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Table: 6, Figs : 6

# Chemical characterization and antimycotic potential of *Azadirachta indica* L. leaf extracts against *Penicillium digitatum* of kinnow fruit

Charu Maini, Ritu Tandon*, Anu Kalia[1] and Harpreet Kaur
Department of Chemistry, Punjab Agricultural University, Ludhiana
Email: ritutandon@pau.edu, charumaini648@gmail.com

## ABSTRACT

We assessed the antimycotic potential of various ethanolic extracts of *Azadirachta indica* leaves against the post-harvest fungus *P. digitatum* of Kinnow (*Citrus reticulata* Blanco). The Gas Chromatography-Mass Spectrometry (GC-MS) analysis revealed the presence of various active compounds [phytol (13.14 %), lycoxanthin (8.55 %) in powdered ethanol extract (PEE) and 9,12,15- octadecatrienoic acid, (Z, Z, Z)- (43.5 %), 9 α-Fluoro-17 α-methyl-4-androsten-3 α,6 β,11 β,17 β-tetra-ol (13.07%) in partitioned fresh ethanol extract (PFEE)]. Absorption spectra were measured in 1000-4000 $cm^{-1}$ domain using Fourier transform infrared spectrophotometer (FTIR). PFEE had greater antimycotic potential against the test pathogen *P. digitatum* than PEE as evident from reduction in colony number and diameter. The optical and scanning electron microscopy (SEM) analysis of extract-pathogen post interaction events also showed a biocidal effect on fungal hyphae showing swollen, perforated and collapsed hyphae with other distortions, probably caused by these allelochemicals.

**Key words:** *Azadirachta indica*, antimycotic potential, chemicals characterization, *Citrus reticulata*, FT-IR analysis, GC-MS analysis, kinnow fruit, *Penicillium digitatum*, SEM (Scanning electron microscopy)

## INTRODUCTION

The use of medicinal plants to control crop pests, is safer than synthetic pesticides which are harmful to humans and environment (13). Among various medicinal plants, *Azadirachta indica* (Meliaceace family) possesses diverse medicinal properties [analgesic, anticancer, antioxidant, antimicrobial, antitumor, anti-inflammatory, anti-oxidant, insecticidal and antipyretic (2)]. It is tropical and semi-tropical plant found in India, Nepal, Pakistan, Bangladesh, Sri Lanka and Maldives. Its different parts contain wide range of bioactive compounds (alkaloids, diterpenes, tetraterpenes, sesquiterpenes, fatty acids, flavonoids, steroids and phytosterols). These compounds are broadly classified in two categories: (i). Isoprenoids: These comprises of diterpenoids and triterpenoids (protomiliacins, limonoids, gedunin) and its derivatives, azadirone and its derivatives, C-secomiliacins such as nimbin, azadiractin and salanin and vilasinin. (ii). Non-isoprenoids: These includes carbohydrates, proteins, sulphur compounds, dihydrochalcone, coumarin, tannins, aliphatic compounds, polyphenolics such as flavonoids and their glycosides, etc. (5). These isoprenoids and non-isoprenoids may act as allelochemicals to inhibit the diverse range of pathogens.

The potential of *A. indica* for pathogen control in agriculture is little explored and only few studies have assessed the bio-efficacy of *A. indica* to control plant pathogens (7). However, some studies on human pathogens are known (13,18). Among pathogens, the *Penicillium digitatum* (fungal pathogen) causes great postharvest losses in kinnow fruit. Many fungicides (Thiabendazole, aromatic hydrocarbons, imazalil, sodium ortho-phenyl

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*Correspondence author, [1]Department of Soil Science.

and sterol biosynthesis inhibitors) are used to control the pathogen but these fungicides and their residues remains in fruits (25). Hence, food and agri-industries are looking for safer alternatives to these synthetic fungicides. Medicinal plants do provide such platform but formulating a product with such attributes of bioactive components is challenge due to low bioavailability and poor solubility of bioactive substances, which reduces their efficacy (12).

This study aimed to evaluate the antimycotic potential of different types of *A. indica* leaf extracts on *in vitro* growth of postharvest fungal pathogen (*P. digitatum*).

## MATERIALS AND METHODS

### Leaf extracts

The fresh leaves of *A. indica* were collected in May 2017 from our Herbal Garden, Department of Agronomy. Plant material was washed with tap water followed by double distilled water, air dried in shade and then in hot oven at 40°C. The dried leaves were pulverized to coarse powder and stored in airtight glass containers.

By solvent extraction method using fresh and powdered leaves: 4-different extracts were prepared; (i). Crude [Powdered ethanol extract (PEE), (ii). Fresh ethanol extract (FEE)], (iii). Partitioned [Partitioned powdered ethanol extract (PPEE), (iv). Partitioned fresh ethanol extract (PFEE)] extracts. For crude extracts, 50 g dried powder or 125 g fresh *A. indica* leaves were soaked in 300 ml ethanol for 72 h with intermittent shaking. The plant extract was filtered through Whatman No.1 filter paper. The residue was re-soaked overnight in ethanol (100 ml) and filtered. The extracts were pooled and concentrated using the rotary vacuum evaporator at 40˚C (22). For partitioned extracts, same quantity of *A. indica* leaves were soaked in 300 ml n-hexane for 72 h with intermittent shaking. The residue was re-soaked overnight in n-hexane (100 ml) and filtered. The extracts were then pooled and concentrated to 150 ml and then partitioned thrice with 95% ethanol. The ethanol layers were pooled, filtered, concentrated and stored in refrigerator at 4˚C (24).

### Physical Characteristics

**(i) Color:** Color analysis was done by Hunter Colour Lab (Colorimeter), DP-9000 (Hunter Lab Associates Laboratory, Inc., Reston, VA).

