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Antifungal Evaluation and Phytochemical Identification of Selected Botanicals against *Ceratocystis manginecans* Causing Mango Sudden Death

Kokab Jabeen[1]*, Shahzad Asad[2] and Muhammad Zakria[2]

[1]*PARC Institute of Advanced Studies in Agriculture, National Agriculture Research Centre, Islamabad, Pakistan*
[2]*Crop Diseases Research Institute, National Agricultural Research Centre, Park Road, Islamabad, Pakistan*

Abstract

The antifungal efficacy of three selected plants (*D. viscosa, Citrullus colocynthis,* and *Ailanthus altissima*) was evaluated against *Ceratocystis manginecans* (causal agent of Mango Sudden Death) using poisoned food technique. Ethanol, methanol and aqueous extracts of selected plants prepared at 2.5 g/100 ml, 05 g/100 ml and 10 g/100 ml concentration were evaluated against *Ceratocystis manginecans*. Most effective botanical extracts were screened for their phytochemical constituents through GCMS. Results revealed that ethanol crude extract of *D. viscosa* exhibited highest antifungal efficacy (85.066%) followed by *Citrullus colocynthis* (82.664%) and *Ailanthus altissima* (69.112%). All botanicals exhibited statistically significant antifungal efficacy from each other ($p<0.05$). The ethanol extracts were most effective than methanol and water extracts of selected plants against *Ceratocystis manginecans*. Botanical treatments resulted in thin, collapsed/damaged hyphae as compared to control. Phytochemical profiling of most effective botanical extracts revealed that 9-Octadecanoic acid and l-(+)-Ascorbic acid 2,6-Hexadecanoate were found common in all three most effective botanical extracts. The present study revealed that these compounds possibly contributed to the antifungal efficacy of botanicals against *C. manginecans*.

Keywords: Botanicals; Antifungal efficacy; Aqueous and organic solvent extracts; Phytochemicals; *Ceratocystis manginecans*

Introduction

Mangifera indica L. (Mango) is one of the principal fruit cash crops of the Pakistan. Mango is national tree of Bangladesh and national fruit of Pakistan and India. It occupies 1987000.38 hectares area in Pakistan with a production of 1846000.0 tonnes [1]. The mango crop is subjected to attack of a number of diseases. In Pakistan, Major diseases of mango include; mango malformation, mango sudden death (MSD), anthracnose, and powdery mildews [2]. The mango sudden death is the most holistic and has caused heavy loss upto 50% in grooves of Punjab and Sindh [3]. Due to MSD, a loss up to one billion Rupees alone has been inflicted to the mango growers of Sindh province. There is a great risk that if MSD couldn't be properly managed, then orchards of mango in Pakistan will be wiped out [4]. MSD symptoms include tip die back, canker, twig blight, stem bleeding, gummosis and complete mortality at the end. Major host of *C. manginecans* is *Mangifera indica* [5]. Other hosts of *C. manginecans* include *Dalbergia sissoo*, *Prosopis cineraria*, *Acacia crassicarpa*, and *Acacia mangium* [6]. The soil-borne conidia of *C. manginecans* are considered as main source of inoculum followed by *Hypocryphalus mangiferae* as a vector of MSD in Pakistan. MSD is usually managed by fungicide application. Different botanicals are known to have natural compounds that exhibitpotential [7]. According to Zhou et al. [8], botanical's mode of action against microbes includes: a) Substrate competition with an essential metabolite; b) Inhibition of cytoplasmic membrane function; c) Control of microbial enzymes; d) Inhibition of cell wall, nucleic acid and protein synthesis. Certain phytochemical compounds exhibit excellent antifungal efficacy. For example, Jojoba oil is obtained from jojoba beans. Jojoba oil controlled powdery mildew and white flies on the grapes and ornamental plants. The jojoba oil has the ability to stay stable at elevated temperatures, which sorts it as a fungicide widely usable in almost all climatic conditions. Its mode of action is the formation of a physical obstacle between leaf surface and the insect pest. The fungicide having final concentration of jojoba oil ≤ 1% is applied by spray method [9]. Similarly, (*Z*)-9-heptadecenoic acid inhibits growth of *Idriella bolleyi* and *Phytophthora infestans* [10]. Liu et al. [11] revealed that Palmitic acid, Myristic acid, Lauric acid, and Linoleic acid exhibited significant antifungal efficacy against selected plant-pathogenic fungi (*Fusarium oxysporum*, *Colletotrichum lagenarium*, and *Aspergillus solani)* under *In-vitro* conditions. Pot experiments revealed that palmitic and oleic acids mixture has enhanced the continuous-cucumber and the continuous-tomato seedling growth. Particularly, Palmitic acid exhibited highest antifungal efficacy against selected plant-pathogenic fungi. Botanicals are substantial sources to replace synthetic fungicides for the management of plant diseases [12]. Natural products are environmentally safe [13]. Botanical fungicides have recently gained importance due to their efficacy against phytopathogens and cost effectiveness. Hence, present study was planned to screen out selected botanicals for the control of *Ceratocystis manginecans* (causal agent of mango sudden death).

Material and Methods

Test organism

Rashid [14] conducted pathogenicity test on mango seedlings and revealed that MLT6 was most aggressive isolate of *Ceratocystis manginecans* among all the isolates studied at Mango Research Laboratory, Crop Disease Research Institute (CDRI) NARC, Islamabad. This highly aggressive isolate of *Ceratocystis manginecans* was obtained from the research fields of Mango Research Laboratory, NARC, Islamabad. Culture of *C. manginecans* was maintained on Malt Extract Agar (MEA).

Plant material collection

Botanicals were selected based on their ethno-medicinal usage

***Corresponding author:** Kokab Jabeen, PARC Institute of Advanced Studies in Agriculture, National Agriculture Research Centre, Islamabad, Pakistan, Tel: 00925190733281; E-mail: kkj_satti@yahoo.com

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as antimicrobial source. Fruit of *Citrullus colocynthis* (L.) Schrad. (*Cucurbitaceae*), leaves of *Ailanthus altissima* Mill. (*Simaroubaceae*), and *Dodonaea viscosa* Jacq. (*Sapindaceae*) were selected for present study. Disease free plant samples were collected from Islamabad, Rawalpindi and Multan and identified by Taxonomist of Department of Botany, Quaid-I-Azam University, Islamabad.

Samples preparation

Sample preparation method described by Ambikapathy [15] was used with slight modifications. Plant samples were washed thoroughly with tap water for removal of soil debris. Clorox (10%) was used for disinfection of these samples. Plant samples were washed with distilled water and shade dried at room temperature. These dried samples were ground to fine powder with mechanical grinder. Each sample was properly labeled and stored in airtight jar for further use.

Aqueous botanical extracts

For aqueous extracts, 2.5 g dried powder of individual plant were separately mixed with 100 ml of water in 250 ml conical flasks and boiled till 25 ml volume was left. Extract was filtered through Whatman's filter paper #1. The extract was made solvent free using water bath. Finally these solvent free botanicals were filled in the glass vials. These glass vials containing botanicals extracts were properly labeled, covered with aluminum foil and stored at 4°C for further use. Similarly, 5 g/100 ml solvent and 10 g/100 ml plant extracts were prepared following same procedure [16].

Organic solvent extracts

For organic extracts preparation Selvamohan et al. [17] method was used with slight modifications. Dried powder (2.5g) of individual plant was separately mixed with 100 ml of ethanol in 250 ml Erlenmeyer flasks. Flasks were placed on mechanical shaker at 60rpm for 3 days. The extracts were filtered through Whatman's filter paper #1. Each botanical was made solvent free using rotary evaporator under reduced pressure. Similarly, 5 g/100 ml solvent and 10 g/100 ml plant extracts were prepared following same procedure. Same procedure was followed for preparation of methanol extracts of selected plants.

