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Muntingia calabura botanical formulation for enhanced disease resistance in tomato plants against *Alternaria solani*

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The present study successfully demonstrate the inhibitory activity of the medicinal plant, *Muntingia calabura* against *Alternaria solani* which causes early blight of tomato and proposes the development of a new botanical formulation (Muntingin 5EC) and its use in plant disease management after package and practice. This ecofriendly botanical formulation was developed from the purified antimicrobial metabolite (Stigmasterol) isolated from the methanol extract of *M. calabura* root. Different concentrations of Muntingin 5EC were examined on seed infection, germination and seedling vigour of tomato and it was found that two percent Muntingin 5EC increased the germination and vigour and reduced the seed infection in tomato to a significant extent. The formulation was found to possess good emulsion stability and also retain its antimicrobial activity (shelf life) for 120 days. Application of Muntingin 5EC increased the activity of enzyme such as peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and phenol content of tomato. Muntingin 5EC (2%) was found to be the optimum concentration for control of early blight of tomato under pot culture conditions. The root of *M. calabura* was found to possess good antioxidant activity. Application of this botanical formulation in plant disease management assumes special significance by being an ecofriendly and cost effective strategy, which can be used in integration with other strategies for a greater level of protection with sustained crop yields after sufficient evaluation.

Key words: *Alternaria solani,* botanical formulation, Muntingin 5EC, plant diseases control.

INTRODUCTION

In agriculture, crop loss due to plant pathogens has become a major concern nowadays. Increased usage of different chemical products to control these pathogens have resulted in problems like residual effect of chemicals in agri-based products, increased resistance for chemicals in target pathogens and environmental pollution. Plant diseases cause considerable losses in crop production and storage. The intensive and indiscriminate use of pesticides/fungicides in agriculture has caused many problems such as pollution of the

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environment such as water, soil, animals and residual contamination of food and many others in addition to social economic problems (Stangarlin et al., 1999). Consequently, there is an increasing demand from consumers and officials to reduce the use of chemical pesticides and fungicides. In this context, biological control through the use of natural antagonistic micro-organisms has emerged as a promising alternative (Strange and Scott, 2005). Botanicals with antifungal compounds have been identified and these can be exploited for the management of diseases (Kagale et al., 2004). Botanicals have low mammalian toxicity, target specificity, biodegradability and contain many active ingredients in low concentrations, thus possess biocidal activity against several insect pests and pathogens (Kalaycioglu et al., 1997; Harish et al., 2008).

Crude extracts of some well known medicinal plants are used to control some of the plant pathogens. During the past few years, there was a growing trend all over the world to shift from synthetic to natural products including medicinal plants (Parimala devi and Marimuthu, 2011). The neglected and little known botanicals should be considered now to cure the plant diseases, which create challenging problems in agriculture and pose real economic and environmental threats.

Early blight disease of tomato by *Alternaria solani* (Jones and Grout, 2005) has become most destructive in India and yield losses due to this pathogen were up to 80% (Shanmugasundaram, 2004). The control of tomato early blight disease has been exclusively based on the application of chemical pesticides. Several effective pesticides have been recommended for use against this pathogen, but they are not considered to be long-term solutions, due to concerns of expense, exposure risks, fungicide residues and other health and environmental hazards. In an attempt to modify this condition, some alternative methods have been adopted. Recent efforts have focused on developing environmentally safe, long lasting and effective biocontrol methods for the management of plant diseases. Use of plant products and biocontrol agents has been shown to be eco-friendly and effective against many plant pathogens. A number of plant species have been reported to possess natural substances that are toxic to many fungi causing plant diseases (Lee et al., 2007). The objective of the present study was to evaluate the antimicrobial activity of root extracts from *Muntingia calabura* against *A. solani* under *in vitro* conditions and to develop a botanical formulation against plant diseases control.

MATERIALS AND METHODS

Preparation of the botanical fungicide

The partially purified methanol extract of *M. calabura* root containing the antimicrobial metabolite Stigmasterol was used to develop emulsifiable concentrates. The condensed material containing the antimicrobial metabolite, obtained after column chromatographic separation of fractions was considered as 100% concentration. The formulation was developed by using recommended quantities of surfactant (Tween 20) and co-surfactant (Ethylmethyl ketone). The 4EC formulation were prepared by adding 4 g of antimicrobial metabolite to 20 ml methanol and made up to 100 ml by adding 10 ml of Tween 20 and 70 ml of Ethylmethyl ketone.

The active fraction from methanol extract of *M. calabura* root was developed into two different emulsifiable concentrates and named as 'Muntingin 4EC' and 'Muntingin 5EC'. The formulations Muntingin 4EC and Muntingin 5EC prepared in different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5%) were tested for the efficacy under *in vitro* conditions. The combined formulation was named as 'Muntingin'.

