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Phytochemical Identification and Comparative Antifungal Efficacy of *Dodonaea viscosa* L. Fractions against *Ceratocystis manginecans*

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Key words: *D. viscosa* Ethyl acetate fraction, *Ceratocystis manginecans*, Mango Sudden Death, Phytochemicals, 9-Octadecanoic Acid.

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Abstract

Mango Sudden Death (MSD) is caused by *Ceratocystis manginecans*. It is an important disease distressing mango production in major mango growing countries of the world. Synthetic fungicide applications help to control MSD but biological control of MSD can be eco-friendly and economically efficient approach. Current study was intended to assess antifungal potential of botanical fractions of *Dodonaea viscosa* L. against *Ceratocystis manginecans*. Liquid extraction of crude ethanol extracts of *D. viscosa* was performed using four solvents with an increasing polarity order i.e. n-Hexane, Dichloromethane (DCM), Ethyl Acetate (EA) and n-Butanol. *In-vitro* Antifungal potential assessment by poisoned food technique revealed that Ethyl Acetate fraction (40mg/ml) exhibited highest potential (75.178%) to control growth of *C. manginecans*. MIC value of Ethyl Acetate fraction was recorded 8mg/ml. Phytochemical analysis of Ethyl Acetate fraction through GCMS identified presence of Oleoyl Chloride ($C_{18}H_{33}ClO$), Octadecanoic Acid ($C_{155}H_{106}O_6$), Oleic Acid ($C_{24}H_{44}O_4$), [I-(+)-Ascorbic Acid 2,6 Hexadecanoate] ($C_{38}H_{68}O_8$), [(9E)-9-Octadecanoic Acid] ($C_{18}H_{34}O_2$), [9,12-Octadecadienoic Acid (Z,Z)] **(**$C_{18}H_{32}O_2$), [1,2-Oxathiane, 6-Dodecyl-, 2,2-Dioxide] ($C_{16}H_{32}O_3S$), and 1,2-Butanediol ($C_{19}H_{14}O_3$). These phytochemical compounds of Ethyl Acetate fraction potentially contributed to control *C. manginecans* growth. According to our knowledge this is first study to explore fractions of *D. viscosa* ethanol crude extract for the control of *C. manginecans*.

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Introduction

Mango (*Mangifera indica* L. of family *Anacardiaceae*) is Pakistan's major fruit cash crops. Over 250 mango cultivars are reported in Pakistan and most important commercial mango cultivars include Chaunsa, Anwar Ratul, Langra, Sindhri, Dasehri, Fajri and Maldha (Javaid *et al.*, 2012). Mango crop is affected by various diseases including Malformation, Anthracnose, Sudden Death, and Powdery Mildews (Malik *et al.*, 2005). MSD is most holistic. (Naqvi and Parveen, 2015)

MSD is an economically important disease distressing mango orchard productivity worldwide. In Pakistan (1995), MSD was first reported from Punjab province and subsequently it spread in Sindh and Khyber Pakhtunkhwa due to movement of the diseased materials (Mahmood *et al.*, 2007). *Ceratocystis manginecans* is the causal agent of MSD (Rashid *et al.*, 2014). First MSD Symptoms is the drooping and drying of leaf, followed by splitting of bark, and liquid oozing in collar region. Gummosis symptom appears on very late stage of MSD and finally mortality of whole mango tree occurs. (Van-Wyk *et al.*, 2007).

