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ANTIFUNGAL ACTIVITY OF HERBAL EXTRACTS AGAINST NEEM DIE-BACK PATHOGEN *PHOMOPSIS AZADIRACHTAE*

Vedashree S, Sateesh MK*, Lakshmeesha TR and Mohammad Shafi Sofi

Molecular diagnostic laboratory, Department of Microbiology and Biotechnology, Bangalore University, Bangalore-560 056, Karnataka, India.

ABSTRACT

Herbal extracts of 26 plants belonging to 20 different families of the plant kingdom were evaluated for antifungal activity against *Phomopsis azadirachtae*, a fungus causing destructive die-back disease in neem. Test fungus was isolated from infected neem seeds. The screening was performed by poisoned food technique. Extracts of *Syzygium aromaticum*, *Lawsonia inermis*, *Cinnamomum verum* and *Allium sativum* exhibited significant antifungal activity in a dose-dependent manner. Relatively potent herbal extracts were further evaluated to determine the obligate lowest concentration required to inhibit visible mycelial growth of the pathogen. *Syzygium* extract exhibited 100% inhibition of the pathogen at minimum concentration of 20% then followed by *Lawsonia* and *Ocimum* extracts thus being strongest antifungal agents. The present study revealed that, these plants could be exploited for the possible control of deadly pathogen *P. azadirachtae*. Accordingly, this is an important step towards preventing the spread of the die-back disease through a more ecofriendly approach.

Key words: Herbal extract, *Phomopsis azadirachtae*, Antifungal activity, Neem, Die-back.

INTRODUCTION

Neem (*Azadirachta indica* A. Juss.) is a divine tree and a sacred gift of the nature. It belongs to the family Meliaceae. It is well known for its medicinal and biopesticidal properties [1-5]. Even with all its antimicrobial properties, it is not free from microbial diseases [6,7]. Die-back of neem caused by *Phomopsis azadirachtae* [8], is a devastating disease resulting in almost always 100% reduction in fruit yield. The annual loss due to this disease could be several million dollars. *P. azadirachtae* is a Deuteromycetes fungus, which belongs to class Coelomycetes. It has branched, septate, profuse and colorless mycelia which turns pale brown later. Conidia are produced in the fruiting body called pycnidia. It produces two types of conidia in a cream to dark yellow coloured slimy cirrhi: alpha-conidia hyaline, fusiform, straight, 2-4 guttulate, smooth, aseptate, germinate readily and beta-conidia hyaline, filiform, hamate, eguttulate, aseptate, germination unknown. The fungus affects leaves, twigs and inflorescence, irrespective of age, size and height of the tree [9].

The plant diseases caused by fungal pathogens are usually controlled by application of chemical based antifungal compounds. *P. azadirachtae* is sensitive to fungicides such as bavistin [9]. The chemical based pesticides are non-biodegradable and extremely toxic [10, 11]. They have various drawbacks in terms of genotoxicity [12], hepatotoxicity [13], reproductive disorders [14] and immunosuppression [15]. Frequent use of fungicides has led to the emergence of resistant strains. Furthermore, environmental pollution caused by excessive use of agrochemicals, has increased the public pressure to reduce the use of synthetic fungicides in agriculture [16]. Hence, the need for effective and safe alternative has increased. Concerns have been raised about both the environmental impact, and the potential health risk related to the use of these synthetic compounds [10, 17]. Thus, it is necessary to search for alternative control measures that are non-toxic, eco-friendly and cost-effective for the management of pathogen *P. azadirachtae*. Plant extracts could be an alternative to toxic fungicides for controlling plant pathogens since they are composed of various bioactive compounds such as alkaloids, flavonoids, glycosides, phenols, saponins, sterols and etc. [9, 18].

Medicinal plants are rich sources of antimicrobial agents and its therapeutic use is becoming popular because of its lesser side effects and resistance. There are more than 270,000 higher plants existing on the planet earth. India has one of the richest plants medical traditions in the world and would not be needed for controlling the pathogen *in vivo*.