Stability, storage and antifungal studies on Muntingin 5EC

Emulsion stability

About 20 to 25 ml of emulsion concentrate of *M. calabura* 5EC was added to 70 ml of standard hard water and made up to 100 ml in a beaker. The contents of the beaker were stirred with a glass rod (4 revolutions per second) during the addition. The contents of the beaker were transferred immediately to a clean and dry measuring cylinder. The measuring cylinder with its contents was kept in a thermostat at room temperature for one hour. After 1 h, the volume of creamed matter at the top or the sediment at the bottom was measured.

Thermo stability test

The formulation was poured into a glass bottle until three fourth was filled. The bottle was closed airtight with colloid oil sealing wax to avoid any loss of volatile solvents and kept in the thermostat (50°C) for 7 days. After 7 days, the volume of the creamed matter at the top or the sediment at the bottom was measured.

Cold stability test

The formulation was poured into a glass bottle until three fourth was filled. The bottle was closed airtight with colloid oil sealing wax to avoid any loss of volatile solvents and kept it in the refrigerator (10°C) for 7 days. After 7 days, the formulation was tested for emulsion stability.

Effect of methanol extract of *M. calabura* root on mycelial dry weight of fungal pathogens

Mycelial discs (9 mm) of the pathogens (*A. solani, Fusarium oxysporum* f.sp. *lycopersici*, *Pythium* sp., and *Phytophthora* sp. were inoculated into respective broth separately containing methanol extract of *M. calabura* root (0.2, 0.4, 0.6, 0.8 and 1.0%). Conical flask without the extract was maintained as control. The treatments were replicated thrice and incubated for 21 days. The mycelium was harvested through filtration with Whatman No. 42 filter paper. The filter paper containing fungal mycelium was oven dried at 70°C for 24 h and the weight of the dried mycelium was determined (Singh and Singh, 1980).

Effect of methanol extract of *M. calabura* root on fungal spore germination

The effect of methanol extract of *M. calabura* root (0.2, 0.4, 0.6, 0.8 and 1.0%) on fungal spore germination was tested by cavity slide

method (Montagomery and Moore, 1938). Spores of the pathogen were transferred to test tubes separately by flooding with sterile water and scrapping the culture with glass rod. The transferred spore suspension was centrifuged at 2000 rpm for 10 min to remove mycelial fragments. The spore suspension was adjusted to a concentration of 10^6 per ml using a haemocytometer. One drop of the methanol extract of *M. calabura* root was added (0.2, 0.4, 0.6, 0.8 and 1.0%) to the cavity slide and allowed to evaporate. One drop of spore suspension (after thorough shaking) was added to the cavity slides and kept in a moist chamber and incubated at room temperature (28 ± 2°C). Sterile distilled water served as control. Three replications were maintained and percentage of spore germination was recorded after 24 h.

Antioxidant activity of methanol extract of *M. calabura* root

Dried powders of *M. calabura* root (10 g) were extracted in 100 ml of 50 per cent ethanol solution at 25°C for 30 min with shaking. The extract was centrifuged at 15000 rpm for 3 min and supernatant was collected. The supernatant was concentrated in a rotary evaporator and then lyophilized. The antioxidant assays carried out were ferric reducing antioxidant power assay (Benzie and Strain, 1996), DPPH scavenging activity (Blois, 1958), reducing power assay (Oyaizu, 1986) and superoxide anion radical scavenging activity (Nishikimi et al., 1972).

Antimicrobial effect against selected plant pathogens

The formulation was tested for its antimicrobial activity at different time intervals against selected plant pathogens by poison plate technique.

Assessing the biocontrol potential of Muntingin 5EC against tomato early leaf blight

Effect of Muntingin 5 EC on seed infection, seed germination and vigour of tomato seedlings were evaluated under *in vitro* condition. The treatments adopted were Muntingin 5EC at five different concentration (0.2; 0.4; 0.6; 0.8 and 1.0%), 0.2% Mancozeb (pesticide control) and standard biocontrol agent (*P. fluorescens* PF1). The tomato seeds were soaked in different concentrations of Muntingin 5EC for 2 h and twenty five seeds of each treatment were placed on moist blotters (ISTA, 1993) in Petri plate and incubated at 20 ± 2°C for 12 h of alternate natural light and 12 h of darkness. The seeds were examined for growth of seed borne pathogens on eighth day of treatment. The seed infection was expressed in percentage. The seedlings were evaluated as total number of normal seedlings and the per cent germination was evaluated. The vigour index was compared (Abdul-Baki and Anderson, 1973) and expressed as whole number.