MSD management practices include the cultural practices, synthetic fungicides usage and host plant resistance. Although Cultural practices vary from one place to other, however, they help to optimize resources for sustainable development of mango orchard production and disease management Mostly farmers use synthetic fungicides but due to the higher cost of synthetic fungicides and environmental concerns eventually limit their usage to the small scale farmers. (Kazmi *et al.*, 2007). The indiscriminate usage of fungicides can develop resistance in pathogens as well. Hence, alternative methods of disease/pathogen control should be explored. Plants are comprised of certain chemical components that are found toxic to the pathogens. These chemical components (botanicals), when extracted and applied on infested crop, act as botanical pesticide. Sonyal *et al.* (2015) assessed selected botanicals for antifungal potential to control pomegranate wilt pathogen- *Ceratocystis fimbriata*. They revealed that *Allium sativum* exhibited 32.96% Colony growth inhibition followed by *Zingiber officinale* 32.90% Colony growth inhibition of *C. fimbriata*. While, *Eucalyptus* botanical was observed least effective (11.47% Colony growth inhibition). Similarly, Poussio *et al.* (2016) assessed the *in-vitro* efficacy of the selected botanicals (*Nicotiana tabacum, Citrullus colocynthis* and *Azadirachta indica*) against *Ceratocystis* species. They revealed that 8ml of *C. colocynthis* botanical was found most effective among botanicals studied against *Ceratocystis fimbriata*. Lawal *et al.* (2013) reported significant antifungal efficacy of *D. viscosa* botanical against selected pathogens (*Alternaria solani*, and *Macrophomina phasiolina*). Prakash *et al.* (2012) also revealed in a study that methanol crude extract of *D. viscosa* had great potential for Colony growth inhibition of *Fusarium oxysporum*. Manjulatha (2012) also observed good *in-vitro* antifungal efficacy of *D. viscosa* botanical extract against selected pathogens. Similarly, Khurram (2009) and Nagabaza (2016) also revealed from a study that *D. viscosa* botanical exhibited high antimicrobial potential against selected human pathogens. Research has strengthened to develop botanical fungicides against pathogens in agriculture sector. These botanical fungicides had potential to inspire present agro-chemical research field. Botanical fungicides can help to diminish the damaging effects of the synthetic fungicides usage like residual effects, pathogen's resistance development and environmental pollution. Liquid-liquid extraction of botanicals results in separation of phytochemical constituents because they migrate differently on the basis of polarity. Botanicals consisted of various unknown phytochemicals (organic in nature) that are identified by GCMS (Stashenko and Martinez, 2014). Keeping in view, current study was aimed to assess selected fractions of *D. viscosa* to control growth of *C. manginecans*. Botanical selection was based on traditional knowledge (an antifungal source) and scientifically known potential to control human pathogens. Objectives of this study included *In-vitro* antifungal efficacy of different fractions of *Dodonaea viscosa* ethanol crude extract and phytochemicals

identification of most effective fraction against *C. manginecans*.

Material and methods

Sample preparation

Disease free, fresh leaves of *D. viscosa* were collected for sample preparation using slightly modified method described by Ambikapathy *et al.* (2011). After collection of *D. viscosa* leaves, sample was washed with water (to remove inert material) followed by surface disinfection (10% Clorox) and again washed with water. Plant material was air dried, filled in paper bags, 24 hours oven dried at 35˚C and then grounded into a fine powder. Ten grams dried plant leaf powder was added in 100 ml of ethanol and flask was placed on a mechanical shaker (at 60 rpm for 3 days). Organic solvents from the sample was filtered using filter paper (Whatman's filter paper #1). Solvent was evaporated from filtrate through rotary evaporator. Solvent free *D. viscosa* leaf extract was kept in glass vials. A 100gm of dried *D. viscosa* ethanol crude extract was pooled for fractionation.

Sequential liquid extraction

An aqueous suspension (500 ml) of *D. viscosa* ethanol crude extract (100gm) was used for fractionation following slightly modified method reported by Mahlo *et al.* (2016). Fractionation was performed using increasing order of solvent's polarity i.e. *n*-hexane → dichloromethane (DCM) → ethyl acetate (EA) → *n*-butanol. Each solvent (500 ml) was poured in separating funnels of 2000ml capacity for fractionation. All botanical fractions obtained in this process were filtered with Whatman's filter paper #1 and subsequently made solvent free through rotary evaporator (at 45 ± 5°C). The last water soluble part of the botanical fraction was evaporated by using a water bath. Finally, all these botanical fractions were stored (at 4°C) for further analysis.

Culture maintenance of fungal pathogen

Ceratocystis manginecans culture was maintained on the MEA (Malt Extract Agar), and one week old fungal culture was utilized for the *in-vitro* experiments.