Antifungal bioassay

Determination of mycelial growth inhibition potential of botanicals: Ethanol, methanol and aqueous extract of *D. viscosa, Citrullus colocynthis,* and *Ailanthus altissima* were tested against *C. manginecans* using poisoned food technique [18]. From botanical concentration, 1 ml was mixed uniformly in 25 ml of MEA (Malt Extract Agar). A 5mm mycelial disc was taken from the periphery of a seven days old culture of *C. manginecans* and was inoculated at the center of each Petri plate. The Petri plates were incubated at 25°C ± 2°C. The experiment was terminated when complete growth in control plates was observed and data was recorded. Control treatment was provided with 5% of respective solvent. A positive control with fungicide treatment was also kept along. Completely Randomized Design of experiment (CRD) was used with five replicates per treatment. The minimum inhibitory concentrations (MICs) were

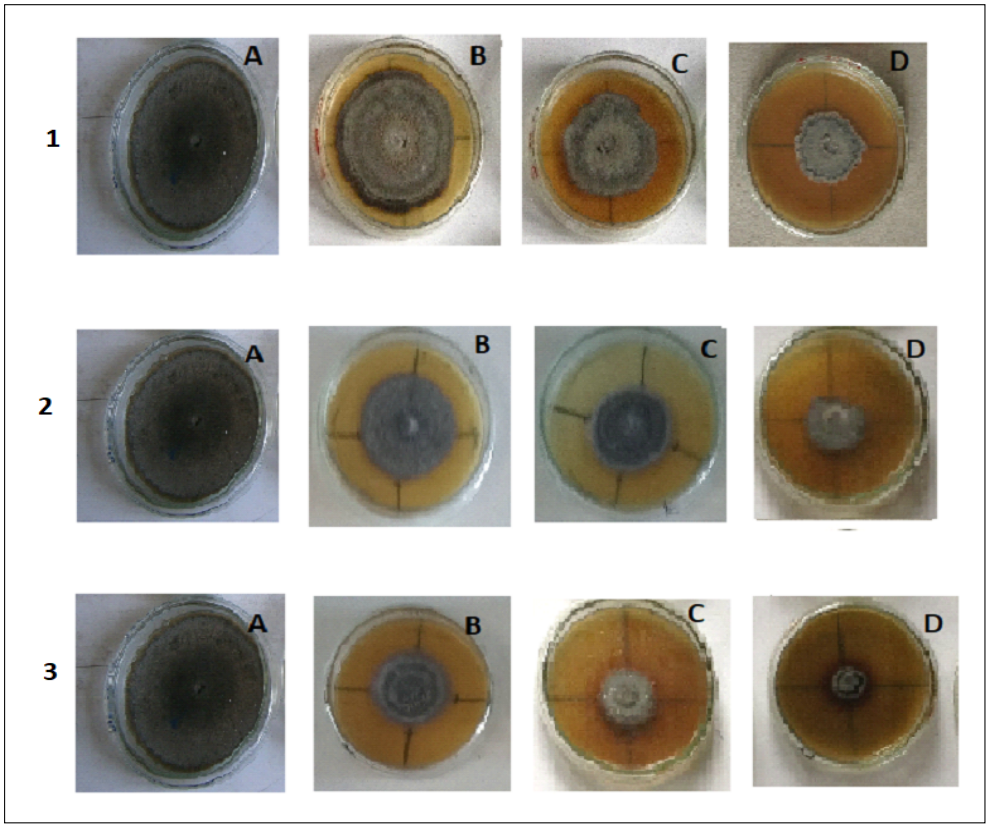

Figure 1: Mycelial growth inhibition of ethanol crude extracts of selected botanicals 1) *A. altissima* treatment; 2) *C. colocynthis* treatment; 3) *D. viscosa* treatment. A) control, B) 2.5 g/100 ml extraction concentration of botanical treatment, C) 5 g/100 ml extraction concentration of botanical treatment and D) 10 g/100 ml extraction concentration of botanical treatment.

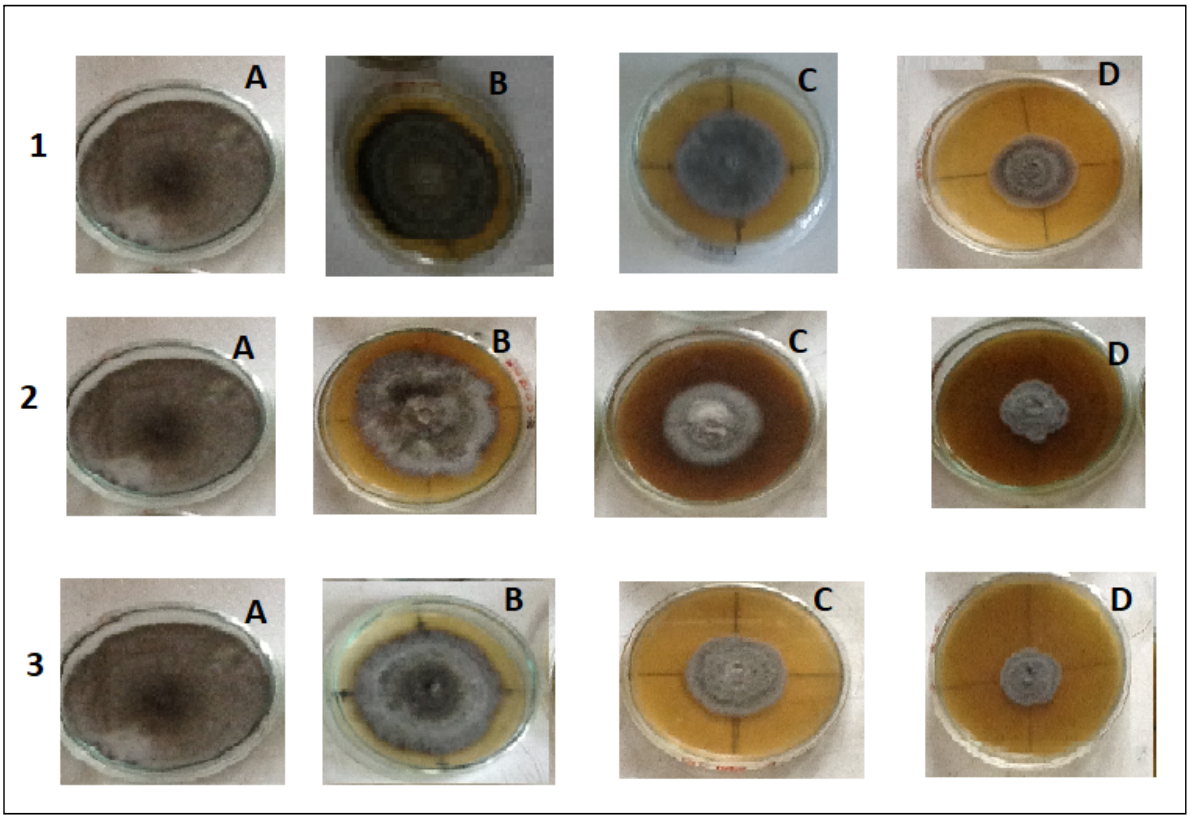

Figure 2: Mycelial growth inhibition of methanol crude extracts of selected botanicals 1) *A. altissima* treatment; 2) *C. colocynthis* treatment; 3) *D. viscosa* treatment. A) control, B) 2.5 g/100 ml extraction concentration of botanical treatment, C) 5 g/100 ml extraction concentration of botanical treatment and D) 10 g/100 ml extraction concentration of botanical treatment.

