

ANTIMICROBIAL ACTIVITY OF SELECTED SPICES AGAINST *Pectobacterium carotovorum* ISOLATED FROM SOME VEGETABLES IN SRI LANKA

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ABSTRACT

Pectobacterium carotovorum is the most common causative organism of bacterial soft rot which gives rise to great economic losses. It is important to implement disease control measures and management strategies on this bacterium in order to prevent further crop loss. Among the various management strategies, using the antibacterial nature of spices offer promising solutions to pesticidal hazards and also to increasing cost of plant protection operations. Although there are many reports on the antibacterial nature of spices on various pathogens, its effect on *P. carotovorum* is limited. Objectives of this study were to determine the antimicrobial activity of selected spices and then to ascertain the minimum inhibitory concentration (MIC) of aqueous and ethanolic extracts of those spices against *P. carotovorum*. Among the nine tested spices, four were active against *P. carotovorum*. As depicted by agar well diffusion assay, the highest inhibitory effect on *P. carotovorum* was shown by the aqueous and ethanol extracts of *Allium sativum* while the aqueous and ethanolic extracts of *Cinnamomum verum* gave the smallest zones of inhibition. *Garcinia cowa* and *Tamarindus indica* displayed moderate antibacterial activity. There was no significant difference ($p>0.05$) in inhibition zones between aqueous and ethanol extract of a particular spice. MIC of the spices, which inhibited the growth of *P.*

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carotovorum was 0.5 g/ml within the test range. Positive extracts can be used as a cost effective and eco-friendly preventive strategy to minimize the effects of *P. carotovorum*. Evaluation of the antibacterial activity of these extracts under field conditions is necessary before a cost-effective formulation is developed.

Keywords: *Pectobacterium caratovorum*, soft rot, spice extract, garlic, inhibition zone

INTRODUCTION

Plant associated diseases are the main constraints in crop cultivation which leads to significant reduction in yield and product quality. Plant pathogenic microorganisms such as bacteria and fungi contribute significantly to this problem. Bacterial soft rot caused by *Pectobacterium carotovorum* (syn. *Erwinia carotovora*) is destructive disease in vegetable crops, which remains a widely unresolved issue, causing heavy burdens in economy (Dhanvantari and Dirks 1987; Dickey and Kelman 1988; Olayemi *et al.*, 2012).

P. carotovorum is a Gram negative, soil borne opportunistic phytopathogen which macerates plant tissues using its pectolytic ability. This bacterium has been classified under the family Enterobacteriaceae (Perombelon 1990) where many phyto and human pathogens are included. Pathogenicity of *P. carotovorum* is known to be through secretion of exoenzymes such as pectinases leading to degradation of the fleshy parts of the plants ranging from root of carrots to leafy green vegetables such as lettuce.

Currently the pathogen and disease management is mainly through cultural and chemical strategies (Feldberg *et al.*, 1988; Wright *et al.*, 2005; Paradza *et al.*, 2012). However, these various management strategies confer innumerable problems such as pesticidal hazards, environmental pollution, pesticide residues in crops and ever growing cost of the plant protection operations.

Recently intense studies have been focused on the antibacterial nature of medicinal plants including spices (Eltaweel, 2014) as a promising solution to these myriad obstacles. Various phytochemicals and secondary metabolites present in spices are responsible for their characteristic aromas and flavours (Avato *et al.*, 2000; Joe *et al.*, 2009). Not only the aroma and flavour, the antimicrobial activity of spices is also governed by these phytochemicals that act as phyto-protectants in plants (Joe *et al.*, 2009). Bioactive compounds such as isoflavones, anthocyanins, flavonoids (Chang 1988; Joe *et al.*, 2009) are found in spices.

Allium sativum (garlic), *Tamarindus indica* (tamarind), *Garcinia cowa* (garcinia), *Cinnamomum verum* (cinnamon), *Piper nigrum* (pepper), *Curcuma longa* (turmeric), *Zingiber officinale* (ginger), *Brassica nigra* (mustard) and *Syzygium aromaticum* (clove) are some of the most commonly consumed spices in Sri Lanka. The antimicrobial potential of these nine spices have already been evaluated on many phytopathogenic fungi and human pathogens of family Enterobacteriaceae. However, literature related to effects of the above spices on *P. carotovorum* is limited.