**(ii) Electrical conductivity and pH:** The electrical conductivity and pH of prepared extracts were determined by conductivity meter (microprocessor-based Helix (Biosciences) LMCM-202) and pH meter (Helix (Biosciences) LMPH-10) at temperature of 25± 2˚C, respectively.

### Phytochemical Analysis

The individual extract was subjected to qualitative phytochemical screening for the presence of different chemical constituents. Phytochemical analysis for steroids, flavonoids, tannins, amino acids, diterpenes, emodins, phytosterol and proteins was done using standard protocols of Dash *et al.* (6), whereas, saponins, reducing sugars and alkaloids were detected using standard procedure of Sawant and Godghate (21).

**(i) Alkaloids:** Extract (3ml) was taken in test tube, HCl (1 ml) was added to it and the mixture was gently heated for 20 min, cooled and filtered. Filtrate was treated with few drops of Meyer's reagent; appearance of yellow precipitate or turbidity indicated the presence of alkaloids.

**(ii) Amino acids:** Ninhydrin reagent (2 ml) was added to extract (2 ml) and boiled for few minutes; formation of blue colour indicated the presence of amino acids (Ninhydrin test).

**(iii) Diterpenes:** Extract (3 ml) was dissolved in distilled water and treated with few drops of copper acetate solution, appearance of emerald green colour indicated the presence of diterpenes (Copper acetate test).

**(iv) Emodins:** $NH_4OH$ (2 ml) and benzene (3 ml) was added to extract (3 ml), occurrence of red colour indicated presence of emodins.

**(v) Flavonoids:** Extract (3 ml) was treated with 10% NaOH solution (2 ml); appearance of dark yellow colour indicated presence of flavonoids.

**(vi) Phytosterol:** Extract (3 ml) was treated with chloroform and filtered. The filtrate was treated with few drops of concentrated sulfuric acid, shaken and was allowed to stand for 5 min. Appearance of golden red colour indicated the presence of phytosterols (Salkowski's test).

**(vii) Proteins:** Extract (3 ml) was treated with few drops of concentrated $HNO_3$, formation of yellow colour indicated the presence of proteins (Xanthoproteic test).

**(viii) Reducing sugars:** Extract (2 ml) was dissolved in 1 ml each of Fehling's solutions A and B. The mixture was shaken and heated for 10-15 min. The formation of red precipitates indicated the presence of reducing sugars.

**(ix) Saponins:** Extract (5 ml) was shaken vigorously with distilled water (5 ml) and heated in a test tube. Formation of stable foam indicated the presence of saponins.

**(x) Steroids:** Extract (1 ml) was dissolved in chloroform (10 ml) and equal quantity of concentrated sulfuric acid was carefully added from the side of the tube. The upper layer showed red and sulfuric acid layer turned yellow with green fluorescence indicating the presence of steroids.

**(xi) Tannins:** Extract (4 ml) was treated with $FeCl_3$ solution (4 ml) and formation of green Colour indicated the presence of condensed tannins.

**GCMS (Gas Chromatography-Mass Spectrometry):** GC-MS analysis of plant extract was done using GC-MS (QP2010 Plus, Shimadzu, Japan) equipped with mass spectrometric detector (MS), equipped with Rtx-5 MS capillary column (30.0 m × 0.25 mm, 0.25 µm film thickness) to separate the components of *A. indica* plant extract. Oven temperature programme with initial temperature of 80˚C to hold for 2 min, raised to 250˚C at 10˚C/min, then to 280˚C at 10˚C/min. The injector temperature of 280˚C and the carrier gas used was helium at a constant pressure of 81.9 kPa. Data acquisition was started 4.0 min after injection. The diluted crude extracts were taken in a syringe and injected into injector with split mode. MS parameters used were; Ionization Voltage (EI) 70 eV, peak width 2 s, mass range 40-600 amu and detector voltage 1.5 V (9). The compounds were characterized with the National Institute of Standards and Technology (NIST) Library, Chem Station software, FFNSC 2 and WILEY8.

**FT-IR Analysis:** Absorption spectra of different extracts was done using NaCl windows and spectrum was recorded using Thermo Nicolet FT-IR- 6700 Fourier transform infrared spectrometer at resolution 3.86 $cm^{-1}$ in the range 1000-4000 $cm^{-1}$.

**Test Fungus**

Pure culture of test fungal pathogen, *Penicillium digitatum* isolated from kinnow fruit was obtained from IARI (Indian Agriculture Research Institute), New Delhi with ITCC No. 7190. These cultures were preserved on 2% PDA (potato dextrose agar) medium, incubated at 28±1° C and kept at 4°C in refrigerator.

**Media Preparation:** The known weight of prepared HiMedia Potato dextrose agar (PDA) was dissolved in 1.0 L lukewarm water and was sterilized for 20 min at 5 psi using wet steam in stainless steel jacketed autoclave. To avoid bacterial contamination, a broad-spectrum antibiotic 'gentamycin' was added to obtain 30 mg $L^{-1}$ working concentration in molten agar media before pouring the medium in pre-sterilized petridish.

**Conidial Suspension:** The fungal inoculum was obtained from lawn culture by flooding a small amount of autoclaved saline solution to the plate and softly rubbing the surface of culture with sterile inoculation loop. The conidia count of pathogen stained with lactophenol cotton blue dye was poured on hemocytometer and was viewed under 20X and 40X objective lenses in Leica DM5000B optical research microscope ($10^6$ CFU/ml).

