Identification and quantification of antifungal compounds from medicinal plants by HPLC method

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ABSTRACT

The quest for plants with medicinal properties continues to receive attention as scientist survey plants, particularly with ethnobotanical, significance for complete range of biological activities ranges from antibiotic to anti-tumor. The anti-microbial compounds present in medicinal plants are degradable and selective in their toxicity and so they are considered as valuable ingredients for controlling plant diseases caused by virus, bacteria and fungi. The antifungal compounds of two medicinal plants *viz.*, *Lantana camara* and *Bougainvillea glabra* was extracted by using ethyl acetate. Active spots identified by thin layer chromatography assay and were further purified using preparative HPLC. The purified compounds were subjected to chemical analysis and found to contain phenolic group of compounds. HPLC purified *Lantana camara* ethyl acetate extract was found to be more active against *Pythium aphanidermatum* than the *Bougainvillea glabra* extract. The compound was further analysis and confirmed by GCMS. The partially purified phenolic compounds can be used against plant pathogens. However further study in the purified compounds must be under taken to validate the results.

Key words: plant species, pathogens, chromatogram, chemical compounds

The presence of inherent antimicrobial compounds in several higher plants has long been recognized as important factor for disease resistance. Such compounds being degradable and selective in their toxicity are considered as valuable ingredients for controlling plant diseases caused by viruses (Balasaraswathi, 1995), bacteria (Akhtar et al., 1997) and fungi (Bindu et al., 1998). During the past few years, there is a growing trend all over the globe to shift from synthetic to natural products including medicinal plants. It is high time now to consider neglected and little known botanicals to cure the diseases, which create challenging problems in agriculture and pose real economic and environmental threats. In spite of the efficacy of plant extracts on plant disease, farmers are skeptical to use the botanicals because these products are not readily available. Preparation of plant extracts is also considered as labour intensive and time consuming. Recently attempts have been made to test the potential benefit of the plant extracts for the control of plant disease in vegetables (Babu *et al.,* 2001). However, these works were limited to in vitro screening of plant products and have not been tested under field conditions. Currently, Integrated Disease Management (IDM) concept is increasingly adapted in many plant disease management programs with a view to protect the environment and to maintain the healthy food chain. The recent research also focuses on searching for safer and more compatible alternative methods for plant disease management. Plant derived bio – fungicides are now emerging as most important eco – friendly component of IDM (Kumbhar et al., 2001). The name HPLC originally referred to the fact that high pressure was needed to generate the flow required for liquid chromatography in packed columns. In the beginning, instrument components only had the capability of generating pressures of 500psi (35 bar). This was called High Pressure Liquid Chromatography (HPLC). The early 1970's saw a tremendous leap in technology. These new HPLC instruments could develop up to 6,000psi (400 bars) of pressure, and included improved detectors and columns. HPLC really began to take hold in the mid to late 1970s. With continued advances in performance, the name was

changed to High Performance Liquid Chromatography (HPLC). High Performance Liquid Chromatography (HPLC) is now one of the most powerful tools in analytical chemistry, with the ability to separate, identify and quantitative the compounds that are present in any sample that can be dissolved in a liquid. Today, trace concentrations of compounds, as low as parts per trillion are easily obtained. HPLC can be applied to just about any sample, such as pharmaceuticals, food, neutraceuticals, cosmetics, environmental matrices forensic samples, and industrial chemicals.

Many fungal diseases affect many crops. Control of fungal disease by use of resistant varieties has been very useful but the pathogen is able to develop into new physiological races over years and thereby they were able to overcome the resistance. This situation forced scientists to search for new antimicrobial substances from the various sources including medicinal plants. Keeping in view of these aspects research was formulated with the following objectives; i) Collection of plant materials ii) Preparation of plant extract and determination of antifungal activity by agar well diffusion method ii)Screening and isolation of antifungal compounds and iv) Purification and identification of antifungal compounds.

MATERIALS AND METHODS

Collection of plant material

The fresh leaves of *Lantana camara* and *Bougainvillea glabra* were collected from the Tamil Nadu Agricultural University, Coimbatore. The collected plant material were shade dried, powdered and stored for conducting antifugal assays.