Antifungal Evaluation

In-vitro assessment of botanical fractions was conducted through poisoned food technique as described by Bahadar *et al.* (2016) with a slight modification. Two concentrations (20 mg/ml and 40 mg/ml) of botanical fraction were amended in the autoclaved MEA (100 ml per flask for each treatment). *Ceratocystis manginecans* culture media disk (5 mm) was placed in the center of the plates and kept in an incubator (at 25 ± 2˚C). Without any botanical fraction (1% of solvent only) was used as control and Topsin-M served as a positive control. *C. manginecans* colony growth and colony morphology (shape and colour) was assessed in botanical treatments with reference of control treatments. CRD (Completely Randomized Design of experiment) was used and data obtained was statistically analyzed with Statistix software (8.1 version). Jorgenson and Ferraro (2009) method was used with slight modification to find MIC value of the most effective botanical fraction of *D. viscosa*. Serial dilutions of botanical fraction (18mg/ml to 4mg/ml) were used. Broth medium (autoclaved) was amended with 1ml of botanical fraction and poured in the sterile glass vials, later, inoculated with standard *C. manginecans* suspension (1ml) was incubated at 25±2 ˚C for 72hrs. Positive and negative control were also used. Three replicates for each treatment were used. Treatments were observed for the broth media turbidity showing *C. manginecans* growth. MIC was denoted by the lowest concentration of botanical fraction at which the growth of *C. manginecans* was inhibited.

Phytochemical identification

Most effective botanicals fraction against *C. manginecans* was subjected to GCMS analysis following slightly modified method (Ezhilan and Neelamegam, 2012). Shimadzu 2010 (GC-MS system) specifications included an auto-sampler (AOC-20i) with a gas-chromatograph interfaced to a mass-spectrometer instrument, DB-5Ms Column having 0.25µm diameter, 30m length, and 0.25µm thickness. GCMS operated at 70eV in Electron Impact mode with 1.73ml/min constant flow of carrier gas (Helium). Botanical fraction (Injection volume -3µl)

was used at 10:1 SR (split ratio) with 270°C (Injector temperature). Ion source temperature was set at 200°C and Mass spectra was taken at 70eV with Scan interval (0.5 seconds) and Fragments (40 - 450Da). Total 52.0 minutes time was set for one complete GC run (Table 1). GC-MS mass spectrum interpretation of was carried out through the database of NIST (National Institute Standard and Technology).

Results

Antifungal evaluation

Botanical fractions of solvents (*n*-hexane, EA, and n-butanol) having density lesser than the water were collected from upper layer of the fraction. While botanical fraction of dichloromethane (having density higher than water) was collected from bottom layer. *In vitro* antifungal evaluation of *D. viscosa* botanical fractions revealed that ethyl acetate fraction (at 40mg/ml and 20mg/ml) exhibited highest colony growth inhibition 75.178% and 58.32 % followed by the n-butanol botanical fractions that exhibited with colony growth inhibition 47.888% and 34.178% respectively. (Fig. 1 and 2). Statistically, botanical fractions showed significantly varying antifungal potential from each other against colony growth of *C. manginecans* (Table 2). MIC techniques was used to determine the definitive antimicrobial properties of highly effective botanical fraction. MIC value for *D. viscosa* ethyl acetate fraction was observed 8mg/ml. Colony morphology of *C. manginecans* after botanical fraction treatments were also observed. It was observed that *C. manginecans* colony in the *D. viscosa* botanical treated plate had initial bright grey color (later turned to greyish brown), irregular (wavy) margins and submerged mycelial growth. While, the control treatment plate was observed with a *C. manginecans* colony having mouse grey colour, regular margins and slightly aerial mycelial growth.

Table 1. The Oven temperature program of GCMS**.**

STEP	Rate (°C/min)	Hold Time (minutes)	Final Temperature (°C)
0	-	02	40
1	08	0.0	150
2	08	0.0	250
3	08	20	280

Table 2. Analysis of Variance for effect of Dodonaea viscosa fractions on Percent Inhibition of *C. manginecans* showing that selected fractions had significantly different antifungal potential against *C. manginecans*.

