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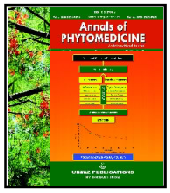

Original article

Antifungal efficacies of plant extracts against *Alternaria solani* (Ellis and Martin) Jones and Grout under *in vitro* condition

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Abstract

The antifungal activity of crude plant extracts of ten plant species, *viz.*, neem (*Azadiracta indica*), prosopis (*Prosopis juliflora*), onion (*Allium cepa*), eucalyptus (*Eucalyptus obliqua*), pungam (*Pongamia pinnata*), curry leaf (*Murrya koenigii*), garlic (*Allium sativum*), henna (*Lawsonia inermis*), turmeric (*Curcuma longa*) and ginger (*Zingiber officinale*) were evaluated against *Alternaria solani* (Ellis and Martin) by using poisoned food technique under *in vitro* conditions. The results showed that turmeric extract recorded minimum radial mycelial growth of 9.50 mm with maximum per cent inhibition of 89.44 over control. It was followed by garlic observed (12.80 mm, 85.77 per cent) and eucalyptus (24.27 mm, 73.03 per cent) respectively. Extract of *C. longa*, *A. cepa* and *E. obliquea* reduced conidial germination to 91.11, 84.44 and 80.06 per cent over control, respectively. Thermal stability of extracts were tested against pathogen from preliminary screening; extracts of tested plant species were found to be heat stable and aqueous extracts of *C. longa* showed the highest antifungal activity (10.78 mm), followed by *A. cepa* (13.42 mm). Results of this study indicated that all plant extracts have the antimicrobial activity among all, both extracts of turmeric and garlic were strong inhibitors of this fungus and to levels comparable to standard fungicides.

Keywords: *Alternaria solani* (Ellis and Martin), crude extracts, poisoned food technique, inhibition, antifungal

1. Introduction

Tomato (*Lycopersicon esculentum* Mill) belongs to the Solanaceae family, is one of the most remunerable and widely grown vegetable crop in the world. In world acreage, tomato ranks next to potato among all the vegetables and ranks first among the processing crops. Tomato is grown for its edible fruits, which can be consumed either fresh or in processed form and is a very good source of vitamins, organic acids, essential amino acids and dietary fibres. It occupies number one in their nutrient contribution to human diet. In India, tomato occupies an area of 809 million hectare, with a production of 19696.9 metric tons and productivity of 24.4 metric tons/ha in the year 2016-2017 (Saxena, 2017).

There are many factors infecting tomato, *viz.*, fungi, bacteria, viruses, nematodes and abiotic factors. Among all diseases, early blight disease caused by *Alternaria solani* (Ellis and Martin) Jones and Grout, is one of the most common and destructive disease of tomato. Early blight disease infecting leaf, stem and fruit are most damaging symptoms and yield losses up to 79% damage by *A. solani* were reported from India, Canada, USA and Nigeria (Basu, 1974; Datar, 1981; Sherf and MacNab, 1986; Mathur and Shekawat, 1986; Gwary and Nahunnaro, 1998).

Chemical fungicides have been used to manage these diseases, but this conduct is associated to negative environmental impacts, potential human exposure to fungicides and residue deposition on the fruits. However, the effectiveness of chemical fungicides has been reduced by development of resistance by the pathogens. Hence, there is a great demand for safer, alternative and effective chemotherapeutic agents (Balakumar *et al*., 2011; Liu *et al*., 2013).

Studies have shown that the extracts of medicinal plants have antimicrobial activity against pathogen (Manilal and Idhayadhulla, 2014). The plant extracts of *Prosopis juliflora*, *Carica papaya*, *Polyalthia longifolia* and *Ricinus communis* are important candidate to develop formulations for the management of *A. solani* (Kalpashree and Raveesh, 2016). Considering inhibition of mycelial growth and spore germination *in vitro* and reduction in the development of early blight symptoms in plants *in vivo* treated with essential oils extracted from *E. staigeriana*, *E. globulus* and *C. camphora* (Tomazoni *et al.*, 2017). In this work, the present investigation was intended to explore *in vitro* antifungal effect of plant extracts against the pathogenic fungi *A. solani*.

