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Cinnamomum zeylanicum extracts and their formulations control early blight of tomato.

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ABSTRACT

Alternaria solani causes early blight in plants belonging to family Solanaceae. It severely infests agriculturally important crops like tomato and potato at different developmental stages. We have screened eighty one extracts of twenty seven medicinally important plants for their antifungal activity against *A. solani*. Twelve extracts of eight plants (*Cinnamomum zeylanicum, Syzygium aromaticum, Ferula foetida, Inula racemosa, Hemidesmus indicus, Rubia cordifolia, Glycyrrhiza glabra* and *Saussurea lappa*) possessed marginal to excellent antifungal activity. Hexane (Hx) and methanol (MeOH) extracts of *C. zeylanicum* showed complete inhibition of *A. solani in vitro* at a dose of 3 ml/lit water. Formulation trials conducted in shade house using MeOH extract of *C. zeylanicum* at a dose of 2 ml/lit water were highly effective against *A. solani* infesting tomato plants. TLC, HPTLC and GC-MS analysis confirmed the presence of Eugenol, Cinnamaldehyde and 2H -1- Benzopyran -2- one in MeOH extract of *C. zeylanicum*. These secondary metabolites were isolated by preparative TLC to further confirm antifungal activity and probable structures by GC-MS. 2H-1-Benzopyran-2-one, a coumarin has been reported for the first time as an antifungal against *A. solani* in present study. An effective lab-scale formulation was developed and tested against *A. solani*.

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Key words: Antifungal, *C. zeylanicum*, *A. solani*, Eugenol, Cinnamaldehyde, 2H -1- Benzopyran -2- one.

INTRODUCTION

India is the fourth largest producer of tomato (*Lycopersicon esculentum* Mill.) globally, contributing around 11.9 MMT. However, its average per hectare production is less (19.6 MT) compared to the world average (28.2 MT) (http://www.novagrim.com/Pages/2000-2011tomato statistics EN.aspx). This yield gap is attributed to various diseases and insect pests associated with tomato cultivation. Amongst these, tomato is highly susceptible to early blight, late blight and *Fusarium* wilt (Panthee and Chen, 2010). Fungal infestations cause deterioration in the quality of tomato, minimize yield and fetch less market value.

To combat attack of these fungi, many synthetic/chemical fungicides are available. Although these fungicides help in increasing crop yield, providing stability in production and market value (Kishore and Pande, 2007), their repeated use leads to development of resistance (benzimidazoles, dicarboximides etc.) in few fungi (Elad *et al*., 1992; Myresiotis *et al*., 2007) and accumulation of chemical residues in the food chain (Gonzalez–Rodriguez *et al*., 2008). Fungicides like chlorothalonil and maneb have been listed as carcinogens by the Environmental Protection Agency (EPA) in USA (Ragsdale, 2000).

Moreover, there is an increased public demand for sustainable and chemical residue-free food production (Arthur, 1996). In response to this, biofungicides, derived either from microbes or plants, emerged as promising alternative strategies. Neem (*Azadirachta indica*), garlic (*Allium sativum*), onion (*Allium cepa)* and few other plants inhibit early blight in potato and tomato (Prasad and Naik, 2003; Mate *et al*., 2005). Leaf extracts of *Prosopis juliflora* and *Cocos nucifera* inhibited proliferation of spores of *A. solani* above 90% (Thiribhuvanamala *et al*., 2001) Plant Growth Promoting Rhizobacteria (PGPR) combined with plant extracts for triggering plant 'immune system' to fight against various diseases and insect pests have been tried (Latha *et al*., 2009). The potential biological activity of plant extracts had been assessed against a wide range of fungal

Table 1. Details of plants used for antifungal assay against *A. solani*.

