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Inhibitory effects of leaf extract of *Lawsonia inermis* on *Curvularia lunata* and characterization of novel inhibitory compounds by GC–MS analysis

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ABSTRACT

Plants produce a high diversity of natural products with a prominent function in the protection against microbial pathogens on the basis of their toxic effect on growth and reproduction. In the present study, effect of partially purified acetone fraction of *L. inermis* leaves on various cytomorphological parameters i.e. mycelium width, conidial size, etc. of test fungi and fraction was subjected to confirming the presence of primary and secondary metabolites by rapid qualitative phytochemical tests, chromatographic methods such as TLC, column chromatography, GC–MS, etc. which were responsible for the inhibition of growth of test pathogen conidial size of *Curvularia lunata* decreased up to 64.76% at 0.039 μg/ml concentration of the extract. Mycelial width of *C. lunata* increased up to 55.91% at 0.312 μg/ml concentration of the extract. Carbohydrate, steroids, volatile oils, flavonoids, and tannins were found to be present in acetone extract of *L. inermis* leaf. Total of 7 bands were observed in TLC fingerprinting of *L. inermis* acetone fraction. Total of 10 fractions were collected from the column chromatography. Fractions which show the most significant antifungal activity against the test fungus was subjected to further GC–MS analysis for the separation and identification of active principle. GC–MS analyses show the presence of total 6 constituents i.e. hexacosane, octadecane, docosane, heptacosane methyl, octacosane, and tetracosane.

1. Introduction

The genus *Curvularia* represents the most widespread filamentous fungi in subtropical and tropical region of the world [1]. It causes brutal losses in the tropical regions but is a minor pathogen in temperate regions [2–4]. The cultural characteristic of hyphal growth of test fungi *Curvularia lunata* is black brown cottony growth in an appearance on the rich medium such as potato dextrose agar (PDA) [5]. The basis of fungal vegetative or hyphal growth is the continued and coordinated expansion of fungal cell/hyphae into a simple linear or complex structure. Inhibition in the hyphae of fungal cells is clearly visible in the Petri plates and can be measured as percent (%) inhibition in growth. As a result of hyphae inhibition reproductive structures also get inhibited. Sporulation is the most common mode of reproduction for diverse groups of fungi, which results in the production of large numbers of mitotically derived spores or conidia, the primary agents for infecting host plants for many plant pathogenic fungi [6]. When there is inhibition in the hyphal growth the number of reproductive structures also get reduced.

Growth and reproduction of microbes are usually denoted by the change in total population rather than an increase in the size or mass of an individual organism. The extreme growth of the pathogens result in various diseases, thus in order to treat a disease, it is very essential to inhibit the growth of microorganisms or their reproductive structures. Plants have an inbuilt capability to synthesize various secondary metabolites which act as main agents for plant defense actions against microorganisms, insects, and herbivores [7]. The use of various plant extracts and essential oils for growth inhibition has been reported by various authors [8–16].

The biological and molecular action of plant secondary metabolites induces various morphological, cytological changes in microorganisms. Banos and Lopez [17] reported that chitosan and various plant extracts induce alterations in conidia and mycelial morphology of *Fusarium oxysporum*, *Penicillium digitatum*, and *Rhizopus stolonifer*. The effect of leaf extracts of *Cassia alata* L., *Cassia fistula* L. and *Cassia tora* L. on mycelial morphology, conidial morphology, and

Abbreviations: PDA, Potato dextrose agar; CC, Column chromatography; TLC, Thin layer chromatography; HPLC, High performance liquid chromatography; DAD, Diode array detector; MFC, Minimum fungicidal concentration; GC–MS, Gas chromatography–mass spectrometry; PE, petroleum ether; RF, Retardation factor; SE, Standard error; CD, Critical difference; CV, Coefficient of variation; MIC, Minimum inhibitory concentration; PE, Petroleum ether; HCl, Hydrochloric acid; $FeCl_3$, Ferric chloride; NaOH, Sodium hydroxide; H_2SO_4, Sulfuric acid.

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germination of *Microsporum gypseum* has been reported by Phongpaichit et al. [18]. Toxigenic *Aspergillus parasiticus* also exhibits morphological and anatomical changes when exposed to neem (*Azadirachta indica*) leaf and seed aqueous extracts [19]. Berdicevsky et al. [20] reported the changes in hyphae and spore structure in dermatophytes due to severe destruction of in fungal cell coat by treatment with *Inula viscosa* extract. Goel and Sharma [21] reported a remarkable decrease in growth and reproduction, changes in cell number, mycelium width, conidial size and conidiophore size of *Aspergillus fumigates* by acetone extract of leaf and alcohol extract of inflorescence of *Euphorbia pulcherrima*. Sharma and Sharma [22] reported the remarkable effect of acetone fraction of *L. inermis* leaves and petroleum ether (PE) fraction of *Corymbia citriodora* leaf, their mixture and essential oils on various cytomorphological parameters i.e. mycelium width, conidial size, hyphal morphology, conidiophore size, etc. of test fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Parveen and Sharma [23] also studied the effect of plant extract on growth and reproduction of *Pythium aphanidermatum* and *Pythium myriotylum*. Hada and Sharma [24] reported the inhibition of growth of *Alternaria solani* using cassia fistula fruit pulp extract.

In order to find out the active compound present in the active fraction of plant extract, the extract is initially subjected to thin layer chromatography (TLC) to find out the number of constituent compounds and then each compound is separated [25]. Separation of an individual compound can be done by various methods like column chromatography (CC), high-performance liquid chromatography (HPLC), gas chromatography (GC), etc. These fractions are further assayed for antifungal activity [26,27].

Many researchers have investigated the antimicrobial activity of active compounds isolated from plants. Ilic et al. [28] detected flavonoids from flowers of *Linum capitatum* using thin layer chromatography. Phenolic compounds were reported in the leaves of *Cymbopogon citratus* using TLC [29]. Vessal et al. [30] reported the detection of steroid and alkaloid glycosides by thin layer chromatographic in fruits of Winter Cherry (*Physalis alkekengi*). Lee [31] used silica gel column chromatography to evaluate the fungicidal activity of volatile compounds isolated from *Acorus gramineus* rhizome, against phytopathogenic fungi. Shiac et al. [32] separated the antioxidants from the extract of *Taraxacum mongolicum* by using HPLC. Gomez-Alonsoa et al. [33] also performed HPLC with UV–vis photodiode array detector (DAD) and fluorescence detection for examination of diverse grape and wine phenolics. Kartal et al. [34] isolated Echimidine N-oxide from the root of *Symphytum sylvaticum* and this compound was found to be inhibitory against *Epidermophyton floccosum*, *Dreschlera rostrata*, *Microsporum canis*, *Nigrospora oryzae*, *Aspergillus niger*, *Allefsheira boydii*, and *Candida albicans*. Drimane sesquiterpene was found to be inhibitory against *Epidermophyton floccosum* and *Trichophyton rubrum* isolated from *Drimys brasiliensis* [35].

In the present study, the effect of partially purified acetone fraction of *L. inermis* leaves on various cytomorphological parameters i.e. mycelium width, conidial size, etc. of test fungi and extract was subjected to rapid qualitative phytochemical tests for confirming the presence of primary and secondary metabolites which were responsible for the inhibition of growth of test pathogen and active fractions were isolated by chromatographic methods such as TLC, column chromatography, and GC–MS, and these fractions were further assayed for antifungal activity.

2. Materials and methods

2.1. Effect of extracts on cytomorphology of test fungi

Effect of selected extract on various morphological and cytological parameters of test pathogen was studied. Test fungi were treated with increasing concentrations of the extract up till minimum inhibitory concentration (MIC). A small fungal biomass consisting of mycelium, vesicle and conidia/spores were removed from each tube and microscopic examination was done after staining with cotton blue and mounting in lactophenol. Change in mycelium width and conidial size morphology were observed with the help of Olympus trinocular research microscope BX-51 and analyzed by Image analysis software Olysia Bioreport 3.2 of Olympus.

2.2. Phytochemical analysis of Lawsonia inermis leaf extract

Qualitative methods were used for the identification of different secondary metabolites or phytochemicals present in the plant extracts. Acetone fractions of leaf were subjected to qualitative test suggested by Kokate et al. [36]. The leaves were shade dried, crushed, powdered and extracted (100 g/ml). Various solvents (methanol, ethanol, hexane, chloroform, acetone, ethyl acetate, and distilled water) were used for extraction. The extract was kept in an orbital shaker for 5–6 days at 25 °C temperature. The supernatant was collected and analyzed for the presence and absence of alkaloids, steroids, volatile oils, carbohydrates, tannins, flavonoids, and saponins.

