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Research Article

ANTIFUNGAL APPRAISAL OF *BURKHOLDERIA GLADIOLI* STRAIN VIMP03 (JQ867372) AGAINST *CERATOCYSTIS PARADOXA*

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ABSTRACT

Objective: The purpose of the present investigation was to assess antifungal characterization of *Burkholderia gladioli* strain VIMP03 (JQ867372), an isolate from sugar beet rhizosphere.

Methods: Antifungal characterization was carried out by biochemical, dual culture, and agar well diffusion methods against *Ceratocystis paradoxa*, a soilborne pathogen of sugarcane and other crops. Culture filtrate and ethyl acetate extract obtained from culture supernatant were analyzed by high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) analyses, respectively.

Results: The culture filtrate and ethyl acetate extract exhibited effective antifungal activity. Organic acid profile of the culture filtrate was determined. Acetic acid was mainly produced by the culture under study. The GC-MS analysis of ethyl acetate extract documented the presence of compounds including tetratetracontane, 9-nonadecene, erucic acid, and other hydrocarbon derivatives.

Conclusion: The GC-MS, HPLC, and biochemical profiles of *B. gladioli* strain VIMP03 (JQ867372) revealed its agro clinical-antifungal potential.

Keywords: Antifungal, *Burkholderia gladioli*, High-performance liquid chromatography, Gas chromatography-mass spectrometry.

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INTRODUCTION

Phytopathogenic fungi such as *Alternaria alternata, Rhizoctonia solani, Aphanomyces chochlioides, Sclerotium rolfsii, and Ceratocystis paradoxa* are the major causes for the soilborne diseases of sugarcane, sugar beet, coconut, pineapples, and other crops. *C. paradoxa* is a causative agent of pineapple disease or black rot of sugarcane. The *C. paradoxa* enters through cut ends and proliferates rapidly in parenchymal tissues of internodes developing brown-black cavities. It thus damages cane setts in the soil, inhibits rooting during the disease development, and affects juice quality by decreasing the level of sucrose and glucose [1-3].

Plant growth-promoting rhizobacteria (PGPR) are the helpful microbes which promote plant growth by solubilizing phosphate, potash, zinc, and sulfur, and fixing nitrogen or decomposing complex organic or inorganic matter as well as act as biocontrol mediators. Several researchers have reported the application of fungi and bacteria as biocontrol agents such as *Trichoderma hamatum, Trichoderma viridae, Saccharomyces unispora, Candida steatolytica, Pseudomonas fluorescens, Burkholderia, Bacillus,* and others [4-8]. Plant growth-promoting microbes can be used as right substitute to chemical pesticides and fertilizers.

Burkholderia species are the PGPR frequently isolated from rhizosphere of various crops [9-13]. They have qualities for potential industrial and agricultural applications including production of antibiotics, bio-surfactants, bio-plastics, and degradation of environmental contaminants [14]. Many *Burkholderia* species have reported as antimicrobial bacteria. *Burkholderia* species inhibited the growth of *Ustilago* and *Fusarium* [15]. Gohar *et al*. [16] characterized antibacterial agents produced by marine *Burkholderia cepacia.* Sultan *et al*. [17] emphasized the role of phthalate-producing *B. cepacia* K87 in antifungal activities against *Candida glabrata* and *R. solani.* Elshafie *et al.* [18] reported antifungal activities of *Burkholderia gladioli pv. agaricicola* against *Fusarium oxysporum* and *R. solani.* The *Burkholderia tropica* was documented to inhibit the growth of phytopathogenic fungi such as *Colletotrichum gloeosporioides, Fusarium culmorum*, *F. oxysporum,* and *S. rolfsii* [19].

Most of the *Burkholderia* species produce bioactive compounds for the suppression of plant diseases, especially antibiotics, siderophores, organic acids, biocidal volatile organic components, phenolic and phthalate derivatives, aromatic complexes, and enzymes including chitinase, cellulase, and protease. Bioactive components were well documented after agar well diffusion, high-performance liquid chromatography (HPLC), and gas chromatography-mass spectroscopy (GC-MS) studies by a number of investigators in extracts obtained from different plants as well as bacterial and fungal cultures [16,20-27]. In the present investigation, antifungal characterization of *B. gladioli* strain VIMP03 (JQ867372), an isolate from sugar beet rhizosphere, was carried out by dual culture, agar well diffusion, HPLC, and GC-MS methods.

