutescens and C annuum

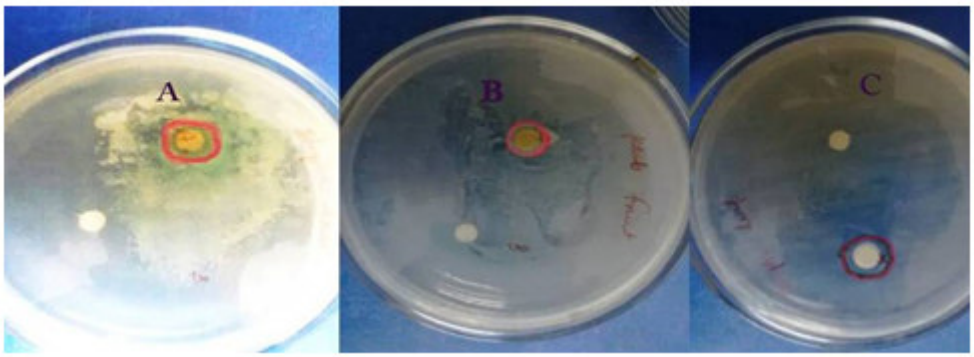
Plant Extract	Test organisms	Zones of growth inhibition (mm)	
		C frutescens	*C annuum*
Fruit	*E coli*	7 mm ± 0.25	Nil
	Staphylococcus aureus	9mm ± 0.5	8mm ± 0.3
	Klebsiella pneumonia	11 mm ± 0.3	Nil
Leaf	*E coli*	3mm ± 0.2	Nil
	Staphylococcus aureus	12 mm ± 0.4	5mm ± 0.4
	Klebsiella pneumonia	8 mm ± 0.5	Nil

Values are mean± standard error of 3 replications

A- E coli
B- Klebsiella pneumonia
C- Staphylococcus aureus

Figure 2

Antibacterial activity screening of purified peptide fraction of Capsicum frutescens by disk diffusion method



The antifungal activities of fruit and leaf extracts of *C frutescens* and *C annuum* are illustrated in Table 2. The fungus *Alternaria* and *Colletotrichum* has shown 100% inhibition of growth and Fusarium showed 66% inhibition of growth at 5 mg/ml concentration of *C frutescens* fruit extract (Graph 1 and Graph 2). *Alternaria, Colletotrichum and Fusarium* showed 75%, 12.5% and 17.5% respectively inhibition of growth when purified *C frutecens* leaf extract was used at 5mg/ml concentration (Graph 3 and Graph 4). The fungi *Alternaria, Colletotrichum and Fusarium* also produced 100%, 75% and 2% respectively, inhibition of growth at

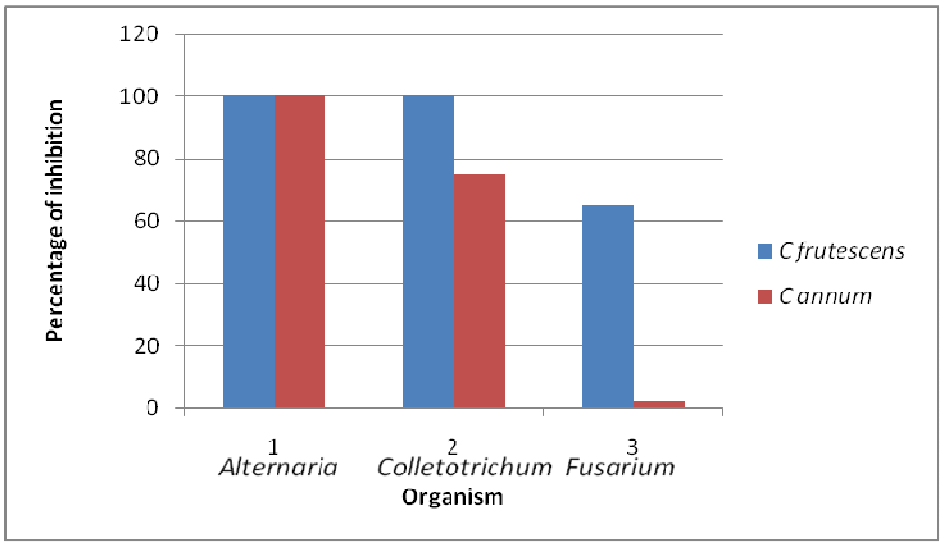
5 mg/ml concentration of *C annuum* fruit extract. The leaf extract of *C annuum* however showed 38%, 20 % and 53% growth inhibition of *Alternaria, Colletotrichum and Fusarium* at 5 mg/ml concentration (Figure 3). The fruit and leaf extract of both the *Capsicum* species did not produce any inhibitory activity against *Phytophthora*.