Source	DF	SS	MS	F	P
Treatment	6	27314.2	4552.36	5530.74	0.0000
Concentration	1	2064.4	2064.38	2508.05	0.0000
Treatment*Concentration	6	563.6	93.94	114.13	0.0000*
Error	56	46.1	0.82		
Total	69	29988.3			
Grand Mean 37.827 CV 2.40					

*Highly significant at α level (0.05).

Phytochemical identification

The phytochemical compounds of *D. viscosa* botanical fraction of ethyl acetate were identified and characterized by GCMS technique and were confirmed by matching with the NIST Library.

It was revealed that the compounds detected and identified in botanical fraction were either Esters or the derivatives of Esters. EA fraction of *D. viscosa* was observed with total eight compounds i.e. I-(+)-Ascorbic acid 2,6 Hexadecanoate; Octadec- 9-enoic acid $$ (9E)-9-Octadecanoic acid; 9,12-Octadecadienoic acid (Z,Z); 1,2-Oxathiane, 6-dodecyl-, 2,2-dioxide; Oleoyl chloride $$ Oleic acid 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 (Table 3). At RT of 26.825 a peak with an area (44.54%) and height (18.99%) was identified as Octadec-9-enoic acid $$

(9E)-9-Octadecanoic acid exhibiting 282g/mol molecular weight and $C_{18}H_{34}O_2$ molecular formula (*Fig*. 3). At 28.89 RT, another peak having 31% area and 22.15%height was identified as 1,2-Oxathiane, 6-dodecyl-,2,2-dioxide exhibiting 304g/mol molecular weight and $C_{16}H_{32}O_{3S}$ molecular formula.

Table 3. Phytochemical Constituents of D. viscosa Ethyl acetate fraction Identified through GCMS and confirmed using NIST Library**.**

Peak No.	Start time	End time	Retention time	Area %	Height %	Name of Compound	Molecular weight	Molecular formula	M/	A/	MAR
1	24.84	25.04	24.905	5.71	14.87	I-(+)-Ascorbic acid 2,6 Hexadecanoate	652	$C_{38}H_{68}O_8$	TI	5	V
2	26.49	26.92	26.826	44.54	18.99	Octadec- 9-enoic acid $$ (9E)-9-Octadecanoic acid	282	$C_{18}H_{34}O_2$	TI	30	
3	27.64	28.04	27.76	5.23	5.41	9,12-Octadecadienoic acid (Z,Z)	280	$C_{18}H_{32}O_2$	TI	12	V
4	28.515	28.99	28.89	31	22.15	1,2-Oxathiane, 6-dodecyl-, 2,2-dioxide	304	$C_{16}H_{32}O_3S$	TI	12	V
5	29.29	29.865	29.688	1.47	4.06	Oleoyl chloride $$ Oleic acid chloride	300	$C_{18}H_{33}ClO$	TI	4	V
6	30.69	31.04	30.949	21.25	17.04	Octadecanoic acid, 1-[(1-oxohexadecyl)oxy]methyl]-1-,2-ethanediyl ester	862	$C_{155}H_{106}O_6$	TI	9	
7	31.765	31.99	31.889	0.5	1.53	1,2-Butanediol, 1-(2-furyl)-3-methyl-1,2-butanediol	170	$C_{19}H_{14}O_3$	TI	4	
8	32.515	32.815	32.685	1.67	3.73	Oleic acid, (2,2-dimethyl-1,3-dioxolan-4-yl)methyl ester	396	$C_{24}H_{44}O_4$	TI	5	

Discussion

It was revealed that Ethyl acetate fraction of *D. viscosa* botanical exhibited highest *In-vitro* antifungal potential against *C. manginecans*. Khurram *et al*. (2009) reported similar findings that the botanical fractions of *D. viscosa* exhibited highly effective for colony growth inhibition of selected pathogens. Nagabaza (2016) reported that n-Hexane/Ethyl Acetate fraction (30:70) fractions obtained from *D. viscosa* exhibited significantly high antimicrobial efficacy against selected human pathogens. Jabeen *et al*. (2018) also observed that ethanol extract of *D. viscosa* showed highest colony growth inhibition (85 %) followed by 82% inhibition potential of *Citrullus colocynthis* and 69% inhibition potential of *Ailanthus altissima* against *Ceratocystis species*.