METHODS

Bacterial culture

Bacterial culture used for the present study was *B. gladioli* strain VIMP03 (JQ867372), the isolate by Mahamuni [11], Mahamuni and Patil [28] from sugar beet rhizosphere having phosphate solubilizing and composting abilities.

Phytopathogenic culture

The phytopathogenic culture of *C. paradoxa* was obtained from the Plant Pathology Section of Vasantdada Sugar Institute, Manjari Bk., Pune, Maharashtra, India.

Culture media

Pikovskaya's broth and agar [29] media were used to cultivate *B. gladioli* strain VIMP03 (JQ867372) at 30°C for 4 days. The Pikovskaya's agar contained (g/L) dextrose 10; NaCl 0.2; $Ca_3(PO_4)_2$ 5; $(NH4)_2SO_4$ 0.5; KCl 0.2; $MgSO_4.7H_2O$ 0.1; $MnSO_4.H_2O$ 0.0001; yeast extract 0.5; and Agar 18. The pH of medium was 7.0 (±0.2). The Pikovskaya's broth contained the same components without agar.

Potato dextrose agar (PDA) was used to cultivate phytopathogenic culture of *C. paradoxa* and also to check *in vitro* antagonistic activity at room temperature for 4-7 days. The PDA contained (g/L) potato infusion 200; dextrose 20; agar 20, and the pH of medium was 5.6 (±0.2).

The pH of both culture media was adjusted using 1N NaOH or 1N HCl. Media were sterilized by autoclaving at 120°C for 15 minutes.

Cellulase activity

On cellulose agar plate containing cellulose as sole carbon source and pH 7.5 (± 0.2), the bacterial culture *B. gladioli* strain VIMP03 (JQ867372) was spot inoculated and incubated at 30 0C (± 0.2°C) for 48 hrs. After incubation plate was repeatedly treated with 0.5% Congo red for 15 min at room temperature and was counterstained with intermittent thorough washing by 1M NaCl solution. The nonappearance of clear zone around the colony was considered cellulase negative activity [30].

Protease activity

For protease test, culture under study was spot inoculated on milk agar having pH of 7 (±0.2) and incubated at 30°C (±0.2°C) for 48 hrs. The development of clear zone around the colony against opaque background was considered protease positive [31].

Chitinase activity

The culture of *B. gladioli* strain VIMP03 (JQ867372) was spot inoculated on colloidal chitin agar and incubated at 30°C (±0.2°C) for 5 days, and chitinase activity was examined as positive if there was zone of clearance around the colony [32].

HPLC analysis for organic acids

Organic acids were detected by HPLC in Pikovskaya's broth, in which *B. gladioli* strain VIMP03 (JQ867372) was cultivated at 30°C (±0.2°C) for 4 days. The culture broth was filtered through 0.2 µm filter (Millipore), and 20 µl of filtrate was injected to HPLC (Model - Waters Alliance Company) equipped with an ultraviolet detector. Organic acid separation was carried out on organic acid (Prevail) column (Make Grace) with specifications such as length 150 cm and internal diameter (ID) 4.6 mm and 25 mM KH_2PO_4 as mobile phase. At a wavelength of 210 nm, retention time (RT) of each signal was recorded. Organic acids from the culture filtrate were identified by comparing RTs of chromatographic peaks with those of reference standards.

Dual culture method

Primary antifungal activity of *B. gladioli* strain VIMP03 (JQ867372) was checked by the dual culture *in vitro* assay method [33,34]. The culture was spot inoculated at one end of the PDA. After 2 days incubation at room temperature, 6 mm agar disc, using growth of fungal pathogen *C. paradoxa* from fresh PDA agar culture, was placed at the other marginal side of the plate and incubated at room temperature for 7 days. The radii of the fungal colony toward and away from the bacterial colony were noted to calculate percent growth inhibition by the following formula:

$$\text{Percent inhibition} = (A - a)/A \times 100$$

Where, "a" is the radius of the fungal colony opposite to the bacterial colony and "A" is the maximum radius of the fungal colony away from the bacterial colony.