Corresponding Author: **Sateesh MK** E-mail: sateeshmkgreen@gmail.com

around 25,000 effective plant-based formulations used in ethanobotanical communities in India [19, 20]. However, only a small portion has been explored phytochemically [21]. In fact, huge efforts are being made to isolate bioactive products from medicinal plants for their possible utility in development of plant based biopesticides. The antimicrobial activity of different plant extracts has been reported by many studies [22-27]. Significant level of antifungal activity has been exhibited by crude extracts of various plants [22, 28]. The use of essential oils for control of *P. azadirachtae* has been reported [29]. The aim of the present work was to evaluate the antifungal efficacy of aqueous plant extract against the neem die-back pathogen, *P. azadirachtae* using the poisoned food technique for determination of the best plant based product for effective management of the pathogen.

MATERIALS AND METHODS

Collection of plant material and preparation of aqueous extract

Twenty six widely available plants (Table 1) were selected based on an ethno-botanical survey. Leaf samples were collected early morning during the flowering season in March/April 2010 and 2011 from Dhanvantrivana, Bangalore University and Gandhi Krishi Vigyan Kendra (GKVK) University of Agricultural Sciences, Bangalore). Flower buds, seeds, bark, fruits, rhizome of different plants (Table 1) were purchased from the local market in and around Bangalore, India.

The freshly collected leaves were washed with running tap water and then rinsed in distilled water. The leaves were shade dried and pulverized using an aseptic electric blender to obtain a powdered form and it was sieved and stored in sterile polyethylene sample bags prior to use [9, 30]. Fifty grams each of different plant material was macerated with 50 ml of sterile distilled water for 10 min, filtered using double layered muslin cloth. The filtrate was centrifuged at 5,000 RPM for 20 minutes and supernatant was filtered through Whatman no. 1 filter paper and was subjected to antifungal assay.

Test organism

P. azadirachtae was isolated from infected neem seeds according to Sateesh [9]. Neem seeds were processed to remove fruit pulp and surface disinfected with 2% sodium hypochlorite solution (10-15% available chlorine) for ten minutes and then rinsed five times with sterile distilled water. Surface sterilized neem seeds were plated onto the potato dextrose agar (PDA) medium amended with 100 µg/ml chloramphenicol at the rate of 8 seeds/plate and incubated at 25^0 C for ten days near bright light. Colonies of *P. azadirachtae* growing from infected seeds were identified based on their colony characteristics, spore morphology [9, 31] and confirmed by the molecular method [32]. The isolated pathogen was sub cultured onto PDA and maintained at 4^0 C for further use.

Preparation of inoculum

P. azadirachtae was cultured on PDA medium amended with 100 µg/ml chloramphenicol at 25^0 C for ten days near bright light. Five mm diameter mycelial disc retrieved from fresh cultures grown on PDA plates served as inoculum [33].

Evaluation of antifungal activity of crude aqueous extracts of botanicals

The antifungal activity of crude extracts of 26 plants (Table 1) was evaluated by poisoned food technique using the Czapek Dox Agar (CDA) medium. The CDA medium amended with 50% aqueous extract of test plants was prepared and autoclaved. Then 15 ml of the medium was poured into each petriplate, allowed to cool and solidify. Five mm mycelial disc from seven-day old culture of *P. azadirachtae* was inoculated aseptically at the center of each plate and incubated at 25 ± 2^0 C for ten days near bright light. Culture grown on CDA plates without plant extract served as control and CDA plates amended with bavistin (1mg/ml) served as positive control. Percentage inhibition of mycelial growth of *P. azadirachtae* by different extracts was evaluated using the formula % inhibition = dc – dt/dc x 100. Where, dc = Average increase in mycelial growth in control, dt = Average increase in mycelial growth in treatment [34]. Crude extract of botanicals showing positive effect among 26 plants on *P. azadirachtae* was further subjected to antifungal activity at different concentrations *viz.,* 5, 10, 15, 20, 25, 30, 40 and 50% against the test fungi *P. azadirachtae* to determine the Minimum Inhibitory concentration (MIC). Each experiment was repeated three times.

Statistical analysis

The effect of plant extracts on the growth of *P. azadirachtae* was evaluated by univariate analysis of variance (ANOVA) using SPSS 19 statistical package.