Test organisms

The fungal cultures used in the study were obtained form Department of Plant Pathology, TNAU, Coimbatore. Totally seven numbers of fungal cultures were used. They are

- *Aspergillus niger*
- *Aspergillus flavus*
- *Fusarium oxysporum*
- *Fusairum udum*
- *Pythium aphnidermatum*
- *Macrophomina phaseolina*
- *Phytophthora infestans*
- *Fusarium monliforme*

Preparation of plant extract

About 30 g of each plant powder was percolated with 150 ml of ethyl acetate separately (at the ratio of 1:5), and kept for overnight for digestion. After overnight extraction the extract was filtered using Whatman No. 44 filter paper and concentrated using rotary vacuum evaporator at 40°C to obtain dried extract, which was then stored at refrigerated condition for further use.

Preparation of test samples

300mg of dried extract of each plant sample were dissolved in 3.5 ml of ethanol and used in the antifungal assays. Ethanol solvent without the test compound was used as the negative control. An antifungal agent ketoconazole is used as positive control at a concentration of 10mg/ml.

Preparation of culture media

The fungi were cultured and maintained on potato dextrose agar (PDA) medium. For the bioassay, a loopful of the organism was inoculated into 100 ml of the respective broth as the medium. The conical flasks were incubated at a temperature of 37° C.

Agar well diffusion assay were carried out as per the procedure given by Iqbal et al., 1998.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC was performed to test the antifungal activity of both the plant extracts and active bands using tube dilution method (Claeys *et al.*, 1988). The MIC was defined as the lowest concentration able to inhibit any visible microbial growth. This test was performed at four concentrations of the plant extract (10 mg ml^{-1}, 1 mg ml^{-1}, 0.1 mg ml^{-1}, 10 µg ml^{-1}).

Inhibitory concentration 50 (IC50)

The inhibitory concentration was performed to test the antifungal activity of both the plant extracts and active bands using tube dilution method IC50 is defined as it is a concentration at which 50% mortality of organisms will be observed (Phongpaichit *et al.*, 2004).

Chromatographic separation of plant extract

Thin layer chromatographic studies were carried out to find out the antifungal compound present in the ethyl acetate extract of the plant material.

Purification of antimicrobial compounds through RP-HPLC

The bands, which possess antifungal activity, were checked for their purity in reversed phase HPLC. The samples were run using the mobile phase acetonitrile:water (60:40) at 1 ml/min flow rate. The detection wavelength was 245 nm.

Identification of compounds using GC-MS

GC-MS analysis was carried out by using Perkin Elmer - Clarus 500 GC-MS unit. The analysis was carried out to detect the possible compounds present in the active fraction of the plant extract. The GC conditions are given below

GC-MS conditions

Column type : PE-5 (equivalent to DB-5)
Column length: 30 m
Carrier gas : Helium
Flow rate : 1 ml/min
Column temperature
Initial : 80^o C
Final : 280^o C
Rate of temperature change : 10^o c
Injector temperature : 230^o c
Detector temperature : 280^o c
Sample injection volume : 2 µl.

Statistical analysis

The data were analyzed using completely randomized design (Panse and Sukhatmae, 1965). If the treatment differences are found significant in F test, critical differences was worked out at 5 per cent probability level.

RESULTS AND DISCUSSION

Antifungal activity by agar well diffusion method

Traditionally used medicinal plants produce a variety of compounds of known therapeutic properties. Those substances, which can either inhibit the growth of pathogens or kill them and have no or least toxicity to host cells are considered candidates for developing new antimicrobial drugs In the present study, ethyl acetate extract of *Lantana camara* exhibit antifungal activity against *Pythium aphanidermatum, Macrophomina phaseolina, Aspergillus niger, Fusarium udum* with the inhibition zone of above 2cm by agar well diffusion method whereas, the positive control ketoconazole produced inhibition zone of above 2.5 cm. the results were compared to the standard antibiotics, it is clear that the extract has strong effect against *Pythium aphanidermatum, Macrophomina phaseolina, Aspergillus niger* and *Fusarium udum*. The ethyl acetate extract of *Bougainvillea glabra* against *Fusarium udum* exhibited 2.7 cm zone of inhibition and in case of *Aspergillus niger* the inhibition zone obtained was 2.2 cm. The ethanol control exhibited 0.3 cm zone of inhibition. It is estimated, that if an inhibition is obtained by 1-10 mg plant extract per ml test solution, the extract can be considered worthy for further investigations.