2. Materials and Methods

2.1 Isolation of the fungus *Alternaria solani*

The infected leaf was first washed with tap water to remove dust and other contaminants. The periphery of the lesions were cut into

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small bits and surface sterilized with 10 per cent sodium hypochlorite for 5-10 min. In order to remove the residue of the chemical, the tissue bits were washed with three changes of sterile distilled water. The surface sterilized bits were placed on potato dextrose agar (PDA) medium in sterilized petri dishes. These plates were incubated at room temperature (28 ± 2°C) for seven days. After incubation, the cultures were purified by hyphal tip method (Dhingra and Sinclair, 1985) and the fungal cultures were maintained separately in agar slants per petri plate. Also, pathogenicity of *A. solani* was proved by Kochs postulates on healthy tomato plants.

2.2 Collection of plants sample and preparations of plant extracts

Bioactive natural products were extracted from different plant species with significant antifungal activity, such as neem (*Azadiracta indica* A. Juss), prosopis (*Prosopis juliflora* (Sw). Dc), onion (*Allium cepa* L.), eucalyptus (*Eucalyptus obliqua* L. Her.), pungam (*Pongamia pinnata* L.), curry leaf (*Murrya koenigii* (L.) Sprengel), garlic (*Allium sativum* L.), henna (*Lawsonia inermis* L.), turmeric (*Curcuma longa* L.) and ginger (*Zingiber officinale* (L.) Roscoe). 100 g (fresh wt) of mature leaves of above selected plants were homogenized separately in a pre-chilled pestle and mortar using chilled, sterilized distilled water. The extract was filtered through four layers of moistened muslin cloth and the volume was adjusted to 100 ml with distilled water at final. The filtrate was centrifuged at 8000 rpm, 48°C for 15 min. The supernatant thus obtained was designated as concentrated leaf extract (Shekhawat and Prasada, 1971).

2.3 *In vitro* effect of plant extracts against pathogen

The efficacy of plant extracts on the growth of *A. solani* was studied by poison food technique (Schmitz, 1930). From the standard plant extract 10 ml was added to 90 ml of sterilized and cooled (warm) complete medium and thoroughly mixed by shaking for making 10 per cent concentration. This was plated into sterile petri plates in 20 ml quantities and allowed to solidify. A nine millimetre actively growing culture disc of *A. solani* was aseptically placed onto the medium at the centre of the plate. Three replications were maintained for each treatment. The complete medium without incorporating the plant extract served as control. The plates were incubated at room temperature (28 ± 2°C). The diameter of the colony was measured and expressed as per cent growth reduction over control.

$$PI = \frac{Dc - Dt}{Dc} \times 100$$

Dc = Average diameter of fungal growth (cm) in control

Dt = Average diameter of fungal growth (cm) in treatment

PI = Per cent inhibition

2.4 Spore germination assay

About 4.5 ml volume of potato dextrose, broth was pipetted into the test tubes containing 0.5 ml of plant extracts and mixed it thoroughly. At the same time, an aliquot (100 µl) of spore suspension (adjusted to 10^6 conidia ml^{-1}) was added into each tube. After 24 h of incubation at 28°C on a rotary shaker, a drop of the mixture from each tube was placed in a microscope slide and slides were fixed in lacto-phenol cotton blue and observed spore germination microscopically. The same volume was added in place of plant extracts in the control samples served as a control. A conidium was considered as germinated, if the length of the germ tube was at least half the length of the conidium. The number of germinated conidia was counted out of 100 randomly selected conidia in three replicate slides. Percentage spore germination was calculated according to the following formula:

$$\text{Spore germination (\%)} = \frac{\text{Germinated spores}}{\text{Total spores}} \times 100$$

2.5 Thermal inactivation of plant extracts

Plant extracts showing potential antifungal activity among the screened plant extracts were further tested for thermal stability. About 1 ml of plant extracts in glass tubes were exposed to 60°C in a water bath for 10 min and cooled to room temperature. Afterwards, the plant extracts were evaluated against *A. solani* by poisoned food technique method.

2.6 Statistical analysis

The data were statistically analyzed (Gomez and Gomez, 1984) and the treatment means were compared by Duncan's Multiple Range Test (DMRT). The package used for analysis was IRRI-Stat version 92-a developed by International Rice Research Institute Biometrics Units, Philippines.