**Antimycotic Activity:** The screening for antimycotic property of *A. indica* leaf extracts against *P. digitatum* was done using the poisoned food technique (4). Different plant extracts were added aseptically in melted PDA medium in appropriate proportions, mixed and solidified. All tests were done with different concentrations of prepared extracts *i.e.* 5 - 40% for PEE and PPEE, 1 - 40% for FEE and PFEE. Stock solution (40%) was made by dissolving 4g extract in minimum amount of ethanol and diluted with distilled water to 10 ml. Rest of the solutions were made by subsequent dilutions. Three replications were taken of each concentration of extract (1 ml) along with solvent control (ethanol) and the standard Carbendazim 50 WP (0.0125%). After solidification, 50 µl of conidial suspension ($10^6$ CFU/ml) was spread over the assay plate and allowed to grow. The inoculated plates were incubated at 25 ± 2˚C and growth of the pathogen was measured on $5^{th}$, $10^{th}$ and $15^{th}$ day of growth. The inhibition (%) was calculated from the mean value of colony numbers of treated and untreated fungus.

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

Where, C: Number of colonies in control and T: Number of colonies in treated fungus.

**Morphological Studies**

**(i) Optical research microscopy:** The morphological changes in the fungal samples collected from the control and treated PDA plates were observed under the optical research microscope Leica DM 5000B. The fungus samples were fixed in lactophenol cotton-blue dye and examined under optical microscope at 20X and 40X to observe morphological changes. Untreated samples taken from the control plates were also stained and examined. Optical micrographs were taken with computer attached camera.

**(ii) Scanning Electron Microscopy (SEM)**: The SEM analysis was done to study the morphological changes in hyphae and conidia of fungal pathogen growing in control and treated PDA plates. The SEM examination of the regions on petri plate exhibiting antagonism of test fungi (4×10 mm segments including periphery) were cut from the colonies growing on PDA plates. The fungal samples were fixed with 2.5% glutaraldehyde and kept at 4°C overnight, rinsed thrice with 0.1 M caco buffer. Then, 1% osmium tetraoxide was added followed by thorough rinsing. Later, the samples were dehydrated by passing through graded aqueous ethyl alcohol series (30%, 50%, 70%, 90% and 95%), placed in 100% ethanol at room temperature for few minutes. It was then dried and mounted on aluminium stubs for carbon sputtering. The stub was then examined under Hitachi Scanning Electron Microscope S-3400N (11).

**Statistical Analysis**

The experimental results were statistically analysed and expressed as mean of three replicates. The data were analyzed using the SAS software 9.0. Differences between means were tested using least significant difference and treatment means were compared with Dunkan's multiple range test (DMRT) ($p \leq 0.05$) and t-grouping where a value of $p \leq 0.05$ was regarded as significant.

## RESULTS AND DISCUSSION

### I. Physical Characterization of Plant Extracts

**(i) Colour:** Crude ethanol *A. indica* extracts (PEE and FEE) were dark green in colour and partitioned ethanolic extracts (PPEE and PFEE) were greenish-yellow exhibiting characteristic, strong odour. Colour of the extracts was expressed in terms of L*, a* and b* values (Table 1). The L* a* and b* values for all the extracts were determined in triplicates. The L* value represent lightness and darkness where lower and higher values indicate dark and light colors respectively. Darkest colour was revealed by FEE (1.19 ± 0.08) and the lightest colour by PFEE (9.59 ± 0.3). Low a* values indicated redness/ greenness index. The PEE was the dark green extract, whereas, PPEE was light green. Higher b* value indicated the blueness/ yellowness index. The b* values of all the extracts exhibited a decreasing order as PFEE > PPEE > FEE > PEE.

Table 1. Colour of *A. indica* leaf extracts

| *A. indica* Extract | *A. indica* extract colour | | |
|---|---|---|---|
| | Light and dark | Redness/greenish | Blueish/ yellowish |
| PEE | 2.47 ± 0.11 | -1.64 ± 0.11 | 0.42 ± 0.08 |
| PPEE | 5.99 ± 0.33 | 3.23 ± 0.07 | 3.64 ± 0.19 |
| FEE | 1.19 ± 0.08 | 2.34 ± 0.31 | 0.78 ± 0.06 |
| PFEE | 9.59 ± 0.30 | 2.63 ± 0.14 | 12.66 ± 0.59 |

Values, average ± Standard error, PEE: Powdered ethanol extract, FEE: Fresh ethanol extract, PPEE: Partitioned powdered ethanol extract, PFEE: Partitioned fresh ethanol extract

**(ii) Electrical conductivity and pH:** Electrical conductivity of extracts ranged from 44.87-71.87 $\mu Scm^{-1}$. Our studies showed that partitioned extracts exhibited lower conductance than crude extracts. This observation may be correlated with greater number of solute molecules present in the crude extracts than in the partitioned ones. pH values of all extracts were from 8.88 to 10.15. However, FEE exhibited maximum pH (10.15 ± 0.50) and PFE showed the minimum (8.88 ± 0.20) (Figure1). Contrary to the results, aqueous *A. indica* powdered chewing sticks extract pH was near neutral (1).