Evaluation of Muntingin 5EC on tomato early leaf blight under pot culture condition

Effect of different concentrations of Muntingin 5EC (0.5, 1.0, 1.5 and 2.0%) together with standard practices of Mancozeb (0.2%) and biocontrol agent (*P. fluorescene* PF1) on early blight (caused by *A. solani*) was tested under pot culture condition in tomato. The extent of disease incidence is expressed as percent disease index and calculated using the disease score card for *A. solani* (Ayyangar, 1928). The peroxidase (Puttur, 1974), polyphenol oxidase (Mayer et al., 1965) and phenylalanine ammonia lyase (Zucker, 1965) were analyzed in the roots of tomato upto 10 days at 2 days interval. The phenol content was also measured at two days interval by standard procedure as described by Spies (1955).

RESULTS

The botanical formulation prepared using partially purified methanol extract of *M. calabura* containing antimicrobial metabolite stigmasterol was assessed in this study for its biocontrol potential. The formulation was found to possess good emulsion stability and also retained its antimicrobial activity (shelf life) for 120 days. The prepared formulation was tested for its efficacy in controlling the early leaf blight disease of tomato under *in vitro* and pot culture studies.

Effect of different concentrations of formulated products on fungal pathogens

The active fraction F21 from methanol extract of *M. calabura* root were developed into two different emulsifiable concentrates and named as 'Muntingin 4EC and Muntingin 5EC'. The formulations Muntingin 4EC and Muntingin 5EC were prepared in different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5%). The formulation Muntingin 4EC at 2.5% and Muntingin 5EC at 2.0 and 2.5% concentration completely inhibited (100%) the growth of *A. solani, F. oxysporum* f.sp. *lycopersici, Pythium* sp., and *Phytophthora* sp. While the formulation Muntingin 4EC at 2.0% level reduced the mycelial growth up to 92.06, 90.98, 91.79 and 90.30% in *A. solani, F. oxysporum* f.sp. *lycopersici, Pythium* sp. and *Phytophthora* sp. respectively (Table 1).

Stability of EC formulations

Emulsion stability of the formulations were tested with standard hard water and the results are presented in Table 2. The standard critical limits of sedimentation of emulsion formulations were taken as 2 ml. For both formulations, *M. calabura* root, Muntingin 4EC and Muntingin 5EC, the sedimentation levels were not exceeding the critical limits of 2 ml.

The 5EC formulation of *M. calabura* root was kept at 10 and 50°C for 7 days and the sedimentation of emulsion was recorded. The visual observations confirmed the temperature stability of the EC formulations, as there was no sedimentation observed in high and low temperatures. The 2.0% solution of EC formulation had a neutral pH (7.0).

Shelf life of formulation and its effect on fungal pathogens

The formulation Muntingin 5EC was tested for its stability at room temperature (28 ± 2°C) for different periods (Table 3). The results show that the EC formulation

Table 1. Effect of different EC formulation of Muntingin (antimicrobial from *M. calabura* root) on growth inhibition of different fungal pathogens.

Formulation	Concentration (%)	*A. solani*		*F. oxysporum* f.sp. *lycopersici*		*Pythium* sp.		*Phytophthora* sp.	
		A	B	A	B	A	B	A	B
Muntingin 4EC	0.50	31.40 (± 0.12)[a]	65.38	38.32 (± 0.06)[a]	56.77	30.60 (± 0.02)[a]	66.51	32.99 (± 0.01)[a]	64.39
	1.00	22.35 (± 0.02)[b]	75.36	24.65 (± 0.02)[b]	72.19	22.00 (± 0.05)[b]	75.92	21.55 (± 0.02)[b]	76.74
	1.50	14.05 (± 0.01)[c]	84.51	16.80 (± 0.01)[c]	81.05	15.75 (± 0.02)[c]	82.76	10.98 (± 0.01)[c]	88.15
	2.00	7.20 (± 0.06)[d]	92.06	8.00 (± 0.09)[d]	90.98	7.50 (± 0.01)[d]	91.79	8.99 (± 0.02)[d]	90.30
	2.50	0.00 (± 0.00)[e]	100.00	0.00 (± 0.00)[e]	100.00	0.00 (± 0.00)[e]	100.00	0.00 (± 0.00)[e]	100.00
Muntingin 5EC	0.50	23.15 (± 0.23)[a]	74.48	23.67 (± 0.12)[a]	73.30	21.10 (± 0.09)[a]	76.90	17.86 (± 001)[a]	80.72
	1.00	12.86 (± 0.12)[b]	85.82	13.46 (± 0.05)[b]	84.82	15.30 (± 0.02)[b]	83.25	13.00 (± 0.06)[b]	85.97
	1.50	6.40 (± 0.05)[c]	92.94	7.23 (± 0.09)[c]	91.84	8.90 (± 0.01)[c]	90.26	9.70 (± 0.09)[c]	89.53
	2.00	0.00 (± 0.00)[d]	100.00	0.00 (± 0.00)[d]	100.00	0.00 (± 0.00)[d]	100.00	0.00 (± 0.00)[d]	100.00
	2.50	0.00 (± 0.00)[d]	100.00	0.00 (± 0.00)[d]	100.00	0.00 (± 0.00)[d]	100.00	0.00 (± 0.00)[d]	100.00
Control		90.70		88.65		91.36		92.65	

A, Diameter of mycelial growth in Petri dish (mm); B, percent reduction over control. *Mean of three replications.

retained its 100% antifungal activity up to 120 days against the four pathogens tested *viz., A. solani. F. oxysporum* f.sp. *lycopersici, Pythium* sp. and *Phytophthora* sp.