Antifungal activity of culture filtrate and ethyl acetate extract

The culture *B. gladioli* strain VIMP03 (JQ867372) grown in Pikovskaya's broth for 4 days at 30°C (±0.2°C) was centrifuged at 3000 rpm for 10 minutes, and the supernatant was sterilized by passing it through a millipore membrane filter (0.45 µm of pore size). The sporulated culture of *C. paradoxa* was inoculated into sterile molten PDA medium (45°C) and poured into sterile Petri dishes. Antagonistic activity of culture filtrate was detected by agar well diffusion technique.

A bioassay-directed practice was followed in the process of isolating and fractionating ethyl acetate extract. Antifungal principles from the cell-free filtrate were extracted by solvent ethyl acetate. Ethyl acetate extract was evaporated at room temperature and concentrated. About 500 ml of ethyl acetate extract was reduced to 15 ml. Antifungal activity of concentrated ethyl acetate extract was detected qualitatively by agar well technique using 100 µl of the extract.

GC-MS analysis of ethyl acetate extract

The GC-MS analysis was done with thermo GC coupled with ITQ 1100 mass detector and X-Caliber software and the National Institute of Standards and Technology (NIST) Spectral data (GCMSMS, Thermo Fisher Scientific). A DB-5 MS capillary column having 30 mm × 0.25 mm ID and coated with 0.25 µm film thickness was injected with 2 µl sample. The carrier gas helium (99.99%) was used at a flow rate of 1 ml/minute in split mode (1:50). The temperature of the column was programed at 60-280°C. The injection port and transfer line temperatures used were 250°C and 280°C, respectively. The temperature program initiated at 60°C for 2 minutes hold, then it was raised at 15°C/min to 160°C, which was held for 0 min, and then at 3°C/min to 200°C, which was held for 1 minute, and again at 8°C/min to 280°C, which was held for 6 minutes. The mass spectrum of compounds present in the sample was recorded with electron impact ionization energy 70 eV over mass range of 50-650 Da amu. The chemical components from ethyl acetate extract of culture filtrate were identified by comparing RT of chromatographic peaks with those of reference standards from database of the NIST library.

RESULTS

The culture under studies, *B. gladioli* strain VIMP03 (JQ867372), was protease and chitinase positive as clear zones were developed around the colonies (growth) on milk agar and chitin agar, respectively; while the culture was avowed cellulose negative due to non-development of clear zone around its colony.

HPLC (peaks) of organic acids is shown in Fig. 1. The organic acid profile of the bacterial culture is presented in Table 1 along with RT and organic acid content in mg per 100 ml (or mg %). In the culture filtrate obtained from Pikovskaya's broth, four organic acids were detected. The highest amount of organic acid produced by the *B. gladioli* strain VIMP03 (JQ867372) was acetic acid (17.22 mg %), which was followed by formic acid (1.15 mg %), oxalic acid (0.27 mg %), and pyruvic acid (0.11 mg %).

Table 1: Organic acid profile based on HPLC

S. No.	Organic acids	RT (minutes)	Content (mg %)	Percent area
1	Oxalic acid	1.855	0.27	22.39
2	Formic acid	2.433	1.15	6.06
3	Pyruvic acid	2.609	0.11	7.94
4	Lactic acid	–	–	–
5	Citric acid	–	–	–
6	Gibberellic acid	–	–	–
7	Acetic acid	4.178	17.22	63.60

