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Original Article

IN VITRO INHIBITORY ACTIVITY OF MEDICINAL PLANTS AGAINST *PHOMOPSIS AZADIRACHTAE*, THE INCITANT DIE BACK DISEASE OF NEEM

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ABSTRACT

Objectives: Plant diseases extend a threat to the global production of herbal medicines. Safer plant protection strategies are in need to meet the demands from the pharmaceutical industry. Neem is a tree with great medicinal values, which is being infected by Phomopsis azadirachtae (PA), causing die back disease. The resulting massive destruction of the tree demands optimum disease management methods and thus, the objective of the present study was to evaluate the inhibitory potential of some plant extracts against the growth of PA.

Methods: In this study, we investigate the in vitro antifungal activity of ether: chloroform (1:1), ethyl acetate, acetone and ethanol extracts from different parts (leaves and roots) of five plant species viz., Ocimum tenuiflorium, Centella asiatica, Solanum nigrum, Apium graveolens and Besella alba against the growth of PA.

Results: All the 40 crude extracts (at concentration of 1000 µg/ml), were active against PA. Among them, the ethanol and ethyl acetate extracts proved optimum with antifungal activity ranging from 65-95% and minimum inhibitory concentration (MIC) values from 75-200 µg/ml. The antifungal activity of the extracts was in the order: ethanol>ethyl acetate>acetone>ether: chloroform (1:1). Also, noteworthy results were observed in all the extracts tested against the spore germination of PA, with concentration and time-dependent kinetics.

Conclusion: The results prove promising in order to develop new antifungal agents against the growth and spread of PA.

Keywords: Neem, *Phomopsis azadirachtae,* Medicinal plants, Antifungal activity, Plant extract.

INTRODUCTION

Neem (*Azadirachta indica* A. Juss), is an indigenous medicinal plant, traditionally known as "the village pharmacy" attributing to its potential uses as antiallergic, antidermatic, antifeedant, antiviral, antifungal and several other biological activities [1,2]. It is facing a great threat because of the die-back disease, caused by the phytopathogenic fungi *Phomopsis azadirachtae* (PA) [3]. The loss as a result of this infection accounts to several million dollars because of which, its protection is of high priority [4]. Previous studies on the disease incidence, reports about 80-95% incidence in all the surveyed areas of Karnataka and Tamil Nadu [5, 6]. Also, the pathogen endures a wide range of climatic conditions [7]. The high disease incidence as well as the wide climatic existence of the pathogen poses greater challenges in designing optimum control of the disease. Some reports on the control of the pathogen *in vitro* using synthetic antifungal agents have established bavistin, to effectively inhibit the growth of PA [8, 9]. While it's *in vivo* studies have also been carried out and proved promising, the health and environmental hazards that are caused by the use of chemical fungicides [10] creates concern in using them as a routine antifungal agent in the control of PA. Resistance that the fungi develop as a result of fungicide administration is another factor that requires consideration while using these chemical agents [11].

Plants prove safer alternatives over chemicals with lesser side effects and thus are gaining popularity in medicinal research. Plant-based antimicrobial agents exert inhibition by various modes *viz*., by affecting the cell membrane permeability, regulating or disabling the enzymatic processes or by inhibiting protein translation [12]. Development of drugs from plant sources has received much attention and they are known to be isolated from various parts of the plants like roots, leaves, shoots and bark. The popular uses of the plants selected for this study have previously been reported in the literature (table 1). However, scientific evidence of their antifungal effects with respect to PA has not been investigated yet. Hence, the aim of the present study was to find out *in vitro* antifungal activity of the extracts from various parts (leaves, and roots) of five Indian traditional plants species named *Ocimum tenuiflorium*, *Centella asiatica*, *Solanum nigrum*, *Apium graveolens* and *Besella alba*. The study also aims to assess the inhibitory effects of the extracts against the germination of the spores and their time-dependent kinetics at different concentrations.

MATERIALS AND METHODS

Chemicals

Reagents and solvents used for extraction were procured from Merck (Mumbai, India). The standard drugs were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). All the other reagents were of analytical grade.