2.3. Tests for detection of secondary metabolites

2.3.1. Alkaloids

Alkaloids are compounds having one or more nitrogen which contain a heterocyclic ring. Wagner's test can be performed to find out the presence of alkaloids in the partially purified fractions [37]. After putting in 2 drops of diluted hydrochloric acid (HCl) in 1 ml amount of extract, it was stirred and filtered. Wagner's reagent results in the formation of a reddish brown precipitate. Various alkaloid reagents were tested on the filtrate and the development of colored precipitate was observed.

2.3.2. Volatile oils

Volatile or essential oils are the odorous volatile chemical constituents of plants. Sudan III test can be used to detect the presence of volatile oils. A modified method of Kokate et al. [37] was used for the detection of volatile oils. Development of red color on mixing with Sudan III would indicate the presence of volatile oils. A small amount of extract and Sudan III dye are mixed for the observation of the development of red color.

2.3.3. Tannins

Chemically, tannins contain a mixture of complex organic substances. Polyphenols are present in these substances. The presence of condensed tannins is indicated by the development of green color and the presence of hydrolyzable tannins is indicated by blue color. Examination of tannins was detected using the slightly modified method of Trease and Evans [38]. About 0.5 ml of extract was dissolved in 5.0 ml of distilled water. The mixture was treated with alcohol ferric chloride ($FeCl_3$) solution and observed for the color development.

2.3.4. Saponin

Saponins are known to be complex glycoside compounds. In these compounds, aglycone is steroidal in nature. Foam test can be used to detect the presence of saponins. Detection of saponin was carried out by the slightly modified method of Kokate [39]. About 0.5 ml of extract was dissolved in 20 ml of distilled water and shaken in a graduated cylinder for 15 min. Formation of persistent frothing on warming indicates the presence of saponins. The ability of saponins to produce frothing in aqueous solution was used as a screening test for the sample.

2.3.5. Carbohydrates

Carbohydrates are widely distributed in plants. They can be detected by Molish's test or Fehling's test [37]. For Fehling's test, 1 ml of extract mixed in 5 ml of distilled water and filtered after dissolution. A few drops of naphthol and concentrated sulfuric acid (H_2SO_4) were added to the filtrate, the color of the filtrate changed to purple. It indicates the presence of sugar. Similarly, when a small quantity of filtrate was heated with an equal amount of Fehling A and Fehling B solution, the color changed to brick red color. It indicates the presence of carbohydrates.

2.3.6. Flavonoids

Flavonoids usually occur in plants as glycosides. In this one or more of phenolic hydroxyl groups are combined with sugar residues. Alkaline reagent test is to be used to detect flavonoids. Detection of flavonoids was done by using the slightly modified method of Evans [40]. 0.5 ml of extract was dissolved in 5 ml of 10% aqueous sodium hydroxide (NaOH) solution. It results in the development of reddish brown color which shows the presence of flavonoids.

2.3.7. Sterols

Sterols are triterpenes and based on cyclopentane perhydroxy phenanthrene ring system. They are also called phytosterols. Liebermann's Burchard test is used for detection of phytosterols [41]. 1 ml of extract was mixed with 1 ml of acetic anhydride and 2 ml $CHCl_3$ followed by the gradual addition of concentrated H_2SO_4 through the side of the test tube. Formation of a ring of brown color at the junction of two layers is the indication of the presence of sterols.

2.4. Isolation and characterization of the active principle from selected leaf extract by using various chromatography techniques

Isolation of active principle from selected acetone extract of *L. inermis* leaf was done by using chromatography techniques i.e. thin layer chromatographic fingerprinting, column chromatography and gas chromatography. Characterization of the active compound was done by mass spectrometry analysis of the extracted phytochemicals.

2.4.1. TLC fingerprinting of acetone fraction of Lawsonia inermis

For TLC fingerprinting, a 10 cm long TLC plate was cut and marked carefully. 10 μl of plant extract was spotted onto the marked plate with the help of a capillary tube or pipette. Acetone: n-hexane: benzene (2.5 ml: 8 ml: 1 ml) was used as mobile phase. The TLC plate was kept in a chromatographic chamber containing the respective solvent system and the chamber was covered with a glass plate to prevent the evaporation of the solvent. The plate was allowed to remain in the chamber until the solvent reached up to 9 cm distance. The plate was then observed in UV-florescence analysis cabinet at short and long wavelengths.

2.4.2. Visualization of TLC plate

The TLC fingerprinting plate was derivatized with anisaldehyde sulphuric acid reagent followed by heating at 100 °C till colored bands of various secondary metabolites appeared. The observations were taken before and after derivatization which visible in ultraviolet light.

Rf values were calculated as follows:

$$\text{Rf} = \frac{\text{Distance traveled by substance}}{\text{Distance traveled by solvent}}$$

2.5. Column chromatography of partially purified acetone fraction of Lawsonia inermis *leaf*

10 gm of dried and partially purified acetone fraction of *Lawsonia inermis* leaf was dissolved in the mobile phase i.e. 25 ml acetone, 80 ml hexane and 1 ml benzene and this solution was subjected to column chromatography. Glass column (Merck: 100–

Table 1
Effect of different concentrations of acetone extract of *Lawsonia inermis* leaf on mycelium width of *Curvularia lunata*.

S. No.	Extract concentration (mg/ml)	Mycelium width (μm) ± SD	% Increase in mycelium width
1.	Control	4.1 ± 0.05	–
2.	0.625	NF	–
3.	0.312	9.3 ± 0.04	55.91
4.	0.156	8.8 ± 0.02	53.40
5.	0.078	8.1 ± 0.05	49.38
6.	0.039	7.7 ± 0.02	46.75
7.	0.019	6.5 ± 0.03	36.92
8.	0.0097	6.2 ± 0.03	33.87
9.	0.0048	5.7 ± 0.05	28.07
10.	0.0024	5.1 ± 0.02	19.60

NF: Not Found.

Table 2
Effect of different concentrations of acetone extract of *Lawsonia inermis* leaf on conidial size of *Curvularia lunata*.

S. No.	Extract concentration (mg/ml)	Conidial size (μm) (L × W)	Conidial size (μm) ± SD (Area)	% Reduction in conidial size
1.	Control	24.16 × 8.1	195.69 ± 0.17	–
2.	0.625	CNF	CNF	–
3.	0.312	CNF	CNF	–
4.	0.156	CNF	CNF	–
5.	0.078	CNF	CNF	–
6.	0.039	14.80 × 4.66	68.96 ± 0.29	64.76
7.	0.019	15.96 × 4.96	79.16 ± 0.19	59.54
8.	0.0097	17.63 × 5.50	96.96 ± 0.28	50.45
9.	0.0048	18.50 × 6.06	112.11 ± 0.39	42.71
10.	0.0024	21.06 × 7.03	148.05 ± 0.18	24.34

CNF: Conidia Not Formed.