Table 2
The antifungal activity of fruit and leaf peptides on phytopathogenic fungi in percentage of growth inhibition at conc. 5 mg/ml

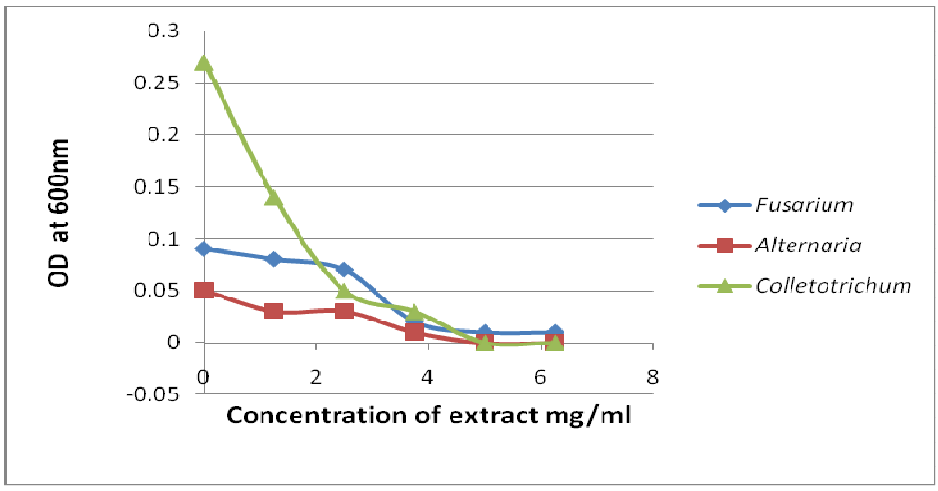
Plant Extract	Fungal strains	*C frutescens*	*C annuum*
Fruit	*Alternaria*	99± 0.5	99±0.12
	Colletotrichum	99± 0.7	75 ±0.36
	Fusarium	65.9 ± 0.56	2.12 ±0.14
	Phytophthora	Nil	Nil
Leaf	*Alternaria*	75 ±0.25	37.5 ±0.65
	Colletotrichum	12.5 ± 0.7	20 ±0.12
	Fusarium	17.5± 0.63	52.5± 0.6
	Phytophthora	Nil	Nil

Values are mean ± standard error of 3 replications

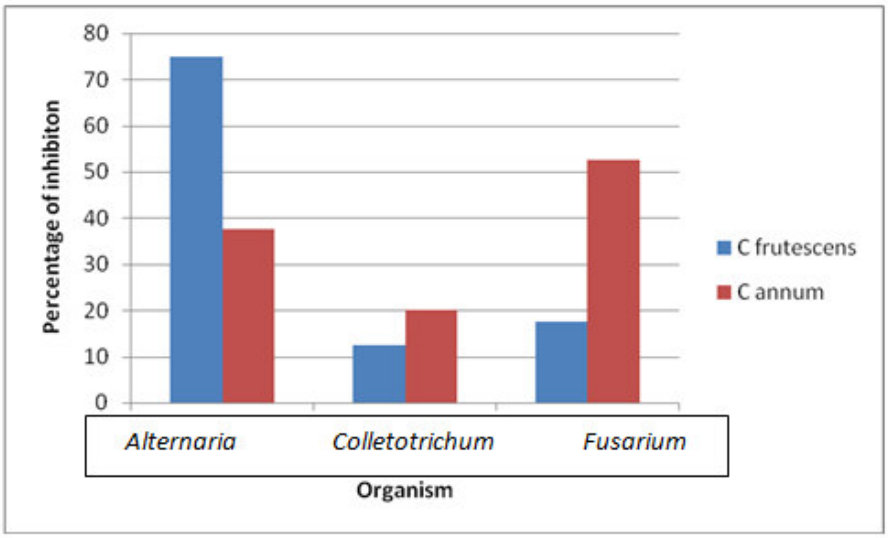
Graph 1
Antifungal activity of fruit peptides on phytopathogenic fungi in percentage of growth inhibition at conc 5mg/ml



Graph 2
Graph showing antimicrobial activity of fruit peptides of C frutescens at different concentrations against phytopathogenic fungi



Graph 3
Antifungal activity of leaf peptides on phytopathogenic fungi in percentage of growth inhibition at 5 mg/ml concentration



Graph 4
Graph showing antimicrobial activity of leaf peptides of C frutescens at different concentrations against phytopathogenic fungi

