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Antimicrobial activity of medicinal plants against *Xanthomonas campestris* pv. *campestris* causing black rot of cabbage

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Abstract
Cabbage is considered as the fifth most important vegetable crop in India and major constraint in its production is black rot caused by *Xanthomonas campestris* pv. *campestris* (*Xcc*). The management of the disease is difficult and the chemicals are least effective and are hazardous. Hence, a study was carried to test the potentiality of few medicinal plant extracts against the pathogen as the effective ones could be exploited as an alternate, cheap and eco friendly method of management. The water extract from only two botanicals *viz*., *Ocimum gratissimum* and *Tylophora asthmatica* were effective in inhibiting the growth of *Xcc*. *O. gratissimum* extract showed highest inhibition zone of 29.33 mm at 1:0 dilution and had inhibitory effect upto 1:10 dilution with 22.33 mm inhibition zone. Alcohol extract of *O. gratissimum* was the most effective in inhibiting the growth of *Xcc* followed by *Calotropis gigentea*, *T. asthmatica*, *O. sanctum*, *Nigella sativa* and *Ruta graveolens*.

Keywords: medicinal plants, *Xanthomonas campestris* pv. *campestris*, cabbage, inhibition zone, blackrot

Introduction
Cabbage (*Brassica oleracea* var. *capitata*) is an important vegetable crop grown throughout India. Its total acreage in India during 2014-15 was 0.38 million ha with a production of 8.60 million tonnes, which makes it fifth important vegetable crop after potato, onion, tomato and egg plant. During the same year, the production of cabbage in Karnataka was 221.27 thousand tonnes from 10.49 thousand ha area with the productivity of 21.09 metric tonnes (Anonymous 2016) [1].

Black rot, caused by the seed-borne bacterium *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson (*Xcc*), is one of the most devastating diseases of cabbage (Vicente & Holub, 2013) [14]. This pathogen is responsible for severe economic losses worldwide (Rimmer *et al*., 2007) [11]. When the pathogen becomes established in the field early in the growing season and favourable environment prevails, it may become extremely destructive leading low-quality crops with reduced market value. Plants affected by black rot are also more susceptible to infection by other pathogens such as the soft rot causing bacteria *Pseudomonas* spp. and *Erwinia* spp., adding to the economic damage already caused by black rot (Hoda Ghazalibiglar *et al*., 2016) [5].

The use of chemicals has not been effective in the control of black rot of cabbage because the pathogen is a seed borne and is systemic in its nature. The use of copper based bactericides and antibiotics seldom gave satisfactory control. Traditional antimicrobial plant materials could be explored as an alternative method in the management of the disease as they are cheap and can be locally produced. The plant kingdom represents an enormous reservoir of biologically active compounds with various chemical structures and disease preventing properties (Kavitha and Satish, 2013) [7]. There has been a growing interest in the research of the possible use of the plant-derived natural pesticides such as plant extracts, which can be relatively ecofriendly for disease control in agriculture (Choi *et al*., 2008) [3]. Botanicals because of their natural origin are biodegradable and they do not leave toxic residues or by-products to accumulate in the environment. Therefore under this scenario, botanical pesticides seem to be ideal candidate to be exploited in management of black rot of cabbage in view of the safety, renewable nature, cost effective and high target specificity. Hence an investigation was conducted to study the effect of extracts from few important medicinal plants against *Xcc*.

Material and methods

Selection of medicinal plants

Medicinal plants mentioned in table 1 which were reported to contain some antibacterial constituents and being used in Indian system of medicine (Kamala Ramachandran *et al*., 1986) [6] were selected to screen for their antibacterial properties against *Xcc* causing black rot of cabbage. These plants were collected from College of Agriculture, Mandya.

Isolation of pathogen

Cabbage leaves showing typical symptoms of black rot were collected and cut into small pieces aseptically from the edge of typical lesion along with healthy tissue. The infected leaf bits were surface sterilized in 70 per cent alcohol and was washed in three series of sterile water to remove traces of alcohol. The bits were suspended in a drop of sterile water taken on a sterilized microscope slide and were allowed for five minutes. When a drop of water became turbid due to oozing of bacterial cells from the cut ends of the leaf bits, a loopful of the bacterial suspension was taken on an inoculation needle aseptically and streaked on the surface of nutrient agar contained in sterilized Petri plates. The inoculated plates were incubated at 30^0C for 48 hours and were observed for the development of well-separated typical *Xanthomonas* colonies.

It was purified by picking the individual colonies and streaked on the surface of yeast extract dextrose calcium carbonate agar (YDCA) medium contained in Petri dishes. Three to four loop ful of well-separated colonies were suspended in sterile distilled water taken in vials. The vials were stored at 5^0C and served as stock culture for further studies.

The bacterium isolated from diseased plant was identified on the basis of morphological, cultural and biochemical characteristics prescribed by Bradbury (1986) [2] and Schaad and Stall (1998) [13].