RESULTS AND DISCUSSION

In the present study, crude aqueous extract of 26 plants (Table 1) were evaluated for antifungal activity against *P. azadirachtae* causing die-back disease in neem by poisoned food technique. Variable level of inhibition was exhibited by different herbal extracts (Fig. 1). Out of the twenty six plants screened, five plants *viz. Allium sativum* L., *Lawsonia inermis* L., *Syzygium aromaticum* L., *Ocimum sanctum* L. and *Cinnamomum verum* J. gave significant results ($p<0.05$) by completely inhibiting the pathogen *P. azadirachtae* (Table 2, Fig. 1 and 2). The plant extracts which exhibited 100% inhibition were further evaluated to determine the least percentage required for complete inhibition of the pathogen. The minimum percentage required to inhibit visible growth of the pathogen was found to be 20% for *Syzygium aromaticum*, 25% for both *Lawsonia inermis* and *Ocimum sanctum*, 30% for *Cinnamomum verum* and 40% for *Allium sativum* (Fig. 2).

The *Syzygium* extract completely suppressed the mycelial growth of *P. azadirachtae* at the low dosage of about 20%, thus a potent phytochemical compared to other positive plant extracts. The main constituent of *S. aromaticum* and *O. sanctum* is eugenol [35-37], which has an aromatic nucleus and a phenolic OH group that can form hydrogen bonds with -SH group in the active site of target enzyme [38]. Eugenol is also known to disrupt the cytoplasmic membrane and results in cell leakage [39-41]. Similarly, the main constituent of *C. verum* is "cinnamaldehyde," which contains an aldehyde group and conjugated double bond outside the ring [42-43]. This compound exhibited antifungal activity mainly by controlling β-(1, 3)-glucan and chitin synthesis in yeasts and molds [44]. The active ingredient in *Lawsonia inermis* is "lawsone" ($C_{10}H_6O_3$), a naturally-occurring naphthoquinone which is well known for its fungicidal properties [45, 46]. *Allium sativum* has allicin as the principal component mainly involved in antimicrobial properties [47, 48]. Thus, antifungal activity of above discussed botanicals against *P. azadirachtae* is perhaps due to the presence of few secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, saponins, sterols and etc. [49].The antifungal activity of plant extracts screened was found to be dose dependent, where the pathogen growth was completely inhibited at higher concentration of the extract as compared to lower concentration, which just slowed down the growth rate of the pathogen. This result is similar to the study conducted by Lakshmeesha *et al*. [10], Parveen *et al*. [50]. Several reports have stated the antifungal activity of medicinal plants against many phytopathogenic fungi [51-54]. The remaining plant extracts did not exhibit significant inhibition against *P. azadirachtae* even at higher concentration (50%). Thus, it cannot be considered as a promising fungicide for control of neem die-back pathogen *P. azadirachtae*. In contrast, the *Syzygium aromaticum*, *Lawsonia inermis*, *Cinnamomum verum* and *Allium sativum* extract supplemented with CDA medium did not allow the growth of the pathogen even after 20 days of incubation. This clearly suggests that the toxic effect of the extract would be retained for a longer period. This would imply that frequent application of the treatment would not be needed for controlling the pathogen *in vivo*.

Table 1. List of plants used to evaluate antifungal activity against *Phomopsis azadirachtae*

Sl. No.	Plant	Family	Part of the Plant Used
1	*Allium cepa* L.	Amaryllidaceae	Bulbil
2	*Allium sativum* L.	Amaryllidaceae	Bulbil
3	*Aloe vera (L) Burm.*	Liliaceae	Leaves
4	*Annona reticulata* L.	Annonaceae	Leaves
5	*Capsicum annuum* L.	Solanaceae	Fruit
6	*Catharanthus roseus* L.	Apocynaceae	Leaves
7	*Cinchona officinalis* L.	Rubiaceae	Leaves
8	*Cinnamomum verum* J.	Lauraceae	Cinnamon
9	*Coriandrum sativum* L.	Apiaceae	Dried fruits
10	*Cuminum cyminum* L.	Apiaceae	Seed
11	*Curcuma longa* L.	Zingiberaceae	Rhizome
12	*Eucalyptus obliqua* (L.) Her.	Myrtaceae	Leaves
13	*Lawsonia inermis* L.	Lythraceae	Leaves
14	*Mangifera indica* L.	Anacardiaceae	Leaves
15	*Mentha spicata* L.	Lamiaceae	Leaves
16	*Ocimum sanctum* L.	Lamiaceae	Leaves
17	*Phyllanthus emblica* L.	Phyllanthaceae	leaves
18	*Phyllanthus emblica* L.	Phyllanthaceae	fruit
19	*Piper nigrum* L.	Piperaceae	Dried fruit
20	*Psidium guajava* L.	Myrtaceae	Leaves
21	*Punica granatum* L.	Punicaceace	Leaves
22	*Syzygium aromaticum* L.	Myrtaceae	Dried flower buds
23	*Tagetes erecta* L.	Asteraceae	Flower
24	*Tamarindus indica* L.	Fabaceae	Leaves
25	*Tinospora cordifolia* (Thunb.) Miers	Menispermaceae	Leaves
26	*Zingiber officinale* Roscoe	Zingiberaceae	Rhizome