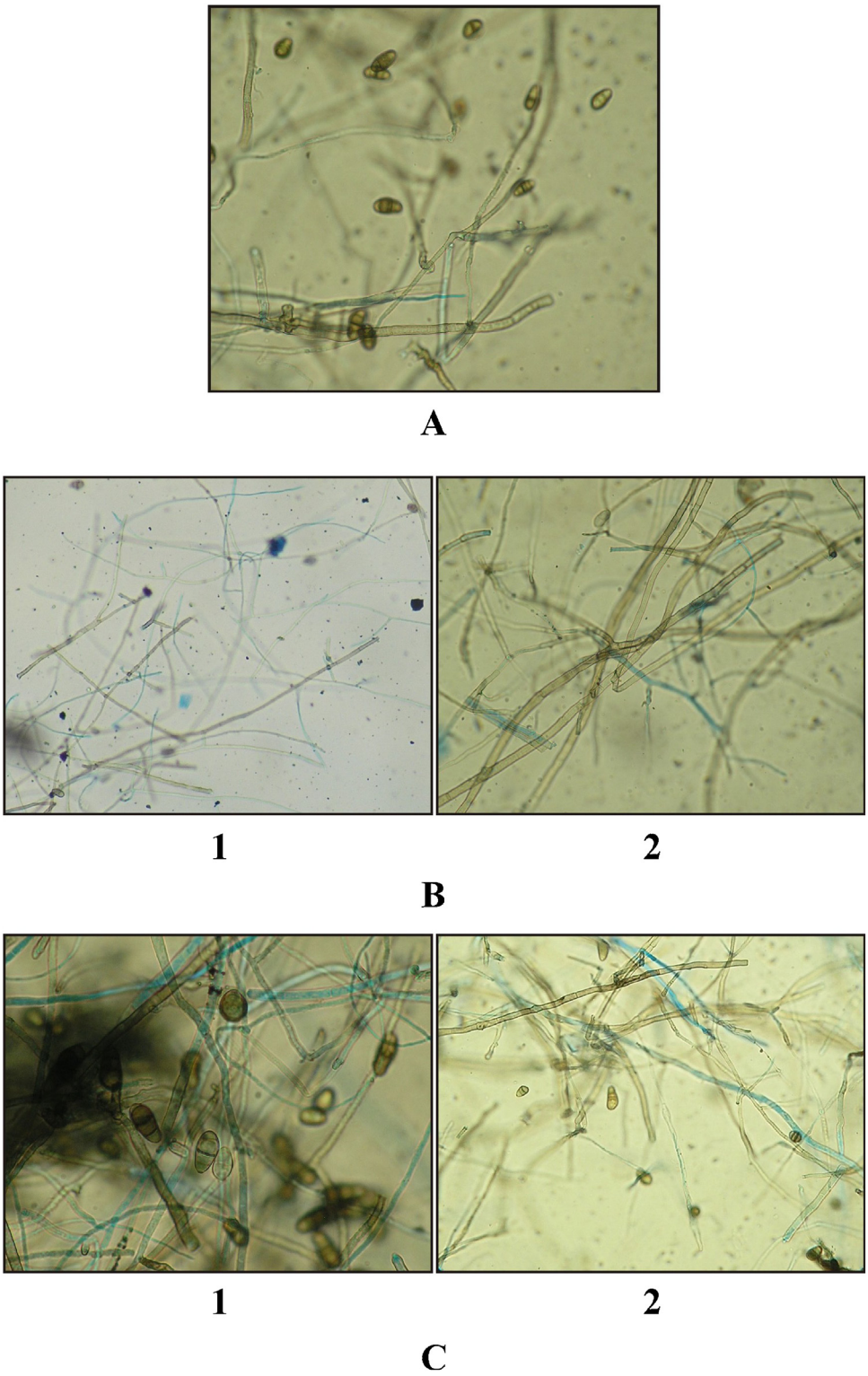


Fig. 1. Morphological alterations in *Curvularia lunata* due to treatment with Acetone extract on different concentrations. (A) Mycelium and conidia of *Curvularia lunata* (Control at 400X). (B) (1) Normal mycelium (Control at 400X); (2) Mycelium showing increasing width (Control at 400X). (C) (1) Normal mycelium (Control at 400X); (2) Conidia showing a decrease in size (Control at 400X).

200 mm) filled with 650 g of silica gel was used for column chromatography. According to the color band developed in column different fractions of the extract containing various secondary metabolites were collected. All fractions obtained from the column were dried in rotary vacuum evaporator under reduced pressure. These fractions were screened for their antifungal activity against *C. lunata*. The fraction showing best antifungal activity against *C. lunata* was subjected to further purification and characterization for active molecule via gas chromatography and mass spectrometry.

2.6. Identification and structure determination by gas chromatography/mass spectrometry (GC–MS)

The GC–MS analysis was performed on a GC (Perkin-Elmer) system coupled to Perkin- Elmer Turbo Mass MS. HP1-MS capillary column (30 m × 0.25 μm × 0.25 μm) was used under the following conditions: oven temperature programmed from 70 °C for 10 min, then gradually increased at 290 °C at 3 min; injector temperature, 250 °C; carrier gas Helium, flow rate 1 ml/min; volume of the injected sample was 0.4 μl; split ratio 1:60; ionization energy 70 eV: Run time 40 min. The relative amount of each component was calculated by comparing its average peak area to the total area. The identification of the separated volatile compounds was done through retention indices and mass spectrometry by comparing mass spectra of the unknown peaks with those stored in the Nist 98/Nbs 75 K GC–MS library.

3. Results and observations

3.1. Effect of extracts on the morphology of test fungi

Study of leaf extracts effect of *L. inermis* on morphology and reproduction of test fungi are presented in Tables 1 and 2 and Figs. 1(A, B, C) & Fig. 2(A, B). The luxuriant growth of test fungi i.e. *C. lunata* was observed in control i.e. thick mycelial mat showed the presence of abundant conidiophores with a large number of conidia. A gradual decrease in conidia size and increase in mycelial width was observed with increasing concentration of the extract till minimum inhibitory concentration (MIC) at 0.625 mg/ml (Supplementary Fig. 1). Therefore, minimum fungicidal concentration (MFC) was also determined, and it was observed at 1.25 mg/ml (Supplementary Fig. 1).

A decrease in size of conidia was directly proportional to the increasing concentration of extract. Conidial size of *C. lunata* decreased up to 64.76% at 0.039 μg/ml concentration of the extract (Table 2). Increasing in mycelia width was directly proportional to the increasing concentration of extract. Mycelial width of *C. lunata* increased up to 55.91% at 0.312 μg/ml concentration of the extract (Table 1 & Supplementary Fig. 1).

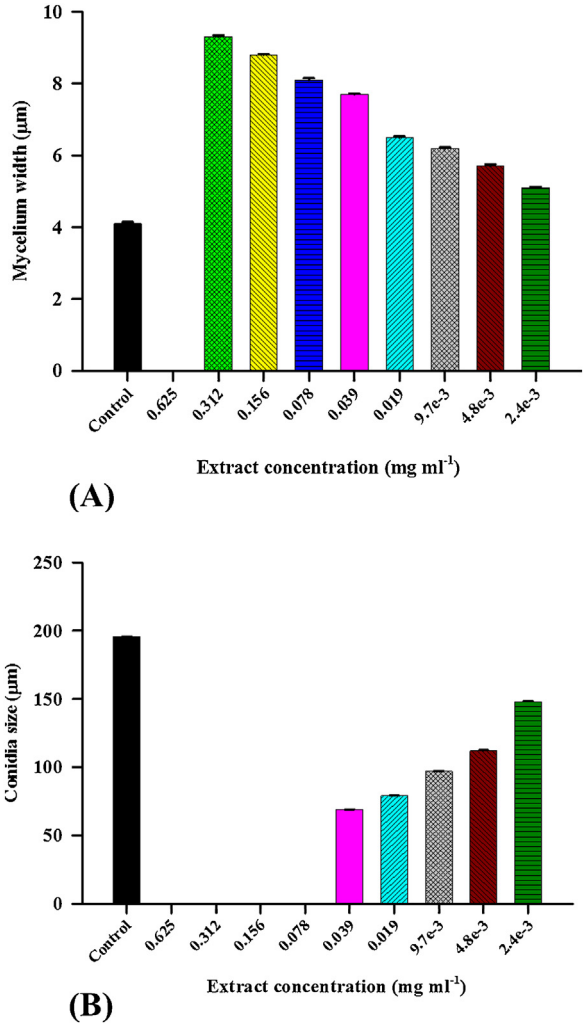


Fig. 2. (A) Effect of different concentrations of acetone extract of *Lawsonia inermis* leaf on mycelium width of *Curvularia lunata*. (B) Effect of different concentrations of acetone extract of *L. inermis* leaf on conidia size of *C. lunata*.

3.2. Phytochemical analysis

Phytochemical tests suggest that carbohydrate, steroids, volatile oils, flavonoids, and tannins were found to be present in acetone extract of *L. inermis* leaf. TLC plates showed clear distinct color bands. After derivatization i.e. spray of anis-aldehyde reagent on TLC plate. Changes in the color of these bands suggest the presence of different secondary metabolites in the extract (Fig. 3)

The Rf values and color of bands on thin layer chromatography fingerprinting are given in Table 3 and Total 7 bands were observed in TLC fingerprinting of *L. inermis* acetone fraction. Rf values were calculated of these bands. The presences of different bands of

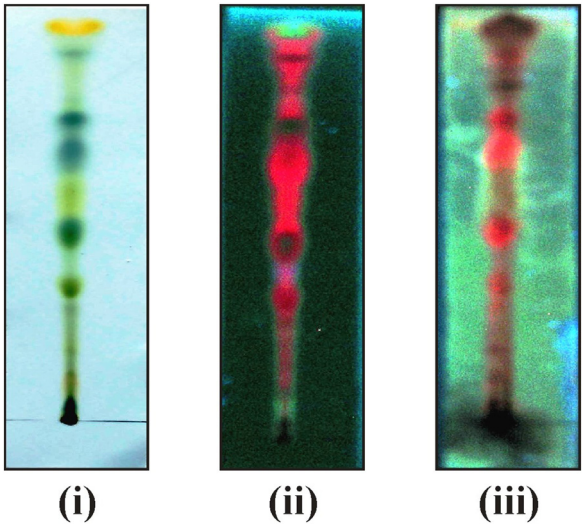


Fig. 3. Thin layer chromatography (TLC) of various fractions of *Lawsonia inermis* leaf. (i) TLC of acetone fraction of leaf under visible light; (ii) TLC of acetone fraction of leaf under UV light (Before derivatization); (iii) TLC of acetone fraction of leaf under UV light (After derivatization).