Common Name	Botanical Name	Family	Part Used	Pharmacological Activity	Chemical Constituents	Reference
Aapta	*Bauhinia variegate* L.	Fabaceae	Bark	Anti-bacterial	Alkaloids	Kumar *et al.*, 2005
Anantmul	*Hemidesmus indicus* (L)	Apocyanceae	Root	Demulcent	Saponins	Gayatri and Kanabiran, 2009
Babchi	*Psoralea corylifolia* L.	Papilionaceae	Seeds	Anti-bacterial, Anti-helminthic, Anti-inflammatory	Alkaloids	Gidwani *et al*., 2010
Bel	*Aegle marmelos* L. Correa	Rutaceae	Fruits, Leaves	Haemostatic, Anti-diabetic	Tannins	Suresh *et al*., 2009
Bharangi	*Cleodedron serratam* L.	Verbenaceae	Roots, Leaves	Asthama, Bronchitis	Tannins	Shrivastava and Patel, 2007
Chakunda	*Cassia tora* L.	Caesalpinaceae	Seed	Skin Care	Emodin	Roopashree *et al*., 2008
Daru halad	*Berberis aristata* R.	Berberidaceae	Bark, Leaves	Anti-inflammatory, Anti-bacterial, Anti-pyretic, Anti-periodic, Anti-septic, Anti-cancer	Alkaloids, Tannins	Shahid *et al*., 2009
Guggul	*Commiphora mukul* Engl.	*Burseraceae* (frankincense)	Gum	Anti-arthritic, Anti-cholesteremic	Sterols	Salamah *et al*., 1999
Gunja	*Abrus precatorius* L.	Fabaceae	Seed	Abortifacient	Glycosides, Alkaloids	Mistry *et al.*, 2010
Hing	*Ferula foetida* regel	Apiaceae	Rhizome Gum	Anti-oxidant, Anti-hemolytic	Alkaloids, Terpenoids	Baldemir *et al.*, 2006
Jeshthamadh	*Glycyrrhiza glabra* L.	Fabaceae	Bark	Anti-microbial	Glycyrrhizin, Flavonoids	Gupta *et al.*, 2006
Kantkari	*Solanum surratence* Burm F.	Solanaceae	Fruit	Anti-bacterial	Steroids, Alkaloids	Liu *et al.*, 2006
Karle	*Momordica Charantia* L.	Cucurbiteaceae	Seeds	Skin Diseases	Flavonoids, Tannins, Alkaloids	Costa *et al.*, 2011
Kulthi	*Dolichos biflorus* L.	Fabaceae	Seed	Diuretic	Saponins	Ray and Majumadar, 1974
Kushta	*Saussurea lappa* ((Decne.) C.B.Clarke.)	Asteraceae	Root	Anti-inflammatory	Terpenoids	Yang *et al.*, 2007
Kutacha	*Holarhina antidysenterica*	Apocyanaceae	Bark, Seeds	Anti-microbial, Anti-cancer	Phytol, Phenolic Compounds	Preethi *et al.*, 2010
Manjishtha	*Rubia cordifolia* L.	Rubiaceae	Root	Skin Care	Tannins	Naidu *et al.*, 2009
Nirgudi	*Vitex negundo* L.	Lamiaceae	Leaves	Anti-rheumatic, Anti-bacterial, Anti-inflammatory	Alkaloids	Panda *et al.*, 2009
Palash	*Butea frondosa* (Lam.) Taubert	Fabaceae	Flowers, Leaves	Diuretic	Glycosides	Gurav *et al.*, 2008
Pushkarmool	*Inula racemosa* Hook	Asteraceae	Leaves, roots	Anti-rheumatic	Flavonoids, Terpenoids	Wang *et al.*, 2004
Salai guggul	*Boswellia serrata* Roxb.	Proteaceae	Gum	Anti-inflammatory	Boswellic acid	Raja *et al*., 2011
Wavding	*Embelia ribes* Burn. F.	Myrsinaceae	Fruit	Astringent, Anti-helminthic, Anti-fertility, Anti-oestrogenic	Benzoquinone	Radhakrishnan *et al.*, 2011

Marigold	*Calendula officinalis* L.	Asteraceae	Seeds	Skin diseases	Alkaloids, Flavonoids, Tannins	Bissa and Bora, 2011
Black pepper	*Piper nigrum* L.	Piperaceae	Seeds	Analgesic, Anti-septic	Linalool, β-pinene	Bowers and Locke, 2004
Cardamom	*Elettaria cardamomum* Maton	Zinziberaceae	Seeds	Anti-cancer, Analgesic, Anti-inflammatory	Protocatechuic Acid	Farah *et al.*, 2005
Cinnamon	*Cinnamomum zeylanicum* Blume	Lauraceae	Bark, Leaves	Anti-cancer, Anti-diabetic	Cinnamaldehyde, Eugenol	Bowers and Locke, 2004
Clove	*Syzygium aromaticum* L.	Myrtaceae	Fruits	Expectorant	Caryophyllene, Eugenol	Kishore and Pande, 2007

Twenty seven plants were screened for antifungal activity against *A. solani.* Description of plants like common and botanical name, family, plant part used, pharmacological activity, active chemical compounds and related references have been mentioned.

phytopathogens (Carson *et al*., 1998; Fiori *et al*., 2000; Letessier *et al*., 2001; Mahmoud, 1999; Ranasinghe *et al*., 2002; Suganda and Yulia, 1998; Yulia, 2005).

In this study, we have selected twenty seven medicinal plants (Table 1) used in the traditional Indian Ayurvedic medicinal system out of which four are common spices. These plant species have been reported to be effective against various skin diseases caused by microorganisms and in treating some other pharmacological implications (Jagtap *et al*., 2010). Activity of these plants has been studied against early blight in tomato caused by *Alternaria solani* (Ellis and Martin) Jones and Grout. Aims of this study were to (i) evaluate bioefficacy of different medicinal plant extracts against early blight, (ii) pin down the probable active ingredient(s) and finally (iii) develop a commercially viable formulation.

MATERIALS AND METHODS

Plant material

Twenty seven medicinally important plant species (Table 1) were selected and their powders procured locally. Chemical fungicide, Cabriotop (BASF, Ludwigshafen, Germany) was used as a positive control. Other chemicals were obtained from SD Fine, Mumbai, India, unless otherwise mentioned.

Fungal Culture

The phytopathogen, *Alternaria solani* (NCIM - 887) was purchased from National Culture of Industrial Microorganisms, National Chemical Laboratory, Pune (MS), India. It was maintained on potato dextrose agar (PDA) and stored at 4°C. The actively growing culture was transferred to fresh PDA and plates were stored at 25 ± 2°C for 3-5 days before starting each experiment.

Preparation of extracts

Plant material was dried in the laboratory at room temperature (RT) and finely powdered with the help of a mixer-grinder (Jyoti, India). Each plant powder (30 g) was separately extracted in three different solvents (each 300 mL) (i) water (95°C) for 4 h (ii) methanol; MeOH (74°C) for 3 h (iii) hexane; Hx (60°C) for 3 h using a Soxhlet apparatus. Extracts (total 81), were filtered with Whatman filter paper 1 and distilled to obtain 10- fold concentrated extract. Extracts thus obtained were subjected to bioefficacy testing either without dilution (X) or diluted (X/2) or (X/10) with respective solvent to a final volume of 5 mL. The remaining extracts were stored at 4°C for further studies.