Table 2. Percentage of mycelial inhibition of *P. azadirachtae* by different plant extracts

Plant	Percentage of inhibition
Allium cepa L.	0±0 a
Allium sativum L.	100±0 h
Aloe vera (L.) Burm.	5.6±0.58 abc
Annona reticulata L.	5.1±0.65 abc
Capsicum annuum L.	14.6±2.5 abcd
Catharanthus roseus L.	0±0 a
Cinchona officinalis L.	26±5.29 def
Cinnamomum verum J.	40±5.63 fg
Coriandrum sativum L.	1.6±0.64 abc
Cuminum cyminum L.	48.6±3.26 g
Curcuma longa L.	40.4±2.2 fg
Eucalyptus obliqua (L.) Her.	16.2±1.7 bcde
Lawsonia inermis L.	100±0 h
Mangifera indica L.	5±1.0 abc
Mentha spicata L.	6.8±0.74 abc
Ocimum sanctum L.	100±0 h
Phyllanthus emblica L.	6.02±1.1 abc
Phyllanthus emblica L.	9.24±5.6 abc
Piper nigrum L.	19.9±2.8 cde
Psidium guajava L.	29.9±8.4 ef
Punica granatum L.	8.3±1.5 abc
Syzygium aromaticum L.	100±0 h
Tagetes erecta L.	0.4±0.05 a
Tamarindus indica L.	6.4±2.6 abc
Tinospora cordifolia (Thunb.) Miers	0±0 a
Zingiber officinale Roscoe	8.9±1.6 abc

Antifungal index are given as mean of three replicates. Mean values within column followed by the same letter are significantly same at 95% confidence level.

Figure 1. Antifungal activity of different herbal extracts on *Phomopsis azadirachtae*