Table 3
Rf values of TLC fingerprinting of acetone fraction of *Lawsonia inermis* leaf.

Band number	Colour of bands		Rf value
	Before derivatization	After derivatization	
1.	Green	Blue green	0.16
2.	Greenish blue	Dark blue	0.35
3.	Yellow	Yellow	0.52
4.	Light yellow	Yellow blue	0.63
5.	Blue	Purple	0.70
6.	Dark blue	Light blue	0.75
7.	Yellow	Dark yellow	0.85

Table 4
Antifungal activity of column fractions (1–10 numbers) of acetone extract of *Lawsonia inermis* leaf against *Curvularia lunata*.

S. No.	Type of extract	Growth diameter after 7 days (mm)	% Mycelial growth inhibition
1.	F1	20.66 ± 0.57	75.00
2.	F2	35.00 ± 1.00	57.66
3.	F3	35.66 ± 1.15	56.86
4.	F4	41.33 ± 1.52	50.00
5.	F5	46.33 ± 0.57	43.95
6.	F6	30.66 ± 0.57	62.91
7.	F7	37.33 ± 1.15	54.84
8.	F8	39.33 ± 0.57	52.42
9.	F9	43.66 ± 0.57	47.18
10.	F10	49.00 ± 1.73	40.72
11.	Control (Water)	82.67 ± 0.57	NI

F: Fraction; NI: No Inhibition.

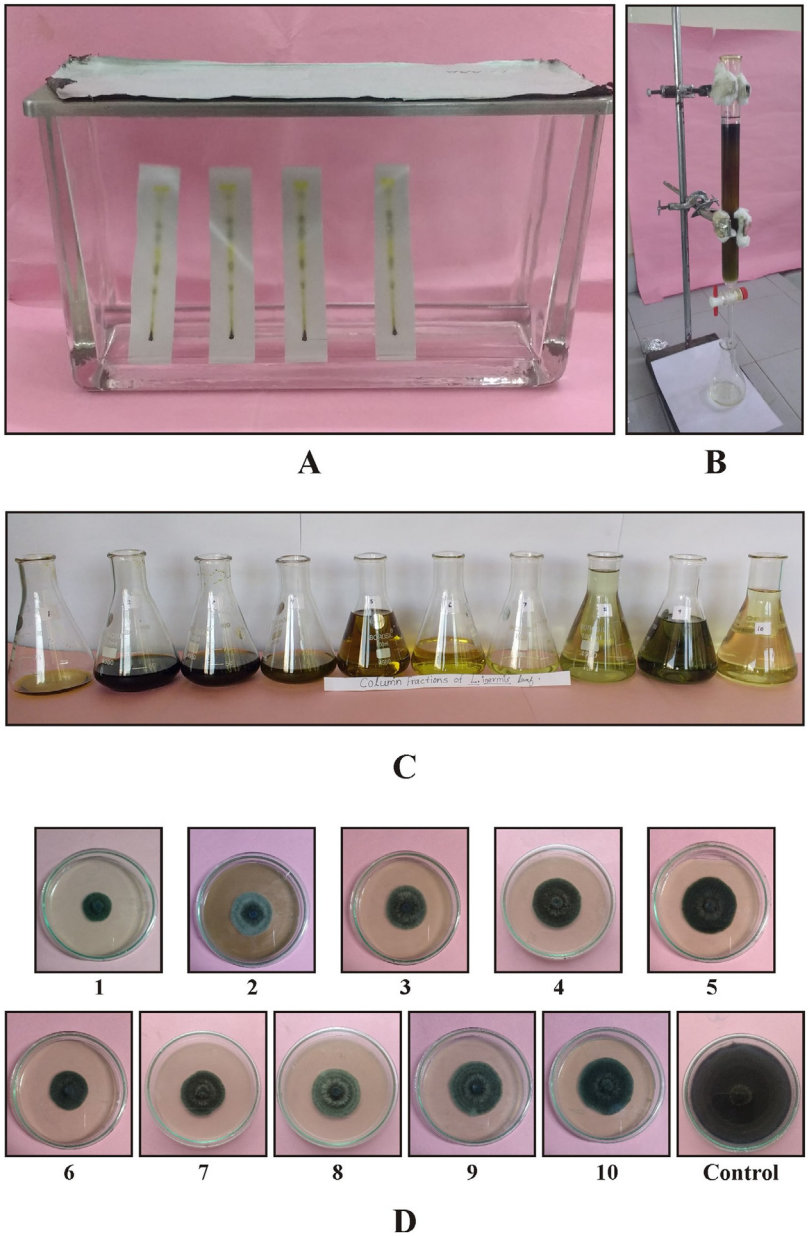


Fig. 4. Phytochemical analysis of extracts. (A) Thin layer chromatographic chamber; (B) Column chromatography; (C) *Lawsonia inermis* leaf fractions collected from column chromatography; (D) Antifungal activity of column fractions (1–10) of acetone extract of *Lawsonia inermis* leaf against *Curvularia lunata*.

different colors show the existence of various secondary metabolites in the fractions.

3.3. Column chromatography and GC–MS analysis of column fractions

All the fractions were analyzed for further antifungal activity against pathogenic fungus *C. lunata*. Results of the antifungal activity of ten column fractions of acetone extract against *C. lunata* are presented in Table 4 and Fig. 4A-D. Column fraction no. F1 showed best maximum inhibition (75.00%) followed by fraction no. F6 (62.91%), F2 (57.66%), F3 (56.86%), F7 (54.84%), F8 (52.42%), F4 (50.00%), F9 (47.18%), F5 (43.95), and F10 (40.72%), respectively.

On the basis of in vitro assay of antifungal activity of all column fractions, fraction no. F1 which exhibited the most significant antifungal activity against the test fungus was subjected to further GC–MS analysis for the separation and identification of active principle. The chromatogram obtained in GC–MS analysis of *L. inermis* fraction is presented as in Fig. 4A. GC–MS analysis of column fraction showed the occurrence of total 6 constituents which are hexacosane (Fig. 4B), octadecane (Beilstein 1770570) (Fig. 4C), docosane (EINECS 211-121-5) (Fig. 4D), heptacosane methyl (14167-66-9) (Fig. 4E), octacosane (RN 630-02-4) (Fig. 4F) and tetracosane (Beilstein 1758462) (Fig. 4G).