Screening of plant extracts against *A. solani*

The antifungal activity of each plant extract was tested with *A. solani* separately using agar well diffusion method (Perez *et al*., 1990). Sterilized PDA (30 ml) was mixed with fungal spore suspension (1 mL) and poured into a petri-plate (100 mm) and allowed to solidify at RT for 1 h. In each plate, four wells of 9 mm diameter were made using a stainless steel borer. Various extracts of different dilutions and respective solvent controls were added (each 100 µL). Plates were incubated at 25 ± 2°C for 5 days. Each experiment was carried out in triplicate, repeated twice and zones of inhibition were measured.

Dose profile study

Plant extracts which showed maximum zone of inhibition (≥10 cm) were used for dose profile studies (Table 2). For this study, fungal suspension culture of *A. solani* (1 mL), ranging from 10^{-1} to

10^{-9} dilutions was added in 30 ml PDA individually in each plate. To these plates, *C. zeylanicum* extracts in Hx and MeOH were added at a dose of 1, 2 and 3 mL/lit. Each plate was gently swirled to mix and allowed to solidify on a plain platform for 1 h at RT. These plates were incubated at 25 ± 2°C and observations were noted after every 24 h until 120 h. Plates of PDA alone served as master control and plates with PDA plus fungal culture served as positive control. Similarly, solvent controls (1–3 mL/lit) were also run in parallel to demonstrate the effect of solvents on fungal growth, if any (Yulia, 2005). The experiment was carried out in triplicate and repeated twice.

Formulation of *C. zeylanicum* extracts

Formulations of *C. zeylanicum* extracts (Hx and MeOH) were prepared using (i) carrier oil (Karanj; *Pongamia pinnata*, Mamta Herbals, Mumbai, India), (ii) emulsifiers (Triton X-100; 5% v/v and sodium lauryl ethyl sulfate; 0.5% v/v) and (iii) stabilizers (Epichlorhydrin; 0.5% v/v).

The treatments used in this experiment for application on tomato plants were categorized as i) master control (untreated tomato plants), ii) *A. solani* treated (tomato plants induced with *A. solani* alone), iii) Cabriotop (synthetic fungicide), iv) Hex-F (*C. zeylanicum* Hx extract formulation),

Table 2. Agar diffusion assay with aqueous, hexane and methanol extracts of eight different plants against *A. solani.*

Plant name	Solvent	Average zone of inhibition (mm) at different dilutions		
		X	X/2	X/10
Cinnamomum zeylanicum	H_2O	-	-	-
	MeOH	69.00 ± 2.65	66.33 ± 2.40	44.00 ± 0.58
	Hx	64.33 ± 4.06	57.00 ± 1.53	41.67 ± 1.76
Ferula foetida	H_2O	-	-	-
	MeOH	20.33 ± 1.45	19.33 ± 0.33	15.00 ± 0.58
	Hx	21.33 ± 1.76	17.00 ± 0.58	-
Glycyrrhiza glabra	H_2O	-	-	-
	MeOH	20.67 ± 1.20	19.33 ± 0.67	9.33 ± 1.76
	Hx	-	-	-
Hemidesmus indicus	H_2O	-	-	-
	MeOH	20.33 ± 1.45	14.67 ± 0.33	12.00 ± 0.58
	Hx	-	-	-
Inula racemosa	H_2O	-	-	-
	MeOH	23.33 ± 4.37	17.33 ± 2.03	12.67 ± 0.33
	Hx	19.67 ± 0.88	20.67 ± 1.20	11.67 ± 1.33
Rubia cordiafolia	H_2O	-	-	-
	MeOH	26.33 ± 1.76	21.67 ± 1.33	11.33 ± 1.20
	Hx	-	-	-
Saussurea lappa	H_2O	-	-	-
	MeOH	-	-	-
	Hx	30.67 ± 1.76	19.67 ± 2.03	12.33 ± 2.03
Syzygium aromaticum	H_2O	-	-	-
	MeOH	46.33 ± 2.40	44.00 ± 2.65	30.33 ± 2.03
	Hx	53.67 ± 2.40	44.33 ± 1.20	20.67 ± 1.76

Data represents average zone of inhibition (mm) ± standard deviation (S.D.) of water (H_2O), hexane (Hx) and methanol (MeOH) extracts of eight different plants against *A. solani*. All extracts (100 µL) were added (i) without dilution (X), (ii) 50 % dilution of extract with respective solvent (H_2O, MeOH and Hx) (X/2) and (iii) 90 % dilution of extract with respective solvent (H_2O, MeOH and Hx) (X/10) along with respective solvent controls to 9 mm diameter wells in PDA consisting of *A. solani* spores. Experiment was conducted in triplicate.

v) Mex-F (*C. zeylanicum* MeOH extract formulation), vi) HexMex-F (mixture of Hx and MeOH extracts of *C. zeylanicum*), vii) Hx control, viii) MeOH control and ix) mixture of Hx and MeOH solvent controls.

Induction of *A. solani* on tomato and its control

For evaluating preventive as well as curative *in vivo* anti-fungal potential of *C. zeylanicum* extracts, induction of *A. solani* infection on tomato (*Lycopersicum esculentum*, Mill variety – Abhinav, Syngenta) plantlets was prime prerequisite. Twenty one days old plantlets were used for inducing the infection. Twenty seven plantlets were used for preventive study and remaining twenty seven for curative study. All plants were fertigated with standard water soluble slow release fertilizers (Nitrogen, Phosphorus, Potassium; 19:19:19).