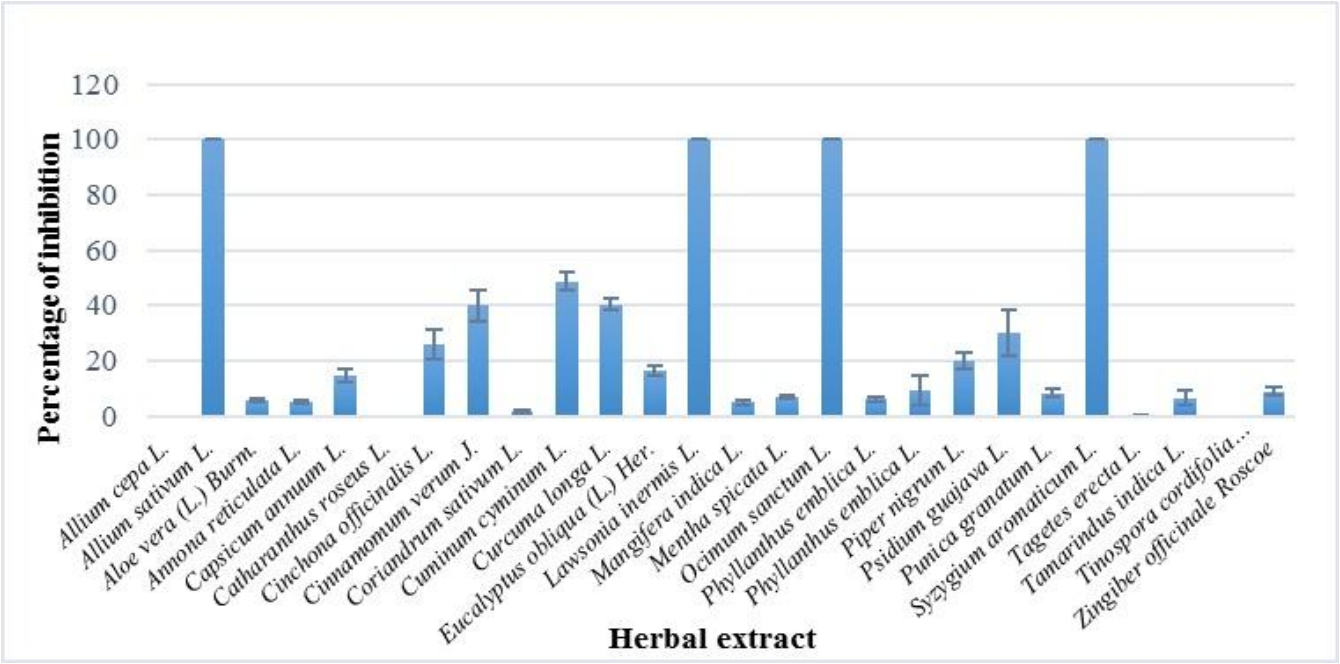
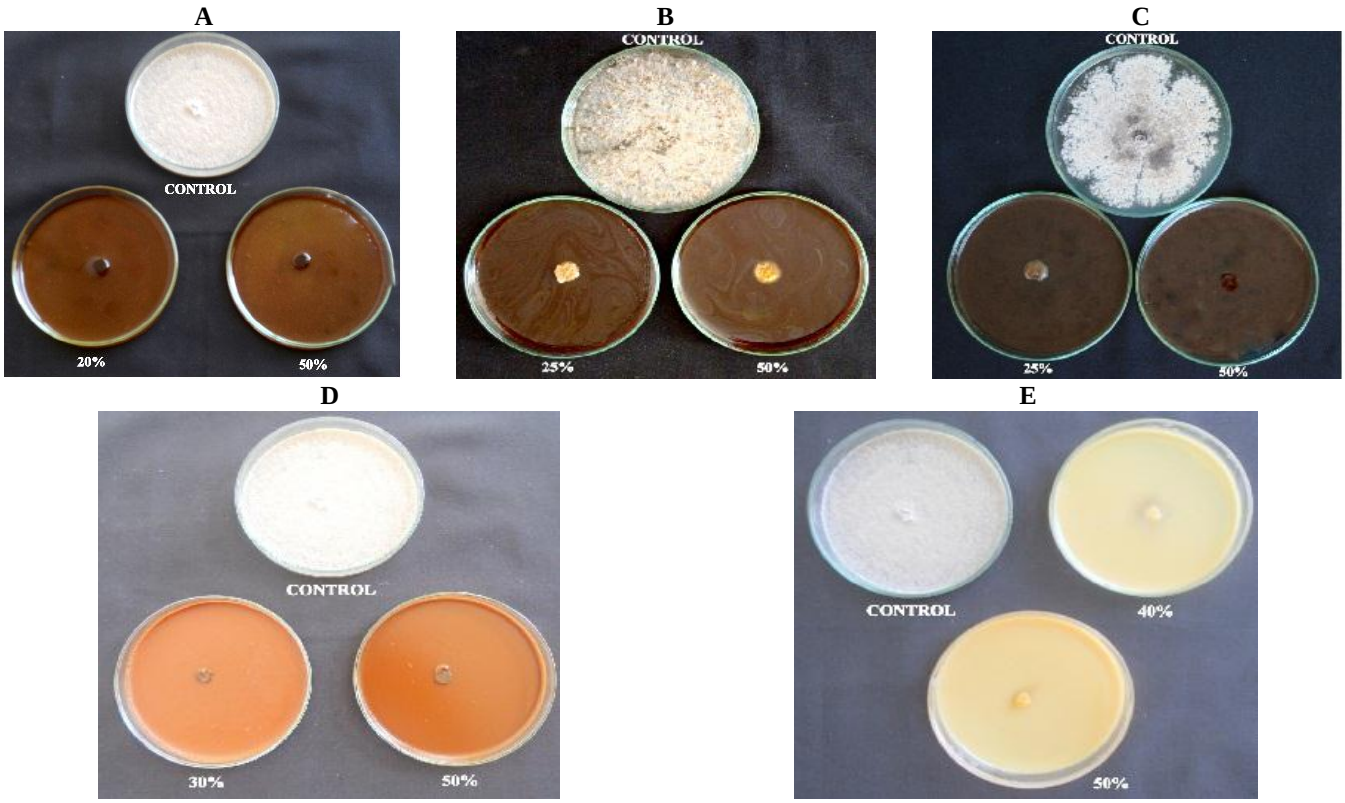


Figure 2. Inhibitory effect of different herbal extracts on *Phomopsis azadirachtae*
a: Syzygium aromaticum, b: Lawsonia inermis, c: Ocimum sanctum, d: Cinnamomum verum* and *e: Allium sativum.