–: Not detected

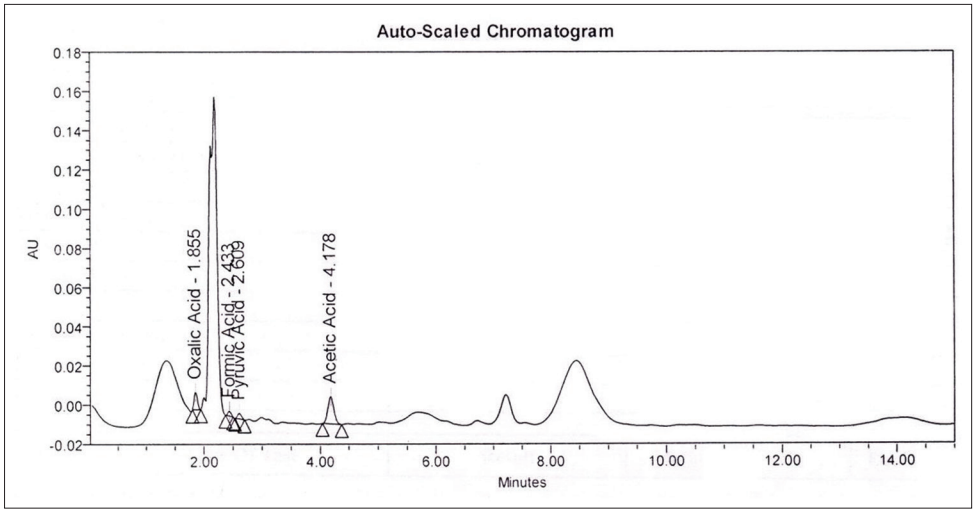


Fig. 1: High-performance liquid chromatogram (peaks) of organic acids

The percent growth inhibition estimated by dual culture method, exhibited by the *B. gladioli* strain VIMP03 (JQ867372), against *C. paradoxa* was 41%. This finding is in accordance with that of Kadir *et al*. [35] who reported 41-81.7% fungal growth inhibition by *B. cepacia.* Both ethyl acetate and culture filtrate extracts under the present investigation inhibited the fungal pathogen growth with zone of inhibitions (ZOIs) of 19 mm (standard deviation [SD] ±2) and 24 mm (SD ±2), respectively.

The GC-MS technique was used to identify the probable antifungal compounds of *B. gladioli* strain VIMP03 (JQ867372) extract that possessed antifungal activity, and 9 compounds were identified. GC of ethyl acetate extract is shown in Fig. 2.

The number of peaks, the RT, and area and matching factor of the compounds present were compared with those of in the NIST database. The compounds identified are shown in Table 2 along with RT, molecular formula (MF), molecular weight (MW), and their chemical structures The first compound identified with less RT (12.20 minutes) was E-2-octadecadecen-1-ol, whereas the last compound which took longest RT (33.33 minutes) to identify was tetratetracontane.

Mass spectra (MS) of the related compounds are shown in Fig. 3a-i.

DISCUSSION

Different solvents have various degrees of solubility for different antimicrobial compounds and also organic solvents have more powerful antimicrobial activity as compared to aqueous extracts [22]. The present study outcomes were not in harmony with the findings of Bhuvaneswari and Gobalakrishnan [22] as more ZOI was recorded by the aqueous culture filtrate extract. Antibiosis is generally mediated by specific or non-specific metabolites of microbial origin, by lytic agents, enzymes, volatile compounds, or other toxic substances [36]. Lavermicocca *et al*. [37] purified and characterized novel antifungal compounds from *Lactobacillus plantarum* and also reported that lactic and acetic acid produced by bacterium played the most significant role in antifungal actions. The VIMP strain produced a variety of organic acids as shown in Table 1 and lytic enzymes such as chitinase and protease. These might be the reasons that the culture exhibited antifungal activities as supported by percent inhibition by dual culture method and ZOIs as shown by both ethyl acetate and culture filtrate extracts. The ZOI exhibited by culture filtrate extract was 26.31% more than the ZOI revealed by the ethyl acetate extract. This might be due to the presence of more amounts of lytic enzymes, volatile compounds, and other metabolites in concentrated and active states in culture filtrate rather than in the ethyl acetate extract. Mahamuni [38,39]