Effect of methanol extract of *M. calabura* root on mycelial dry weight of fungal plant pathogens

At a concentration of 0.8%, the methanol root extract exhibiting mycelial growth reduction of 95.28, 96.39, 94.86 and 95.03% was observed in *A. solani*, *F. oxysporum* f.sp. *lycopersici*, *Pythium* sp. and *Phytophthora* sp. respectively (Table 4). Whereas in 1.0% concentration, methanol root extract of *M. calabura* and ketoconazole 100% inhibition of mycelial growth was observed.

Effect of methanol extract of *M. calabura* root on spore germination of fungal plant pathogens

At a concentration of 0.8%, the methanol root extract exhibit, inhibition of spore germination of 97.38, 96.94, 96.98 and 97.73% in *A. solani*, *F. oxysporum* f.sp. *lycopersici*, *Pythium* sp and *Phytophthora* sp., respectively (Table 5). Whereas in 1.0% concentration, methanol root extract of *M. calabura* and ketoconozole 100% inhibition of spore germination was observed.

Antioxidant activities of methanol extract of *M. calabura* roots

The methanol extract of *M. calabura* root showed good antimicrobial activity against selected plant pathogens at a concentration of 10 mg/ml. Hence, the same concentration was also used for the study of antioxidant activity (Table 6).

Effect of Muntingin 5EC on seed infection, seed germination of tomato seedlings

The seed infection by *A. solani* was reduced by 99.28% in Muntingin 5EC (2%) treated seeds. The treatments which received Mancozeb (0.2 %) and *P. fluorescens* recorded 89.28 and 90.21% reduced seed infection respectively over the control. The germination percent was increased by 16.50% in Muntingin 5EC (2%) treated seeds as compared to the control (Table 7).

The effect of various concentrations of Muntingin 5EC on growth of tomato seedlings is presented in Table 8. The maximum shoot (13.06

Table 2. Emulsion stability of different EC formulations.

Formulation	Creamy appearance/100 ml measuring cylinder		Sedimentation/100 ml measuring cylinder	
	Below 2 ml	Above 2 ml	Below 2 ml	Above 2 ml
Muntingin 4EC	+	-	+	-
Muntingin 5EC	+	-	+	-

+, Positive; -, negative.

Table 3. Shelf life of Muntingin 5EC formulation and its effect on fungal pathogens.

Days after inoculation	*A. solani*		*F. oxysporum* f.sp. *lycopersici*		*Pythium* sp.		*Phytophthora* sp.	
	A	B	A	B	A	B	A	B
30	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0
60	0.00	100.0	0.00`	100.0	0.00	100.0	0.00	100.0
90	0.01	100.0	0.01	100.0	0.01	100.0	0.01	100.0
120	0.01	100.0	0.01	100.0	0.01	100.0	0.01	100.0
150	7.54	91.28	7.23	92.42	7.75	91.95	6.36	91.70
180	12.00	85.32	10.50	85.84	11.95	85.56	10.97	85.91
Control	86.76		92.60		93.50		90.00	

ME- Methanol extract of *M. calabura*; A- Diameter of mycelial growth in petridish (mm); B- Percent reduction over control. *Mean of three replications.

Table 4. Effect of different concentrations of methanol extract of *M. calabura* root on mycelial dry weight of fungal pathogens.

Treatment	Mycelial dry weight							
	A. solani		*F. oxysporum* f.sp. *lycopersici*		*Pythium* sp.		*Phytophthora* sp.	
	A	B	A	B	A	B	A	B
ME 0.2%	34.50 (±0.72)^{b}	83.81	31.82 (±0.64)^{b}	82.84	40.92 (±0.59)^{b}	81.55	39.80 (±1.15)^{b}	84.37
ME 0.4%	28.25 (±0.43)^{c}	86.74	24.62 (±0.59)^{c}	81.48	32.50 (±0.75)^{c}	85.13	30.20 (±0.69)^{c}	88.14
ME 0.6%	13.16 (±1.16)^{d}	93.82	15.23 (±1.15)^{d}	95.41	17.65 (±0.61)^{d}	91.92	23.58 (±1.17)^{d}	90.74
ME 0.8%	10.05 (±1.18)^{e}	95.28	10.15 (±1.24)^{e}	96.39	11.23 (±1.16)^{e}	94.86	12.65 (±0.61)^{e}	95.03
ME 1.0%	0.00 (±0.00)^{f}	100	0.00 (±0.00)^{f}	100	0.00 (±0.00)^{e}	100	0.00 (±0.00)^{f}	100
Ketaconazole 0.2%	0.00 (±0.00)^{f}	100	0.00 (±0.00)^{f}	100	0.00 (±0.00)^{e}	100	0.00 (±0.00)^{f}	100
Control	213.20(±1.85)^{a}		186.70 (±1.73)^{a}		218.70(±1.79)^{a}		254.78 (±0.59)^{a}	