A loopful culture of freshly revived *A. solani* was inoculated into 300 mL of sterile Potato Dextrose Broth (PDB) and incubated on a rotary shaker at 120 rpm for 7 days at RT. Broth containing mycelia and spores was homogenized with a sterile glass rod, diluted 9 times with sterile distilled water and spore count was estimated around 2 x 10^4 spores/mL (Arunkumara, 2006). This mycelial and spore suspension was used as an inoculum for infecting tomato plants by foliar spray method (Arunkumara, 2006). Plantlets were covered with polyethylene bags for almost 72 hrs to create favorable conditions for the induction of *A. solani* infection on tomato plants. After removal of polyethylene bags plantlets were transferred in the soil and monitored for symptoms of early blight for next 25 to 30 days. Whereas, for preventive studies, formulations were sprayed on plants after 24 h of exposure to *A. solani*, for curative studies, though lesions were formed, the visual disease index of plants was very low. Hence, fifty day old plantlets were again exposed to *A. solani* suspension of 2 x 10^4 spores/mL approximate spore count (Arunkumara, 2006). This second exposure triggered the speed of lesion development with considerable increase in the disease index in seven days.

Preventive

After 24 h of exposure to *A. solani*, polyethylene bags were removed and formulations were sprayed on plants. Nine treatments as mentioned earlier with every treatment in triplicate at a dose of 2 mL/lit water were tried. Plantlets were again covered with polyethylene bags for next 48 h as discussed in the curative study to avoid ambiguity in induction of infection. Efficacy of formulations were evaluated after every seven days in terms of (i) total number of lesions developed, (ii) total number of infected as well as healthy leaves, (iii) girth of stem and (v) height of plants etc.

Curative

In this study, experiments were carried out in triplicate at a dose of 2 ml/lit water for each treatment and observed after every five days of spraying. The efficiency of formulations was measured in terms of per cent increase in fungal infection. Calculations were done as mentioned below.

Measurement of total leaf area

Three young leaves of approximately same size and age from a single plantlet were selected and total area of each leaf was calculated ---

Total leaf area (cm^2) = Length (cm) of a leaf * breadth (cm) of a leaf

Infected area of leaf

Area of lesion (cm^2) = Length (cm) of a lesion* breadth (cm) of a lesion

a. number of lesions developed on the same leaf was calculated along with area
b. Sum of area of all developed lesions

$$\text{Per cent control of fungal infection} = \frac{(\text{Infected area after 144 h} - \text{Initial infected area}) \times 100}{\text{Initial Infected area}}$$

***C. zeylanicum* extracts analyses**

For this study, standard silica gel 60 F 254 (Merck, Darmstadt, Germany) plates were used. Initially, TLC studies were carried out for standardizing the solvent system. Toluene: Ethyl acetate: Formic acid (90:9:1) was used for determining the active principle in Hx and MeOH extract of *C. zeylanicum* probably responsible for antifungal activity against *A. solani*. Standard eugenol was applied simultaneously as a suspected active metabolite. For band application on HPTLC plates, Linomat 5 was used (CAMAG, Switzerland). Plates were developed in a twin-trough chamber using the above mentioned solvent system and standard. After

drying, plates were scanned (CAMAG TLC scanner 4) at 254 and 365 nm. Most prominent spots from Hx (H 1, H 2, H 3, H 4 and H 5) and MeOH (M 1, M 2, M 3 and M 4) extracts of *C. zeylanicum* were isolated by preparative TLC from 40 TLC plates (20 x 10 cm), dissolved in respective solvents and further filtered through Whatman filter paper 1. The filtrate was collected, evaporated and used to test antifungal activity against *A. solani*. Methanolic extract of *C. zeylanicum* and spots isolated (M3 and M4) by preparative TLC, were subjected to Gas Chromatography - Mass Spectroscopy analysis. The GC-MS (Shimazdu QP 2010 Ultra Mass Spectrometer) was fitted with electron impact (EI) mode. Helium was used as a carrier gas at a flow rate of 1.78 mL/min. The temperature was programmed at 40°C for 1 min then increased to 200°C at a rate of 6 °C/min and to 280°C at a rate of 30°C/min. The temperature of injector and EI detector (70eV) was adjusted to 250°C. Each sample (1µL) was injected in to GC by Split mode and allowed to analyze on a fused silica column RTX- 5 (30 m in length X 0.25 mm in i.d, film thickness 0.25µm). Suspected compounds were identified by matching their mass spectra and retention time with compounds present in the NIST 08 (National Institute of Standards and Technologies) library.

Statistical analyses

All experiments were performed in triplicate and repeated twice. Data were analyzed using Single factor ANOVA and considered statistically significant when the F value obtained was higher than F-critical at $p < 0.01$.