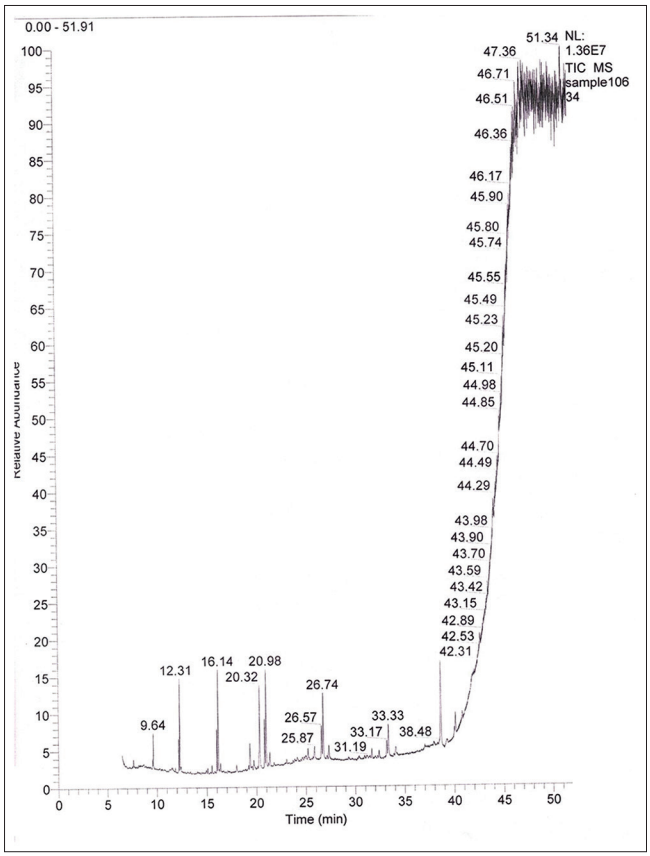


Fig. 2: Gas chromatogram of ethyl acetate extract

also reported large ZOI for aqueous culture filtrate extracts against fungal phytopathogens such as *A. alternata* and *C. paradoxa*. Degree of solubility for all antimicrobial components in ethyl acetate extract might not be so much extensive.

Many previous reports stated antifungal activities of ethyl acetate extracts obtained from the supernatants of different microbial cultures as well as of different plant extracts and listed the identified possible antifungal compounds based on GC-MS analyses. Many researchers reported the antimicrobial role of phenolic, alkaloid, carboxylic acid, hydrocarbon, ketone, ester, and phthalate derivatives [17]. Presence of tetratetracontane and diethyl phthalate having antimicrobial