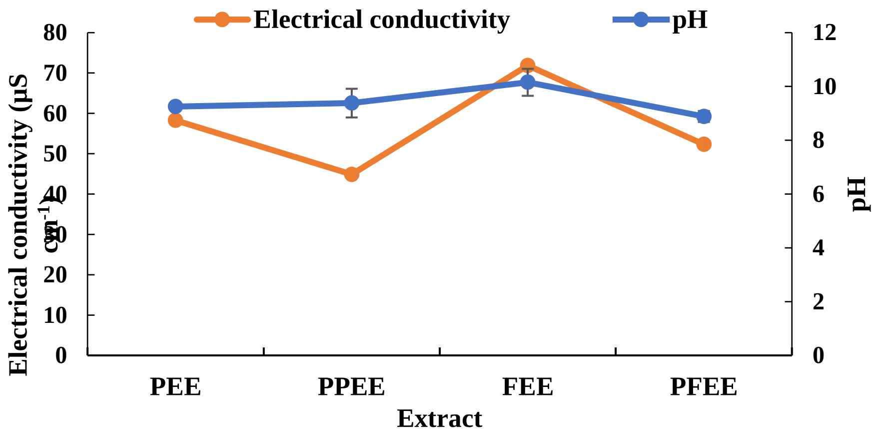


Figure 1. Electrical conductivity and pH of *A. indica* leaf extracts

**II. Phytochemical Analysis:** The Alkaloids, flavonoids, diterpenes, tannins, steroids and phytosterols were present in all ethanolic extracts of *A. indica* (Table 2). The alkaloids, phenolics, flavonoids, terpenoids, steroids, cardiac glycosides, reducing sugars and non-reducing sugars are present in powdered dry leaf ethanolic extracts (17). All extracts gave negative test for presence of reducing sugars except PEE. Moreover, proteins and amino acids were present only in PPEE. The extracts gave negative test for emodins, whereas, saponins were present in crude extracts only and not in partitioned ones. These results were in agreement with Dash *et al* (6) as alkaloids, saponins, tannins, glycosides, flavonoids and reducing sugars were found in both aqueous and methanolic extracts.

Table 2. Phytochemical analysis of different *A. indica* extracts

| Phytochemicals | *A. indica* extracts | | | |
|---|---|---|---|---|
| | PEE | PPEE | FEE | PFEE |
| Alkaloids | + | + | + | + |
| Saponins | + | - | + | - |
| Steroids | + | + | + | + |
| Tannins | + | + | + | + |
| Emodins | - | - | - | - |
| Proteins | - | + | - | - |
| Amino acids | - | + | - | - |
| Flavonoids | + | + | + | + |
| Diterpenes | + | + | + | + |
| Phytosterols | + | + | + | + |
| Reducing sugars | + | - | - | - |

(+): Presence; (-): Absence; PEE: Powdered ethanol extract, FEE: Fresh ethanol extract, PPEE: Partitioned powdered ethanol extract, PFEE: Partitioned fresh ethanol extract

**III. GC-MS Analysis:** Gas Chromatography- Mass Spectrometry data of PEE leaf extract identified total of 55 compounds and the major ones were : Phytol (13.14%), lycoxanthin (8.55%), n-hexadecanoic acid (5.31%), 9,19-cyclolanostan-3-ol, acetate (3 β)- (4.76%), neophytadiene (4.32%) and 9,12,15-octadecatrienoic acid, (Z, Z, Z)- (4.3%). Most of the compounds found in this study are anti-fungal (17, 19, 22, 25). Table 3 shows various constituents of PEE and PFEE with their per cent area of compounds present.

The GC-MS data of PFEE identified 19 compounds with 9,12,15- octadecatrienoic acid, (Z, Z, Z)- (43.5%), 9α-fluoro-17α-methyl-4-androsten-3α,6β,11β,17β-tetra-ol (13.07%), 1H-cyclopropa[3,4]azulene-5,7b,9,9a-tetrol, 1a,1b,4,4a,5,7a,8,9- octahydro-3-(hydroxymethyl)-1,1,6,8-tetramethyl-,5,9,9a-triacetate, [1aR-1aα,1bβ,4aβ,7aα ,7bα,8α,9 beta,9aα)]- (9.197%), 16 hydroxy ingenol (5.965%), pentatriacontane (4.446%), stigmastan-6,22-dien,3,5-dedihydro (3.874%) (Table 3). Harikrishnan *et. al.* (2010) reported 24 compounds from the ethanolic powdered leaf extract of *A. indica* by Soxhlet extraction method, of these 4-compounds were : n-hexadecanoic acid (14.34%), phytol (19.96%), 9,12,15-octadecatrienoic acid, (Z, Z, Z)- (18.57%), and vitamin E (11.37%) (8). Hexadecanoic acid (20.6%), a fatty acid methyl ester was found in the ethanol leaf extract (prepared by maceration) and 16.6% and 25.8% of n-hexadecanoic acid was found by maceration and refluxing of leaves in ethanol, respectively (20). The results of present studies are in agreement with published literature. However, the per cent composition of compounds varied with climatic conditions, time of sample collection and extraction methods used.

Table 3. GC-MS analysis of *A. indica* extracts showing per cent areas of compounds present