RESULTS AND DISCUSSION

C. zeylanicum and *S. aromaticum* extracts are effective against *A. solani*

Eighty one extracts and three different concentrations of each extract [without dilution (X), X/2 and X/10 in respective solvents; total 243] were prepared and tested against *A. solani*. Out of the 27 plants screened, 8 plants and their extracts in various solvents exhibited marginal to excellent antifungal activity (Table 2). Hexane and MeOH extracts of *C. zeylanicum, S. aromaticum, I. racemosa, F. foetida,* methanol extracts of *R. cordifolia, H. indicus, G. glabra,* and Hx extract of *S. lappa* showed *in vitro* anti-fungal activity against *A. solani.* Hexane and MeOH extracts of *C. zeylanicum* and *S. aromaticum* were found to be most promising amongst all tested extracts. Diameter of zones of inhibition of *C. zeylanicum* was almost 2- fold as compared to other plant extracts (Table 2). Aqueous extracts of all plants under study did not show any activity against *A. solani.*

Dose profile studies of extracts

Excellent activities of MeOH and Hx extracts of *C. zeylanicum* prompted us to study its dose profile against *A. solani*. Both solvent extracts showed similar inhibition pattern against growth of *A. solani* at all doses tested (Table 3). MeOH and Hx extracts at a dose of 1 mL/lit showed 50% inhibition of *A. solani* after 10^{-4} colony count, whereas 2 mL/lit dose showed complete inhibition of *A. solani* after 10^{-7} colony count. At 3 mL/lit dose, *A. solani* was completely inhibited at all dilutions (Table 3).

Table 3. *In vitro* dose profile studies of *C. zeylanicum* extracts against *A. solani.*

Plant Extract	Extract Dose (mL/L)	Serial dilutions of *A. solani* suspension								
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
MeOH	1	++++	++++	+++	++	++	+	+	-	-
	2	+++	++	++	+	+	+	-	-	-
	3	-	-	-	-	-	-	-	-	-
Hx	1	++++	++++	+++	++	+	+	-	-	-
	2	+++	++	++	+	+	+	-	-	-
	3	-	-	-	-	-	-	-	-	-

Three doses *viz.* 1 to 3 mL/L of hexane and methanol extracts of *C. zeylanicum* were prepared and tested against serial dilutions of *A. solani* suspension. ++++, +++, ++, + and – indicate 100, 75, 50, 25 and 0 % growth of *A. solani*, respectively. Experiment was conducted in triplicate.

Table 4. Phytotonic effect of *C. zeylanicum* formulations on early blight of tomato.

Treatment	Number of leaves	Number of infected leaves	Number of lesions	Plant height (cm)	Stem girth (cm)
Master control	46.67 ± 1.53	0	0	21.07 ± 0.52	1.5 ± 0.10
A. solani treated	13.00 ± 1.73	9.00 ± 3.61	2.0 ± 1.0	7.67 ± 0.35	1.1 ± 0.53
Cabriotop	46.0 ± 2.65	5.33 ± 3.06	1.0 ± 0.1	14.37 ± 0.4	1.17 ± 0.40
Hex-F	46.33 ± 1.15	7.00 ± 2.00	1.33 ± 0.5	17.80 ± 0.5	1.4 ± 0.44
Mex-F	49.67 ± 2.08	3.67 ± 1.15	0.93 ± 0.15	22.10 ± 1.05	1.37 ± 0.25
HexMex-F	30.67 ± 2.08	7.67 ± 0.57	1.13 ± 0.15	21.33 ± 0.42	1.15 ± 0.3
Hx control	31.67 ± 3.21	10.0 ± 1.73	3.67 ± 1.15	12.83 ± 0.80	1.23 ± 0.29
MeOH control	29.33 ± 0.58	8.67 ± 2.08	1.67 ± 1.15	10.97 ± 0.91	1.13 ± 0.25
Hx + MeOH control	33.00 ± 1.00	5.67 ± 2.31	2.0 ± 1.0	11.60 ± 0.46	1.1 ± 0.52

Table represents phytotonic effect of *C zeylanicum* formulations on tomato plants after controlling *A. solani* infection. Different treatments were (i) Master control (untreated tomato plants), (ii) *A. solani* treated (infected tomato plants; positive control), (iii) Cabriotop (tomato plants treated with chemical fungicide), (iv) Hex-F (*C. zeylanicum* Hx extract formulation), (v) Mex-F (*C. zeylanicum* MeOH extract formulation), (vi) HexMex-F (*C. zeylanicum* Hx and MeOH extract combined in formulation), (vii) Hx solvent control, (viii) MeOH solvent control and (ix) Hx and MeOH mixed solvent controls.

In vivo* antifungal activity of *C. zeylanicum* formulations against *A. solani

In vivo studies of Hx and MeOH extracts of *C. zeylanicum* on *A. solani* showed variations in the growth of tomato plants in terms of number of healthy leaves, infected leaves, lesion developed, plant height and stem girth (Table 4).

Preventive study

Tomato plants were monitored almost for 60 days after spraying fungicidal formulations and observed every seventh day. Considerable differences were recorded twenty eight days after spraying. These studies indicated that in Mex-F extract number of infected leaves were less as compared to *A. solani* treated positive control as well as Cabriotop treated. Also, number of lesions developed were least in number in Mex-F extract treated plants. The number of lesions developed in Cabriotop treated plants were 1 ± 0.1. Prominent phytotonic effect was noted 28 days after spraying in Mex-F. It was evaluated by the total number of leaves and average height of plants which was better than the master control and Cabriotop treated plants. Whereas, the average stem girth of Mex-F extract treated plants was less than master control and Hex-F but more than all other treatments (Table 4).

Curative study

In vivo studies indicated that the infection of early blight varied with the treatments Figure 1.