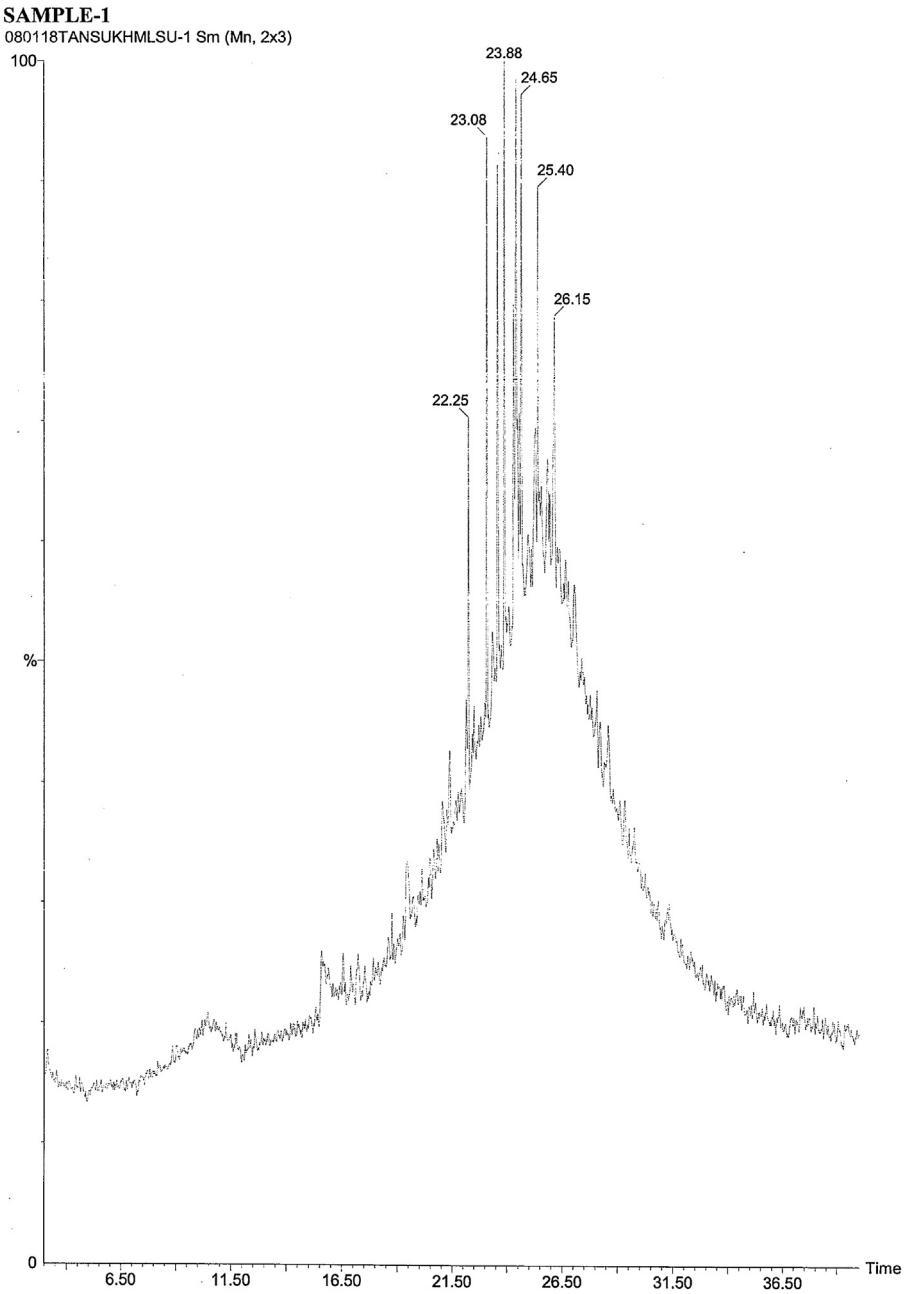


Fig. 5. (A) Gas Chromatography–Mass spectrometry analysis of Fraction no.1 of acetone fraction of *Lawsonia inermis* leaf; (B) Mass spectrum of Hexacosane (EINECS 211-124-1); (C) Mass spectrum of Octadecane (Beilstein 1770570); (D) Mass spectrum of Docosane (EINECS 211-121-5); (E) Mass spectrum of Heptacosane methyl (14167-66-9); (F) Mass spectrum of Octacosane (RN 630-02-4); (G) Mass spectrum of Tetracosane (Beilstein 1758462).

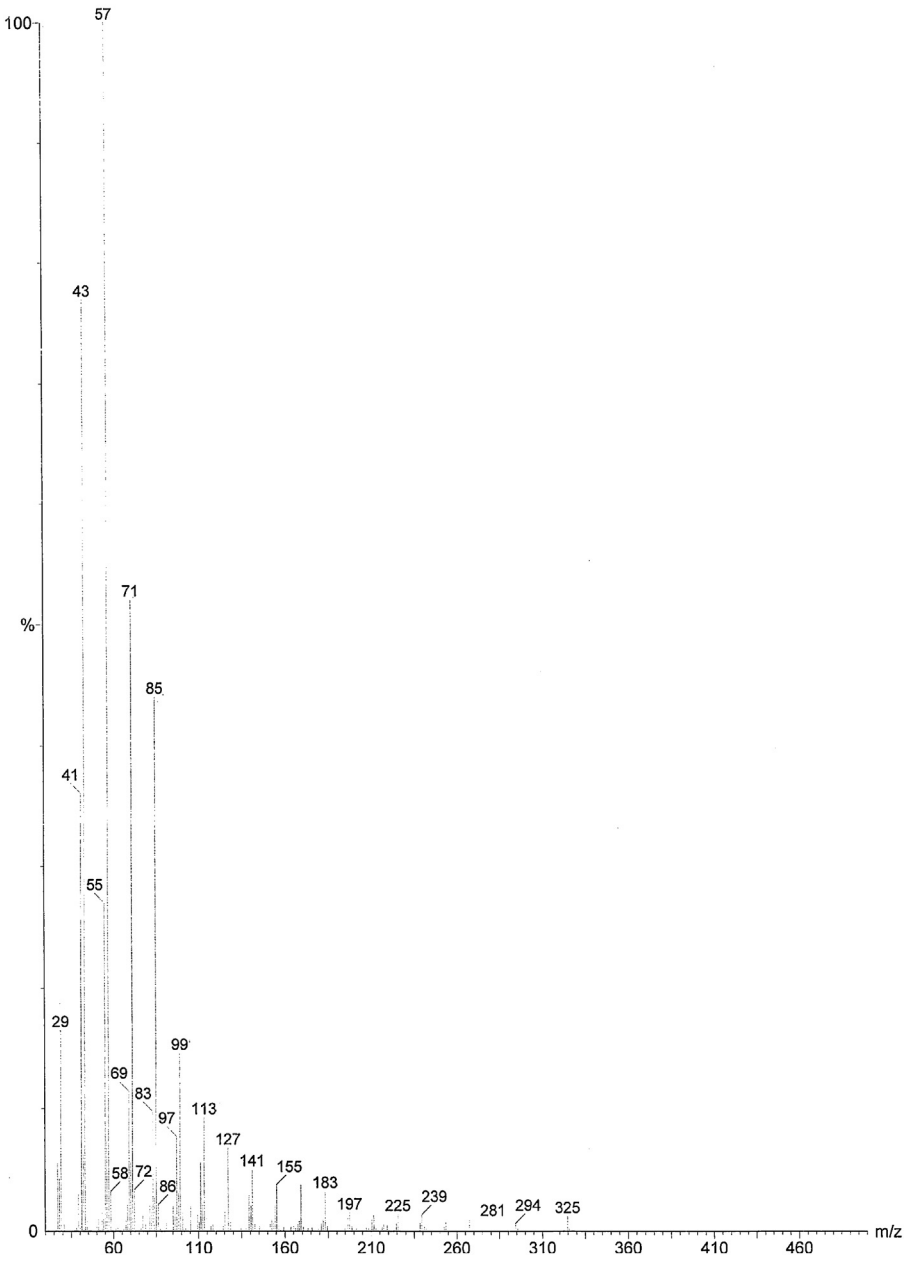


Fig. 5. (Continued)

4. Discussion

Microorganisms are able to reproduce tremendously within a relatively short time when they find favorable conditions such as an abundant supply of nutrients, optimum temperature, pH, etc. The extreme growth of the pathogens results in various diseases, thus in order to treat a disease, it is very essential to inhibit the growth of microorganisms. Use of plants as a source of medicine is as old as humanity. It is estimated that about 7500 plants are used in local health practices in India. Plants have an immense ability to synthesize aromatic substances which are the master agents for plant defense mechanisms against microorganisms, insects, and herbivores. Thus, plant extracts and their products are now being exploited as agents of disease control.