Method of Extraction

Two most common methods used in extraction were followed to extract the antimicrobial components contained in eight different plant species in order to screen for their antimicrobial property against tomato bacterial wilt pathogen were (1) Water extract method (2) Alcohol extract method.

Protocol for water extract

The economic parts of the plants noted in table 1 were used for the purpose of extraction. 50g of leaves or seed as the case may be were taken and cut into small pieces under aseptic condition. The sample was put into waring blender containing 50ml sterilized distilled water at a ratio 1:1 (water: plant material). The sample was spun at low speed for 10-15 minutes in a coffee warring blender till the material formed to fine texture. The blended material was then squeezed through a sterilized muslin cloth so as to get a crude liquid extract. The crude extract was filtered through Whatman no 1 filter paper followed by sterilized Seitz filter. The sterilized filtrate was collected in sterilized glass tubes and the tubes were sealed under aseptic condition and labelled as "WE". The water extract was kept at 5ºC in a refrigerator for further use.

Table 1: List of medicinal plants used to test their antimicrobial properties against *X. campestris* pv. *campestris*

Sl. No	Common Name	Scientific Name	Family	Part used for extraction
1	Antamul	*Tylophora asthmatica W. & A.*	Asclepiadaceae	Leaves
2	Mudar	*Calotropis gigentea L*	Asclepiadaceae	Shoot
3	Holy basil	*Ocimum sanctum L*	Lamiaceae	Leaves
4	Clocimum	*Ocimum gratissimum L*	Lamiaceae	Leaves
5	Tinospora	*Tinospora cardifolia Willd.*	Menispermaceae	Leaves
6	Black Cumin	*Nigella sativa L.*	Ranunculaceae	Seeds
7	Garden Rue	*Ruta graveolens L.*	Rutaceae	Shoot
8	Meswak	*Salvadora persica L.*	Salvadoraceae	Stem

Protocol for alcohol extract

Fifty gram of the economic parts of the respective plant was mixed with a small quantity of 70 per cent ethyl alcohol and macerated in a pestle and mortar under aseptic condition. The material was blend to fine texture, transferred to a beaker and the final volume was made up to 50ml with 70 per cent ethyl alcohol in the ration of 1:1 (plant material: alcohol). The beaker was kept overnight under refrigerated condition. Alcohol extract was squeezed through muslin cloth, then passed and finally sterilized through Seitz filter apparatus. The sterilized filtrate was collected in sterilized glass tubes and the tubes were sealed under aseptic condition and labelled as "AE". The alcoholic extract was stored at 5ºC in a refrigerator for further use.

In-vitro evaluation of plant extracts

Both water and alcohol extracts of the medicinal plants were screened at different dilutions viz., 1:0 (undiluted), 1:1, 1:10, 1:100, 1:1000. The efficacy of the extracts was tested by the zone of inhibition assay technique against *Xcc* causing black rot of cabbage. A heavy suspension of the test bacteria ($7x10^8$cfu/ml) was seeded to the sterilized nutrient agar medium by mixing the bacterial cultural with the cooled nutrient agar (45-50ºc) in a 500ml Erylenmeyer flask. The seeded medium was poured on sterilized Petri plates and allowed to solidify.

Sterilized filter paper disc (Whatman no.1) measuring 10mm diameter were soaked for 10 minutes in undiluted (1:10) and diluted (1:1, 1:10, 1:100 and 1:1000) plant extracts and placed on the surface of seeded nutrient agar medium contained in the Petri plates in marked position. The inoculated plates were incubated first at 4ºC for 4 hours so as to allow the diffusion of the extract into the medium. The plates were then transferred to incubator maintained at 30ºC and incubated for 48 hours. Observations were recorded on the zone of inhibition produced around the filter paper disc in each plant extract at different dilutions, by measuring the diameter of the inhibition zone. The data was statistically analysed by using factorial design.

Results and Discussion

Effect of water extract of medicinal plants against *X. campestris* pv. *campestris*

Water extracts of *O. gratissimum* and *T. asthmatica* had inhibitory effect against *Xcc* and rest of plant extracts tested had no effect at all. *O. gratissimum* extract showed highest inhibition zone of 29.33 mm at 1:0 dilution and had inhibitory effect upto 1:10 dilution (22.33 mm) which was far better

than the control (15.66 mm). Whereas, *T. asthmatica* was also effective upto 1:10 dilution and was superior to control upto 1:0 dilution with inhibition of 18.66 mm (table 2). Saha *et al*. (2013) [12] reported inhibitory effect of extracts of five *Ocimum* species against Gram-positive and Gram-negative bacteria and few plant pathogenic fungi.