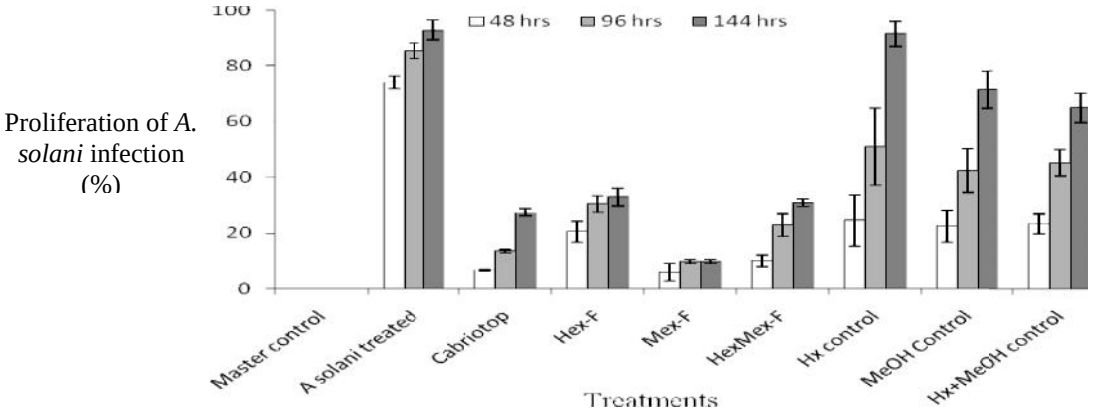

Fig 1. Control of early blight of tomato with *C. zeylanicum* formulations.

An impressive influence of Hex-F and Mex-F individually and even in the blend (He x Me x-F) was observed on *A. solani* when compared with Cabriotop (Figure 1). In *A. solani* treated plants, approximately 90% infection was observed after 144 h. Solvent controls also showed control of infection initially but after 144 h more than 65-91% infection was developed. This was 6-9 times more than Hex-F and Mex-F of *C. zeylanicum*. Plants treated with Cabriotop showed better control when compared to Hex-F while Mex-Fextract of *C zeylanicum* showed maximum control of *A. solani* at 48, 96 and 144h.

TLC, HPTLC and GC-MS analysis of *C. zeylanicum* extracts

Aqueous extract of *C. zeylanicum* showed 2 peaks (R_f 0.30 and 0.43; Figure 2A), Hx extract showed 5 peaks from H 1 to H 5 (R_f 0.03, 0.30, 0.42, 0.53 and 0.71 respectively; Figure 2B) while MeOH extract showed seven separated peaks but peak areas of 4 compounds M 1 to M 4 (R_f 0.21, 0.31, 0.45 and 0.52 respectively, Figure 2C) were prominent. Compound corresponding to R_f 0.42 (hexane) and 0.45 (MeOH) was matching standard eugenol (R_f 0.40; Figure 2D). This was confirmed on the basis of HPTLC by scanning the plates under deuterium (254 nm) and mercury lamp (366 nm) with flourescence quenching as detection mode using CAMAG 4 scanner (Figure 2B and 2C). When the separated spots were scanned in UV region (200-400 nm), spots present in Hx and MeOH at R_f 0.42 and 0.45 showed almost characteristic UV-spectrum showing λ_{max} at 201 and 280 nm with a typical shoulder at around 230 nm. Spot corresponding to standard eugenol and three other compounds, showing R_f 0.21, 0.31, and 0.52 from MeOH extract of *C. zeylanicum*, were further isolated by preparative TLC. These compounds exhibited antifungal activity against *A. solani*. Zone of inhibition of spot corresponding to R_f 0.52 (16 mm) was maximum followed by spot of R_f 0.45 (14 mm), R_f 0.21 (12 mm) and R_f 0.31 (11 mm).

Being proved excellent antifungal amongst the chosen treatments, GC MS analysis of MeOH extract of *C. zeylanicum* was performed. The identified compounds of the extract and two prominent spots (M 3 R_f 0.45 and M 4 R_f 0.52) separated using TLC are presented in Table 5 with their retention indices, molecular formulae and nature of compound. The identification / prediction of these compounds is based on the NIST 08 library. The GCMS profile of isolated compounds M 3 (R_f 0.45) and M 4 (R_f 0.52) showed the presence of 2H-1-Benzopyran-2-one, eugenol and cinnamaldehyde. Presence of cinnamaldehyde and eugenol in oil of *C. zeylanicum* has also been confirmed earlier (Schmidt *et al*., 2006; Gende *et al*., 2008). There is limited study about the presence of 2H-1-Benzopyran-2-one and its antifungal activity (Uma *et al*., 2009). Here, we report the presence as well as antifungal activity of this compound from *C. zeylanicum*.

Amongst twenty seven plants screened for activity against *A. solani*, our initial results showed that *C. zeylanicum* is excellent. Although the antifungal activity of *C. zeylanicum* has been reported many years before, there is not much information on *A. solani*. Therefore, we have tried to study this aspect in detail and find an economical and eco-friendly alternative to chemical fungicides. Plant parts, powders, oils and extracts have gained importance as safer antifungal agents to control agricultural phytopathogens (Mendki, 2002). Almost 7500 different plant species are found in India (Preethi *et al*., 2010), producing an array of secondary metabolites like alkaloids, flavonoids, terpenoids etc.

With the advent of modern chemistry, many secondary metabolites have been identified and their roles probed. Spices are known to contain essential oils, mixture of volatile as well as non-volatile terpenes and their oxygenated derivatives. Essential oils have been reported for their broad spectrum antifungal activity against different plant pathogens (Kishore and Pande, 2007; Isman, 2000) by reducing hyphal growth and inducing lysis and cytoplasmic evacuations in fungi (Fiori *et al*., 2000). Based on this information, we hypothesized that extracts or active metabolites from these plants might possess antifungal activity (Table 2). Further, these extracts can be formulated to meet the growing demands of sustainable organic agriculture and find a place as an effective alternative strategy in the grim scenario of chemical fungicides.