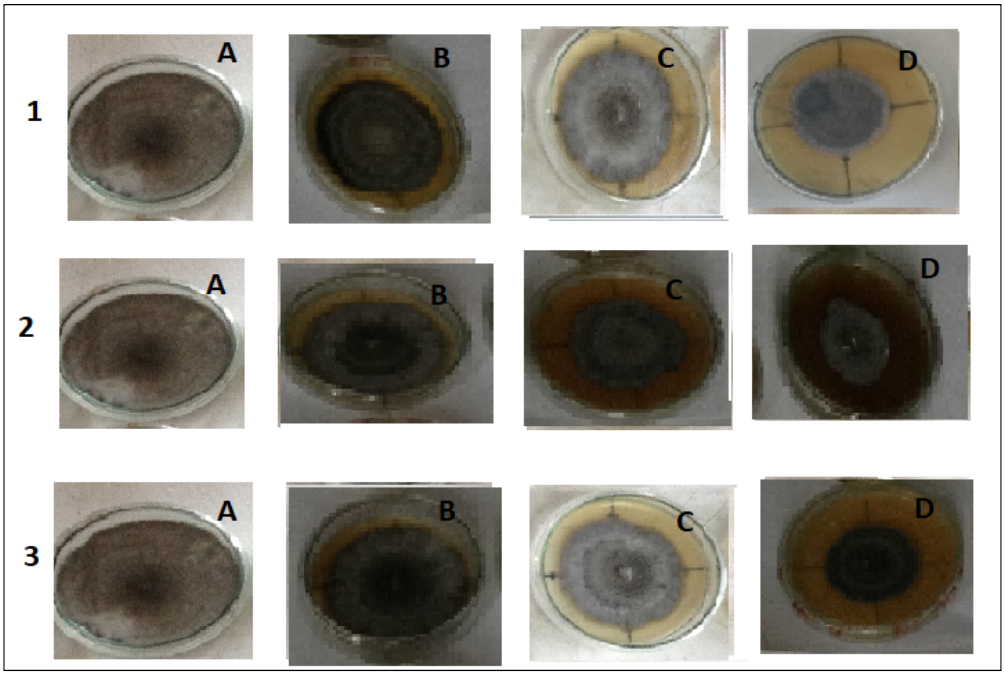

Figure 3: Mycelial growth inhibition of aqueous crude extracts of selected botanicals 1) *A. altissima* treatment; 2) *C. colocynthis* treatment; 3) *D. viscosa* treatment A) control, B) 2.5 g/100 ml extraction concentration of botanical treatment, C) 5 g/100 ml extraction concentration of botanical treatment and D) 10 g/100 ml extraction concentration of botanical treatment.

Source	DF	SS	MS	F	P
Replicates	4	3	0.8		
Solvents	2	7235	3617.5	2820.92	0.0000
Concentrations	2	5785	2892.5	2255.58	0.0000
Treatments	4	230733	57683.2	44981.3	0.0000
Solvents × Concentrations	4	112	27.9	21.75	0.0000
Solvents × Treatments	8	5190	648.8	505.93	0.0000
Concentrations × Treatments	8	3927	490.8	382.74	0.0000
Solvents × Concentrations × Treatments	16	314	19.7	15.33	0.0000
Error	176	226	1.3	--	--
Total	224	253525	--	--	--
Grand Mean-54.812, CV-2.07					

Table 1: Completely Randomized Factorial design of AOV for Percent Inhibition of colony growth of *C. manginecans* by three most effective botanicals at 2.5, 5 and 10% w/v concentration using three solvents.

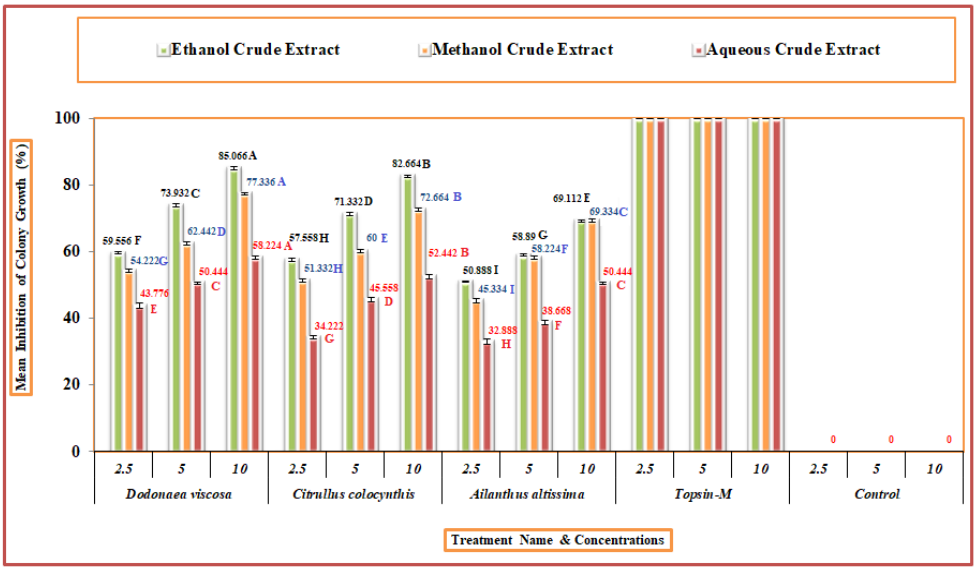

Figure 4: Efficacy of three botanical extracts at 2.5 g/100 ml, 5 g/100 ml and 10 g/100 ml concentration against percent inhibition of radial mycelial growth of *C. manginecans*.

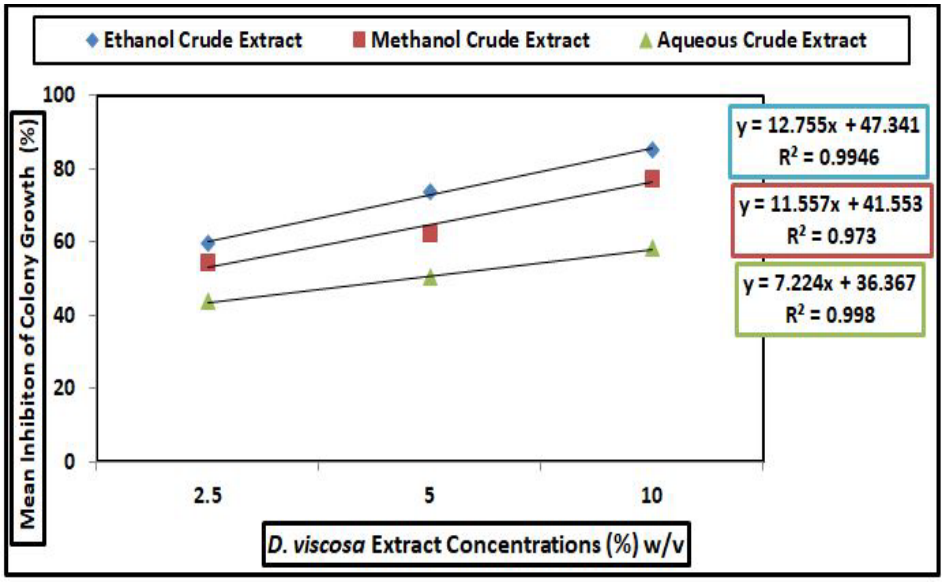

Figure 5: Correlation expression of Dose (*D. viscosa* Extracts) and response (Colony Growth Inhibition of *C. manginecans*) relationship.