Plant materials

The five plants, namely *Ocimum tenuiflorium*, *Centella asiatica*, *Solanum nigrum*, *Apium graveolens* and *Besella alba* were collected in the month of February and March 2013, in Chamundi hill region of Mysore, Karnataka, India. The specimens were identified by the Department of Horticulture, Government of Karnataka, Mysore, India.

Preparation of plant extracts

The leaves and roots of the collected plant materials were separated, washed with tap water and dried at room temperature. These were powdered using a homogenizer and stored at 4^0C until use. The coarse powder was subjected to successive extraction with solvents ranging from non-polar to highly polar in series, namely ether: chloroform (1:1), ethyl acetate, acetone, and ethanol (all solvents at 95% concentration) using the Soxhlet apparatus. Extraction was done for fifteen cycles with each solvent (300 ml). Solvents of individual extracts were filtered and the filtrate was concentrated under vacuum using rotary evaporator (Rotavapor R-200, Buchi, Switzerland) to remove the organic solvent. The stock solution (samples) was prepared by suspending each of the extract with their respective solvent such that the final concentration of the solvent was 0.5% (v/v).

Fungal pathogen

The pathogen was isolated from various infected sites in Mysore region, according to Nagendra et al. [5] with slight modifications. Briefly, infected neem twigs were identified using the characteristic middle transition zone and terminal drying of the bark. Such samples were collected and cut into 4-5 cm pieces. The twigs were further washed using running tap water for about 20 min and surface sterilized using 5% sodium hypochlorite. Finally, they were washed with sterile distilled water and placed on petri plates containing solidified potato dextrose agar (PDA) medium amended with 100 ppm chloramphenicol. The plates were incubated at 12 h photoperiod for 14 days. Morphological identification was done by cultural and microscopic observations as suggested by Nagendra et al. [5]. The fungal cultures were maintained on PDA slants at 4 ^{0}C.

Preparation of spore suspension

Spore suspension of PA was prepared from the mono-conidial culture by spreading 2 ml of sterile distilled water over the axenic culture. The conidial ooze was dislodged from the pycnidia with the aid of a sterile needle, into the sterile distilled water to obtain a suspension of conidia. This suspension was aseptically transferred into sterile tubes and further diluted using sterile distilled water such that the final concentration was 10^6 conidia/ml, which was obtained by counting the spores using hemocytometer.

Antifungal activity

The antifungal activity was evaluated by poisoned-food technique [21]. Briefly, 1000 µg/ml of the extracts were added to 15 ml PDA medium taken in the petri plate before solidification. The extracts were mixed well with the medium and allowed to solidify. One disc of mycelial plug taken from an actively growing culture was inoculated in the centre of each petri plate. The inoculated plates were incubated at 12 h photoperiod for 5-8 days till the growth in the control plate reached the edge of the petri plate. The plates without the plant extracts were used as negative control, and all the experiments were carried out in triplicates. The percentage of inhibition were calculated by measuring the radial growth of the fungus (cm) in the control (C) and treated (T) plates using the formula,

$$\text{Inhibition (\%)} = [(C-T)/C] \times 100,$$

Minimum inhibitory concentration

The susceptibility of PA towards the extracts was evaluated by determination of the respective minimum inhibitory concentration (MIC) [22]. In brief, the test samples (extracts) were sterile filtered using 0.45 µm filter and added to individual Erlenmeyer flasks (25 ml) containing 10 ml Potato dextrose broth (PDB) such that the final concentration ranged from 75-1500 µg/ml of extracts in the culture medium. To the flasks containing the extracts, an inoculum of 5 µl (10^6 spores/ml) spore suspension was added and incubated at 28^0 C for 5-8 days. MIC values were determined as the minimum concentration of the extracts at which no visible growth of the fungal spores was observed.

Spore germination assay

Spore germination assay was conducted by testing the inhibitory role of the potent extracts which exhibited a better antifungal activity with relatively lower MIC (table 2). The extracts namely, ethanol extracts of *Ocimum tenuiflorum* leaves, *Besella alba* leaves, *Centella asiatica* leaves, *Apium graveolens* leaves and ethyl acetate extract of *Solanum nigrum* leaves, were assayed for their inhibitory potential against the generation of germ tube by the fungal spores [23]. The extracts (75-1000 µg/ml) were added to the fungal spore (10^6 spore/ml) suspension and mixed well. Further, a 10 µl aliquot from each sample was placed on a glass slide and incubated in a moisture chamber at 28^0 C for 24 h. After the incubation, each slide was fixed with lactophenol in cotton blue and observed under the microscope to count the number of spores that had generated germ tube. On the other hand, the spore germination assay was performed with the solvents (ethyl acetate and ethanol) without the extracts which served as control and all the tests were carried out in triplicates.