3. Results

3.1 Plant extracts against *A. solani* in *in vitro*

Of the tested samples obtained from plant species showed antifungal activity against *A. solani* early light disease in tomato plants which shown in Table 1. Among the ten plant extracts, turmeric (*Curcuma longa*) showed minimal mycelial growth of 9.50 mm with highest inhibition over control (89.44 per cent). This was followed by garlic (*A. sativum*) and eucalyptus (*E. globulus*) which observed mycelial growth of 12.80 and 24.27 mm with inhibition area of 85.77 and 61.60 per cent. Radial mycelial growth and inhibition area over control was observed in ginger (*Z. officinale*) (28.35 mm, 68.50 per cent), onion (*A. cepa*) (34.56 mm, 61.60), curry leaf (*M. koenigii*) (46.31 mm, 48.54 per cent), neem (*A. indica*) (51.76 mm, 42.48) and henna (*L. inermis*) (57.18 mm, 36.46 per cent).

Maximum radial mycelial growth and least inhibition was recorded in prosopis (*P. juliflora*) (65.57 mm, 27.14 per cent), pungam (*O. glabra*) (71.18 mm, 20.91 per cent) and the control showed full growth of *A. solani* without any inhibition area (Table 1) (Figure 1).

3.2 Conidial germination assay

The plant extract from *C. longa, A. sativum* and *E. globulus* showed potent antifungal activity in reducing the spore germination to 91.11, 84.44 and 80.06 per cent with spore germination of 8.16, 14.28 and 18.36 per cent, respectively. Extract from *Z. officinale, A. cepa, M. koenigii* and *A. indica* showed reduction in condial germination to 75.55, 66.67, 57.78 and 48.89 per cent with conidia germination percentage of 22.45, 30.61, 38.77 and 46.93. Lowest conidal germination was observed in *L. inermis, P. juloflora* and *P. glabra*

showed 55.10, 63.26 and 75.51 per cent conidial germination with reduction in 39.97, 31.12 and 17.77 per cent over control (Table 2.) (Figure 2).

3.3 Thermal stability of aqueous extracts on *A. solani*

Aqueous extracts of *C. longa, A. sativum, E. globulus, Z. officinale, A. cepa, M. koenigii* and *A. indica* that showed high activity against *A. solani* in the preliminary screening were further tested for their thermal stability and for the potency of their aqueous extracts. The results showed that there was no significant difference (p>0.05) between heated (at 60°C) and unheated plant extracts in their efficacy against *A. solani* (Table 3). It indicates that the hot water treatment (HWT) temperature range had no effect on the antimicrobial activity of the plant extracts.

Table 1: Antimicrobial effect of different plant extracts on growth of *A. solani*

S.No.	Botanical name	Common name	Parts used	Mycelial growth (mm)*	Per cent inhibition over control (%)
1.	*Azadirachta indica* A. Juss.	Neem	Leaf	51.76 ± 0.33^{g}	42.48
2.	*Prosopis juliflora* (Sw.) DC	Prosopis	Leaf	65.57 ± 0.24^{i}	27.14
3.	*Allium cepa* L.	Onion	Bulb	34.56 ± 0.16^{e}	61.60
4.	*Eucalyptus globulus* Labill.	Eucalyptus	Leaf	24.27 ± 0.34^{c}	73.03
5.	*Pongamia glabra* (L.) Pierre	Pungam	Leaf	71.18 ± 0.19^{j}	20.91
6.	*Murrya koenigii* (L.) Sprengel	Curry leaf	Leaf	46.31 ± 0.35^{f}	48.54
7.	*Allium sativum* L.	Garlic	Bulb	12.80 ± 0.42^{b}	85.77
8.	*Lawsonia inermis* L.	Henna	Leaf	57.18 ± 0.24^{h}	36.46
9.	*Curcuma longa* L.	Turmeric	Rhizome	9.50 ± 1.08^{a}	89.44
10.	*Zingiber officinale* (L.) Roscoe	Ginger	Rhizome	28.35 ± 0.85^{d}	68.50
11.	Control			90.00 ± 0.14^{k}	-
		CD (p=0.05)		**2.215**	**1.021**

*Mean of three replications
± represents SE of mean
Means in a column followed by same superscript are not significantly different by Duncan's Multiple Range Test at $p < 0.05$.