CONCLUSION

The overall result of the present study has generated information about the possible use of the tested plants in the control of *P. azadirachtae*. However, further phytochemical investigations will be done to isolate the active constituents from *Syzygium aromaticum*, *Ocimum sanctum*, *Lawsonia inermis*, *Cinnamomum verum* and *Allium sativum.*

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REFERENCES

1. National Research Council. NEEM : A Tree For Solving Global Problems Report of an Ad Hoc Panel of the Board on Science and Technology for International Development National Research Council. Washington, DC. National Academy Press, 1992.
2. Rembold H. Neem and its general development for pest control. In: Singh RP, Chari MS Raheja AK, Kraus W Eds. Neem and Environment Vol I. New Delhi: Oxford & IBH Publishing Co Pvt Ltd, 1996.
3. Hashmat I, Azad H, Ahmed A. Neem (*Azadirachta indica* A. Juss) – A nature's drugstore: an overview. *Int Res J Biol Sci*, 1, 2012, 76-79.
4. Ogbuevo IP, Odoemenam VU, Obikaonu HO, Opara MN, Emenalom OO, Uchegbu MC, Okoli IC, Esonu BO, Floeje MU. The growing importance of neem (*Azadirachta indica* A. Juss) in agriculture, industry, medicine and environment: A Review. *Res J Medic Plant*, 5, 2011, 230-245.
5. Serrone PD, Nicoletti M. Antimicrobial Activity of a Neem Cake Extract in a Broth Model Meat System. *Int J Environ Res Public Health*, 10, 2013, 3282-3295.
6. Anonymous. Intellectual Piracy and the Neem Patents. The Research Foundation for Science, Technology and Natural Resource Policy, India. 1995.
7. Tewari DN. Monograph on neem (*Azadirachta indica* A. Juss.). Dehra Dun, India; International Book Distributors. 1992.
8. Sateesh MK, Shankara Bhat S, Devaki NS. *Phomopsis azadirachtae* sp. *nov*. from India. *Mycotaxon*, 65, 1997, 517-520.