Table 2: Evaluation of water extracts of medicinal plants against *X. campestris* pv. *campestris*

Dilution	Zone of inhibition (mm)							
	O. gratissimum	***O. sanctum***	***T. asthmatica***	***R. graveolens***	***C. gigentea***	***N. sativa***	***S. persica***	***T. cardifolia***
1:0	29.33 (5.51)	0.00 (1.00)	24.33 (5.03)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
1:1	24.33 (5.03)	0.00 (1.00)	23.00 (4.90)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
1:10	22.33 (4.83)	0.00 (1.00)	18.66 (4.43)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
1:100	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
1:1000	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Streptocycline 400 ppm. (control)	15.66 (4.08)	15.66 (4.08)	15.66 (4.08)	15.66 (4.08)	15.66 (4.08)	15.66 (4.08)	15.66 (4.08)	15.66 (4.08)

(Figures in parenthesis are square root transformed values)

	S. Em+	C. D. (1%)
Factor A	0.0088	0.0248
Factor B	0.0088	0.0248
A x B	0.0215	0.0607

Effect of alcohol extract of medicinal plants against *X. campestris* pv. *campestris*

The alcohol extracts of six medicinal plants viz., *O. gratissimum*, *O. sanctum*, *T. asthmatica*, *R. graveolens*, *C. gigentea*, and *N. sativa* exhibited inhibitory effect whereas, *S. persica* and *T. cardifolia* had no such effect (table 3). Among the botanicals tested, *O. gratissimum* was the most effective in inhibiting the growth of *Xcc* followed by *C. gigentea*, *T. asthmatica*, *O. sanctum*, *N. sativa* and *R. graveolens*. Extract of *O. gratissimum* was effective up to 1:100 dilution and produced inhibition zones of 31.66, 26.33, and 22.00 mm at 1:0, 1:1 and 1:100 dilution respectively which was superior when compared streptocycline (15.66 mm) which served as control. *T. asthmatica* and *O. sanctum* extracts were inhibitory up to 1:10 dilutions whereas, *R. graveolens*, *C. gigentea* and *N. sativa* extracts were effective upto 1:10 dilutions. But *S. persica* and *T. cardifolia* extracts showed no inhibitory effect at all the dilutions tested against the pathogen. Murthy *et al* (2014) [8] observed solvent extracts of *Ocimum sanctum* inhibited the growth of *R. solanacearum*. Similarly, Ponnanikajamideen *et al*., (2013) [10] found that *T. asthmatica* extracts obtained from different extracts showed inhibitory effect against different strains of bacteria. The alcohol and water extracts of *R. graveolens* exhibited inhibitory activity against many Gram negative bacterial and plant pathogenic fungi tested (Pandey *et al*., 2011) [9]. The strong antibacterial activity of essential oil of *N. sativa* seeds was demonstrated against both Gram-positive and Gram-negative bacteria and maximum inhibitory activity was recorded against *Bacillus subtilis* (El-Kamali *et al*., 1998) [4]. Vijai Pal *et al*. (1993) [15] also observed inhibitory activity against three *Erwinia* spp. causing soft not of potato by the extract of *Calotropis procera*, thus confirmed the result obtained of present investigation.

Table 3: Effect of alcohol extracts of medicinal plants against *X campestris* pv. *Campestris*

Dilution	Zone of inhibition (mm)							
	O. gratissimum	***O. sanctum***	***T. asthmatica***	***R. graveolens***	***C. gigentea***	***N. sativa***	***S. persica***	***T. cardifolia***
1:0	31.66 (5.71)	21.33 (4.72)	25.33 (5.13)	15.00 (4.00)	25.66 (5.16)	18.00 (4.36)	0.00 (1.00)	0.00 (1.00)
1:1	26.33 (5.23)	19.33 (4.51)	23.33 (4.93)	12.00 (3.61)	22.33 (4.83)	13.33 (3.78)	0.00 (1.00)	0.00 (1.00)
1:10	22.00 (4.79)	15.33 (4.04)	19.66 (4.55)	0.00 (1.00)	19.33 (4.51)	12.00 (3.61)	0.00 (1.00)	0.00 (1.00)
1:100	18.66 (4.43)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
1:1000	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Streptocycline 400 ppm. (control)	15.66 (4.08)	15.66 (4.08)	15.66 (4.08)	15.66 (4.08)	15.66 (4.08)	15.66 (4.08)	15.66 (4.08)	15.66 (4.08)

(Figures in parenthesis are square root transformed values)

	S. Em+	C. D. (1%)
Factor A	0.0164	0.0463
Factor B	0.0164	0.0463
A x B	0.0402	0.1134

Conclusion

The present investigation revealed that extracts of many medicinal plants had inhibitory effect against *Xcc*. Further, alcohol extracts were more effective than water extracts as in alcohol extract, more number of phytochemicals liberated and their efficacy were enhanced against the pathogen. Hence these could be exploited as an alternate management strategy for chemical pesticides in the management of black rot of cabbage. The future studies should focus on identification and elucidation of the active principles present in medicinal plants having potential antimicrobial properties.

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