Table 2: Effect of plant extracts on conidial germination of *A. solani*

S.No.	Botanical name	Conidial germination (%)*	Per cent inhibition over control
1.	*Azadirachta indica*	46.93(43.24)^{g}	48.89
2.	*Prosopis juliflora*	63.26(52.69)^{i}	31.12
3.	*Allium cepa*	30.61(33.59)^{e}	66.67
4.	*Eucalyptus globulus*	18.36(25.37)^{c}	80.06
5.	*Pongamia glabra*	75.51(60.37)^{j}	17.77
6.	*Murrya koenigii*	38.77(38.50)^{f}	57.78
7.	*Allium sativum*	14.28(22.20)^{b}	84.44
8.	*Lawsonia inermis*	55.10(47.92)^{h}	39.97
9.	*Curcuma longa*	8.16(16.59)^{a}	91.11
10.	*Zingiber officinale*	22.45(28.28)^{d}	75.55
11.	Control	91.83(73.41)^{k}	-
	CD (p=0.05)	**1.532**	**2.566**

*Mean of three replications

*Values in the parenthesis are arcsine transformed

*Means in a column followed by same superscript are not significantly different by Duncan's Multiple Range Test at $p < 0.05$.

4. Discussion

The investigation of plants containing natural antimicrobial metabolites for plant protection had been identified as a desirable method of disease control (Seema *et al.*, 2011; Dwivedi and Neetu, 2012). Plants were being used against many plant pathogenic fungi. The plant extracts serve as eco-friendly and environment safe bio-control agents (Swami and Alane, 2013). Efficacy of some plant extracts on seed borne fungi of mungbean seed were reported by Swami and Alane (2013) and found that all the extracts were significantly reduced the fungi at higher concentration.

Table 3: Thermal effect on activity of plant extracts against *A. solani*

S.No.	Plant extract	Thermal effect(mm)*	Without thermal Effect (mm)*
1.	*Curcuma longa*	10.78^{a}	9.96^{a}
2.	*Allium sativum*	13.42^{b}	14.12^{b}
3.	*Eucalyptus globulus*	26.89^{c}	25.16^{c}
4.	*Zingiber officinale*	27.32^{d}	26.10^{d}
5.	*Allium cepa*	37.46^{e}	36.71^{e}
6.	Control	89.90^{f}	90.00^{f}
	CD (p=0.05)	**2.340**	**1.118**

*Mean of three replications

*Means in a column followed by same superscript are not significantly different by Duncan's Multiple Range Test at $p < 0.05$.

The leaf extract from neem showed high efficacy to inhibit the radial mycelial growth of *A. solani* (43.3 and 26.7% at 0.1% and 0.01%, respectively) (Sharma *et al.*, 2007). Various plant products like plant extracts, essential oils, gums, resins, *etc.*, were shown to exert biological activity *in vitro* and *in vivo* and were used as bio-fungicidal compounds (Fawzi *et al.*, 2009; Al-Askar and Rashad, 2010). *A. indica, A. cepa* and *A. sativum* showed antifungal property against *Alternaria alternata* (Lakshman and Ahir, 2011).

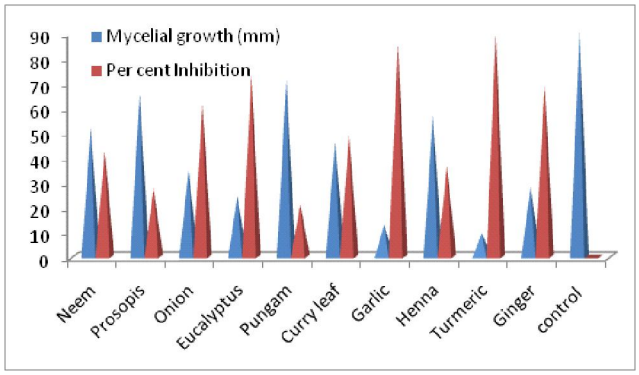

Figure 1: Effect of plant extracts on growth and inhibition of *A. solani*.