9. Sateesh MK. Microbiological investigations on die-back disease of neem (*Azadirachta indica* A. Juss.). PhD thesis, Mysore: University of Mysore. 1998.
10. Lakshmeesha TR, Sateesh MK, Vedashree S, Sofi MS. Antifungal activity of some medicinal plants on Soybean seed-borne *Macrophomina phaseolina*. *J Appl Pharma Science*, 3, 2013, 84-87.
11. Yadav SK. Pesticide applications-Threat to ecosystems. *J Hum Ecol*, 32, 2010, 37-45.
12. Bolognesi C. Genotoxicity of pesticides: a review of human biomonitoring studies. *Mutat Res*, 543, 2003, 251-272.
13. Cecchi A, Rovedatti MG, Sabino G, Magnarelli GG. Environmental exposure to organophosphate pesticides: Assessment of endocrine disruption and hepatotoxicity in pregnant women. *Ecotox Environ Safe*, 80, 2012, 280-287.
14. Richard S, Moslemi S, Sipahutar H, Benachour N, Gilles-Eric Seraline. Differenential effects of glyphosate and roundup on human placental cells and aromatase. *Environ Health Persp*, 113, 2005, 716-720.
15. Singh ND, Sharma AK, Dwivedi P, Kumar M, Patil RD. Immunosuppressive effect of combined citrinin and endosulfan toxicity in pregnant wistar rats. *Veterinarski Arhiv*, 81, 2011, 751-763.
16. Manasi K. Bhagwat, Ajit G. Datar. Antifungal activity of herbal extracts against plant pathogenic fungi. *Arch Phytopathology Plant Protect*, 2014.
17. Abad MJ, Ansuategui M, Bermejo P. Active antifungal substances from natural sources. *Arkivoc*, 7, 2007, 116-145.
18. Lakshmeesha TR, Sateesh MK, Vedashree S, Sofi MS, Umesha S. Efficacy of botanicals on soybean seed-borne *Fusarium equiseti*. *VCFL Sciences*, 3, 2013a, 10-16.
19. Verma S, Singh SP. Current and future status of herbal medicines. *Vet World*, 1, 2008. 347-350.
20. Pathak K, Das RJ. An overview on medicinal herbs in the treatment of skin diseases. *J Pharm Pharm Sci*, 2, 2013, 437-449.
21. Helton LR. Folk medicine and health beliefs: an Appalachian perspective. *J Cult Divers*, 3, 1996, 123-128.
22. Agbenin ON, Marley PS. *In vitro* assay of some plant extract against *Fusarium oxysporum, F. sp. lycopersici* causal agent of tomato wilt. *J Plant Prot Res*, 46: 2006, 215-220.
23. Mackeen MM, Ali AM, Lajis NH, Kawazu K, Hassan Z, Amran M, Habsah M, Mooi LY, Mohamed SM. Antimicrobial, antioxidant, antitumour-promoting and cytotoxic activities of different plant part extracts of *Garcinia atroviridis* Griff. ex T. Anders, *J Ethnopharmacol*, 72: 2000, 395-402.
24. Kuri SK, Islam RM, Mondal U. Antifungal potentiality of some botanical extracts against important seedborne fungal pathogen associated with brinjal seeds, *Solanum melongena* L. *J Agricultural Technol*, 7, 2011, 1139-1153.
25. Ponnusamy K, Petchiammal C, Mohankumar R, Hopper W. In vitro antifungal activity of indirubin isolated form a South Indian ethnomedicinal plant *Wrightia tinctoria* R. *J Ethopharmacol*, 132, 2010, 349-354.
26. Shivalingaiah, Umesha S, Sateesh MK. *Cocculus hirsutus* extract inhibits the *Xanthomonas oryzae* pv. *oryzae*, the bacterial leaf blight pathogen in rice. *Arch Phytopathology Plant Protect*, 46, 2013, 1885-1894.
27. Sukatta U, Haruthaithanasan V, Chantarapanont W, Dilokkunanant U, Suppaku P. Antifungal activity of Clove and Cinnamon oil and their synergistic against postharvest decay fungi of grape *invitro*. *Kasetsart J (Nat Sci)*, 42, 2008, 169-174.
28. Satish S, Raghavendra MP, Raveesha KA. Antifungal potentiality of some plant extracts against *Fusarium* sp. *Arch Phytopathology Plant Protect*, 42, 2009, 618-625.
29. Prasad MNN, Shankara Bhat S, Sreenivasa MY. Antifungal activity of essential oils against *Phomopsis azadirachtae*- the causative agent of die-back disease of neem. *J Agricultural Technol*, 6, 2010, 127-133.
30. Sofi MS, Sateesh MK, Bashir M, Harish G, Lakshmeesha TR, Vedashree S, Vedamurthy AB. Cytotoxic and pro-apoptotic effects of *Abrus precatorius* L. on human metastatic breast cancer cell line, MDA-MB-231. *Cytotechnology*, 65, 2012, 407–417.
31. Bhat SS, Sateesh MK and Devaki NS. A new destructive disease of neem trees incited by *Phomopsis azadirachtae*. *Curr Sci India*. 74, 1998, 17-19.
32. White T, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sininsky JJ, White TJ, Eds. PCR protocols: a guide to methods and applications, San Diego: Academic Press, 1990, 315-322.
33. Vedashree S, Sateesh MK, Lakshmeesha TR, Sofi MS, Vedamurthy AB. Screening and assay of extracellular enzymes in *Phomopsis azadirachtae* causing die-back disease of neem. *J Agricultural Technol*, 9, 2013, 915-927.
34. Singh J, Tripathi NN. Inhibition of storage fungi of blackgram (*Vigna mungo*) by some essential oils. *Flavour Frag J*, 14, 1999, 1-4.
35. Khan A, Ahmad A, Manzoor N, Khan LA. Antifungal activities of *Ocimum sanctum* essential oil and its lead molecules. *Nat Prod Commun*, 5, 2010, 345-349.
36. Kumar A, Rahal A, Chakraborty S, Tiwari R, Latheef S, Dhama K. *Ocimum sanctum* (Tulsi): a miracle herb and boon to medical science – A Review. *Intl J Agron Plant Prod*, 4, 2013,1580-1589.
37. Rana IS, Rana AS, Rajak RC. Evaluation of antifungal activity in essential oil of the *Syzygium aromaticum* (L.) by extraction, purification and analysis of its main component eugenol. *Braz J Microbiol*, 42, 2011, 1269-1277.