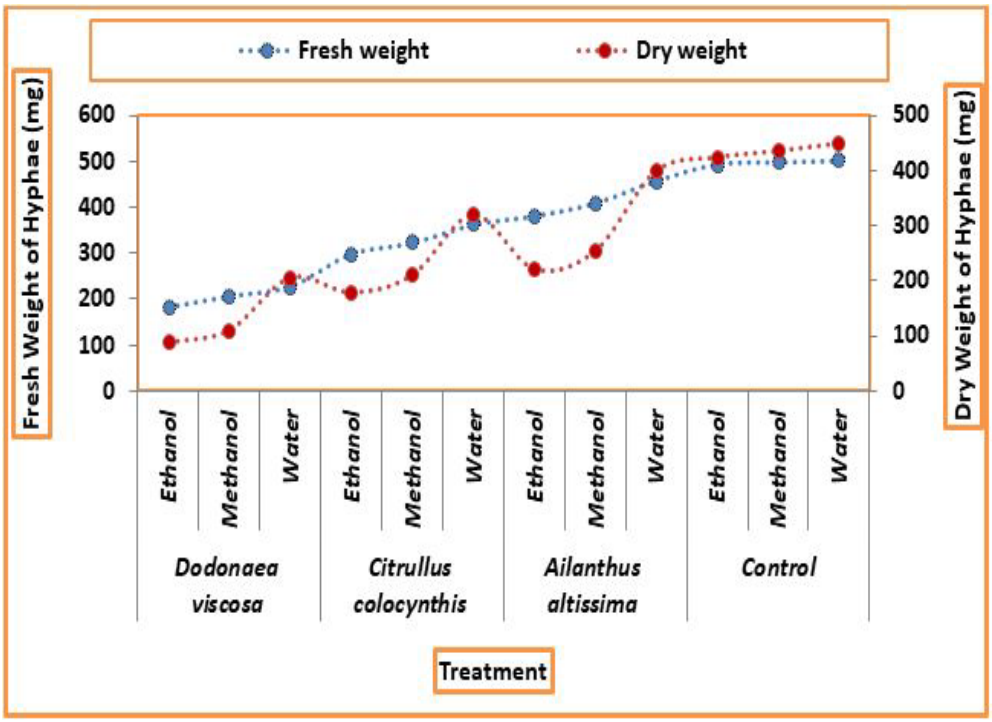

Figure 6: Effect of different treatments on Biomass of *C. manginecans.*

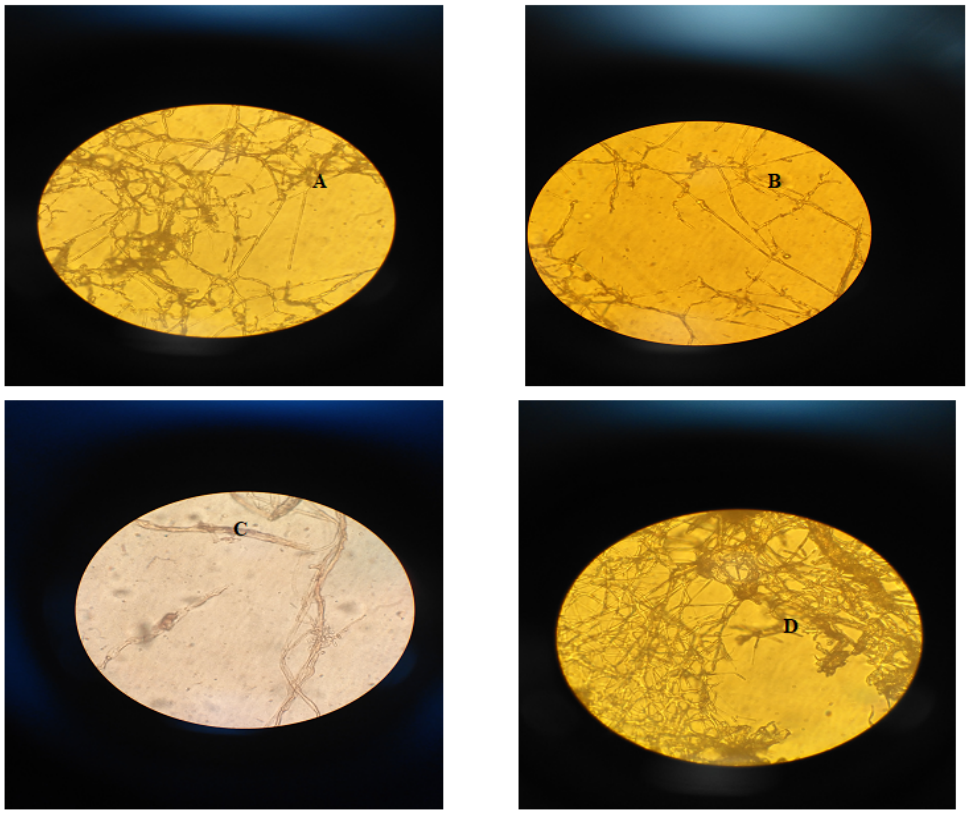

Figure 7: Hyphae of *C. manginecans* after different treatments observed under compound microscope A) *A. altissima* treatment, B) *C. colocynthis* treatment, C) *D. viscosa* treatment, and D) Control.

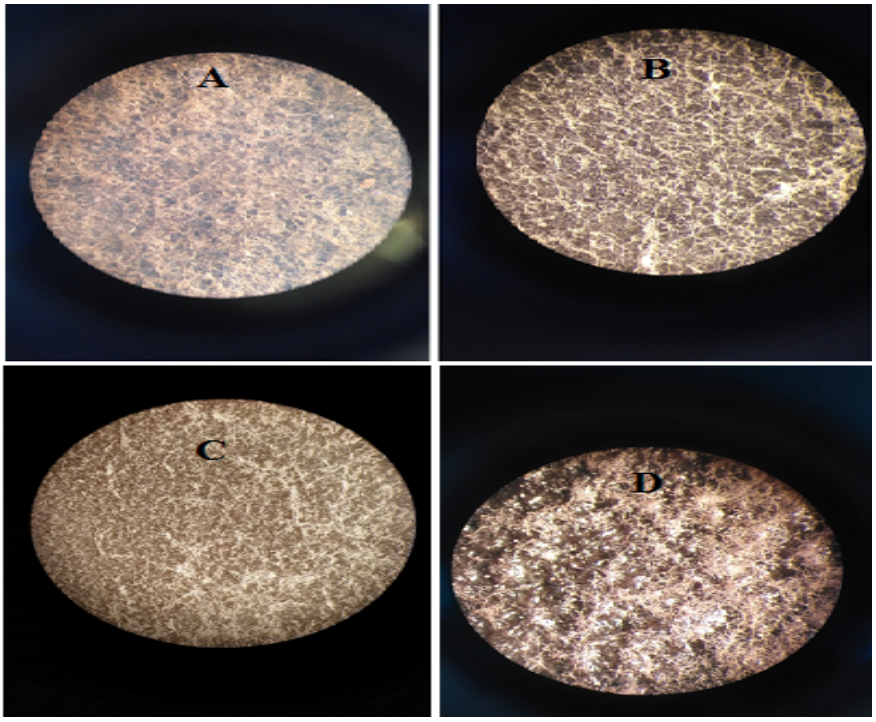

Figure 8: Colony characteristics of *C. manginecans* after different treatments observed under Stereoscope A) *A. altissima* treatment, B) *C. colocynthis* treatment, C) *D. viscosa* treatment, and D) Control.

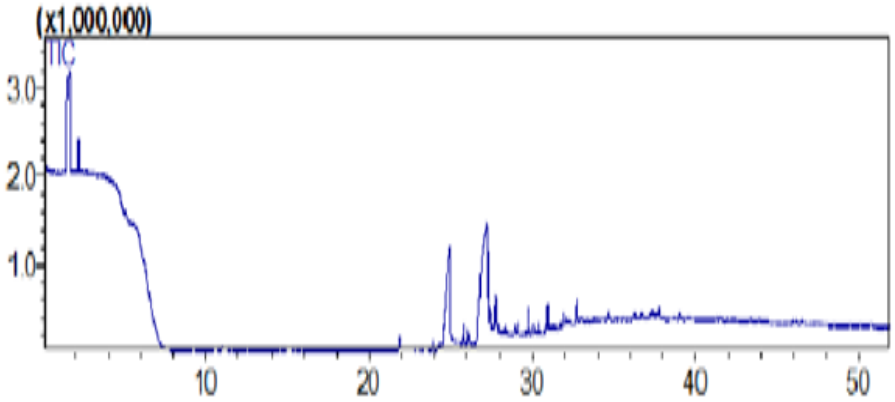

Figure 9: GCMS Chromatogram of phytochemical compounds in *D. viscosa* ethanol extract.