Interestingly, Mex-F controlled *A. solani* infection

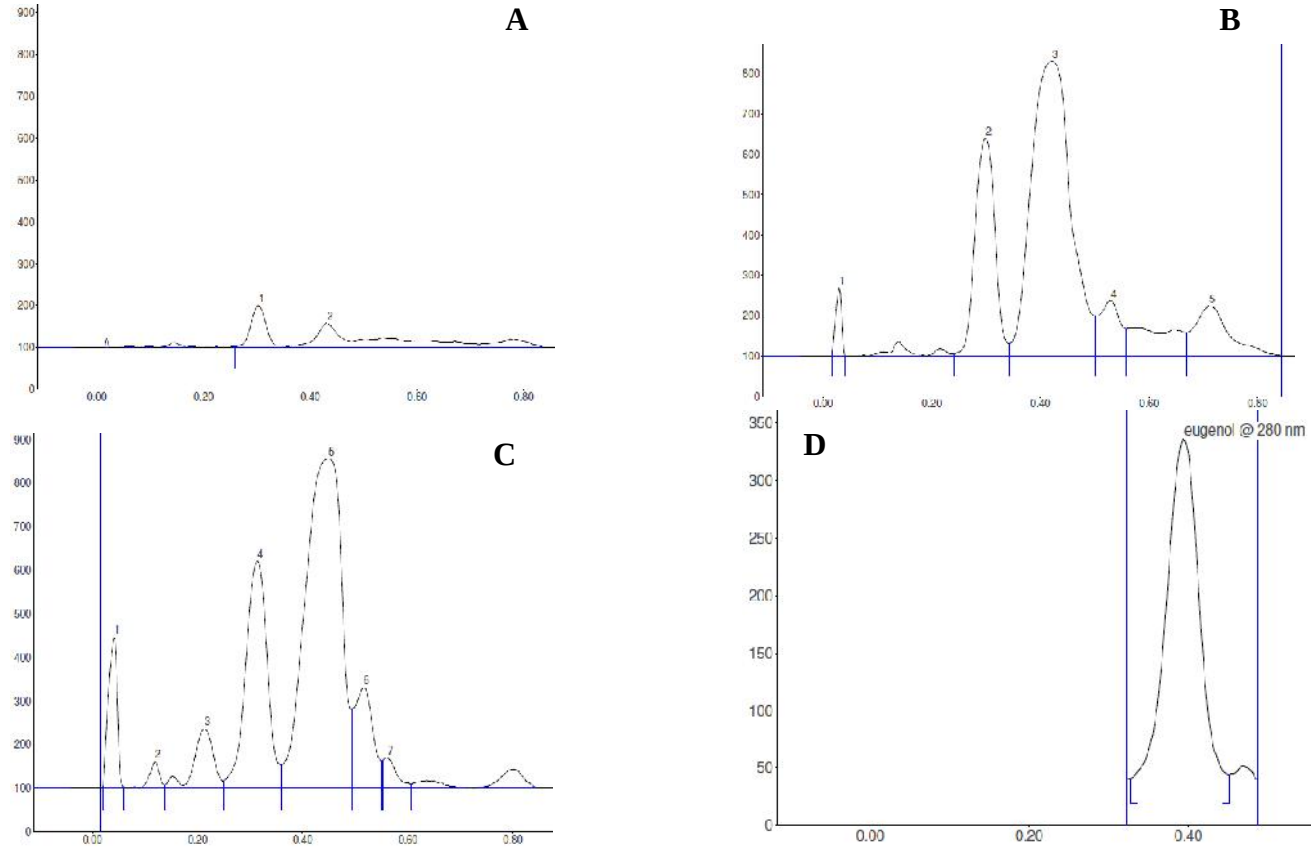

Fig 2. HPTL chromatography of *C. zeylanicum* extracts.

almost equally effective at 48, 96 and 144 hrs as compared to *A. solani* treated tomato plants (positive control) (Figure 1). For developing a commercially viable and acceptable formulation, we thought of screening the most effective MeOH extract of *C. zeylanicum*. A dose of 2 mL of *C. zeylanicum* extract per liter of water can control *A. solani* infection with maximum efficiency (Table 3). This optimized dose of every formulation / treatment was used for evaluating *in vivo* bioefficacy against *A. solani* in tomato plants. Eugenol and cinnamaldehyde have been reported for their antifungal activity against different animal pathogens as well as few phytopathogens (Barrera-Necha *et al*., 2009; Faria *et al*., 2006). On the basis of this, we have isolated these secondary metabolites by preparative TLC and tested their 'anti-early blight' activity individually. HPTLC and GC-MS provide quick and reliable identification of suspected plant secondary metabolites (Figure 2 and Table 5).

Limited work has been done on 2H-1-Benzopyran-2-one and its derivatives as prominent anti-fungals against phytopathogens (Uma *et al*., 2009; Brooker *et al*., 2008). To our knowledge, the antifungal activity of 2H-1-Benzopyran-2-one, a coumarin has been reported for the first time against *A. solani*. Comparatively higher activity of hexane and methanol extracts of *C. zeylanicum* than other plants under study might be attributed to active components present in them (Table 5).