Minimum Inhibitory Concentration method for evaluation of antifungal activity

The minimum inhibitory concentration is the concentration at which 50% of the organisms get killed by the compound. Sabitha and Suranarayana, (2006) reported that different concentration of *Spilanthes cemella* flower head extract was evaluated for antifungal activity at concentration of 0.1 and 0.2 mg. Rao *et al.* (2006) reported that the minimum inhibitory concentration of *Achyranthes aspera* against *Fuarium udum* and *Aspergillus niger* are 30 µg and 40 µg respectively. The lowest MIC was observed in the aqueous extract of *Myrtus communis* and *Terminalia chebula* as 0.31mg against *Candida albicans* and *Candida utilis* (Bonjar, 2004).

In the present study ethyl acetate extract of *Lantana camara* exhibit MIC against *Pythium aphanidermatum, Macrophomina phaseolina, Aseprgillus niger, Fusarium udum* at a concentration of 10 mg. It was clear that band 11 from the ethyl acetate extract of *Lantana camara* have also observed for their maximum inhibition at10 mg concentration in MIC assay. In other concentration *i.e* lesser than 10 mg of band 11 no inhibition was observed against all the tested four pathogens.the study further revealed that 10 mg concentration of ethyl acetate extract of *Bougainvillea glabra* was found to be active against the three fungal pathogens *viz., Aspergillus niger, Aspergillus flavus and Fusarium udum* in MIC assay. While band 6 of *Bougainvillea glabra* obtained from TLC separation showed inhibition at 10 mg concentration against the fungal pathogens such as *Aspergillus niger, Aspergillus flavus, Fusarium udum*. The growth was observed in all

other concentration of band 6 lesser than 10 mg in MIC assay. The MIC assays concludes the possibility of IC 50 values ranging between 1-10 mg for the band 11 of *Lantana camara* and as well as band 6 of *Bougainvillea glabra*.

Inhibitory concentration (IC50) for Lantana camara and Bougainvillea glabra

Inhibitory concentration 50 was evaluated for band 11 of *Lantana camara* and band 6 of *Bougainvillea glabra* against fungal plant pathogens. Band 11 of *Lantana camara* showed IC 50 values of 1mg, 1mg, 0.95mg and 0.87 mg against *Pythium aphanidermatum*, *Macrophomina phaseolina*, *Aspergillus niger* and *Fusarium udum* respectively.

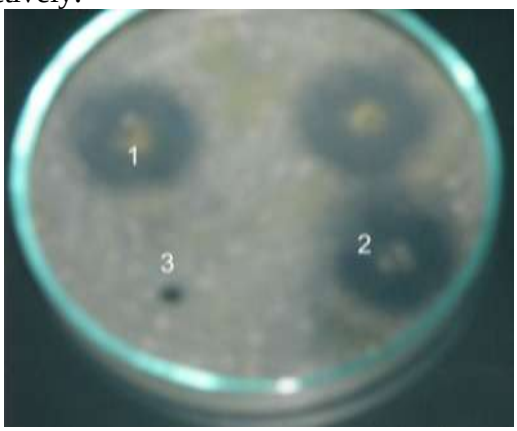

Fig 1. Inhibition zone for *Lantana camara* leaf extract against *Phythium aphanidermatum*

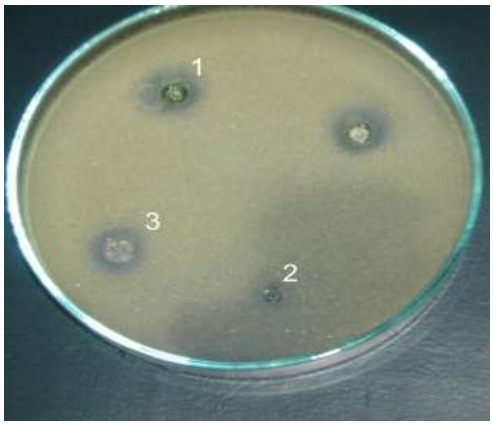

Fig 2. Inhibition zone for *Lantana camara* leaf extract against *Macrophomina phaseolina*