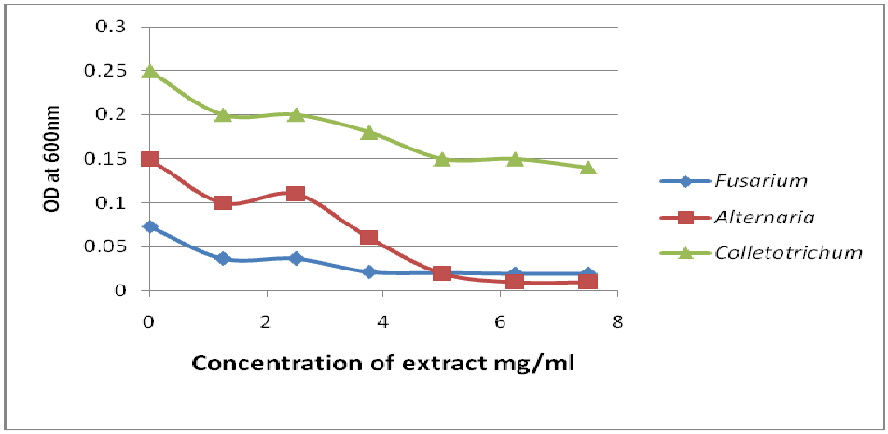
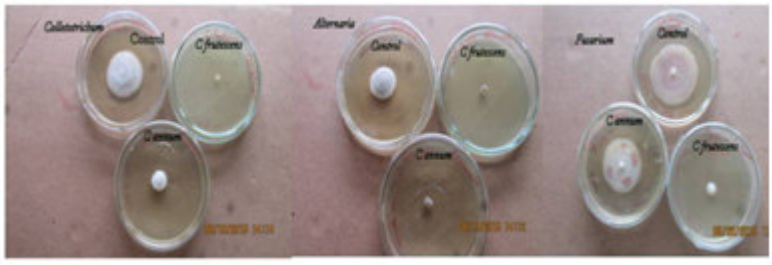


Figure 3
Antifungal activity of fruit peptides of C frutescens and C annuum against phytopathogenic fungi (Colletotrichum, Erwinia, Fusarium) by agar incorporation method



DISCUSSION

Antimicrobial peptides (AMPs) are small molecular weight proteins with broad spectrum antimicrobial activity, isolated from various organisms and effective against bacteria, viruses, and fungi. These can be exploited for disease control in plants in a way that complies with the strict regulations on the efficacy and safety of disease control strategy. There have already been reports regarding the antimicrobial properties of various chilly species. Nascimento et al [10] have reported good inhibitory activity by the ethanolic extract of *Pimenta malagueta (C. frutescens)* against both Gram-positive bacteria as well as Gram-negative bacteria. They also reported that among the various phytochemical compounds studied in this species, the antimicrobial property was mainly due to chrysoeriol [11]. The antifungal potential of aqueous leaf and fruit extracts of *Capsicum frutescens* against four major fungal strains associated with groundnut storage showed strong activity (nearly 88% at 10 mg/ml)

against *Aspergillus flavus* and *A niger* [12]. A small-fruited pungent pepper accession, *Capsicum frutescens* 'BG2814-6' was reported to be resistant to several isolates of *Cucumber mosaic virus* (CMV).This could facilitate the development of commercially acceptable pepper varieties with adequate levels of CMV resistance [13]. The results of the present study also highlight the antimicrobial property of Kanthari mulaku (*C frutescens).* The purified fruit and leaf extracts showed only weak inhibitory action against *Ecoli, Staphylococcus aureus* and *Klebsiella pneumonia* as it did not comply with the zone diameter interpretive standards of these bacteria. The peptide fraction from the fruit exhibited strong inhibitory action against the fungi, *Alternaria* and *Colletotrichum* which are main pathogens of various crop plants especially vegetables. The purified leaf extract also showed good fungicidal activity against *Alternaria*.

CONCLUSION

AMPs could play strong roles in agriculture as plant protection agents. Majority of the studies were at the *invitro* level and no tests have been carried on plant pathosystems. Over the years much effort has been devoted to the search for naturally derived fungicides that are believed to be eco friendly with no side-effects. Exploitation of naturally available chemicals from plants, which has antimicrobial activity can play a prominent role in the development of future commercial pesticides for crop protection. Many studies on the *Capsicum* genus have been reported including *Capsicum frutescens*. However in the studies with *Capsicum frutescens* there are no details regarding the purification and antimicrobial screening of Sephadex G50 column purified fruit and leaf extract of the local chilly variety, Kanthari mulaku (*C. frutescens*). The strong inhibitory action against *Alternaria*, a major phytopathogen of crop plants offers much scope for the development of a biofungicide. The antimicrobial potency of the plant may be attributed to the single or combined effect of many bioactive compounds. Further studies are being carried out to identify and isolate the specific compounds responsible for the antifungal activity exhibited by these extracts of *C. frutescens.*

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