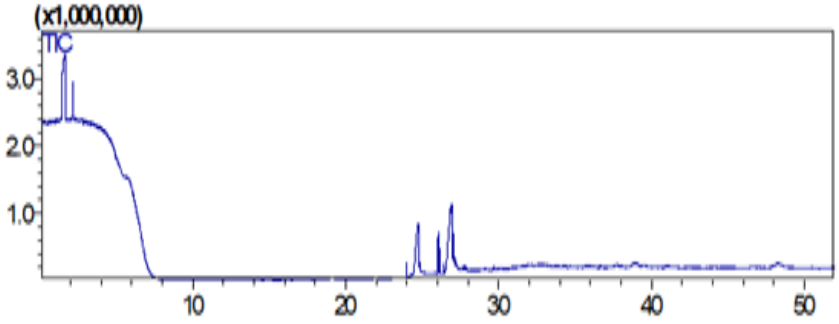

Figure 10: GCMS Chromatogram of phytochemical compounds in *C. colocynthis* extract.

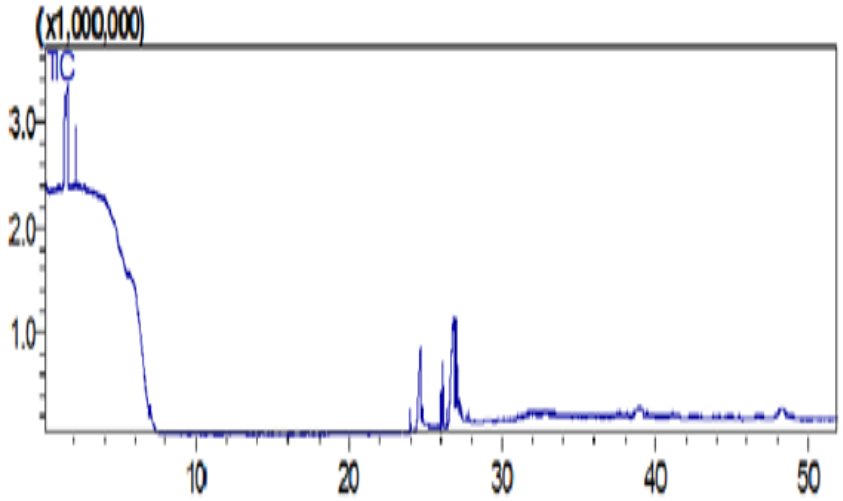

Figure 11: GCMS Chromatogram of phytochemical compounds in *A. altissima* ethanol extract.

determined using serial dilution method by Espinel-Ingroff et al. [19] with slight modification.

Determination of effect of botanical treatments on biomass production of *C. manginecans*: For determination of the effect of selected botanical extracts on the dry weight of *C. manginecans*, 1 ml of each treatment was added to 20 ml sterilized malt extract broth media in 100 ml flask and inoculated with a 5mm disc of *C. manginecans*. Ethanol and methanol control treatment contained 1 ml of respective 5% solvent. The experiment was terminated when complete growth in control plates was observed. Fresh and dry weights of mycelia of *C. manginecans* were determined [20].

Determination of effect of botanical treatments on sporulation and morphological characteristics of *C. manginecans*: Effect of botanical treatments on rate of sporulation was measured at the end of experiment and was compared with control. For conidial count, the conidia were harvested from different treatment plates (botanical and control) by using 10 ml of sterilized distilled water containing Tween 20 (0.05%) and filtered through 8-fold sterile cotton gauze for the removal of residues of growth medium and mycelial content. Conidial count was done using haemocytometer. The effect of botanical treatments on morphological characteristics of *C. manginecans* was determined by a comparative study of structures of Hyphae and conidia in each botanical treatment compared with control under an optical microscope at (40x). Conidial suspension (10µl) was placed on slides under the microscope for determination of the size and shape of conidia. Ten measurements per botanical treatment were recorded.

Statistical analysis

The data was analyzed using Statistix 8.1 software. 3 way factorial design of analysis of variance (AOV) was used to conduct statistical analysis. Mean, standard error of mean, P value, CV, grand mean and LSD were calculated. Data was tested for acceptance or rejection of null hypothesis based on P value.

Phytochemical profiling

Quantitative phytochemical determination using GC-MS: Only highly effective botanicals extracts were used for phytochemical evaluation using method described by Ezhilan and Neelamegam [21] with slight modifications. GC-MS analysis was carried out on Shimadzu comprising of AOC-20i auto-sampler and a gas chromatograph interfaced to a mass spectrometer instrument (Figure 3). GC-MS system consisted of a Column DB-5Ms with 0.25 µm (column diameter), 30 m (column length), and 0.25 µm (column thickness). GC-MS was operating at 70 eV in EI (Electron Impact) mode. Helium gas was used as a carrier gas with constant flow (1.73 ml/min). Injection volume of botanical extract (3 µl) was employed with 10:1 split ratio. Injector temperature was kept 270°C. Ion source temperature was kept 200°C. Mass spectra were taken at 70 eV. Scan interval was 0.5 seconds. Fragments from 40-450Da were used. Total time for one complete GC run was 52.0 minutes. Interpretation on mass spectrum of GC-MS was done by using the database of National Institute Standard and Technology (NIST).

Results and Discussion

Effect of selected botanicals on percent inhibition of *C. manginecans*

In the present study, ethanol, methanol and aqueous botanical crude extracts of *D. viscosa*, *C. colocynthis* and *A. altissima* were evaluated against *C. manginecans* at 2.5 g/100 ml, 5 g/100 ml and 10 g/100 ml extraction concentration. The results revealed that ethanol extracts of *all selected botanicals* produced very significant antifungal activity against *C. manginecans*. However, *D. viscosa* ethanol extract was found the most effective in controlling *C. manginecans* growth at all concentrations tested (Figures 1-3). Ethanol crude extract of *D. viscosa* showed highest antifungal potential (85.066%) causing inhibition of radial mycelial growth at 10 g/100 ml extraction concentration followed by ethanol crude extract of *C. colocynthis* and *A. altissima* with 82.664% and 69.112% inhibition potential respectively. At highest concentration tested, *D. viscosa* methanol crude extract exhibited 77.336% inhibition of colony growth, while, its aqueous crude extract exhibited 58.224% inhibition of fungal growth. Aqueous extract of *A. altissima* was found least effective among all botanical extracts studied against *C. manginecans*. Efficacy of these three botanicals ranged between 50.888% - 85.06% for ethanol extract, 45.334% - 77.36% for methanol extract, and 32.888% - 58.224% for aqueous extracts (Figure 4). These findings were supported by Lawal [22] who revealed that *D. viscosa* exhibited significant antifungal