Therefore, the present study was aimed at evaluation of the antimicrobial activity of above nine spices against *P. carotovorum*. The minimum inhibitory concentration (MIC) of aqueous and ethanolic extracts of selected spices against *P. carotovorum* was also determined.

MATERIALS AND METHODS

Sample Collection

Vegetables with soft-rot were collected from different geographical regions in Sri Lanka (Table 1). The samples were transported in an icebox to laboratory at the Faculty of Applied Sciences, Rajarata University of Sri Lanka for experiments.

Table 1: Vegetables selected for the study and regions in Sri Lanka for sample collection.

Botanical name of vegetable	Vernacular name of vegetable	Family	Geographical Regions
<i>Allium cepa</i>	Onion	Liliaceae	Ambalanthota
<i>Allium porrum</i>	Leeks	Liliaceae	Welimada
<i>Brassica caulorapa</i>	knolkhol	Brassicaceae	Thalawakale
<i>Brassica oleracea</i> var. <i>capitata</i>	Cabbage	Brassicaceae	Galewela, Ambalanthota
<i>Daucus carota</i>	Carrot	Apiaceae	Thalawakale, Welimada
<i>Phaseolus vulgaris</i>	Common beans	Fabaceae	Wellawaya
<i>Raphanus sativus</i>	Radish	Brassicaceae	Thalawakale, Nuwaraeliya
<i>Solanum tuberosum</i>	Potato	Solanaceae	Nuwaraeliya, Welimada

Pathogen isolation, purification and identification

The vegetables were washed with running tap water to remove soil particles attached to the surface. Then the vegetable parts with symptoms of soft-rot disease were separated and surface sterilized with 0.5% sodium hypochlorite for three minutes followed by three repeated washings in sterilized distilled water (SDW), respectively. The scrapings of symptomatic tissues of vegetables were obtained using a sterile scalpel and suspended in

1 ml of SDW separately. A loop-full of the prepared suspension was streaked on MacConkey agar plates and incubated at 29 °C for 24 hours to obtain bacterial colonies (Al-Zomor *et al.*, 2013). After incubation, pink color pure colonies observed to be *P. carotovorum* were used to obtain pure colonies on Nutrient Agar (NA)

P. carotovorum colonies were further confirmed using conventional biochemical tests namely Gram staining, KOH string test, oxidase test, catalase test, indole production, H₂S production and starch hydrolysis according to the procedure followed by Marwaha *et al.*, (2015).

Pathogenicity testing

Fresh, disease-free carrot and potato tissues were used to determine the pathogenicity of isolated bacterial colonies. Carrot and potato tissue segments were washed with running water to remove surface dirt. Then surface sterilization was done using 3% sodium hypochlorite as described before. The surface sterilized vegetables were sliced to a thickness of 7-8 mm and kept on sterile glass slides. Equidistant three spots were injured on the surface of the tissues using a sterilize needle. Subsequently, a loopful of pure bacteria was introduced into two spots using a sterile tooth pick and the remaining spot was maintained as the control. The inoculated tissues were placed in individual moist chambers at room temperature for 24 – 48 hours and observed for the appearance of the symptoms of soft-rot (Perombelon, 2002).

Preparation of spice extracts

Garlic, garcinia, tamarind, cinnamon, clove, turmeric, pepper, mustard and ginger were purchased from local market. Different plant parts of these spices were selected (Table 2) to evaluate their antibacterial activity against the phyto-pathogenic strain *P. carotovorum*.

Table 2: Spices selected against *P. carotovorum*.