ME, Methanol extract; , Mycelial dry weight (mg); B, percent reduction over control. *Mean of three replications; Control, without methanol extract.

Table 5. Effect of different concentrations of methanol extract of *M. calabura* root on spore germination of fungal pathogens.

Treatment	Spore germination							
	A. solani		*F. oxysporum* f.sp. *lycopersici*		*Pythium* sp.		*Phytophthora* sp.	
	A	B	A	B	A	B	A	B
ME 0.2%	4.33 (±0.59)^{b}	94.60	6.72 (±0.62)^{b}	91.96	7.05 (±0.61)^{b}	91.81	4.18 (±0.59)^{b}	94.74
ME 0.4%	3.90 (±0.64)^{bc}	95.13	4.31 (±1.16)^{c}	94.85	4.40 (±0.81)^{bc}	94.89	3.50 (±0.64)^{b}	95.60
ME 0.6%	3.20 (±0.12)^{d}	96.00	3.90 (±0.06)^{c}	95.33	3.30 (±0.69)^{c}	96.16	3.10 (±0.06)^{c}	96.10
ME 0.8%	2.10 ± (0.06)^{c}	97.38	2.56 (±0.59)^{d}	96.94	2.60 (±0.69)^{d}	96.98	1.80 (±0.64)^{d}	97.73
ME 1.0%	0.0 ± (0.00)^{e}	100.0	0.0 (±0.00)^{e}	100.0	0.0 (±0.00)^{e}	100.0	0.0 (±0.00)^{e}	100.0
Ketaconazole 0.2%	0.00 (±0.00)^{e}	100	0.00 (±0.00)^{e}	100	0.00 (±0.00)^{e}	100	0.00 (±0.00)^{e}	100
Control	80.12 (±1.17)^{a}		83.65 (±0.57)^{a}		86.10 (±0.64)^{a}		79.50 (±1.21)^{a}	

ME, Methanol extract; A, spore germination (%); B, percent reduction over control. *Mean of three replications; Control, without methanol extract.

Table 6. Antioxidant activities of methanol extract of *M. calabura* roots.

Antioxidant activity (10 mg/ml)	Root samples
Ferric reducing antioxidant power (µM/g)	0.63 (± 0.02)
DPPH scavenging activity (%)	21 (±1.73)
Reducing power activity (%)	2.42 (± 0.02)
Superoxide anion radical scavenging activity (%)	20 (± 1.15)

Values are mean (± SE)

Table 7. Effect of Muntingin 5EC on disease infection of seed and seed germination of tomato (PKM1).

Treatment	Disease infection of Seed		Seed germination	
	Infection (%)*	Reduction over control	Germination (%)*	Increase over control
0.50% Muntingin 5EC (T_1)	10.20 (0.05)^{b}	88.79	83.00	3.75
1.00% Muntingin 5EC (T_2)	7.30 (0.01)^{cd}	91.97	85.80	7.25
1.50% Muntingin 5EC (T_3)	4.00 (0.06)^{cd}	95.60	89.65	12.06
2.00% Muntingin 5EC (T_4)	0.65 (0.02)^{d}	99.28	93.20	16.50
Mancozeb 0.2% (T_5)	9.75 (0.01)^{bc}	89.28	83.50	4.37
P. fluorescens (T_6)	8.90 (0.09)^{cd}	90.21	84.10	5.12
Control (T_7)	91.00 (0.02)^{a}		80.00	

Values are mean (± SE) of five replicates and values followed by the same letter in each column are not significantly different from each other as determined by Duncan's multiple range test ($p \leq 0.05$).

Table 8. Effect of Muntingin 5EC on vigour of tomato seedlings.