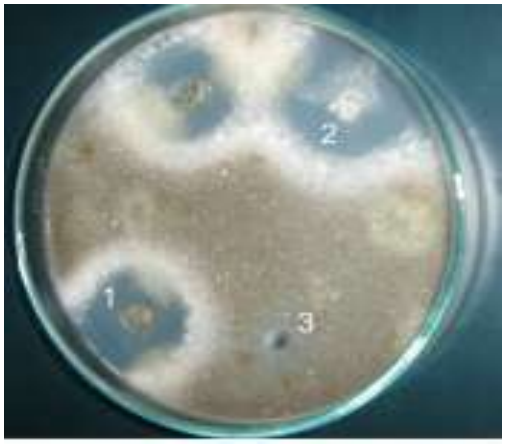

Fig 3. Inhibition zone for *Bougainvillea glabra* leaf extract against *Aspergillus niger*

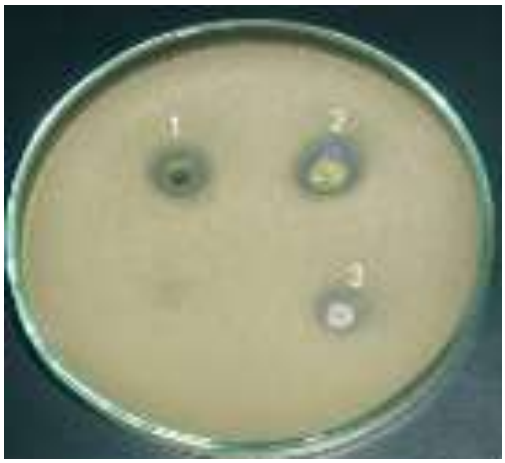

Fig 4. Inhibition zone for *Bougainvillea glabra* leaf extract against *Fusarium udum*

Band 6 of *Bougainvillea glabra* was found to be active against test microorganisms like *Aspergillus niger*, *Aspergillus flavus*, and *Fusarium udum* with IC 50 values ranging from 0.79, 0.90 and 0.79 respectively. Anil *et al.* (2006) also reported that two compounds oleanonic acid and leanolic acid isolated from hexane and chloroform fraction collected from *Lantana camara* showed IC 50 at 100mg and 31.5 mg respectively against *Bacillus* under *in vitro* condition (Fig 1 to 4; 1. Plant extract 2. Ketoconazole (possitive control) 3. Ethanol (Negative control**)**).

HPLC studies

Oleanolic and ursolic acids were isolated for the first time from the alcoholic extract of *Mitracarpus scaber* possessing antimicrobial effects on *Dermatophilus congolensis*. These two

triterpenic acids were also active (MIC 15 µg/ml) on this causative agent of dematophillosis in African animals. To quantify oleanolic and ursolic acids in *Mitracarpus scaber*, a new, simple and rapid high performance liquid chromatography (HPLC) method compatible with MS detection was developed and validate. Hahn *et al.* (1993) separated and quantified ten substituted benzoic and cinnamic acid (phenolic acids) of pea by using reversed phased high performance liquid chromatography.

In the present study, using Shimadzu HPLC the active bands was analyzed. The mobile phase acetonitrile:water (60:40) was pumped through a C_{18} ODS (Octa Decyl Silyl) column at a flow rate of 1.0 ml/min and the eluate was monitored by UV detector at 245 nm for 30 minutes. Five peaks were observed in HPLC chromatogram of band 11 of *Lantana camara*, which indicates the presence of more than one molecule. Seven peaks were observed in HPLC chromatogram band 6 of *Bougainvillea glabra* observed. The maximum area of peak obtained at retention time of 7.0. Presence of many peaks indicates impurities present along with the molecule that exhibits the antifungal activity against the tested plant pathogens (Fig 5 and 6).