Botanical name	Common name/ English name	Family	Plant part used
<i>Allium sativum</i>	Garlic	Alliaceae	Bulb
<i>Garcinia cowa</i>	Garcinia	Clusiaceae	Fruit
<i>Cinnamomum verum</i>	Cinnamon	Lauraceae	Bark
<i>Tamarindus indica</i>	Tamarind	Fabaceae	Fruit
<i>Syzygium aromaticum</i>	Clove	Myrtaceae	Flower bud
<i>Brassica nigra</i>	Mustard	Brassicaceae	Seed
<i>Curcuma longa</i>	Turmeric	Zingiberaceae	Rhizome
<i>Piper nigrum</i>	Black pepper	Piperaceae	Fruit
<i>Zingiber officinale</i>	Ginger	Zingiberaceae	Rhizome

The plant extracts were prepared according to the procedure followed by Indu *et al.* (2006). Five gram (5.0 g) of each spice was weighed. Each spice sample was crushed in a sterilized mortar and pestle and mixed with SDW and 5% ethanol at a ratio of 1:2 (w/v) in order to prepare extracts of spices. The extracts were filtered first using muslin cloth and then through Whatman No. 1 filter paper. The filtrates were used as the standards to formulate series of dilutions from 0.5 g/ml to 0.005 g/ml.

Antibacterial Sensitivity Screening of Spice Extracts

Freshly streaked colonies of *P. carotovorum* were transferred to SDW under aseptic conditions and the concentration of the bacterial cells in the suspension was adjusted according to 0.5 MacFarland standard (equal to 10^6 CFU/ml). Agar well diffusion assay, which was used to determine the antibacterial activity and MICs, was performed using the standard bacterial suspension. Twenty (20) μ l of different concentrations of aqueous and ethanolic crude extracts were introduced into agar wells on NA plates separately. Sodium hypochlorite was used as positive control while SDW and 5% ethanol were used as negative controls. The culture plates were incubated at room temperature for

18 – 24 hours. The inhibition zones were measured as entire diameter (including the wells) according to the antimicrobial susceptibility testing manual (Coyle, 2005) and recorded in millimeters.

Statistical Analysis

The significance of data was analyzed by T-test, Mann-Whitney U test and one-way ANOVA using Minitab 16.0.

RESULTS

Pathogen isolation, purification and identification

Colonies isolated on MacConkey agar were pink, convex, circular with entire margins. They were 1 to 3 mm in diameter. The colonies transferred to NA plates produced light cream color, convex, circular, 1 - 3 mm diameter colonies with entire margin. The biochemical tests confirmed the identity of *P. carotovorum* (Table 3).

Table 3: Results of phenotypic tests of bacteria isolated from vegetables.

Phenotypic test	Result
Gram staining	Gram negative
Cell shape	Rod
Colony color	Light cream
KOH string test	-
Catalase test	+
Oxidase test	-
Indole production	-

Antibacterial sensitivity screening of spice extracts

Among the nine tested spices, only four spices; garlic, garcinia, cinnamon and tamarind were active against *P. carotovorum* according to the agar well diffusion assay (Table 04). The aqueous extract of garlic displayed the largest zone of inhibition (40 mm). The aqueous and ethanolic suspensions of cinnamon showed lowest zones of inhibition (16 mm) among the active spices. Tamarind and garcinia exhibited moderate action against *P. carotovorum*.

The ethanol extract of garlic had the highest diameter of zone of inhibition (37 mm) among all ethanolic extracts of spices. There was no significant difference ($p>0.05$) in inhibition zones between aqueous and ethanol extract of a particular spice.

The antibacterial sensitivity of both aqueous and ethanolic extracts of spices was evaluated using series of concentrations ranging from 0.005 g/ml to 0.5 g/ml. Among these concentrations, visible growth of pathogenic bacteria was hindered at 0.5 g/ml in both aqueous and alcoholic extracts. Therefore, Minimum inhibitory concentrations of the spices, against *Pectobacterium* was 0.5 g/ml.

Table 4: Antibacterial activity of spice extracts against *Pectobacterium carotovorum*.

Name of the spice	Average diameter of zone of inhibition (mm) at 0.5 g/ml	
	Aqueous extract	Ethanolic extract
Garlic	40 ^a	37 ^a
Tamarind	33 ^b	32 ^b
Garcinia	31 ^b	34 ^b
Cinnamon	16 ^c	16 ^c

*mean separation denoted by same letter group has no significant difference ($p>0.05$)

DISCUSSION

In recent years, the applications of eco-friendly organic farming which utilize botanicals gained popularity as an alternative to the hazardous agrochemicals which cause severe issues such as pathogen resistance, diminishing efficacy of agrochemicals in agricultural and environmental settings (Slusarenko *et al.*, 2008). The use of botanicals expands over the time due to its low toxicity especially on mammalian systems (Bankole, 1996).