Treatment	Shoot length (cm)*	Per cent increase over control	Root length (cm)*	Per cent Increase over control	Vigour index*	Per cent Increase over control
0.50% Muntingin 5EC (T_1)	10.08 (0.02)^{b}	32.63	9.00 (0.01)^{ab}	25.87	1583.64	39.45
1.00% Muntingin 5EC (T_2)	11.50 (0.12)^{ab}	51.31	10.40 (0.12)^{a}	45.45	1879.02	55.78
1.50% Muntingin 5EC (T_3)	12.75 (0.01)^{a}	67.76	10.55 (0.02)^{a}	47.55	2088.84	70.08
2.00% Muntingin 5EC (T_4)	13.06 (0.02)^{a}	71.84	10.25 (0.01)^{a}	43.35	2172.49	76.29
Mancozeb 0.2% (T_5)	8.18 (0.06)^{c}	7.63	8.00 (0.02)^{b}	1.18	1351.03	7.70
P. fluorescens (T_6)	8.30 (0.23)^{c}	9.21	8.25 (0.06)^{b}	1.53	1391.85	11.59
Control (T_7)	7.60 (0.01)^{c}		7.15 (0.09)^{b}		1180	

Values are mean (± SE) of five replicates and values followed by the same letter in each column are not significantly different from each other as determined by Duncan's multiple range test ($p \leq 0.05$).

cm) and root length (10.25 cm) were observed in the seeds which received 2.0% Muntingin 5EC. The 2.0% Muntingin 5EC treated plants recorded 71.84 and 43.35% increased shoot and root length, respectively over the control. The vigour index was also maximum with 2.0% Muntingin 5EC treatment. The treatments which received Mancozeb and *P. fluorescens* recorded 7.70 and 11.59% increased vigour index respectively over control.

Effect of Muntingin 5EC on early blight control in tomato

The tomato plants sprayed with various concentrations of Muntingin 5EC under pot culture studies were observed for the early blight disease incidence. The percent disease index of the Muntingin 5EC treated plants was significantly lesser as compared to the control. The

Table 9. Effect of Muntingin 5EC on percent disease index of early blight of tomato under pot culture condition.

Treatment	Percent disease index*	Percent reduction over control
0.50% Muntingin 5EC (T_1)	10.83 (± 0.01)[b]	83.96
1.00% Muntingin 5EC (T_2)	6.56 (± 0.02)[c]	90.28
1.50% Muntingin 5EC (T_3)	3.45 (± 0.26)[d]	94.89
2.00% Muntingin 5EC (T_4)	0.36 (± 0.02)[e]	99.46
Mancozeb 0.2% (T_5)	1.30 (± 0.03)[e]	98.07
P. fluorescens (T_6)	4.18 (± 0.01)[d]	93.81
Uninoculated control (T_7)	-	
Control (T_8)	67.55 (± 0.03)[a]	

Values are mean (± SE) of five replicates and values followed by the same letter in each column are not significantly different from each other as determined by Duncan's multiple range test ($p \leq 0.05$).

treatment which received 2% Muntingin 5EC recorded 99.4% reduced PDI as compared to control. This was followed by the treatment 1.5% Muntingin 5EC which recorded 98.07% reduced PDI, whereas the treatment which received mancozeb (0.2%) and *P. fluorescens* recorded 94.89 and 93.81% reduced PDI, respectively (Table 9).

Effect of Muntingin 5EC spray on plant defence enzymes in roots of tomato

The changes in activities of various enzymes were monitored on 0, 2, 4, 6 and 10 days after the inoculation of *A. solani*. The PO activity reached maximum at 6 days after inoculation with 2.0% Muntingin 5EC treated plants (2.40 units/g of leaf tissue) and then declined thereafter. Though the peroxidase activities increased in inoculated control, the values were significantly lower than the plants treated with various concentrations of Muntingin 5EC, Mancozeb (0.2%) and *P. fluorescens*. In inoculated control, the activity reached maximum (1.80 units/g of leaf tissue) on second day of inoculation and then declined (Figure 1a).

Similarly, the polyphenol oxidase activity increased in plants treated with Muntingin 5EC when compared with inoculated control. Muntingin 5EC at 2.0% concentration significantly increased the activity of PPO to maximum level (2.72 units/g of leaf tissue) on the tenth day of inoculation (Figure 1b). Among the treatments, maximum phenylalanine ammonia lyase activity was observed (49.00 units/g of leaf tissue) with 2.0% Muntingin 5EC (Figure 1c). The concentration of phenol was significantly higher in plants treated with 2.0% Muntingin 5EC (188.4 µg of catechol/g of leaf tissue) than all other treatments on sixth day of challenge inoculation. In all the treatments, maximum phenol content was reached on sixth day after inoculation. However, all the treatments retained the content of phenol without much reduction even on the tenth day, but the phenol content reduced drastically in inoculated control (Figure 1d).

DISCUSSION

Plant diseases are the major biotic constraints to crop growth and cause variety of damage and significant yield loss. The disease management requires effective integration of approaches to reduce the crop loss effectively. Several strategies have been developed based on genetic, chemical, biological, cultural methods and also combined integrated diseases management framework (Arora and Kaushik, 2003).