Treatment	Extraction concentration (g/100 ml)	Conidial Features	Conidial count	Conidial Size Length (µm) ± S.E Mean × Breadth (µm) ± S.E Mean	Conidiophore Character	Conidiophore Size Length (µm) ± S.E Mean × Breadth (µm) ± S.E Mean
Ailanthus altissima	Ethanol 2.5	Hyaline, Cylindrical, with Truncated ends	223 ± 0.6074	46.87 ± 0.3476 × 6. 8 +0.2102	Hyaline, short, tube-like, flaring at mouth	148 ± 1.1247 × 6.7 ± 1.1357
	Ethanol 5	Hyaline, Cylindrical, with Truncated ends	219 ± 0.5774	43. 7 ± 0.2379 × 6.6 ± 0.2123	Hyaline, short, tube-like, flaring at mouth	146 ± 0.4646 × 5.9 ± 0.5126
	Ethanol 10	Hyaline, Cylindrical, with Truncated ends	210 ± 1.2047	41.2 ± 0.2900 × 6.4 ± 0.2004	Hyaline, tube-like, flaring at mouth	149 ± 0.3446 × 6.9 ± 0.2226
	Methanol 2.5	Hyaline, Cylindrical	235 ± 1.1547	47.87 ± 0.3642 × 6. 8 ± 0.2871	Hyaline, tube-like, flaring at mouth	149 ± 0.2786 × 7.6 ± 0.1986
	Methanol 5	Hyaline, Cylindrical	231 ± 0.5774	45.57 ± 0.3516 × 6.6 ± 0.2641	Hyaline, tube-like, flaring at mouth	146 ± 0.6004 × 6.7 ± 0.1190
	Methanol 10	Hyaline, Cylindrical	223 ± 1.1549	41.01 ± 0.2906 × 6.4 ± 0.3645	Hyaline, tube-like, flaring at mouth	148 ± 0.3246 × 7.3 ± 0.2326
	Water 2.5	Hyaline, Cylindrical	236 ± 0.6784	49.87 ± 0.2301 × 6. 8 ± 0.2437	Hyaline, tube-like, flaring at mouth	150 ± 0.2086 × 8.4 ± 0.1091
	Water 5	Hyaline, Cylindrical	238 ± 1.2657	48. 7 ± 0.3986 × 6.6 ± 0.3091	Hyaline, tube-like, flaring at mouth	149 ± 0.2612 × 7.7 ± 0.1346
	Water 10	Hyaline, Cylindrical	241 ± 1.7638	46.2 ± 0.2387 × 6.4 ± 0.2234	Hyaline, tube-like, flaring at mouth	146 ± 0.2106 × 6.7 ± 0.1568
Dodonaea viscosa	Ethanol 2.5	Hyaline, Cylindrical, with Truncated ends	168 ± 1.7321	33.9 ± 0.0623 × 4.7 ± 0.1126	Hyaline, short, tube-like, flaring at mouth	145 ± 0.2546 × 6.2 ± 0.1729
	Ethanol 5	Hyaline, Cylindrical, with Truncated ends	140 ± 2.8868	32.4 ± 0.0523 × 4.6 ± 0.0983	Hyaline, short, tube-like, flaring at mouth	144 ± 0.2343 × 5.8 ± 0.1806
	Ethanol 10	Hyaline, Cylindrical, with Truncated ends	135 ± 2.9068	30.6 ± 0.0882 × 4.5 ± 0.1202	Hyaline, short, tube-like, flaring at mouth	142 ± 0.3245 × 5.1 ± 0.1026
	Methanol 2.5	Hyaline, Cylindrical, with Truncated ends	183 ± 1.1547	33.07 ± 0.1136 × 5.9 ± 0.3180	Hyaline, short, tube-like, flaring at mouth	147 ± 0.4532 × 6.7 ± 0.1978
	Methanol 5	Hyaline, Cylindrical, with Truncated ends	169 ± 0.5974	33.28 +0.2082 × 5.5 +0.3150	Hyaline, short, tube-like, flaring at mouth	145 ± 0.2123 × 5.7 ± 0.1698
	Methanol 10	Hyaline, Cylindrical, with Truncated ends	150 ± 0.8819	37.25 +0.1739 × 5.3 +0.4661	Hyaline, short, tube-like, flaring at mouth	144 ± 0.3125 × 5.5 ± 0.3987
	Water 2.5	Hyaline, Cylindrical	196 ± 0.5974	37.41 ± 0.7234 × 6.2 ± 0.4927	Hyaline, short, tube-like, flaring at mouth	145 ± 0.4632 × 7.8 ± 0.3216
	Water 5	Hyaline, Cylindrical	187 ± 0.9019	37.58 ± 0.3215 × 5.9 ± 0.4612	Hyaline, tube-like, flaring at mouth	148 ± 0.2367 × 7.5 ± 0.4127
	Water 10	Hyaline, Cylindrical	170 ± 0.5774	36.25 ± 0.4485 × 5.7 ± 0.2404	Hyaline, tube-like, flaring at mouth	150 ± 0.2974 × 7.3 ± 0.1432
Citrullus colocynthis	Ethanol 2.5	Hyaline, Cylindrical, with Truncated ends	224 ± 0.8819	38.7 ± 0.2360 × 5.9 ± 0.2913	Hyaline, tube-like, flaring at mouth	150 ± 0.2231 × 7.8 ± 0.1121
	Ethanol 5	Hyaline, Cylindrical, with Truncated ends	210 ± 0.6004	37.2 ± 0.2243 × 5.5 ± 0.3626	Hyaline, short, tube-like, flaring at mouth	146 ± 0.2346 × 6.8 ± 0.2123
	Ethanol 10	Hyaline, Cylindrical, with Truncated ends	198 ± 0.5774	36.5 ± 0.2162 × 5.2 ± 0.3146	Hyaline, short, tube-like, flaring at mouth	145 ± 0.2690 × 5.3 ± 0.1892
	Methanol 2.5	Hyaline, Cylindrical	239 ± 1.1547	48 ± 0.2543 × 6.7 ± 0.3125	Hyaline, tube-like, flaring at mouth	150 ± 0.2046 × 8.5 ± 0.1123
	Methanol 5	Hyaline, Cylindrical	232 ± 1.1607	45 ± 0.2343 × 6.6 ± 0.3276	Hyaline, tube-like, flaring at mouth	147 ± 0.2231 × 7.7 ± 0.1781
	Methanol 10	Hyaline, Cylindrical	220 ± 1.1557	40 ± 0.2126 × 6. 5 ± 0.3298	Hyaline, tube-like, flaring at mouth	149 ± 0.2006 × 5.6 ± 0.1934
	Water 2.5	Hyaline, Cylindrical	243 ± 1.1597	39.09 ± 0.2213 × 6.8 ± 0.2109	Hyaline, tube-like, flaring at mouth	148 ± 0.2902 × 7.5 ± 0.2134
	Water 5	Hyaline, Cylindrical	238 ± 1.1547	43.06 ± 0.2568 × 6.5 ± 0.2378	Hyaline, tube-like, flaring at mouth	147 ± 0.2398 × 6.9 ± 0.3478
	Water 10	Hyaline, Cylindrical	230 ± 2.8868	41.9 ± 0.2903 × 6.2 ± 0.2267	Hyaline, tube-like, flaring at mouth	150 ± 0.3241 × 7.4 ± 0.1604
Control	Ethanol	Hyaline, Cylindrical	244 ± 1.1497	50.87 +0. 2154 × 6.98 ± 0.3676	Hyaline, long tube-like, flaring at mouth	148 ± 0.2213 × 6.8 ± 0.1187
	Methanol	Hyaline, Cylindrical	247 ± 1.1550	50.57 ± 0. 3646 × 7.01 ± 0.2646	Hyaline, long tube-like, flaring at mouth	147 ± 0.2987 × 7.9 ± 0.1456
	Water	Hyaline, Cylindrical	250 ± 1.4530	51.01 ± 0.2846 × 7.01 ± 0.4646	Hyaline, long tube-like, flaring at mouth	150 ± 0.4512 × 8.5 ± 0.2126

Table 2: Effect of botanical treatments on characteristics of conidia of *C. manginecans.*