In this study, we have studied the effect of acetone extract of *L. inermis* on the growth and reproduction of test fungus *C. lunata*. Many researchers have been applied different plant extracts to study the effect on growth and reproduction of different pathogenic fungi but few reports are found on the effect of leaf extract of *L. inermis* on growth and reproduction of *C. lunata* which is the causal agent of leaf spot disease in maize. However, reports are available on the inhibitory effect of *L. inermis* plant extract on other plant pathogenic fungi. Sharma and Sharma [22] reported the remarkable effect of acetone fraction of *L. inermis* leaves and PE

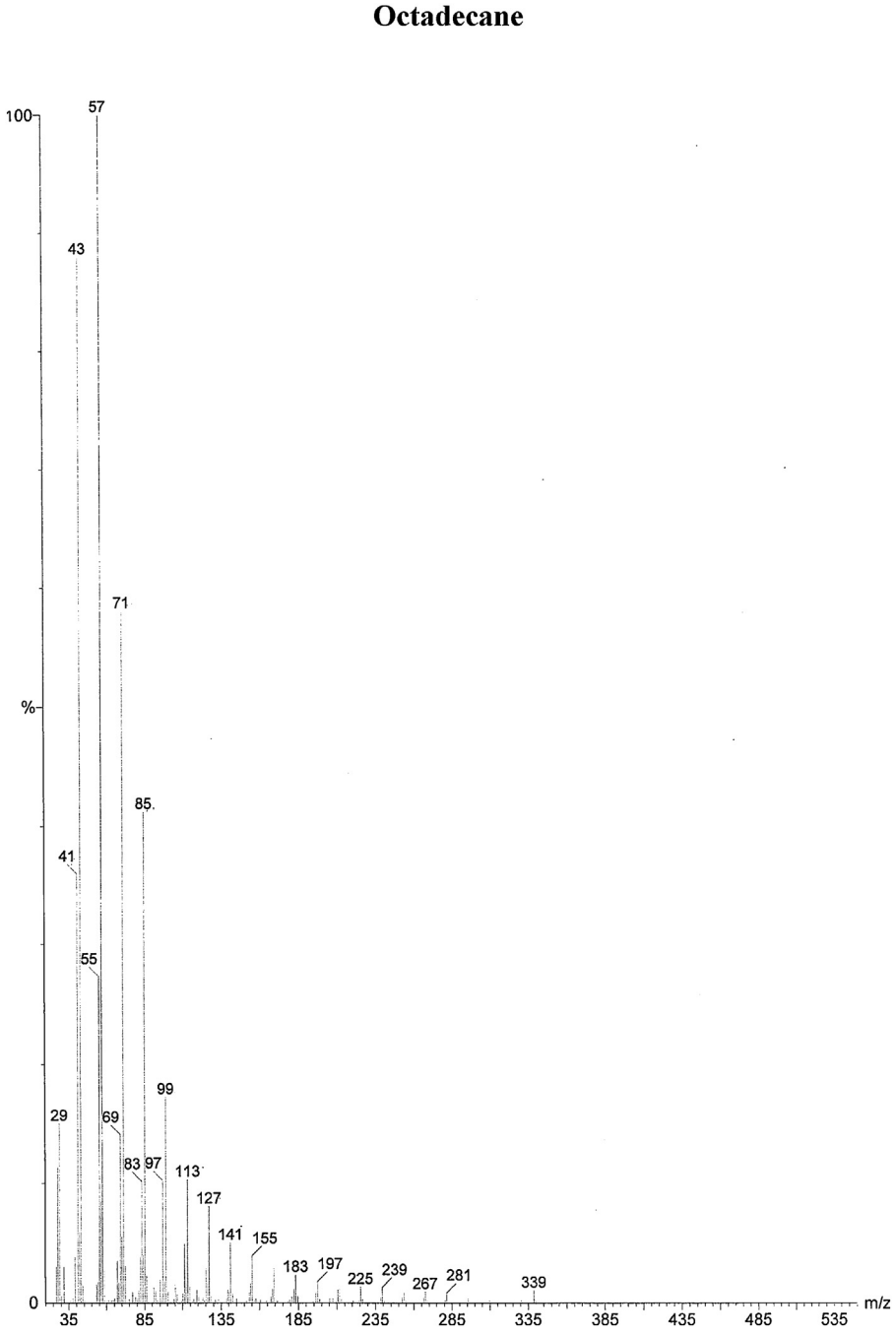


Fig. 5. (Continued)

fraction of *E. citriodora* leaf, their mixture and essential oils on various cytomorphological parameters i.e. mycelium width, conidial size, conidiophore size, etc. of *A. flavus* and *A. parasiticus*. Martin et al. [42] studied the effect of alcoholic and aqueous extracts of jabuticaba (*Myrciaria cauliflora*), guava (*Psidium guajava*) and jambolan (*Syzygium cumini* L.) on different biological parameters against fungus *Beauveria bassiana* (Bals.) Vuill. Results showed concentration-dependent plant extract inhibition of fungal growth which may be due to increase in the concentration of secondary metabolites/active components on increasing the concentration. The same pattern was also reported by Bonjar [43], Maresa et al. [44], Thanaboripat et al. [8], Reddy et al. [9], Reddy et al. [10], and Goel and Sharma [21] (Fig. 5).

The major changes observed due to extracting treatment were increased width of mycelium and decreased conidial size. This increase in width of hyphae, decreased conidial size may be because of the presence of secondary metabolite/s in the plant extract. These may inactivate the microbial adhesions, may change the permeability of the membrane allowing water to enter in and /or may get accumulated in the hyphae of test fungi that results in the increase in the width of hyphae and decreased conidial size, respectively.

Ya et al. [45] and Tsuchiya et al. [46] also reported that the plant secondary metabolites have the ability to inactivate microbial adhesions by disrupting fungal membrane, fungal enzymes, cell envelope and transport proteins which ultimately alter the