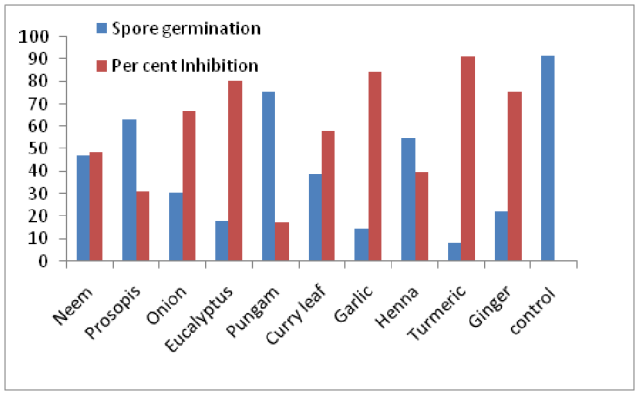

Figure 2: Effect of plant extracts on conidial germination and inhibition of *A. solani*.

The leaf extract of *A. osyrensis* showed highest inhibition of 25 mm against *E. coli*. The *Acronychia pedunculata* extracts tested offered maximum RSA (Radical Scavenging Activities) of 65.94 % against *E. coli*. The antimicrobial activity of *M. koenigii* was estimated by well diffusion method; the maximum antibacterial activity was observed against *Pasteurella multocida* at 75% concentration (Mahendra *et al.*, 2016; Gireesha and Raju, 2016; Aslam *et al.*, 2017).

The results showed that the different antimicrobial forms varied in their effectiveness in inhibiting fungi growth. The diversity in the biocomposition of chemical components of plant extracts, *i.e.*, the secondary metabolites of plants, even those obtained from the same species, may result in different responses, especially with regard to the potential for microorganism inhibition (Talibi *et al.*, 2012).

In the preliminary *in vitro* studies, we observed different crude extracts to completely inhibit the germination of *A. solani*. Similar potent antagonistic activity of plant extracts has been reported against *A. solani* and several other fungi (Pinto *et al.*, 1998; Kishore *et al.*, 2001). Extract of *P. juliflora* and *A. cepa* remained highly antifungal even at 1% (w/v) concentration. These two plant spp. were well known for their antifungal activity. Extract of *P. juliflora* inhibited the germination of sclerotial *Rhizoctonia solani* (Ezhilan *et al.*, 1994). The antifungal activity of turmeric extract could be largely due to the presence of alkaloids. Alkaloid fractions of turmeric extract were known for their antifungal activity (Ahmad *et al.*, 1997).

The results are in line with the results of Masih *et al.* (2015) who observed that the aqueous extracts of *C. longa* showed inhibitory effect on the growth of *Aspergillus fumigates*, *Fusarium solani*, *Alternaria solani* and *Helminthosporium* spp. Also, the results supported by the findings of Raza *et al.* (2016) tested five different plant extracts against *A. solani in vitro* and found that all botanical extracts, including *P. hysterophorus*, significantly inhibited mycelial growth of the pathogen. All botanicals have promising antimicrobial activity, among this turmeric extracts showed maximum inhibition of *A. solani* at 10% concentration after 8 days (Sarfaraz *et al.*, 2018).

The results of thermal inactivation study were supported by previous works (Adegoke *et al.*, 2010) that natural extract components are heat stable under moderate (50-80°C) heat treatment. In addition, the degree of inhibition of fungal growth was increased from 3.5 to 4 scales in the case of *A. indica*. This is in agreement with the earlier findings (Doughari and Manzara, 2008; El-Mahmood, 2010) that heat treatments increased the activity of plant extracts. This may be due to an increase in the release of active compounds and free radicals (Majumder *et al.*, 1998).

5. Conclusion

The antimicrobial activity of the plant extracts against *A. solani* indicates the potential of some plant species as a natural source of fungicidal material. Antifungal activity was confirmed in all the tested plant species, although the results showed that different plant extracts varied in the effectiveness in inhibiting the mycelial growth of *A. solani*. The results of the present study revealed that extracts from turmeric and garlic have maximum inhibition and minimum conidial germination of *A. solani*. Based on the study, we could conclude that plant extracts serve as a good alternative to chemical fungicides for future eco-friendly antifungal agents, controlling early blight disease of tomato and minimizing risks and hazards for the environment.

Acknowledgments

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Conflict of interest

The authors declare that no conflict of interest exists in the course of conducting this research. All the authors had final decision regarding the manuscript and the decision to submit the findings for publication.

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