Kinetic studies

For evaluation of the antifungal kinetics, 10 µl of spore suspension (10^6 spores/ml) of PA was inoculated in tubes containing diverse concentration (300, 600, 1200 µg/ml) of all the five extracts that were previously tested for spore germination assay. The suspension was homogenized by inverting the tubes gently and incubated at 27^0 C at time intervals of 0, 30, 60, 90, 120, 150 minutes. Further, each suspension was filtered (Whatman No. 1 filter paper) and the resulting filtrate containing the spores were washed (3-4 times) with sterile water. The filter was detached and the spores were washed off into 10 ml sterile waters to obtain a spore suspension. Subsequently, 100 µl of this suspension, was placed on a glass slide and incubated at 27^0 C for 24 h. After incubation, the percentage of spore germination was calculated by enumerating the number of spores that generated the germ tube. Control for each solvent was performed as described above in spore germination assay.

RESULTS

Antifungal activity and minimum inhibitory concentration studies

In the present study, 40 distinct crude extracts from the roots and leaves of *Ocimum tenuiflorium*, *Centella asiatica*, *Solanum nigrum*, *Apium graveolens* and *Besella alba* were assayed for mycelia inhibition against the growth of *Phomopsis azadirachtae* (PA). The potential activity was qualitatively and quantitatively assessed by the presence or absence of inhibition zones and MIC values. Solvents were used as negative control did not show any inhibition zone. As shown in table 2, the distinct extracts from 5 potential medicinal plants tested, displayed antifungal activity with variable sensitivity against PA depending on the plant species and the type of the extract. PA was sensitive to 31 extracts out of 40 extracts tested ranging from 65-95% optimum activity.

Table 1: Plant species selected for antifungal activity and their biological activities

Plant species	Family	Common name	Traditional uses	Plant part used	Previously studied activities.
Ocimum tenuiflorum	Lamiaceae	Holy basil	Treatment of Asthma and respiratory disorders [13]. Ayurvedic remidies for cold, cough, headaches, stomach disorders [14].	Leaves, stem, flower, roots and seeds.	Antidiabetic, cardio-protective, antimicrobial, radio-protective, immunomodulatory, antinociceptive and antifertility [15].
Centella asiatica	Apiaceae	Asiatic pennywort/ Indian pennywort	Treatment of inflammation, wound healing, memory enhancing property [16].	Roots and leaves	Neuroprotective, antioxidant, antimicrobial, anti-ulcer, antidiabetic, anti-inflammatory and anti-tumor [16].
Solanum nigrum	Solanaceae	Black nightshade	Treatment of pain, inflammation, ringworm infection and liver disorders [17].	Leaves and berries.	Liv 52 for liver disorders. Anti-seizure, antioxidant, antiproliferative, hepatoprotective and wound healing [17].
Apium graveolens	Apiaceae	Celery	Treatment of arthritis, back pain, rheumatism [18].	Roots, leaves and dried fruits.	Antifungal, antiseptic, diuretic and blood pressure modulatory activities [19].
Besella alba	Basellaceae	Vine spinach/ red vine spinach	Treatment of skin diseases, diarrhea, anemia and malaria [20].	Root, leaves, stem, flower, and seeds.	Wound healing, antimicrobial, anti-ulcer, anti-inflammatory, antidiabetic and hepatoprotective [20].