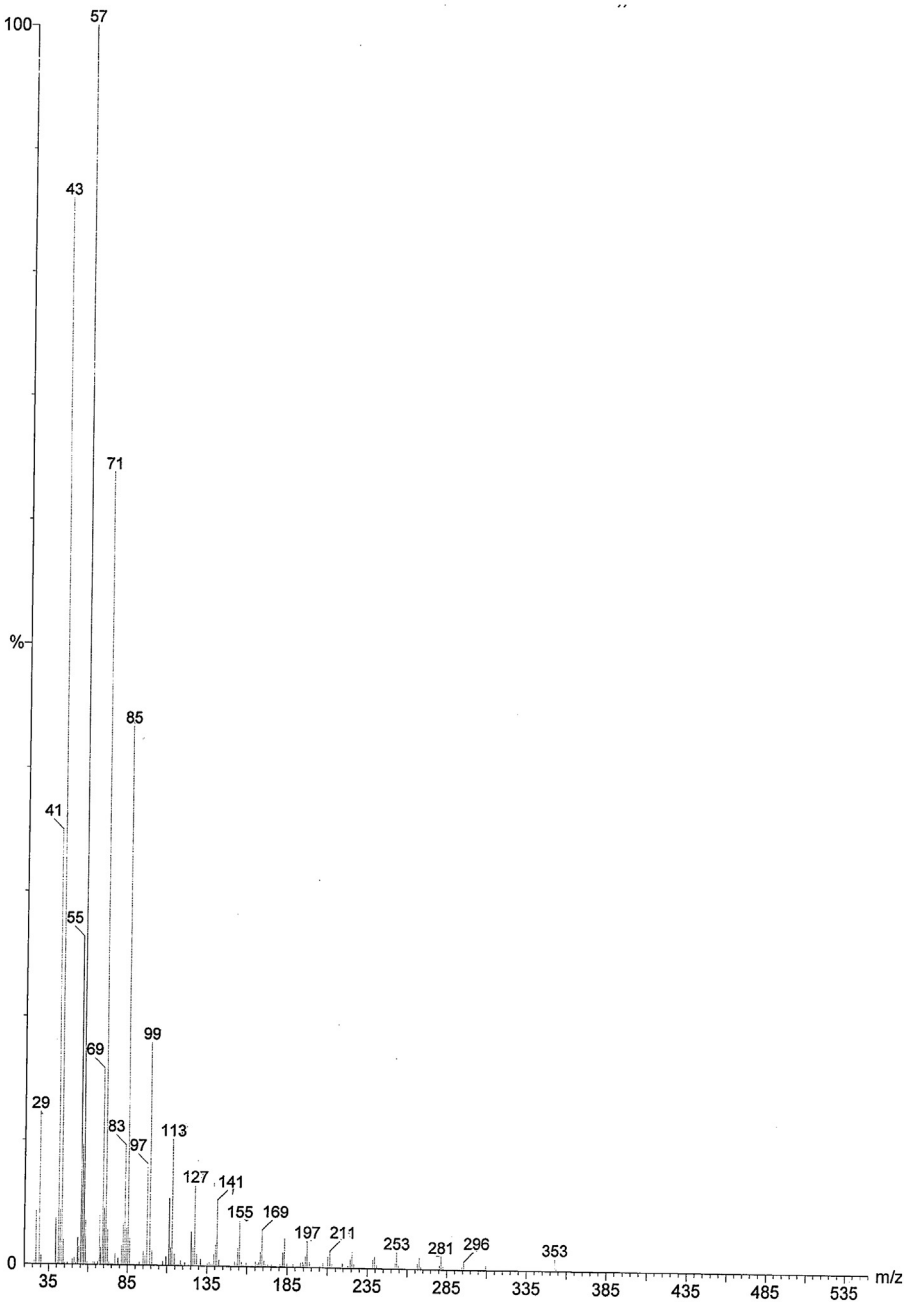
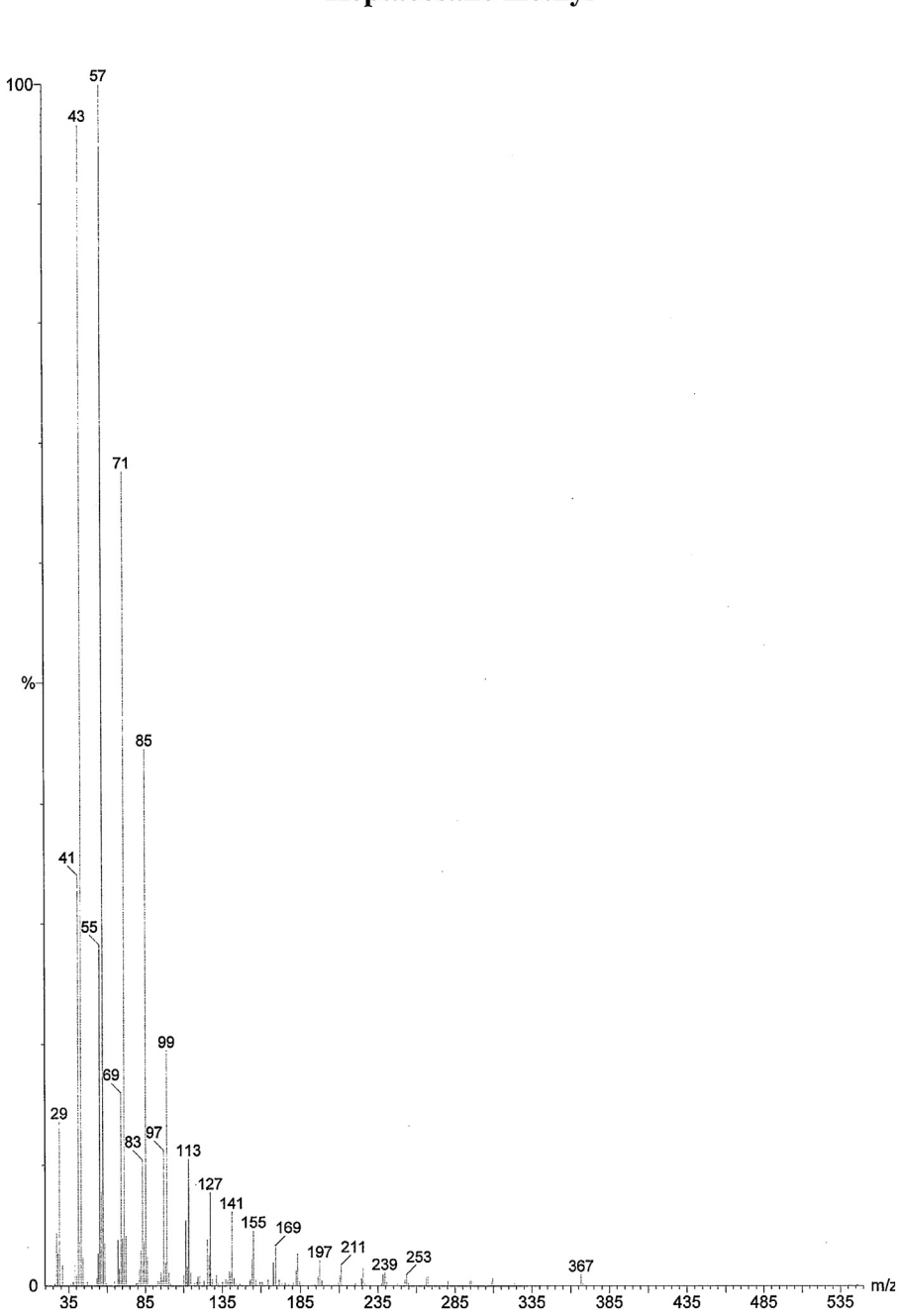


Fig. 5. (Continued)

arrangement of the membrane and hence change in the fungal cell/hyphae morphology as well as other structures. Abnormal filamentation in the fungus has been reported after treatment with plant extract by Rath et al. [47] and Pattnaik et al. [48]. Nakamura et al. [49] also suggested that change in the morphology of hyphae of pathogenic fungi *Candida* spp. could be due to the loss of integrity of the cell wall and the consequent effect on plasma membrane permeability by the active metabolites from *Ocimum gratissimum*. Pornsuriya et al. [50] reported that secondary metabolites from *Chaetomium* caused abnormal mycelial growth in *P. aphanidermatum*. He also reported abnormal swellings and increased the diameter of sporangium, oospores as well as the diameter of oogonia. Many scientists also have studied the effect of different plant extracts on growth and reproduction of different plant pathogenic fungi [22–24,51–53] and presently it is a very interesting area for finding a novel inhibitory agent to control diseases in the eco-friendly ways.

Plants produce a huge miscellany of secondary compounds that are synthesized through secondary metabolism as protection against a wide variety of microorganisms (fungi, viruses, bacteria), herbivores (arthropods, vertebrates), and insect attack. Secondary metabolites such as alkaloids, essential oils (lower terpenoids and



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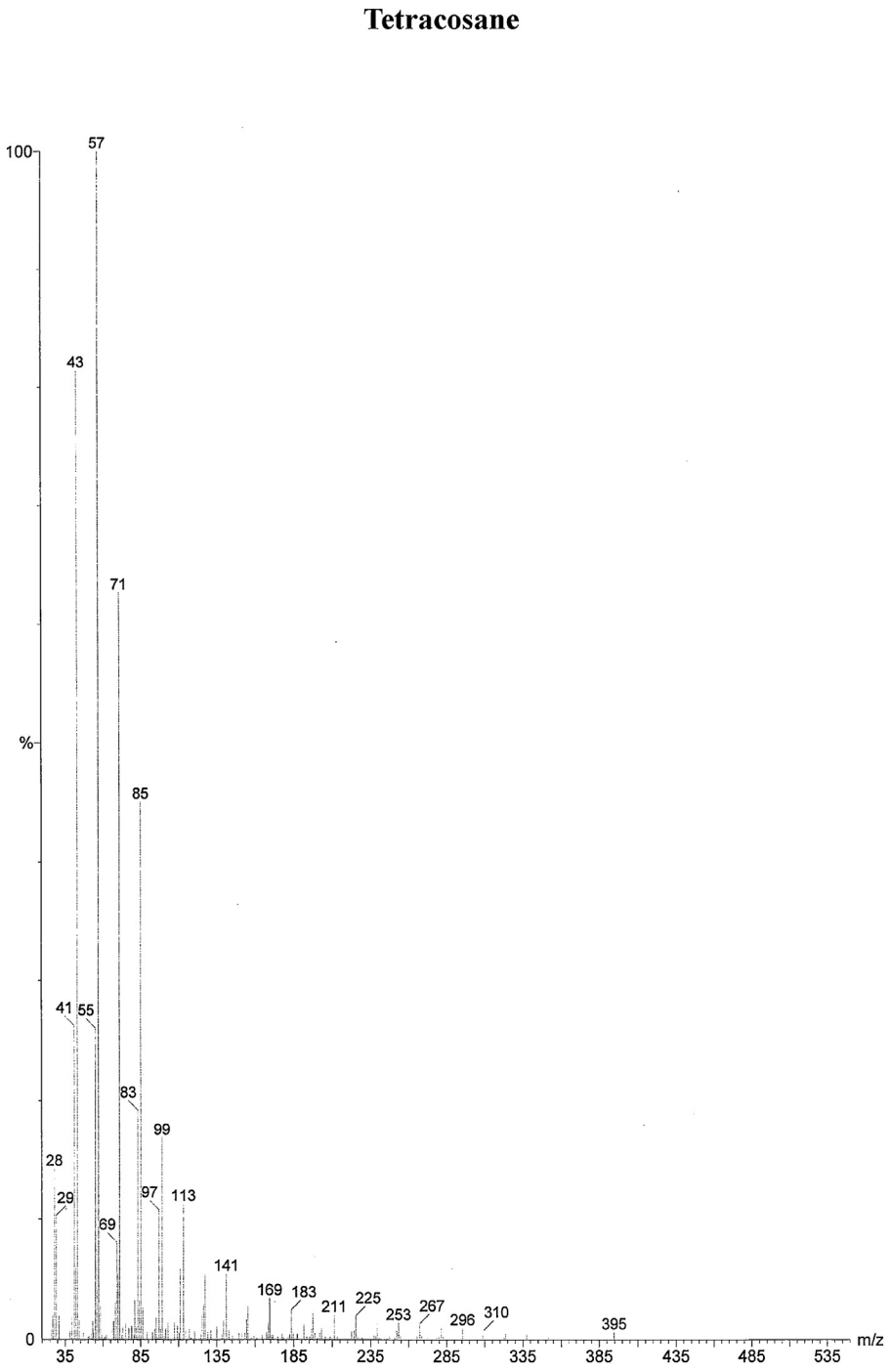
phenylpropanoids), coumarins, anthraquinones, flavonoids, terpenoids (cardenolides, diterpenes, monoterpenoids, iridoids, sesquiterpenoids, and triterpenoids) and steroids have a pervasive history of use as therapeutic metabolites [54–58]. Secondary metabolites are also useful for humans for controlling various diseases. Hence, there has been an amazing insurgence of interest in research of natural products over the past decade or so.