Botanicals are materials or products of plants origin valued for their pesticidal, medicinal or therapeutic properties. Phytopesticide materials range from whole fresh plants to purely isolated bioactive phytochemicals or their formulations which are effective against pests and pathogens (Prakash and Rao, 1996). Preparation and application of botanicals for crop protection are linked to the folklores and tradition of the farmers (Anjorin, 2008). Management of disease through fungicides alone leads to soil residual problem and health hazards, besides involving higher input cost.

One of the recent approaches for plant disease management is exploitation of plant products. Inspite of the wide recognition that many plants possess antimicrobial properties, only a handful of products have been developed, because of their less persistence nature in the crop ecosystem (Kumbhar et al., 2001). According to Nagarajan (1996), the emulsifiable concentrates were the most popular formulations in India and the benefits were greater than other formulations. Reports suggest that emulsifiable concentrates are the most desirable for botanical formulations.

As part of the present study, emulsifiable concentrate (EC) formulation was developed from the partially purified antimicrobial compound obtained from methanol root extract of *M. calabura* tested for their stability under laboratory conditions. EC formulation of Muntingin was

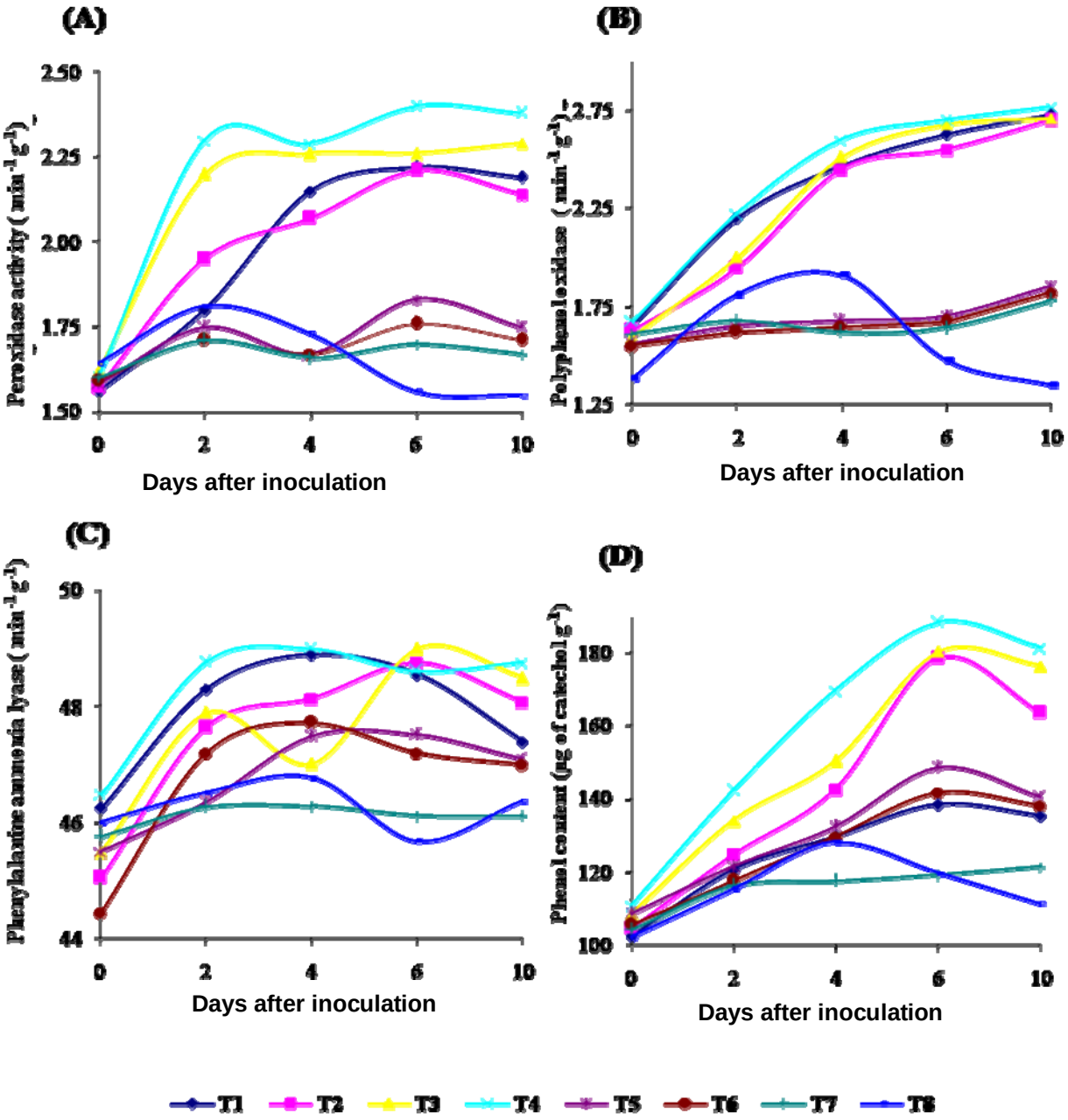

Figure 1. Effect of Muntingin 5EC on changes in plant defense metabolites of tomato under pot culture condition. A- Peroxidase; B- polyphenol oxidase; C- phenylalanne ammonia lyase; D- phenol content.