Table 2: Antifungal activity at 1000 µg/ml concentration of the extracts

S. No.	Plants	Parts used	Solvents	CD[x] (in "cm")	I[y] (%)	MIC[z] (in "µg/ml")
1.	*Ocimum tenuiflorum*	Roots	E: C	9.69±0.36	39.98±0.56	1000
			Ea.	NA	NA	NT
			Ac.	6.02±0.48	53.68±0.87	600
			Et.	3.69±0.96	78.6±0.69	125
		Leaves	E: C	NA	NA	NT
			Ea.	6.95±0.65	51.02±1.03	500
			Ac.	5.52±0.24	65.58±0.46	300
			Et.	2.56±0.25	94.85±0.29	75
2.	*Centella asiatica*	Roots	E: C	NA	NA	NT
			Ea.	NA	NA	NT
			Ac.	9.65±0.58	44.12±0.15	750
			Et.	3.33±0.54	81.02±0.36	100
		Leaves	E: C	8.85±0.02	35.56±0.99	1000
			Ea.	7.86±0.11	50.05±0.33	500
			Ac.	4.75±0.68	60.58±0.75	250
			Et.	2.89±0.54	89.64±0.47	75
3.	*Solanum nigrum*	Roots	E: C	8.58±0.36	52.25±0.58	500
			Ea.	2.97±0.21	88.63±1.02	75
			Ac.	3.65±1.05	75.58±1.14	150
			Et.	4.21±0.25	65.44±0.52	150
		Leaves	E: C	7.24±0.12	50.84±0.77	500
			Ea.	2.58±0.98	95.05±0.14	75
			Ac.	3.12±0.22	89.54±3.02	125
			Et.	3.00±0.17	88.04±1.11	100
4.	*Apium graveolens*	Roots	E: C	7.24±0.12	59.29±1.00	500
			Ea.	NA	NA	NT
			Ac.	4.89±0.74	75.05±0.36	100
			Et.	4.65±0.21	76.52±0.88	125
		Leaves	E: C	NA	NA	NT
			Ea.	NA	NA	NT
			Ac.	5.25±1.24	65.14±0.13	300
			Et.	3.65±0.79	89.05±0.47	100
5.	*Besella alba*	Roots	E: C	NA	NA	NT
			Ea.	8.05±0.55	40.25±1.05	75
			Ac.	4.20±0.11	69.05±0.09	750
			Et.	3.55±0.40	75.02±0.77	100
		Leaves	E: C	NA	NA	NT
			Ea.	5.02±0.89	56.24±0.47	650
			Ac.	4.10±0.47	80.01±0.78	100
			Et.	2.85±1.05	92.05±0.88	200

[x]Colony diameter (CD) of 10 day old culture of *Phomopsis Azadirachtae.* Values are expressed as mean ± SE. (E: C): chloroform: ether (1:1) extract; (Ea.): ethyl acetate; (Ac.): acetone; (Et.): ethanol; (NA):inactive; (NT): Not tested since extracts did not showed antifungal activity diffusion method. [y]Percent inhibition, [z]Minimal inhibitory concentration.

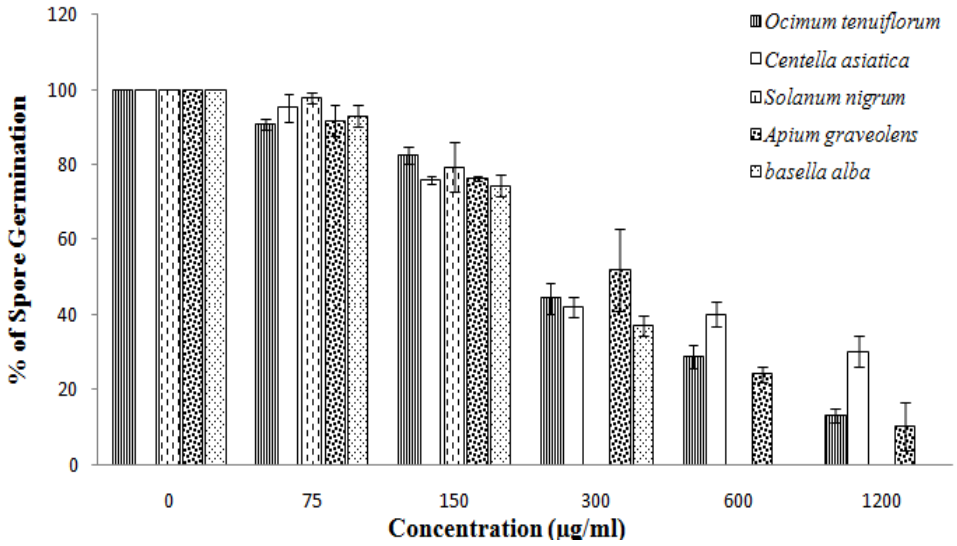

Fig. 1: Effect of different concentrations (µg/ml) of plant extracts on spore germination of *Phomopsis azadirachtae*. Each experiment was performed in triplicates and the data averaged (n=3)