In the present study nine common Sri Lankan spices were selected to investigate their antimicrobial activity on phytopathogenic *P. carotovorum*. Two types of preparations, namely aqueous extract and ethanolic extract of selected spices with different concentrations were tested on the pathogen. Among all the crude suspension of spices tested, *P. carotovorum* was highly sensitive to the aqueous and ethanolic extracts of *Allium sativum* (garlic). Raju *et al.*, (2007) reported that garlic conferred moderate inhibitory effect on *P. carotovorum*. Simeon and Abubakar (2014) recorded that the application of

0.5% aqueous extract of *A. sativum* on the surface of a diseased potato tuber showed inhibition of rot. These reports support the fact that the garlic extracts are effective against the causative agent of soft rot. However, according to Paradza *et al.*, (2012), 0.25 g/ml aqueous solution of garlic did not exhibit any inhibitory effect towards *P. carotovorum*. Our preset study shows 0.5 g/ml is the minimum inhibitory concentration for the soft rot bacterium. However, two other *Pectobacterium* sub species namely *Dickeya dadantii* and *P. atroseptica* had previously responded to 10% and 25% aqueous preparation of garlic cloves (Paradza *et al.*, 2012).

The antibacterial activity of *A. sativum* could be attributed to the action of bioactive compounds, allicin or diallyl thiosulfinic acid or diallyl disulfide (Avato *et al.*, 2000). Allicin is known to inhibit RNA synthesis totally and inhibit DNA and protein synthesis partially of both Gram positive and Gram-negative bacteria (Feldberg *et al.*, 1988; Harris *et al.*, 2001).

Although, the antibacterial activity of tamarind (fruit), garcinia (fruit) and cinnamon (bark) have been confirmed by this study, only limited research indicated similar results on *P. carotovorum*.

Both aqueous and ethanolic extracts of clove, turmeric, pepper, mustard and ginger showed no effect against *P. carotovorum* in this study. According to Simeon and Abubakar (2014) 0.05 g/ml aqueous extract of ginger exhibited some inhibitory effect on the rot induced by *P. carotovorum*. Antimicrobial potential of ginger may be due to the active principles such as sesquiterpenoids (Malu *et al.*, 2009; Gull *et al.*, 2012), zingiberol, zingiberine and bisabolene (Derrida 1999; Joe *et al.*, 2009). The current research work is in conformity with the study of Rahman *et al.*, (2012) in that *P. carotovorum* has showed resistance to 1% extract of *Curcuma longa* (turmeric). Akbar *et al.*, (2014); Vishwanath *et al.* (2018) reported that 10 % extract of turmeric rhizome was active against *Erwinia carotovora*. However, as emphasized by previous studies, all spices mentioned above exhibited antimicrobial activity on many other pathogenic bacteria including bacteria in family enterobacteriaceae especially *E. coli*.

Pectobacterium species elicit their pathogenicity via production of four types of pectolytic enzymes to degrade the soft tissues in the host plant (Perombelon, 2002). Therefore, suppression of the production of pectic enzymes is vital in the approach to inhibit this pathogen. As Sagdic (2003), Gull *et al.*, (2012) described, the antimicrobial activity depends on many factors; the nature and number of bioactive compounds within the spice, concentration of the spice used and species of microorganism used, pH of the

test medium and environmental factors such as temperature. Therefore, evaluation of the antimicrobial activity of these spice extracts against *P. carotovorum* in the field is needed prior to development of a cost-effective formulation.

CONCLUSION

According to the present study, the aqueous extract of garlic is the most effective spice against *P. carotovorum* among the tested spices. Tamarind and garcinia also possess substantial effectivity against *P. carotovorum* while cinnamon is the least effective among the test spices. The effective MIC of test spices is 0.5 g/ml. Therefore, these extracts have the potential to be used as cost-effective and eco-friendly preventive strategy to eliminate *P. carotovorum* from susceptible plant material.

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