studied upto 180 days. The formulations were found to retain their maximum activity up to 120 days. The 100% reduction of fungal growth was observed upto 120 days of storage. The optimum pH of the formulation was neutral and it did not exhibit any phytotoxicity when sprayed on to the plants. The formulation was prepared as 4EC and 5EC with the name 'Muntingin'. Whereas the 5EC formulation exhibited 85% reduction of the mycelial growth against the tested fungal plant pathogen after 180 days of storage. The botanical formulation was prepared with 5EC concentration with account of 2% formulation should contain 1 mg of the active compound per milliliter of the product which is the actual MIC value for the antimicrobial compound stigmasterol.

The methanol extract of *M. calabura* root showed significant reduction in mycelial growth and spore germination of the selected fungal plant pathogens - *A. solani, F. oxysporum* f.sp *lycopersici*, *Pythium* sp. and *Phytophthora* sp. The results of the present study indicate one percent methanol root extract of *M. calabura* inhibited the spore germination of *A. solani, F. oxysporum* f.sp. *lycopersici*, *Pythium* sp. and *Phytophthora* sp. by percent.

The inhibition of spore germination by medicinal plants has been reported by many workers (Thirupathi et al., 1999). From this, it is evident that, the *M. calabura* possess antibacterial activity and capable of counteracting reactive oxygen species (ROS), which are responsible for various oxidative damages in the living system. Whereas methanol extract of *M. calabura* root exhibited 21% DPPH activity and 20% SOD activity.

Parimala devi and Marimuthu (2011) reported that the botanical formulation, Polymin 40 EC at various concentrations (0.5, 1.0, 1.5 and 2.0%) controlled *A. solani*. The p-40 at 2.0%effectively controlled the pathogens under pot culture conditions and was considered as the optimum concentration. The botanical formulation at 2% level was found to reduce the seed infection of *F. oxysporum* f.sp. *lycopersici* in tomato and also increased the vigour of tomato (76.29%) as compare to the control.

In the present investigation, the botanical formulation**,** Muntingin 5 EC at various concentrations (0.50, 1.00, 1.50 and 2.00%) was tested under pot culture conditions in controlling *A. solani* in tomato. The Muntingin 5EC at 2.0% level effectively controlled the pathogen under pot culture conditions and was considered as the optimum concentration. The increase in germination percentage and vigour of seedlings may be due to the fact that the application of Muntingin promoted the activity of seed enzymes such as amylase, catalase, etc. and also increased the metabolic activities of the seed.

The biotic and abiotic inducers play an important role in activating the defense genes in plants. Induction of defense proteins makes the plant resistant to pathogen invasion (Ramanathan et al., 2000). The results of the present study revealed that the tomato applied with Muntingin 5EC significantly induced the defense compounds (PO, PPO, PAL and phenol) as compared to control. The resistance of plants induced against the pathogens, due to the application of botanicals has been widely reported (Straub et al., 1986; Rajeswari, 2002; Kagale, 2001). Kagale et al. (2004) observed an increase in antioxidant enzymes in rice plants sprayed with *Datura metal* leaf extract. Increase in PO activity has been observed in the sesame plants treated with *Azadirachta indica* leaf extract (Guleria and Kumar, 2006). Plant phenolics are well known antifungal, antibacterial and antiviral compounds that increase the physical and mechanical strength of the host cell wall. Phenylalanine ammonia lyase is the first enzyme of phenyl propanoid metabolism in higher plants and has been suggested to play a significant role in regulating the accumulation of phenolics (Daayf et al., 1997). The formulation Muntingin 5EC containing the antimicrobial compounds led to increased biosynthesis of PO, PPO, PAL and phenols, which in turn were responsible for disease resistance in plants.

It has been concluded that the botanical formulation "Muntingin" increased the tomato germination percentage by reducing the seed infection by *A. solani*. It has also been shown, the potential inhibitory effect on the selected plant pathogen under pot culture conditions. The botanical formulation is capable of inducing the resistance in tomato plants through enhancement of defense compounds. Thus, the formulation Muntingin showed the potential for managing the early leaf blight disease in tomato. The future thrust and follow-up research efforts may aim to study the effect of Muntingin on plant disease control under field conditions and this will provide an opportunity for eco-friendly disease management on a variety of crops.

Conflict of interest

The authors declare that they have no conflict of interest.

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