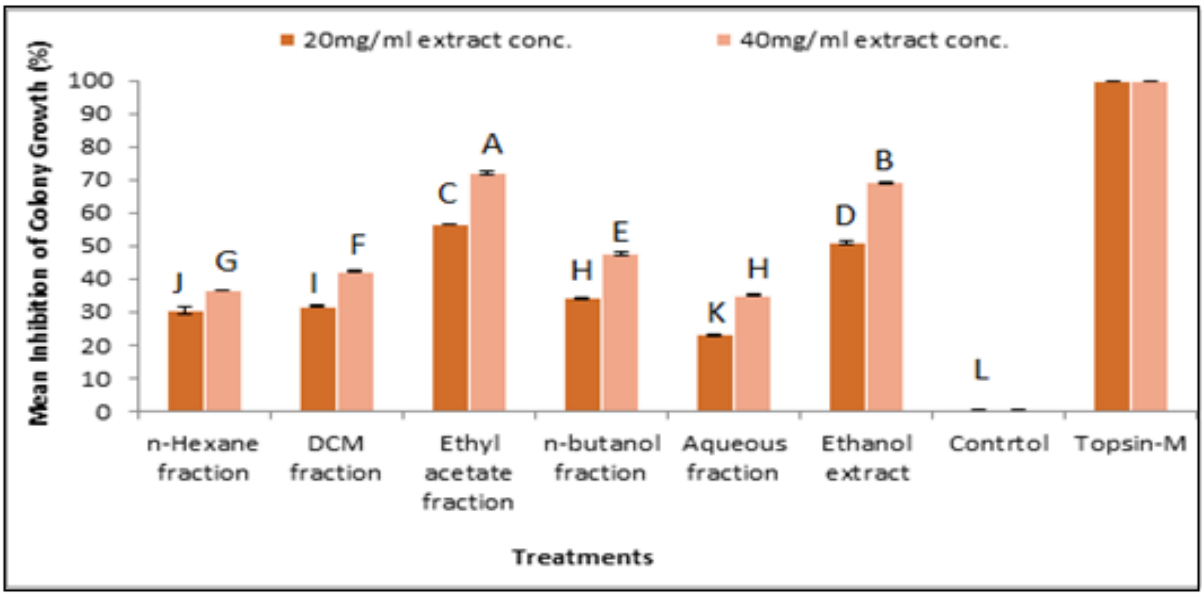

Fig. 1. Efficacy of *D. viscosa* fractions for *C. manginecans* mycelial growth inhibition indicating highest colony growth inhibition by Ethyl acetate fraction.

*At LSD $_{(0.05)}$ = 1.1494, Means followed by same letter are not significantly different at 5% level.

In present study, MIC value for *D. viscosa* botanical (ethyl acetate fraction) was recorded 8mg/ml. However, Esmaeel and Al-Jaburi (2011) reported MIC values for *D. viscosa* ethanol crude extracts ranged between 2.5mg/ml - 10mg/ml against selected pathogens. Present study revealed that botanical fraction treatments altered the *C. manginecans* colony morphology as compared to control. Hashem *et al.* (2016) also reported that several phytochemical compounds present in botanicals synergistically act to destruct the fungal cell structure/function resulting in death of pathogen. Present study findings were also agreed with the observations reported by Khan and Zhihui (2010); Singh *et al.* (2011); Hasan *et al.* (2012); and Perello *et al.* (2013) who revealed that the phytochemicals present in botanicals affect the colony morphology of various pathogenic fungi.