Phytochemical screening of extracts reveals the presence of various secondary metabolites in it. Several researchers have evaluated the phytochemical property of plant extracts by the qualitative methods [59–61,23,62–65]. Results of phytochemical testing of different fractions of *L. inermis* showed the presence of various secondary metabolites. Tannins, carbohydrate, saponins, and flavonoids were present in PE fraction of *L. inermis* leaf. Chloroform and benzene fraction of leaf showed the presence of flavonoids and tannins. Carbohydrate, steroids, volatile oils, flavonoids, and tannins were found to be present in acetone extract of *L. inermis* leaf. Methanol fraction of leaf gave positive results for tannins, steroids, carbohydrates, saponins, and flavonoids whereas aqueous fraction exhibited the presence of carbohydrates, tannins, and steroids.

The next steps include the purification and identification of secondary metabolites present in active fractions. It has been achieved by thin layer chromatography (TLC), column chromatography (CC), gas chromatography (GC), etc. TLC separates the ingredient fractions present in the extract [66–71]. In the present



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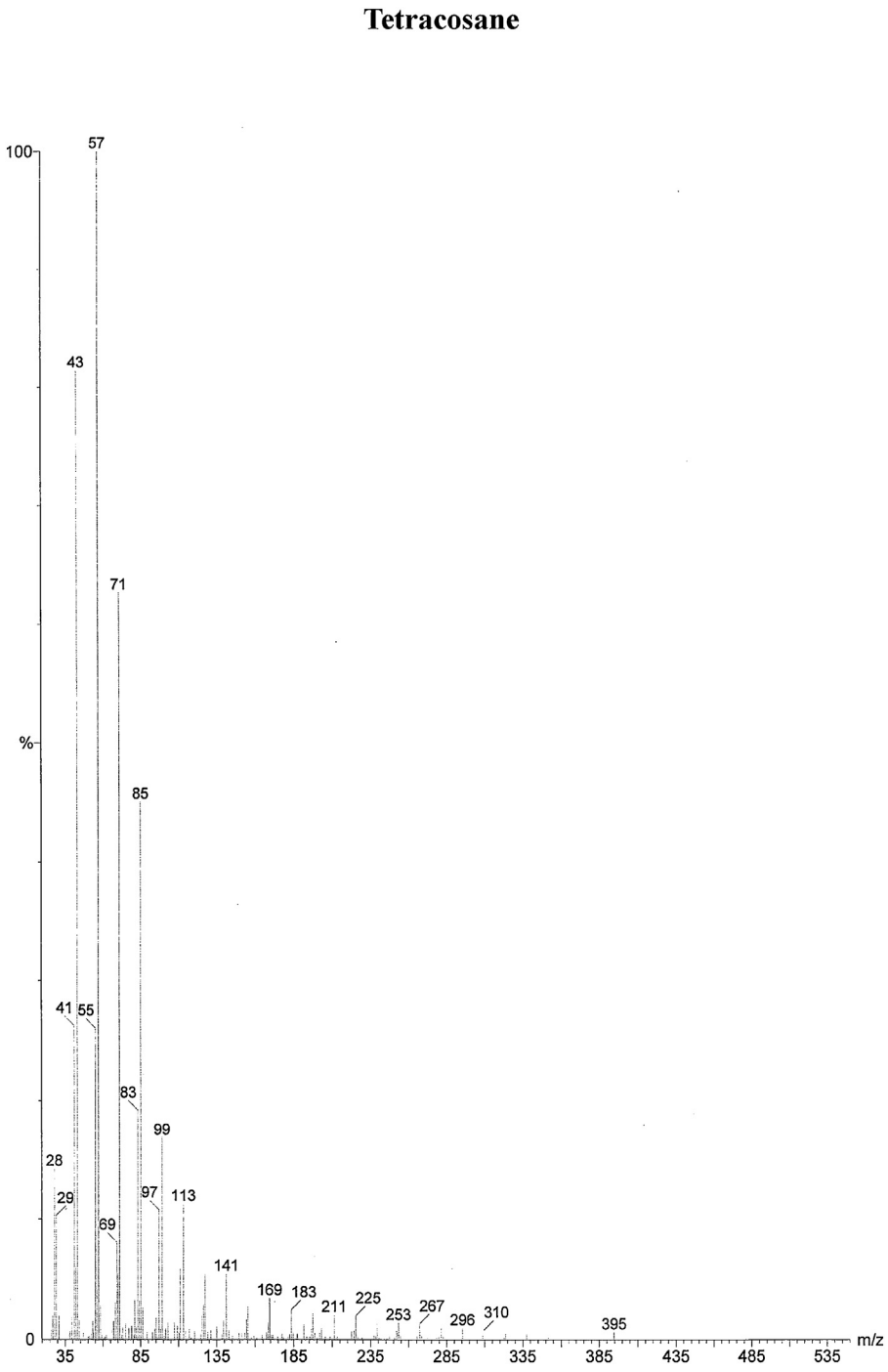
study, TLC fingerprinting of acetone extract of *L. inermis* leaf was done. The Rf values and colors of the resolved bands of TLC fingerprinting of leaves are given in Table 3. After derivatization of the TLC plate, the obtained bands showed the presence of various secondary metabolites in the extract. TLC fingerprinting of acetone extract of *L. inermis* leaf showed the development of bands of light blue, dark blue colors which are characteristics of triterpenoids and pink and red color bands indicates the presence of flavonoids and tannins respectively.

Column fractions of acetone extract of *L. inermis* leaf were also collected. The fractions showed different colors which indicate the presence of different secondary metabolites. Thus, the phytochemical study reveals that a broad category or group of secondary metabolites is present in these plant extracts or fractions which may be responsible for its antifungal activity. The fractionation by TLC and column chromatography suggested the presence of triterpenoids, tannins, and flavonoids in leaf extract. Further separation and characterization of the active principle from column fractions of *L. inermis* using GC–MS analyses reveal the presence of hexacosane, octadecane, docosane, heptacosane methyl, octacosane, and tetracosane.

Hexacosane and heptacosane methyl belong to the class of organic compounds and show medicinal properties. Hexacosane has been detected from many plant species like *Glycyrrhiza glabra*



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and *Vanilla planifolia* but heptacosane methyl has been found only in *G. glabra* [72] and octadecane was identified as a volatile component in the extract of Korean Chamchwi (*Aster scaber* Thunb) [73]. Remaining three components i.e. octacosane, docosane and tetracosane are not identified as having medicinal properties. *L. inermis* also shows the vast medicinal properties and many other researchers also detected other compounds which show the medicinal properties. Mikhaeil et al. [74] reported that *L. inermis* contains p-coumaric acid, lawsone, apigenin, luteolin, 2-methoxy-3-methyl-1,4- naphthoquinone, cosmosiin and apiin. The antifungal activity of 1,4- α naphthoquinone and hydroxyl-carbons was reported by many researchers [22,75,76].

Author's contributions

TSB: Planned and performed the experiments, wrote the manuscript and prepared the final version of the manuscript. MM: planned to design the manuscript format, data analyzed,

analyzed the results, helped for statistical analysis and drafting the manuscript. KS: supervised the whole work. All authors have read and approved the final version of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2019.e00335.

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