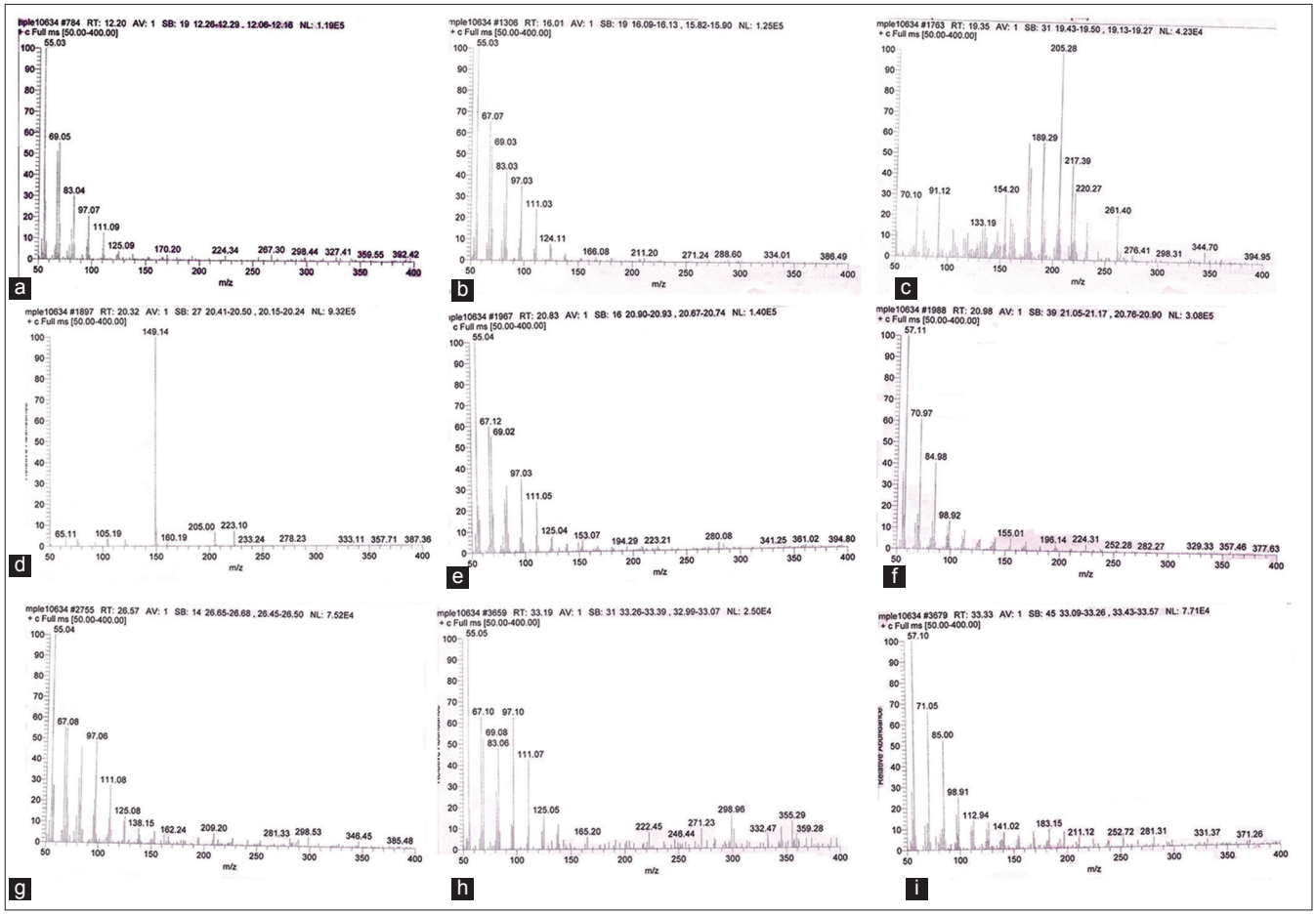
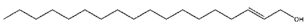
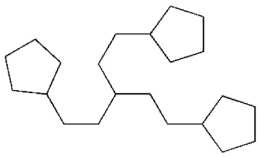
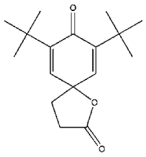
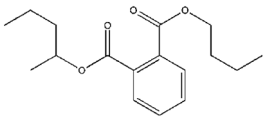


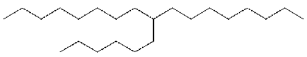
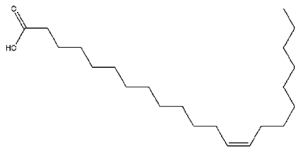
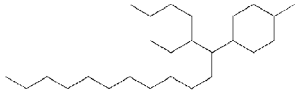
Fig. 3: Mass spectra (MS) of the compounds. (a) MS of E-2-octadecadecen-1-ol, (b) MS of cyclopentane 1, 1(3-(2-cyclopentyl ethyl) 1, 5-pentadiyl, (c) MS of 7, 9-Di-ter-butyl-1-oxaspiro (4, 5) deca-6, 9-diene-2, 8-dione, (d) MS of phthalic acid butyl 2-pentyl ester, (e) MS of 9-nonadecene, (f) MS of heptadecane 9-hexyl, (g) MS of erucic acid, (h) MS of cyclohexane 1, 1-dodecylidenebis (4-methyl), (i) MS of tetratetracontane

Table 2: GC-MS profile of ethyl acetate extract

Sr. No.	Name and chemical structure of the compound	MF	MW	RT (minutes)
1	E-2-octadecadecen-1-ol 	$C_{18}H_{36}O$	268	12.20
2	Cyclopentane 1,1 (3-(2-cyclopentyl ethyl) 1,5-pentadiyl 	$C_{22}H_{40}$	304	16.01
3	7,9-Di-ter-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione 	$C_{17}H_{24}O_3$	276	19.35
4	Phthalic acid butyl 2-pentyl ester 	$C_{17}H_{24}O_4$	292	20.32

(Contd...)