The highest inhibitory effect was attained by the ethyl acetate extract of *Solanum nigrum* leaves (95.05%) followed by ethanol extract of *Ocimum tenuiflorum* leaves (94.85%) and ethanol extract of *Besella alba* leaves (92.05%).

Among all the other tested plant samples, no inhibitory activity was observed in case of the petroleum ether: chloroform (1:1) extract of *Ocimum tenuiflorum* (leaves), *Centella asiatica* (roots), *Apium graveolens* (leaves) and *Besella alba* (leaves and roots).

While, ethyl acetate extracts of *Ocimum tenuiflorum* (roots), *Centella asiatica* (roots) and *Apium graveolens* (leaves and roots) were not active against PA. However, ethyl acetate extract of *Solanum nigrum* leaves has the best antifungal activity with colony diameter of 2.58±0.98 cm and MIC value of 75 µg/ml.

A mycelial growth inhibition exhibited by the extracts against the growth of PA was in the order ethanol>ethyl acetate>acetone> ether: chloroform (1:1). On the other hand, while the MIC in case of the petroleum ether: chloroform (1:1) extract of all the plant samples tested were in the concentration range of 500-1000µg/ml, the ethyl acetate extracts exhibited MIC in the range of 75-650 µg/ml and ethanol extract, being most potent were in the range of 75-200 µg/ml.

In general, ethanol and ethyl acetate extracts of all the plant species taken in the study were most active and ether: chloroform (1:1) extracts were mostly inactive against PA.

On the whole, *Solanum nigrum* showed the greatest inhibitory effects, followed by *Ocimum tenuiflorium*, *Besella alba*, *Centella asiatica* and *Apium graveolens* leaves respectively.

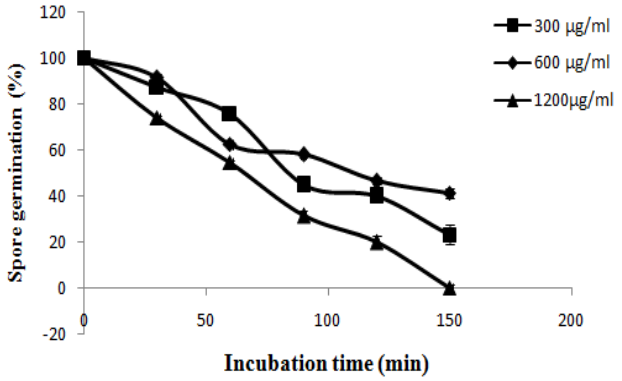

Fig. 2a: Effect of different concentrations of ethanol extract of *Ocimum tenuiflorum* leaves, on time dependent kinetics of the inhibition of *Phomopsis azadirachtae*

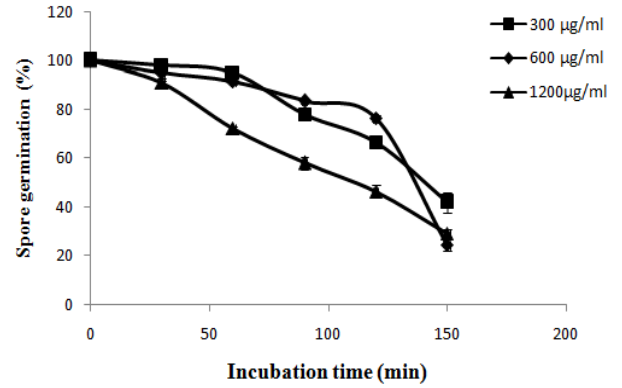

Fig. 2b: Effect of different concentrations of ethanol extract of *Besella alba* leaves, on time dependent kinetics of the inhibition of *Phomopsis azadirachtae*