Treatment	Extract Concentration	Colony color	Colony margins	Hyphae Character
Dodonaea viscosa	Ethanol 2.5	Whitish grey	Irregular, Wavy	Thin, segmented
	Ethanol 5	Whitish grey	Irregular, Wavy	Thin, segmented
	Ethanol 10	Whitish grey	Irregular, Wavy	Thin, segmented
	Methanol 2.5	Whitish grey	Irregular, Wavy	Thin, segmented
	Methanol 5	Whitish grey	Irregular, splitted	Thin, segmented
	Methanol 10	Whitish grey	Irregular, splitted	Thin, segmented
	Water 2.5	mouse grey	Regular	Slightly thin, and segmented
	Water 5	mouse grey	Regular	Slightly thin, segmented
	Water 10	mouse grey	Regular	Slightly thin, segmented
Citrullus colocynthis	Ethanol 2.5	Mouse grey	Irregular, Wavy	Slightly thin, segmented
	Ethanol 5	Mouse grey	Irregular, splitted	Slightly thin, segmented
	Ethanol 10	Mouse grey	Irregular, Wavy	Thin, segmented
	Methanol 2.5	mouse grey	Irregular, Wavy	Slightly thin, segmented
	Methanol 5	mouse grey	Irregular, Wavy	Slightly thin, segmented
	Methanol 10	mouse grey	Irregular, Wavy	Slightly thin, segmented
	Water 2.5	Greyish brown	Regular	Thick, segmented
	Water 5	Greyish brown	Regular	Thick, segmented
	Water 10	Greyish brown	Regular	Thick, segmented
Ailanthus altissima	Ethanol 2.5	Mouse grey	Irregular, Wavy	Slightly thin, segmented
	Ethanol 5	Mouse grey	Irregular, Wavy	Slightly thin, segmented
	Ethanol 10	Mouse grey	Irregular, splitted	Slightly thin, segmented
	Methanol 2.5	mouse grey	Regular	Thick, segmented
	Methanol 5	mouse grey	Regular	Thick, segmented
	Methanol 10	mouse grey	Regular	Thick, segmented
	Water 2.5	mouse grey	Regular	Thick, Smooth, segmented
	Water 5	mouse grey	Regular	Thick, Smooth, segmented
	Water 10	mouse grey	Regular	Thick, Smooth, segmented
Control	Ethanol	Mouse grey	Regular	Thick, Smooth, segmented
	Methanol	Mouse grey	Regular	Thick, Smooth, segmented
	Water	Mouse grey	Regular	Thick, Smooth, segmented

Table 3: Effect of different treatments on colony and hyphae character of *C. manginecans*.

Peak #	R.T	Area %	Height %	Compound Name	Molecular Weight	Molecular formula
1	24.79	14.32	13.07	Hexadecanoic acid, methyl ester $$ Palmitic acid	284	$C_{18}H_{36}O_2$
2	24.905	5.71	14.87	l-(+)-Ascorbic acid 2,6 Hexadecanoate	652	$C_{38}H_{68}O_8$
3	27.159	44.54	18.99	Octadec- 9-enoic acid $$ (9E)-9-Octadecanoic acid	282	$C_{18}H_{34}O_2$
4	27.415	4.14	5.22	cis-11,14-Eicosadienoic acid, methyl ester	322	$C_{21}H_{38}O_2$
5	27.76	5.23	5.41	9,12-Octadecadienoic acid (Z,Z)	280	$C_{18}H_{32}O_2$
6	28.315	2.45	1.76	Glycidol Stearate $$ Glycidol Octadecanoate	340	$C_{21}H_{40}O_3$
7	28.89	1.72	1.65	1,2-Oxathiane, 6-dodecyl-, 2,2-dioxide	304	$C_{16}H_{32}O_3S$
8	29.09	1.02	2.31	Eicosanoic acid $$ Arachic acid	312	$C_{20}H_{40}O_2$
9	29.688	1.47	4.06	Oleoyl chloride $$ Oleic acid chloride	300	$C_{18}H_{33}ClO$
10	30.949	1.41	3.82	Octadecanoic acid, 1-[[(1-oxohexadecyl)oxy]methyl]-1-,2- ethanediyl ester	862	$C1_{55}H_{106}O_6$
11	31.889	0.5	1.53	1,2-Butanediol, 1-(2-furyl)-3-methyl-1,2-butanediol	170	$C1_9H_{14}O_3$
12	32.685	1.67	3.73	Oleic acid, (2,2-dimethyl-1,3-dioxolan-4-yl)methyl ester	396	$C_{24}H_{44}O_4$
13	37.831	1.82	1.6	Stigmast-5-en -3-ol, Oleate	678	$C_{47}H_{82}O_2$

Table 4: Phytochemical compounds in ethanol crude extract of *D. viscosa*, Identified and characterized through GCMS and confirmed by matching with NIST Library.

Peak #	R.T.	Area %	Height %	Compound Name	Molecular Weight	Molecular formula
1	23.967	1.02	3.85	Hexadecanoic acid, methyl ester $$ Palmitic acid	270	$C_{17}H_{34}O_2$
2	24.741	16.81	12.62	l-(+)-Ascorbic acid 2,6 Hexadecanoate	652	$C_{38}H_{68}O_8$
3	24.815	0.78	2.24	Octadecanoic acid, methyl ester $$ Stearic acid	312	$C_{20}H_{40}O_2$
4	26.049	3.91	10.71	9,12-Octadecadienoic acid (Z,Z)	294	$C_{19}H_{34}O_2$
5	26.116	1.77	6.75	9-Octadecenoic acid, methyl ester, (E)-	296	$C_{19}H_{36}O_2$
6	26.404	0.7	2.66	Methyl Strearate	298	$C_{19}H_{38}O_2$
7	26.815	21.23	16.28	n-propyl 9,12-Octadecadienoate	322	$C_{21}H_{38}O_2$
8	26.93	21.25	17.04	9-Octadecenoic acid	282	$C_{18}H_{34}O_2$

Table 5: Phytochemical compounds in ethanol crude extract of *C. colocynthis*, Identified and characterized through GCMS and confirmed by matching with NIST Library.

Peak #	R.T	Area %	Height %	Compound Name	Molecular Weight	Molecular formula
1	24.666	15.79	17.37	I-(+)-Ascorbic acid 2,6 Hexadecanoate	652	$C_{38}H_{68}O_8$
2	26.826	31	22.15	Oleic acid, $$ 9-Octadecenoic acid (Z)-	282	$C_{18}H_{34}O_2$
3	26.987	2.83	8.32	Octadecanoic acid $$ Stearic acid	284	$C_{18}H_{36}O_2$

Table 6: Phytochemical compounds in ethanol crude extract of *A. altissima*, Identified and characterized through GCMS and confirmed by matching with NIST Librar.

efficacy and inhibited the colony growth of *Alternaria solani* followed by *Macrophomina phasiolina*, and *Rhizoctonia solani*. Similarly, Vijaya et al. [23] evaluated the effectiveness of extracts of some botanicals against *Ceratocystis sp.* causing sett rot of the sugarcane. They found that at concentration of 10% garlic extract was most effective with 53.13% inhibition of mycelial growth of *Ceratocystis sp.* followed by neem extract 48.35%, durantha extract 45.62%, pongamia extract 43.22% and glyricidia extract 40.33%. Haripyaree et al. [24] reported methanolic extract of *Mimosa pudica* showed the highest and significant inhibitory effect against *Ceratocystis sp*. with MIC values of of M. *pudica* distilled water, methanol and n-hexane extracts as 2.50 mg/ml, 1.25 mg/ml, and 0.62 mg/ml respectively. Completely randomized design of experiment was used for all experiments. Three way factorial design of analysis of variance (AOV) was used to conduct statistical analysis using Statistix version 8.1software. Mean, standard error of mean, P value, CV, grand mean and LSD were calculated. Data was tested for acceptance or rejection of null hypothesis based on P value. Data obtained from *in-vitro* antifungal evaluation of botanicals revealed that all the treatment means were significantly different from each other. P value less than 0.05 rejected null hypothesis and accepted researcher hypothesis. An interaction of antifungal efficacy of different botanicals and their solvents and treatments was highly significant (Table 1). Dose-response relationship of *D. viscosa* extracts and colony growth inhibition of *C. manginecans* presented very strong positive correlation. Strongest correlation was observed in *D. viscosa* ethanol crude extract concentrations and percent inhibition of colony growth of *C. manginecans*. It indicates that per unit increase of *D. viscosa* ethanol crude extract concentration resulted in 12.755 times increase in percent inhibition of colony growth of *C. manginecans*. R^2=0.9946 indicates that 99.46% relationship was correctly explained. Straight line represented that increase in extract concentration also brings respective increase in inhibition percentage (Figure 5). Present study revealed that MIC and MFC values of *D. viscosa* crude ethanol extract were 12 mg/ml and 14 mg/ml respectively. MIC values of current study were also supported by Esmaeel and Al-Jaburi [25] who reported that *D. viscosa* leaves ethanol extracts exhibited MIC values ranging from 2.5 mg/ml to 10 mg/ml. The percent inhibition and MIC values revealed that *D. viscosa* ethanol extract is most effective among all the botanicals studied against *C. manginecans*. It was observed that an increase in botanical concentration in MEA (substrate) increases the percent inhibition of *C. manginecans*. Comparable effects of various botanicals against other phytopathogens have been observed and reported by Perello et al. and Bahadar et al. [26,27].