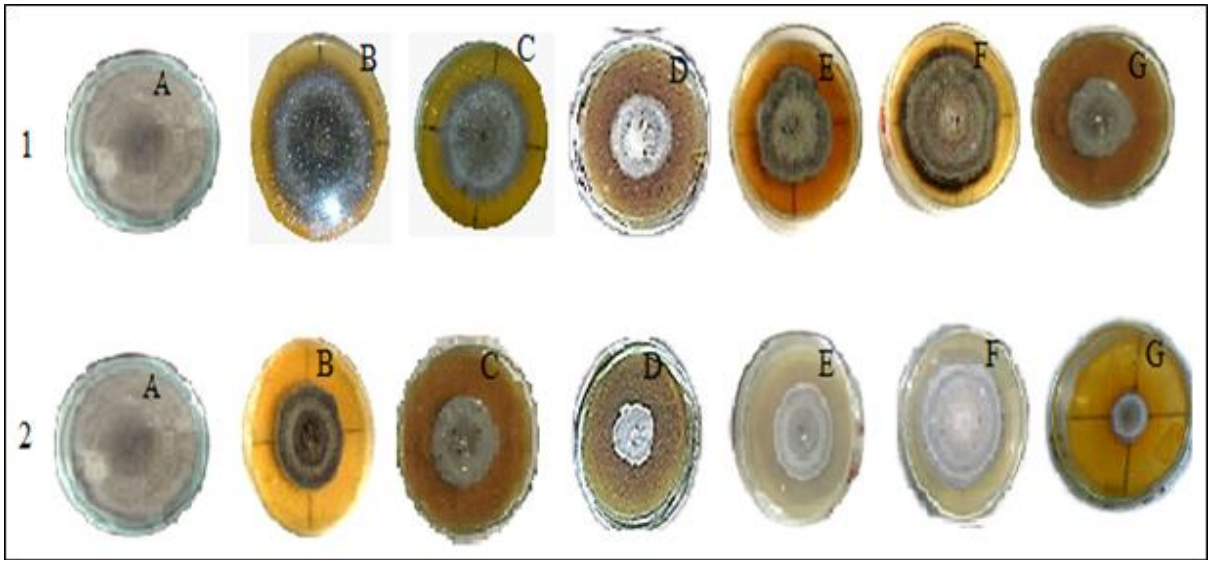

Fig. 2. Efficacy of *D. viscosa* fraction treatments for mycelial growth inhibition of *C. manginecans* at selected concentrations of botanicals 1) 20mg/ml and 2) 40mg/ml concentrations A) Control; B) n-hexane fraction, C) DCM fraction, and D) Ethyl Acetate fraction (Highly effective); E) n-butanol fraction; F) Aqueous fraction (Least effective); and G) Ethanol crude extract.

This supports and justify that the phytochemical compound present in *D. viscosa* ethyl acetate fraction had a similar kind of action for the morphological modifications in pathogen causing MSD (*C. manginecans*). Phytochemicals like [(9E)-9-Octadecanoic acid] ($C_{21}H_{38}O_2$), [I-(+)-Ascorbic acid 2,6 Hexadecanoate] ($C_{38}H_{68}O_8$), [9,12-Octadecadienoic acid (Z,Z)] ($C_{18}H_{32}O_2$), [1,2-Oxathiane, 6-dodecyl-, 2,2-dioxide] ($C_{16}H_{32}O_3$), [Oleoyl chloride] ($C_{18}H_{33}ClO$), [Octadecanoic acid, 1-{(1-oxohexadecyl)oxy]methyl}-1-,2-ethanediyl ester] ($C_{155}H_{106}O_6$), [1,2-Butanediol, 1-(2-furyl)-3-methyl-1,2-butanediol] ($C_{19}H_{14}O_3$), and [Oleic acid, (2,2-dimethyl-1,3-dioxolan-4-yl)methyl ester] ($C_{24}H_{44}O_4$) were first time detected and identified in *D. viscosa* ethyl acetate fraction against MSD causal agent (*C. manginecans*). This study showed that all these phytochemical compounds of ethyl acetate fraction of *D. viscosa* contribute to an overall antifungal potential. Moreover, the contribution of above mentioned phytochemicals *D. viscosa* ethyl acetate fraction for antifungal potential against *C. manginecans* is supported by finding of Jabeen *et al.* (2018) who reported that 9-Octadecanoic acid and I-(+)-Ascorbic acid 2, 6 Hexadecanoate were found common in crude ethanol extract of *Citrullus colocynthis*, *Ailanthus altissima* exhibiting significant antifungal potential against *C. manginecans*. So due to the commonalty of phytochemical exhibiting antifungal potential as reported by other researchers it's an indication that these phytochemical compounds of *D. viscosa* ethyl acetate fraction had possibly contributed (synergistically) to control the colony growth of *C. manginecans*. However, Palmitic