| Sr. No. | Compound | Area (%) | |
|---|---|---|---|
| | | PEE | PFEE |
| 1 | Phytol | 13.14 | 0.35 |
| 2 | 9.α.-Fluoro-17. α.-methyl-4-androsten-3. α.,6. β.,11. β.,17. β.-tetra-ol | | 13.07 |
| 3 | 1H-Cyclopropa [3, 4] azulene- 5,7b,9,9a-tetrol, 1a, 1b, 4, 4a, 5, 7a,8, 9- octahydro-3-(hydroxymethyl)-1,1,6,8-tetramethyl-,5,9,9a-triacetate, [1aR-1a.α.,1b.β.,4a.β.,7a.α.,7b.α.,8. α.,9. beta.,9a.α.)]- | | 9.92 |
| 4 | Lycoxanthin | 8.55 | |
| 5 | 16 hydroxy ingenol | | 5.97 |
| 6 | n-Hexadecanoic acid | 5.31 | |
| 7 | 9,19-Cyclolanostan-3-ol, acetate, (3 β.)- | 4.76 | |
| 8 | Pentatriacontane | | 4.45 |
| 9 | Neophytadiene | 4.32 | 0.8 |
| 10 | 9,12,15-Octadecatrienoic acid, (Z, Z, Z)- | 4.3 | |
| 11 | Silane, 2-butenyltrimethyl-, (E)- | 3.18 | |
| 12 | Ergosta-7,22-dien-3-ol, acetate, (3 β,5. α.)- | 2.97 | |
| 13 | Heneicosane | | 2.77 |
| 14 | 9,12,15-Octadecatrien-OL | 2.44 | |
| 15 | 1,8,15,22-tricosatetrayne | | 2.42 |
| 16 | γ -Sitosterol | 2.29 | |
| 17 | 2,6,10-Trimethyl,14-Ethylene-14-Pentadecane | 2.17 | |
| 18 | Vitamin E | 1.99 | |
| 19 | 5-Hydroxymethylfurfural | 1.79 | |
| 20 | Stigmasta-5,22-dien-3-ol | 1.78 | 3.87 |
| 21 | Bufa-20,22-Dienolide, 14,15-Epoxy-3,5,16-Trihydroxy-, (3 β,5 β,15 β,16 β)- | 1.65 | |
| 22 | Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester | 1.55 | |
| 23 | Trans (β) - Caryophyllene | 1.4 | |
| 24 | 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- | 1.39 | |
| 25 | Ethyl (9Z,12Z)-9,12-octadecadienoate | | 1.22 |
| 26 | Retinol, Acetate | 1.21 | 2.93 |
| 27 | γ -Elemene | 1 | |
| 28 | Doconexent | | 0.98 |
| 29 | (2E)-3,7,11,15-Tetramethyl-2-Hexadecene | 0.75 | |
| 30 | 2,6,10,14,18,22, -Tetracosahexaen, 2,6,10,15,19,23-Hexamethyl- | 0.71 | |
| 31 | Germacrene D | 0.66 | |
| 32 | Andrographolide | | 0.62 |
| 33 | Docosane,11-decyl | | 0.61 |
| 34 | Octadecanoic acid | 0.61 | |
| 35 | Nonadecane | | 0.59 |

| | | | |
|---|---|---|---|
| 36 | 9,12,15-Octadecatrienal | | 0.53 |
| 37 | Dodecanoic acid | 0.52 | |
| 38 | 6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one | 0.51 | |
| 39 | Lauryl acetate | 0.48 | |
| 40 | Bicyclogermacrene | 0.44 | |
| 41 | α-humulene | 0.44 | |
| 42 | Hexatriacontane | 0.42 | |
| 43 | Tetradecanoic acid | 0.41 | |
| 44 | (2E,6E,10E)-3,7,11,15-Tetramethylhexadeca-2,6,10,14-tetraen-1-yl formate | 0.4 | |
| 45 | β -Elemene | 0.4 | |
| 46 | Geranyl linalool isomer | 0.39 | |
| 47 | 1,2-Benzenedicarboxylic acid | 0.37 | |
| 48 | (E)-4-(3-Hydroxyprop-1-en-1-yl)-2-methoxyphenol | 0.37 | |
| 49 | Hexadecanoic acid, ethyl ester | 0.35 | 0.27 |
| 50 | α -Copaene | 0.36 | |
| 51 | δ-elemene | 0.28 | |
| 52 | Spirio-10-(2,11-dioxabicyclo [4.4.1] undeca-3,5-diene)-2'-(oxirane), 1,3,7,7-tetramethyl | 0.28 | |
| 53 | 3-Cyclopentylpropionic acid, 2-dimethylaminoethyl ester | 0.27 | |
| 54 | Pentacosane | 0.2 | 0.37 |

PEE: Powdered ethanol extract, FEE: Fresh ethanol extract, PPEE: Partitioned powdered ethanol extract, PFEE: Partitioned fresh ethanol extract

**IV. FT-IR Analysis:** The FT-IR spectrum of *A. indica* leaf extracts indicated the presence of diverse functional groups (Figure 2) : alcohol and phenol (-N-H, O-H stretching vibration of amide, alcohol, alkane and H bonded to phenols at 3425 to 3384 cm$^{-1}$), aliphatic alkanes (alkyl C-H stretching vibrations at 2975 cm$^{-1}$), alkynes (C-H bond stretching for alkynes at 2362 cm$^{-1}$), aromatic amines (C-N stretching at 1255 cm$^{-1}$), amides (N-H bond vibrations at 2323.04 cm$^{-1}$), carboxylic acid (C=O bending vibrations at 669.1 cm$^{-1}$), and amino acid groups. Most of bands were found in both extracts but with variable intensities.

**V. Antimycotic Activity of Leaf Extracts:** The four *A. indica* leaf extracts exhibited great antimycotic potential against *P. digitatum* but PFEE was the best. Different concentrations of extracts were evaluated for 15 days and monitored on 5$^{th}$, 10$^{th}$ and 15$^{th}$ days after inoculation (DAI). Among all extracts, FEE and PFEE showed high antifungal activity at 5 and 1 % concentrations, respectively (Table 4).

Table 4. Antifungal activity of different concentrations of fresh *A. indica* leaf extracts

| Extract | Concentration (%) | Days | | |
|---|---|---|---|---|
| | | 5 | 10 | 15 |
| Control | | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| FEE | 1.0 | 7.92± 0.80 | 5.33 ± 0.95 | 5.18 ± 0.57 |
| | 1.5 | 43.17 ± 0.47 | 40.53 ± 0.58 | 32.91 ± 0.78 |
| | 2.5 | 53.83 ± 0.59 | 52.00 ± 0.75 | 49.13 ± 0.86 |
| | 5.0 | 81.42 ± 0.89 | 75.73 ± 0.95 | 74.38 ± 0.94 |
| | 10.0 – 40.0 | 100.00 ± 0.00 | 100.00 ± 0.00 | 100.00 ± 0.00 |
| PFEE | 1.0 | 70.04 ± 0.80 | 66.67 ± 0.89 | 62.49 ± 0.54 |
| | 1.5 – 40.0 | 100.00 ± 0.00 | 100.00 ± 0.00 | 100.00 ± 0.00 |
| Carbendazim 50 WP | 0.0125 | 100.00 ± 0.00 | 100.00 ± 0.00 | 100.00 ± 0.00 |