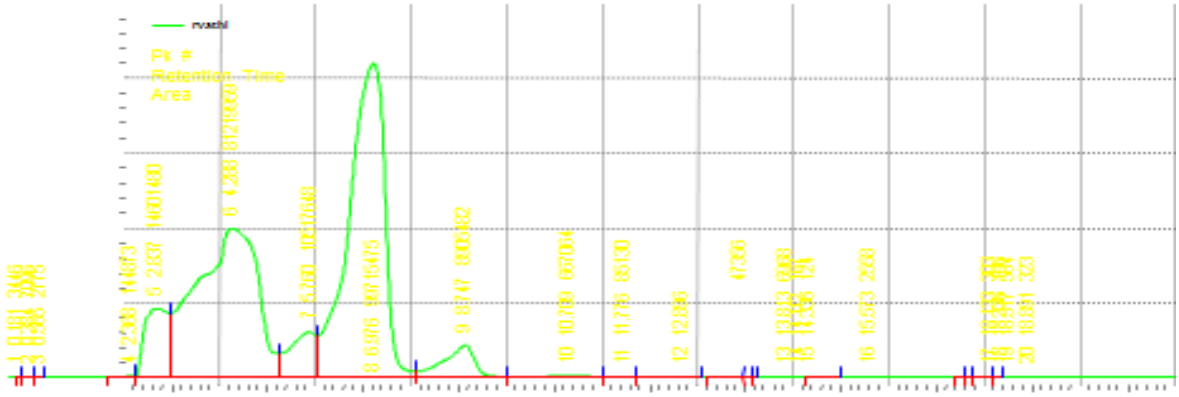

Fig 5. HPLC chromatogram for band of *lantana camera* leaf extract

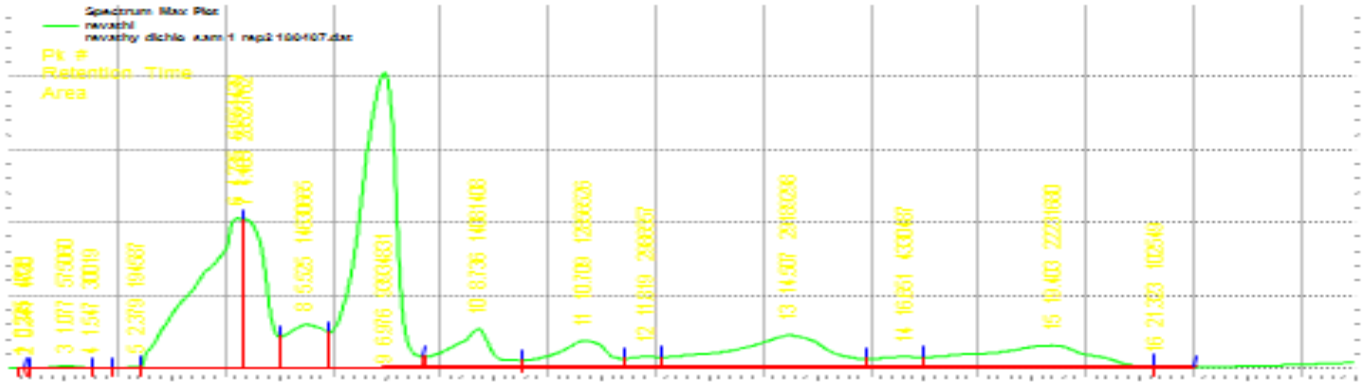

Fig 6. HPLC chromatogram for band 11 of *Bougainvillea glabra* leaf extract

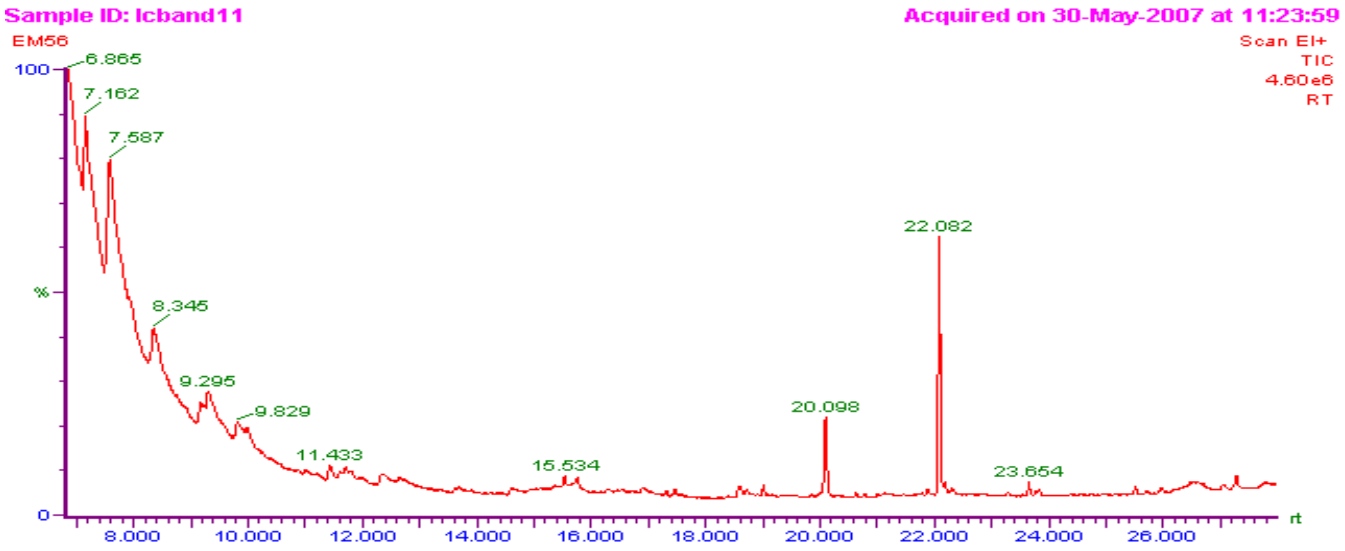

Fig 7.Gc-Ms- Analysis of band 11 0f *Latana camara* plant extract

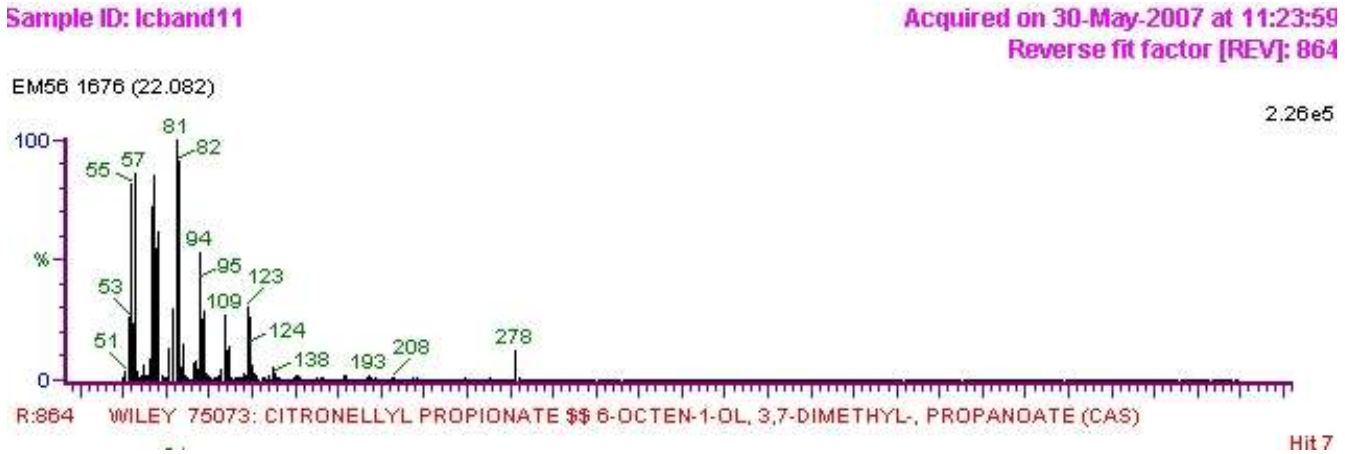

Fig 8 .Mass spectrum of inhibitory band 11

GCMS studies of band 11 of *Lantana camara*

Gas chromatography mass spectrometry identify the compounds based on their retention indices (determined with reference to a homologous series of normal alkanes), and by comparison of their mass spectral fragmentation patterns. *Lantana camara* essential oil is high in α-humulene and β-caryophyllene, and *Lantana aculeate* essential oil is high in β-caryophyllene. *Lantana xenica* is also rich in β-caryophyllene and germacrene D. the essential oils of *Lantana camara*, *Lantana aculeate*, *Lantana xenica* and *Lantana achyranthifolia* have exhibited weak antimicrobial activity, consistent with the results of *Lantana involucrata* leaf oil (Barre *et al*., 1997).

In our present investigation, gas chromatography mass spectrometry studies were carried out for the band 11 of ethyl acetate extract of *Lantana camara*. Seven peaks were observed in GC chromatogram, which indicates the presence of more than one molecule. The major peak was obtained in the retention time of 22.08. The mass spectrum for the peak produced at the retention time of 22.08 was taken. The presence of phenolic type of compound is inferred from the mass spectral data when it was compared with the compound list obtained from GCMS library (Fig 7 and 8).

CONCLUSION

HPLC purified *Lantana camara* ethyl acetate extract was found to be more active against *Pythium aphanidermatum* than the *Bougainvillea glabra* extract. The compound was further analysis and conformed by GCMS. The partially purified phenolic compounds can be used against plant pathogens. However further study in the purified compounds must be under taken to validate the results.

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