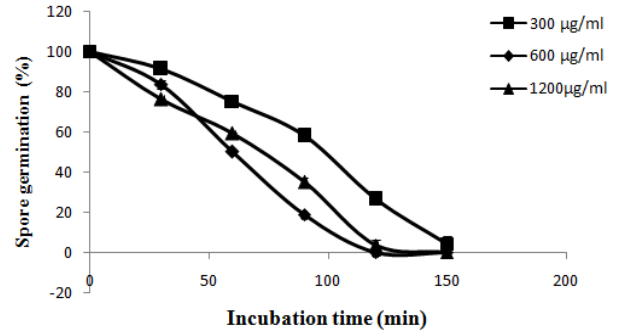

Fig. 2c: Effect of different concentrations of ethanol extract of *Centella asiatica* leaves, on time dependent kinetics of the inhibition of *Phomopsis azadirachtae*

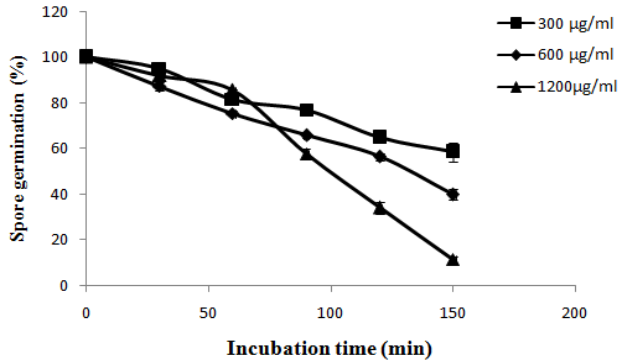

Fig. 2d: Effect of different concentrations of ethanol extract of *Apium graveolens* leaves, on time dependent kinetics of the inhibition of *Phomopsis azadirachtae*

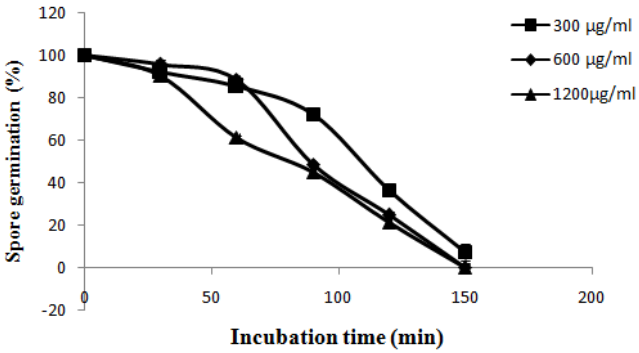

Fig. 2e: Effect of different concentrations of ethyl acetate extract of *Solanum nigrum* leaves, on time dependent kinetics of the inhibition of *Phomopsis azadirachtae*

Spore germination assay

Based on the antifungal activity assay, five best/potent extracts were chosen for spore germination studies *viz*., ethanol extracts of *Ocimum tenuiflorum*, *Besella alba*, *Centella asiatica*, *Apium graveolens* leaves and ethyl acetate extract of *Solanum nigrum* leaves. Ethanol and ethyl acetate solvents (0.5 % v/v) served as control did not inhibit the spore germination as shown in fig. 1, while a varied range of inhibition was visible when tested against the samples at different concentration. Spore germination was inhibited to a level of 100 % by *Solanum nigrum* extract at the concentration of 300 µg/ml followed by *Basella alba* extract at the concentration of 600 µg/ml. In general, the extracts tested inhibited the spore germination to an extent of approximately 50 % at a concentration of <300 µg/ml.

Antifungal kinetics

The extracts tested for inhibitory effects on spore germination, were tested for their antifungal kinetics in 3 diverse concentrations (300, 600 and 1200 µg/ml). As showed in fig. 2 (a-e), varying pattern of spore germination inhibition was observed in individual extract at diverse concentrations. From the graph, it is also evident that an increase in exposure time and concentration of the extracts elevated fungicidal activity. At a concentration of 300 µg/ml, the tested extracts did not result in rapid killing, but exhibited antifungal activity and at 150th minute of exposure time, at least 50% inhibition was observed in all the extracts tested. Conversely, there was a significant rise in the killing rate at 600 and 1200 µg/ml after 30 min of exposure and 95% and 100% inhibition of spore germination was noticed on 150 min exposure, respectively.