Values, average ± Standard error, FEE: Fresh ethanol extract, PFEE: Partitioned fresh ethanol extract

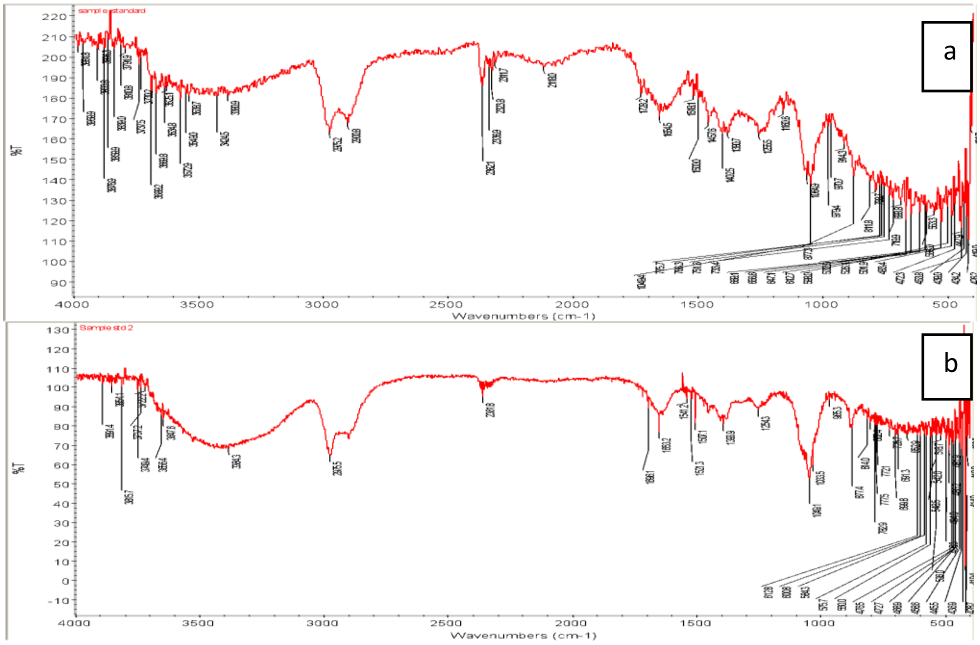


Figure 2. FT-IR spectrum of *A. indica* leaf extracts a) PEE b) PFEE

*P. digitatum* was completely controlled at higher concentrations (10 - 45%) of both FEE and PFEE. Fresh leaf extracts had better antifungal activity than powdered extracts. This may be due to occurrence of some volatile allelochemicals that may have exhibited some synergistic effects with the non-volatile ones. In case of PEE and PPEE, higher concentrations (5-30%) were ineffective against *P. digitatum*. However, the activity was observed at higher concentration of PEE (35%) with 100% inhibition even on day 10 (Table 5)**.**

Table 5. Effects of application of *A. indica* extracts on the colony count of *P. digitatum*

| *A. indica* extract conc (%) | 5 DAI | | | | 10 DAI | | | | 15 DAI | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PEE | PPEE | FEE | PFEE | PEE | PPEE | FEE | PFEE | PEE | PPEE | FEE | PFEE |
| Control | 5.90 | 5.89 | 5.86 | 5.92 | 5.91 | 5.90 | 5.87 | 5.93 | 5.92 | 5.91 | 5.88 | 5.94 |
| 1 | - | - | 2.35 | 4.79 | - | - | 2.35 | 4.79 | - | - | 2.35 | 4.79 |
| 1.5 | - | - | 2.81 | 0.00 | - | - | 2.81 | 0.00 | - | - | 2.81 | 0.00 |
| 2.5 | - | - | 3.16 | 0.00 | - | - | 3.16 | 0.00 | - | - | 3.16 | 0.00 |
| 5 | 5.42 | 5.36 | 5.60 | 0.00 | 5.53 | 5.59 | 5.60 | 0.00 | 5.53 | 5.59 | 5.60 | 0.00 |
| 10 | 4.87 | 4.43 | 0.00 | 0.00 | 5.43 | 5.10 | 0.00 | 0.00 | 5.48 | 5.21 | 0.00 | 0.00 |
| 20 | 3.08 | 3.33 | 0.00 | 0.00 | 4.90 | 5.07 | 0.00 | 0.00 | 4.99 | 5.21 | 0.00 | 0.00 |
| 25 | 2.56 | 3.05 | 0.00 | 0.00 | 4.44 | 4.82 | 0.00 | 0.00 | 4.77 | 5.12 | 0.00 | 0.00 |
| 30 | 0.00 | 0.00 | 0.00 | 0.00 | 1.26 | 4.49 | 0.00 | 0.00 | 4.47 | 4.71 | 0.00 | 0.00 |
| 35 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 4.14 | 0.00 | 0.00 | 4.25 | 4.49 | 0.00 | 0.00 |
| 40 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 2.25 | 0.00 | 0.00 | 1.75 | 3.92 | 0.00 | 0.00 |

DAI: Days after incubation, PEE: Powdered ethanol extract, FEE: Fresh ethanol extract, PPEE: Partitioned powdered ethanol extract, PFEE: Partitioned fresh ethanol extract.