38. Velluti A, Sanchis AJ, Ejido JR, Marln S. Inhibitory effect of cinnamon, clove, lemongrass, oregano and palmth and fumonisin B1 production by *Fusarium proliferatum* in maize grain. *J Food Microbiolol*, 89, 2003, 145-154.
39. Carrasco H, Raimindi M, Svetaz L, Liberto MD, Rodriguez MV, Espinoza L, Madrid A and Zacchino S. Antifungal activity of eugenol analogues. Influence of different substituents and studies on mechanism of action. *Molecules*, 17, 2012, 1002-1024.
40. Darvishi E, Omidi M, Bushehri AKS, Golshani A, Smith ML. The antifungal eugenol perturbs dual aromatic and branched-chain amino acid permeases in the cytoplasmic membrane of Yeast. *PLoS ONE*, 8, 2013, e76028.
41. Pinto E, Vale-Silva L, Carlos C, Salgueiro L. Antifungal activity of the clove essential oil from Syzygium aromaticum on *Candida, Aspergillus* and dermatophyte species. *J Med Microbiol*, 58, 2009, 1454-1462.
42. Mariappan PM, Sabesan G, Koilpillai B, Janakiraman S, Sharma NK. Chemical characterization and antifungal activity of methanolic extract of *Cinnamomum verum* J. Presl bark against *Malassezia* spp. *J of Phar*, 5, 2013, 197-204.
43. Sfeir J, Lefrancois C, Baudoux D, Derbre S, Licznar P. *In Vitro* Antibacterial Activity of Essential Oils against *Streptococcus pyogenes*. *Evid Based Complement Alternat Med*, 2013, 1-9.
44. Bang KH, Lee DW, Park HM, Rhee YH. Inhibition of fungal cell wall synthesizing enzymes by tras-cinnamaldehyde. *Biosci Biotech Biochem*, 64, 2000, 1061-1063.
45. Gull I, Sohail M, Aslam MS, Athar MA. Phytochemical, toxicological and antimicrobial evaluation of *Lawsonia inermis* extracts against clinical isolates of pathogenic bacteria. *Ann Clin Microbiol Antimicrob*, 12, 2013, 1-6.
46. Rahiman FA, Mahmad N, Taha RM, Elias H, Zaman FH. Antimicrobial properties of *Lawsonia inermis* syn. *Lawsonia alba in vivo* and *in vitro*. *J Food Agr Environ*, 11, 2013, 502-504.
47. Aala F, Yusuf UK, Nulit R. Electron microscopy studies of the effects of garlic extract against *Trichophyton rubrum*. *Sains Malaysiana*, 42, 2013, 1585-1590.
48. Ankri S, Mirelman D. Antimicrobial properties of allicin from garlic. *Microbes Infect*, 2, 1999, 125-129.
49. Cowan MM. Plant Products as Antimicrobial Agents. *Clin Microbiol Rev*, 12, 1999, 564-582.
50. Parveen S, Wani AH, Ganie AA, Pala SA, Mir RA. Antifungal activity of some plant extracts on some pathogenic fungi. *Arch Phytopathology Plant Protect*, 47, 2014, 279-284.
51. Arya A, Chauhan R, Arya C. Inhibition of growth of 200 pathogenic fungi by garlic extract. *Mycologia*, 67, 1995, 882-885.
52. Choi GJ, Jang KS, Kim JS, Lee SW, Cho JY, Cho KY, Kim JC. In vivo antifungal activities of 57 plant extracts against six plant pathogenic fungi. *Plant Pathol J*, 20, 2004, 184-191.
53. Raji R, Raveendran K. Antifungal activity of selected plant extracts against phytopathogenic fungi *Aspergillus niger*. *Asian J Plant Sci Res*, 3, 2013, 13-15.
54. Znini M, Cristofari G, Majidi L, Mazouz H, Tomi P, Paolini J, Costa J. Antifungal activity of essential oil from *Asteriscus graveolens* against postharvest phytopathogenic fungi in apples. *Nat Prod Commun*, 6, 2011, 1763-1768.