acid ($C_{16}H_{32}O_2$), 9,12-Octadecadienoic acid (Z,Z)-, methyl ester ($C_{19}H_{34}O_2$), and Octadecanoic acid ($C_{18}H_{36}O_2$) were reported by Ansarli *et al.* (2018) in *D. viscosa* methanol extract showing significant antimicrobial efficacy against selected human pathogens. Additionally, Bokhari *et al.* (2013) also revealed that phytochemicals like Octadeca-9, 11, 13-triynoic acid and *trans*-octadec-13-ene-9, 11-diynoic acid exhibited significant inhibition potential against selected plant pathogenic fungi.

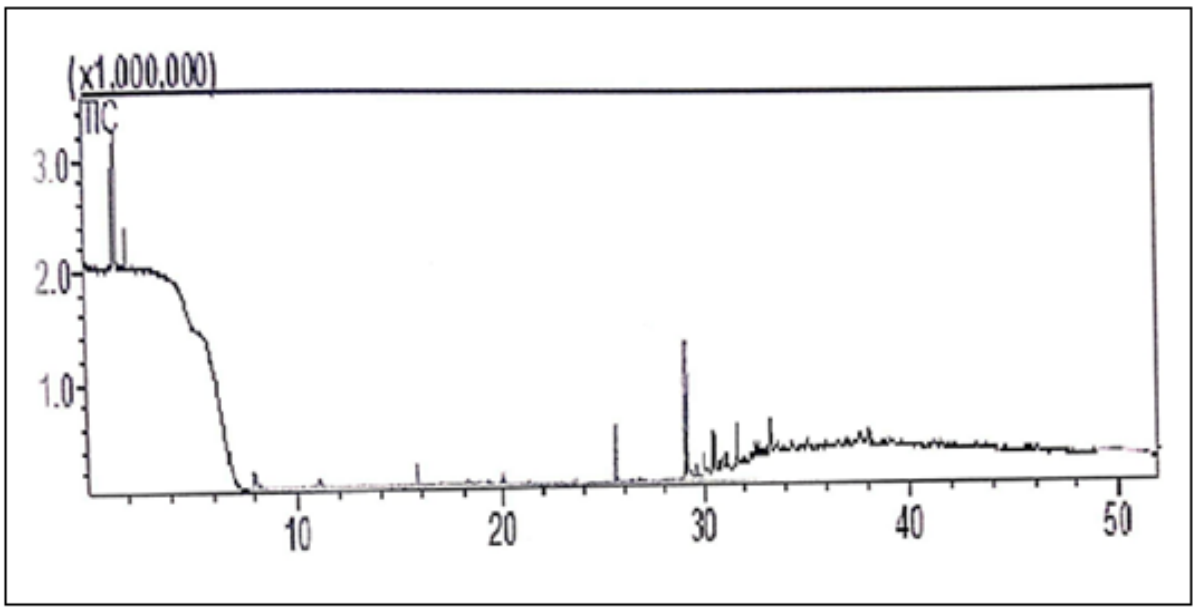

Fig. 3. GCMS Chromatogram of Phytochemical compounds in *D. viscosa* fraction of Ethyl Acetate.

These phytochemicals can serve as an alternative method for integrated disease/pathogen management (Yoon *et al.*, 2010).

Conclusion

Present study concluded that *D. viscosa* ethyl acetate fraction had the highest antifungal potential against *C. manginecans* causal agent of MSD. Phytochemicals identified in ethyl acetate fraction possibly exhibited a synergistic antifungal effect against MSD causal agent (*C. manginecans*).

Recommendation

Further detailed study is recommended for isolation of all these phytochemicals of *D. viscosa* ethyl acetate fraction.

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