Thus, minimum inhibition concentration (MIC) for PEE and PPEE was 35% and 40%, respectively. The MIC for FEE and PFEE was comparatively very low, 5% and 1%, respectively. The inhibitory effects of extracts were evident from the reduction in conidia forming region of colonies growing on test plates and the parameters (conidia number and colony diameter) were decreased. However, in FEE at lower concentrations, there were less number of fungal colonies with large diameter. With increased concentration (till MIC), the number of colonies decreased the colony diameter. These extracts might have hyphaecidal effects, thereby, reducing the vegetative growth but it induced the conidia production. The extract might have affected the hyphal wall integrity and thereby, resulting in weakening of the cell wall barrier, hence its components might even have entered the cell leading to greater fungal toxicity potentials. The effects of supplementation of FEE and PFEE on *P. digitatum* were depicted in Figure 3 and 4 respectively.

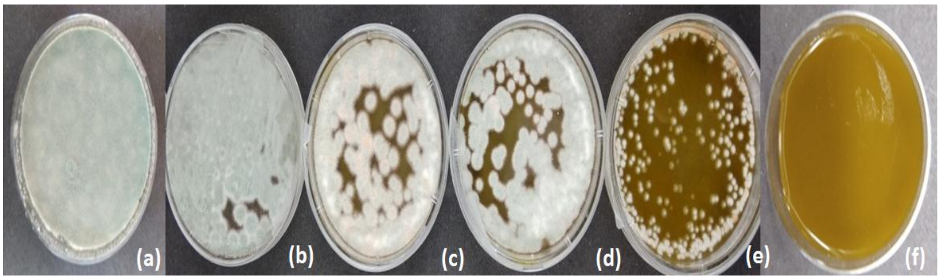


Figure 3. Effects of adding FEE on *P. digitatum* growing on PDA medium (a) Control, (b) 1% FEE, (c) 1.5% FEE, (d) 2.5% FEE, (e) 5 % FEE, (f) 10% FEE

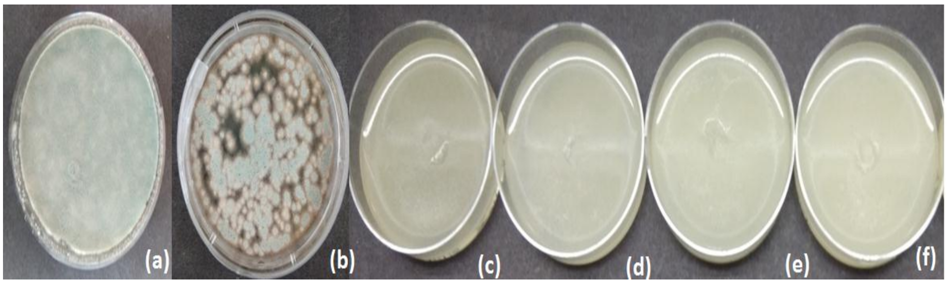


Figure 4. Effects of supplementation of PFEE on *P. digitatum* growing on PDA medium (a) Control, (b) 1% PFEE, (c) 1.5% PFEE, (d) 2.5% PFEE, (e) 5 % PFEE, (f) 10% PFEE

The results of our studies were more effective than earlier studies. The methanol extract of neem leaves was fungitotoxic against *P. digitatum* at 60% concentration than control (22). Similar *in vitro* study (16) indicated the efficacy of 5% extract caused 62% inhibition of *P. digitatum.* However, our study improved efficacy (70.4% inhibition) of PFEE extract. The phytochemical, TLC and GC-MS analysis of extracts showed the presence of various antifungal metabolites in all extracts. However due to their synergistic/antagonistic interactions among each other, variable results of their antifungal potential were obtained. Further, the IR spectra of PEE and PFEE depicted similar bands but their intensities varied with different functional groups which may have affected the interactions among the components of extract. Interactions of such metabolites must have directly or indirectly affected the hyphal growth and sporulation in *P. digitatum,* thereby inhibiting the pathogen.

**VI. Morphological Studies**

**(i) Optical research microscopy:** The optical microscopy analysis of test pathogen treated with various *A. indica* leaf extracts revealed their effects on the fungal morphology and on conidia numbers. The 35% PEE reduced the conidiophore branching and number than control. The 1% PFEE proved most inhibitory to *P. digitatum* with little or no conidiophore formation. Hyphae with no septa and disorganized cytoplasm were observed. The 5% FEE exhibited mild impact than PFEE, as it did not have any effect on hyphal branching although the conidia number and chains were drastically reduced (Figure 5).

The *in vitro* assessment of antifungal effects of *A. indica* leaves and its oil on mycelial growth, morphology, sporulation and ochratoxin A production by *P. brevicompactum* and *P. verrucosum* have been reported (15). They revealed differences at the macroscopic levels between *A. indica* oil and *A. indica* extract treatments but at microscopic level, the size of conidia and conidiophores remained unchanged. There were no modifications in asexual reproduction, appearance of conidiophores and mycelial branching among the two test solutions (15).