Table 2: ***(Continued...)***

Sr. No.	Name and chemical structure of the compound	MF	MW	RT (minutes)
5	9-nonadecene	$C_{19}H_{38}$	266	20.83
6	Heptadecane 9-hexyl 	$C_{23}H_{48}$	324	20.98
7	Erucic acid 	$C_{22}H_{42}O_2$	338	26.57
8	Cyclohexane 1,1-dodecylidenebis (4-methyl) 	$C_{26}H_{50}$	362	33.19
9	Tetratetracontane	$C_{44}H_{90}$	618	33.33

GC-MS: Gas chromatography-mass spectrometry

activity was recorded based on GC-MS studies of endophytic fungal extracts [40]. Antifungal *B. cenocepacia* strain VIMP01 (JQ867371) produced tetratetracontane, heptadecane-9-hexyl, and 7,9-Di-ter-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione [38], whereas *B. gladioli* strain VIMP02 (JQ811577) produced compounds such as 9-nonadecene, 7,9-Di-ter-butyl-1-oxaspiro(4,5) deca-6,9-diene-2,8-dione, phthalic acid butyl 2-pentyl ester, and tetratetracontane [39]. Usha *et al*. [26] documented antimicrobial compounds of marine *Streptomyces cacaoi* strain SU2 (JF730119) in the ethyl acetate extract by GC-MS such as phthalic acid butyl ester and 1 nonadecene derivatives. El-Baz *et al*. [23] listed the presence of antimicrobial tetratetracontane in ethyl acetate extract of *Jatropha cureas* leaves. The presence of antimicrobial heptadecane was also reported by Khairy and El-Kassas [41] in ethyl acetate extract from blue green algae such as *Anabaena flos-aqua, Anabaena variabilis,* and *Oscillatoria angustissima*. Taha *et al*. [42] reported antifungal activity of seed oil from *Eruca sativa* containing erucic acid, a major fatty acid, along with garlic oil against dermatophytes causing hair diseases. Gopalakrishnan *et al*. [43] reported that ethanolic extract of stem of *Cayratia trifolia* contained bioactive tetratetracontane. Rabha *et al*. [44] detected oxalic acid and extracellular hydrolytic protease and chitinase enzymes in the extract obtained from endophytic fungus, *C. gloeosporioides,* having antifungal traits. Results of the present investigation are in accordance with metabolites reported by the above researchers. It may be the first documentation highlighting the production of antimicrobial formic acid and erucic acid by any *Burkholderia* culture. Combination of organic acids, enzymes such as chitinase and protease, and antimicrobial compounds as identified by GC-MS exerted antifungal activity in the present investigation. These components may affect fungal cell wall, proteins, and nucleic acids. However, differences at the level of antifungal activities, HPLC, and GC-MS profiles can be explained on the basis of cultural differences, media composition, growth conditions, and diversity in antifungal metabolites.

CONCLUSION

The present study outcomes highlighted antifungal activity in the culture filtrate and ethyl acetate extract of *B. gladioli* strain VIMP03 (JQ867372) due to the presence of antimicrobial principles. On the basis of biochemical, HPLC, and GC-MS analyses, the number of bioactive principles was detected such as lytic enzymes such as protease and chitinase; organic acids such as acetic, formic, oxalic, and pyruvic acids; and other compounds including erucic acid, 9-nonadecene, heptadecane 9-hexyl, and tetratetracontane. Many of the bioactive principles were not reported earlier for any *Burkholderia* species. Regarding the culture under the study, the GC-MS and HPLC profiles in combination may be unique. The culture may have agroclinical potential to develop biofertilizers having fungicidal activity, especially against pineapple disease causing *C. paradoxa*. Field studies should be conducted in the future to access the impact of *B. gladioli* strain VIMP03 (JQ867372) in declining the incidence of pineapple disease.

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