Many reports are available on antifungal activity of plant extracts or secondary metabolites isolated from them (Sohn *et al*., 2009). To further enhance activity of these secondary metabolites, they must be formulated carefully. This will help in popularizing herbal fungicides against popularly used and vastly available synthetic or chemical counterparts. Promising results of MeOH extract of *C. zeylanicum* prompted us to develop its user friendly and economical formulation. We have incorporated, Karanj oil, as a carrier, Triton X-100

Table 5. GC MS of *Cinnamomum zeylanicum* methanol extract

Extract	Peak	Retention time	Area %	Compound	Molecular formula	Molecular weight	Nature of compound
M1	1	3.022	4.25	Trichloroethylene	C_2HCl_3	130	Halogenated Alkenes
	2	14.838	5.37	Benzeneacetic acid, .alpha.-methox	$C_9H_{10}O_3$	166	Acidic compound
	3	14.924	37.42	Cinnamaldehyde, (E)-	C_9H_8O	132	Aldehyde
	4	16.859	0.80	Eugenol	$C_{10}H_{12}O_2$	164	Phenol
	5	17.768	12.57	5-Methoxyindane	$C_{10}H_{12}O$	148	
	6	18.644	15.37	2H-1-Benzopyran-2-one	$C_9H_6O_2$	146	Ketone compound
	7	20.521	1.36	2-Propenal, 3-(2-methoxyphenyl)-	$C_{10}H_{10}O_2$	162	Aldehyde
	8	28.127	1.18	Pentadecanoic acid	$C_{15}H_{30}O_2$	242	Saturated fatty acid
	9	29.329	1.97	2,6,10,14,18,22-Tetracosahexaene	$C_{30}H_{50}$	410	Trans-isoprenoid
	10	29.415	15.70	2,6,10,14,18,22-Tetracosahexaene	$C_{30}H_{50}$	410	Trans-isoprenoid
	11	29.580	0.63	1,E-11,Z-13-Octadecanriene	$C_{18}H_{32}$	248	
	12	29.610	0.74	cis-Vaccenic acid	$C_{18}H_{34}O_2$	282	Fatty acids
	13	30.245	2.65	gamma.-sitosterol	$C_{29}H_{50}O$	414	Isoprenoid
M3	1	14.842	4.76	2-Propenal, 3-phenyl-	C_9H_8O	132	
	2	16.816	0.43	Eugenol	$C_{10}H_{12}O_2$	164	Phenol
	3	17.267	0.46	Biphenyl	$C_{12}H_{10}$	154	
	4	17.758	0.40	Diphenyl ether	$C_{12}H_{10}O$	170	
	5	18.149	0.62	Benzaldhyde dimethyl acetal	$C_9H_{12}O_2$	152	
	6	18.720	93.11	2H-1-Benzopyran-2-one	$C_9H_6O_2$	146	Ketone compound
	7	21.730	0.20	Diethyl Phthalate	$C_{12}H_{14}O_4$	222	
M4	1	14.871	90.87	Cinnamaldehyde, (E)-	C_9H_8O	132	Aldehyde
	2	16.809	1.93	Eugenol	$C_{10}H_{12}O_2$	164	Phenol
	3	17.712	2.05	9-Methoxybicyclo(6.1.0)nona-2,4,	$C_{10}H_{12}O$	162	
	4	18.577	3.01	2H-1-Benzopyran-2-one	$C_9H_6O_2$	146	Ketone compound
	5	20.470	1.00	2-Propenal, 3-(2-methoxyphenyl)-	$C_{10}H_{10}O_2$	162	Aldehyde
	6	21.722	1.14	Diethyl Phthalate	$C_{12}H_{14}O_4$	222	

GC-MS analysis of MeOH extract of *C. zeylanicum* and spots isolated M3 and M4 from it by preparative TLC was performed on Shimazdu QP 2010 Ultra Mass Spectrometer which showed identification of two important secondary metabolites 2H-1-Benzopyran-2-one and Cinnamaldehyde respectively.

(t-octylphenoxypolyethoxyethanol; non-ionic detergent) and sodium lauryl ethyl sulphate as emulsifiers and EPC (epichlorhydrin) as a stabilizer. During *in vivo* shade-house studies, we found phytotonic effect of *C. zeylanicum* formulations on tomato (Table 4).

This was evident from more number of leaves, less infected leaves and higher plant height in Mex-F treated tomato plants than other treatments. This may be due to pronounced effect of *C. zeylanicum* extract in controlling *A. solani* thus sequestering energy by tomato plants for better vegetative growth.

Popularity of chemical pesticides is largely due to its minimum dose of application and considerable persistence in the environment. On the contrary, plant secondary metabolites have higher doses of application and lesser persistence in the environment due to their sensitivity to temperature and light (Turek *et al*., 2013). Our ongoing experiments aim to increase bioefficacy of the formulation by using additives like oils, essential oils, etc. and minimize the dose around 0.5 to 1.0 mL/lit along with the frequency of application under field/harsh environmental conditions.

Out of 81 extracts tested for *in vitro*, two extracts have been evaluated for *in vivo* control of *A. solani*. Methanolic extract of *C. zeylanicum* was found excellent preventive as well as curative antifungal in the control of early blight on tomato plants. Further documentation of this extract using TLC and GC-MS indicated the presence of three prominent compounds namely Eugenol, Cinnamaldehyde and 2H -1- Benzopyran -2- one. This is the first report which showed antifungal potential of 2H -1- Benzopyran -2- one, against phytopathogen *A. solani*.

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