Effect of botanical treatments on biomass production of *C. manginecans*

Results of present *in-vitro* evaluation of botanical extracts revealed that ethanol crude extract of *D. viscosa* brings significant dry biomass reduction of *C.* manginecans (Figure 6). It was found that greater the colony growth inhibition potential of extract, more reduced biomass of fungus. These findings were in accordance with Hassan et al. [28] that different botanicals treatments significantly reduce fungal hyphae mass.

Effect of botanical treatments on sporulation rate and morphological characteristics of *C. manginecans*

It was observed that *C. manginecans* sporulation rates as well as conidial count, size of conidia and conidiophores were also affected by botanical treatments. Conidiophores and conidial size were significantly reduced by ethanol crude extracts of *D. viscosa* treatment application (Table 2). The sporulation rate decreases with an increase in concentration of botanical treatments. This result is supported by the findings of Elisabeth Bach et al. [29]; and sing et al. [30] who studied some different microbes with different botanical extracts and reported variations in conidiophores and conidial characteristics due to botanical extract treatments. Kessler et al. [31]; Omidbeygi et al. [32] described that secondary metabolites present in botanicals can pass through cell membranes and interact with critical sites (intracellular enzymes and proteins), resulting in structural and functional variations of fungal pathogen. It was observed during *in-vitro* evaluation that an increase in concentration of different botanical extract treatments results in variation of colony color, margin, thickness and texture of hyphae of *C. manginecans*. Present study revealed that ethanol crude extracts of *D. viscosa* treatment application resulted in initial bright grey colony color which turned greyish brown later, submerged mycelial appearance and irregular (wavy or splitted) colony margins as compared to control treatments which had mouse grey colony color, slightly aerial mycelial appearance and regular colony margins (Table 3). The *C. manginecans* hyphae of botanical treatments plates were thin, collapsed/damaged as compared to the hyphae of control treatment plates (Figures 7 and 8). Similar findings were observed by Hashem et al. [33] who revealed that several compounds present in each botanical extract act synergistically to destruct fungal cell structure and function by causing their death. They also observed untreated mycelia were well-developed, inflated having smooth wall), while, treated mycelia were plasmolyzed, distorted, squashed and collapsed hyphae and completely dead. Current findings were also agreed with the observations of Khan and Zhihui [34] who reported that natural compounds affect hyphae morphology of different fungi and result in collapsed and thin hyphae. This supported and justified that the phytochemical compounds of *D. viscosa* had the similar kind of mechanism for morphological modifications in *C. manginecans*.

Quantitative phytochemical determination using GC-MS

Identification and characterization of phytochemical compounds through GCMS technique in present study revealed that most of the detected compounds were either esters or derivatives of ester compounds. Total 13 compounds were detected in *D. viscosa* crude ethanol extract (Figure 9). *D. viscosa* crude ethanol extract was found having Hexadecanoic acid, methyl ester ; I-(+)-Ascorbic acid 2,6 Hexadecanoate; (9E)-9-Octadecanoic acid; cis-11,14-Eicosadienoic acid, methyl ester; 9,12-Octadecadienoic acid (Z,Z); Glycidol Octadecanoate; 1,2-Oxathiane, 6-dodecyl-, 2,2-dioxide; Eicosanoic acid; Oleoyl chloride; Octadecanoic acid, 1-[[(1-oxohexadecyl)oxy] methyl]-1-,2-ethanediyl ester; 1,2-Butanediol, 1-(2-furyl)-3-methyl-1,2-butanediol; Oleic acid, (2,2-dimethyl-1,3-dioxolan-4-yl)methyl ester; and Stigmast-5-en -3-ol, Oleate**.** Details of all 13 compounds

detected are elucidated in Table 4. Similarly, *C. colocynthis* crude ethanol extract was found with eight compounds (Figure 10). These compounds were identified as Hexadecanoic acid, methyl ester; I-(+)-Ascorbic acid 2,6 Hexadecanoate; Octadecanoic acid, methyl ester; 9,12-Octadecadienoic acid (Z,Z); 9-Octadecenoic acid, methyl ester; Methyl Strearate; n-propyl 9,12-Octadecadienoate;9-Octadecenoic acid. Details of all O_8 compounds detected are elucidated in Table 5. In addition, *A. altissima* was found with only three compounds (Figure 11). These three compounds were I-(+)-Ascorbic acid 2,6 Hexadecanoate; 9-Octadecenoic acid (Z); and Octadecanoic acid. Details of all O_3 compounds detected are elucidated in Table 6. 9-Octadecanoic acid (molecular weight 282g/mol and molecular formula $C_{18}H_{34}O_2$) and I-(+)-Ascorbic acid 2,6 Hexadecanoate (molecular weight 652g/mol and molecular formula $C_{38}H_{68}O_8$) were observed in all three ethanol crude botanicals indicating their possible antifungal role against *C. manginecans* causing MSD. These findings were also supported by Hou and Forman [35] who described that 12,13,17-trihydroxy-9(*Z*)-Octadecenoic acid exhibited antifungal efficacy against *Phytophthora infestans*, *Botrytis graminis*, and *Phytophthora recondite (phytopathogenic fungi)*. Similarly, Bokhari et al. [36] who revealed that GC-MS analysis of crude ethanol extract of *C. colocynthis* contained the major components Eicosanoic acid, 2-Heptadecenal and l-(+)-Ascorbic acid 2, 6-dihexadecanoate, were responsible for antifungal efficacy. Moreover, Yoon et al. [37] reported that Octadeca-9,11,13-triynoic acid and *trans*-octadec-13-ene-9,11-diynoic acid exhibited colony growth inhibition of selected phytopathogenic fungi. These fatty acids can be used as alternative methods for integrated management of phytopathogenic microbes.

Conclusion

All botanical treatments (ethanol, methanol and aqueous extracts of *D. viscosa, Citrullus colocynthis,* and *Ailanthus altissima*) were effective for inhibition *Ceratocystis manginecans* colonies growth and conidial germination. The ethanol extracts were highly effective as compared to methanol and water extracts of selected plants against *Ceratocystis manginecans*. *D. viscosa* ethanol crude extract exhibited highest antifungal efficacy followed by *Citrullus colocynthis* and *Ailanthus altissima*. Botanical treatments resulted in thin, collapsed/damaged hyphae as compared to control. Phytochemical profiling of highly effective botanicals revealed that 9-Octadecanoic acid and I-(+)-Ascorbic acid 2, 6 Hexadecanoate were found common in all three most effective botanicals. The present study revealed that these compounds possibly contributed to the antifungal efficacy of botanicals against *C. manginecans.*

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