DISCUSSION

A substantial increase in the traditional or precisely herbal medicinal research is witnessed in both developed as well as developing countries [24]. On the global scale, the market for herbal medicine has been receiving high acclaim resulting in a risen concern over their safety and quality both by health authorities as well as public. Herbal medicines are marketed as formulated finished products, quality of which can be affected by intrinsic (genetic) or extrinsic (environment, collection methods, cultivation, harvest, post-harvest processing, transport and storage practices) factors [25]. One of the most common reasons for the drop of the quality could be the inadvertent microbial contamination either during the production stages or its presence as an infection to the plant. More often, the fungal contaminants secrete the toxic metabolites, mycotoxins, with powerful mutagenic and carcinogenic effect which reduce the value for the herbal formulations [26]. Neem, being a rich source of bioactives and medicinally important components, is among the popular medicinal plants. However, its medicinal value is being lowered due to the infection caused by PA leading to a very destructive disease known as die-back disease of neem. The potential of neem in terms of its enormous biological activities demands an optimum control of the disease. Though synthetic antifungal agents are well known to effectively inhibit the fungal growth they also lead to some of the irreversible damages caused to the environment, development of pest resistance, as well as toxic effects caused to the non-target organisms [27]. The development of protective products with natural origin as alternatives to synthetic fungicides is presently the need of the hour.

The 5 potent plant species tested in this study, exhibited a broad range of antifungal activity by inhibiting the growth of PA. Also, the variation of antifungal activity within the plants selected was evident. Yet, the results are in agreement with [28], suggesting that the variation might be due to the nature of the plant material or its origin. Likewise, the activity of the plant may perhaps be influenced by plant parts used, agro climatic conditions and also the solvent used for extraction.

Our results did not illustrate identical response between the plant species or the extracts in terms of mycelial inhibition. The differences in inhibition of PA among the plant species and its extracts may be attributed to the varying in cell wall composition or the amount and quality of the phyto components in the plant extracts [29]. The inhibitory activity varied significantly among the plant species and its extracts with MIC values ranging from 75–1000 µg/ml. The MIC values gradually increased from non-polar to polar solvents with values ranging from 75-200 µg/ml. In general, ethyl acetate and ethanol extracts showed fairly better inhibition in comparison to all the other solvent extracts which can also be attributed to the chemical composition of the plants. Ethanol and ethyl acetate are found to be the most potent solvent since it has an intrinsic ability to solubilize a large number of secondary metabolites. The present study also confirms the previous reports which establish the ethanol as the best solvent used for extraction of antimicrobial substances relative to most of the other solvents [30].

The study also determined the complex role of the plant extracts tested against the fungal spore germination and the results excelled a 100 % inhibition of the spores and illustrated a broad range of antifungal activity at a concentration of 600-1200 µg/ml of extracts. These results suggest the presence of potential antifungal agents which could be due to the presence of various secondary metabolites like the alkaloids, glycosides, saponins, tannins, volatile oil and ascorbic acid and these finding are in agreement with a previous report (Balakumar et al, 2011). All through the kinetic study of PA, it appeared that the exposure time of the extracts had a diminutive effect on the fungicidal activity at lower concentration. On the whole, a substantial rate of inhibition of PA was acknowledged by the extracts in low concentration and this was a typical feature recognized for the first time in these plants.

In summary, neem has a significant economic importance in the medicinal industry and hence its protection implicates priority. In this regard, antimicrobial activity of *Ocimum tenuiflorium*, *Centella asiatica*, *Solanum nigrum*, *Apium graveolens* and *Besella alba* against the fungal pathogen PA has been represented for the first time in this study. Each extract had a different degree of inhibitory activity and specificity against the growth of PA. The activities demonstrated by the different types of extracts may be attributed to the diversity of structures and/or the differences of chemical constituents within these extracts. Thus from our findings, it is concluded that medicinal plant extracts could become an alternative to synthetic fungicides for using in agro industries and also to screen and develop such novel types of selective and natural fungicides in the treatment of PA causing severe destruction to neem.

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CONFLICT OF INTERESTS

Declared None

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