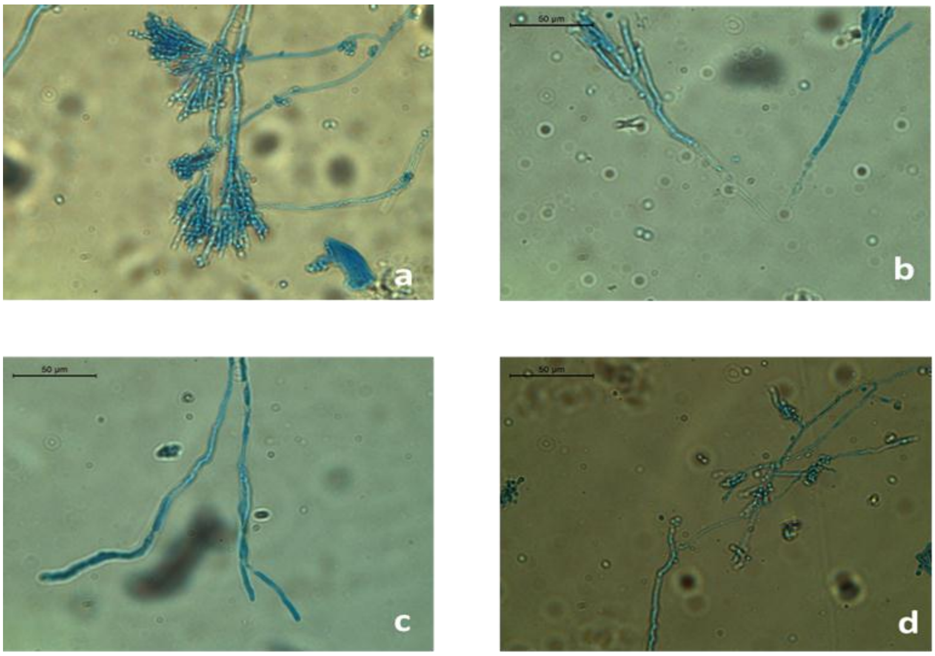


Figure 5. Optical research microscopy of untreated and treated fungus (a) Control, (b) Fungus treated with 35 % PEE, (c) Fungus treated with 1% PFEE, (d) Fungus treated with 5% FEE

**(ii) Scanning Electron Microscopy (SEM):** Scanning electron micrographs of the PEE treated fungal colonies revealed the occurrence of curled, collapsed hyphae with loss of smoothness and unusual bulges than smooth and organized hyphal mass in control. However, the PFEE (1%) treated fungal biomass showed the perforated walls with hyphal distortion than control (Figure 6). The SEM analysis were also similar to our inhibition (%) observations, depicting the varied antifungal potential of different extracts.

Similar studies (26) showed the anomalous branching and notching of terminal hyphae of *P. digitatum* when treated with different oils (*Foeniculum vulgare*, *Thymus vulgaris*, *Eugenia caryophyllata* and *Salvia officinalis*). Also, SEM micrographs of *Alternaria solani* treated with ethanol extract of *Calotropis procera* collapsed the hyphal growth compared to intact hyphae in control (3).

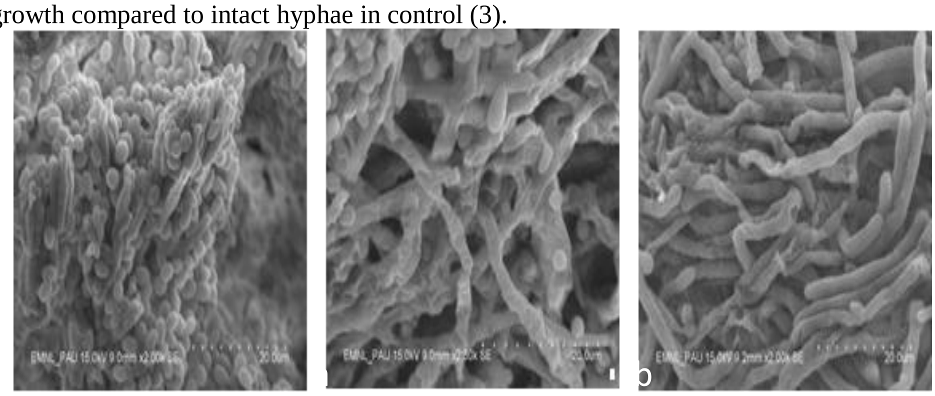

Figure 6. Scanning electron microscopy of untreated and treated fungus (a) Control, (b) Fungus treated with 35 % PEE, (c) Fungus treated with 1 % PFEE

Table 6. Antifungal activity of FEE and PFEE on 5,10 and 15 DAI at different concentrations

| *A. indica* Extract | Radial mycelial growth (mm) | Conidia forming region (mm) |
|---|---|---|
| Ethanol extract | | |
| FEE | 1.691[a] | 1.538[a] |
| PFEE | 0.691[b] | 0.623[b] |
| DAI | | |
| 5 | 1.126[c] | 1.035[b] |
| 10 | 1.197[b] | 1.100[a] |
| 15 | 1.252[a] | 1.109[a] |
| Ethanol extract Concentration (%) | | |
| Control | 6.220[a] | 6.098[a] |
| 1 | 3.958[b] | 3.475[b] |
| 1.5 | 1.903[c] | 1.733[c] |
| 2.5 | 1.504[d] | 1.331[d] |
| 5 | 0.711[e] | 0.339[e] |
| 10 - 40 | 0.000[f] | 0.000[f] |

CV for colony count, radial mycelial growth and conidia forming region of FEE and PFEE was 0.622, 4.538 and 15.231 respectively. The values with different superscripted alphabet are significantly different from each other.

The different concentrations of fresh extracts, FEE and PFEE significantly influenced the colony count, radial mycelial growth and conidia forming region with PFEE showing better results. However, the days of incubation had no significant impact on colony count and on radial mycelial growth and conidia forming region (Table 6).

## CONCLUSIONS

Among all types of *A. indica* leaf extracts, fresh extracts were more effective against *P. digitatum* than powdered leaf extracts. Further, PFEE and PEE at 1% and 35% concentrations, respectively exhibited maximum antimycotic potential. Comparison of GC-MS data of PFEE and PEE showed less number of allelochemicals in PFEE. Higher efficacy of PFEE even at 1% concentration may be due to presence of some selective allelochemicals in the extract with higher antimycotic potential. The optical and scanning electron microscopic studies interpreted the harmful effects of *A. indica* extracts on the morphology of phytopathogen at nano scale.

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## CONFLICT OF INTERESTS

The author declares that there is no conflict of interests regarding the publication of this paper.

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