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GC-MS analysis for the potential bioactive compounds and *in vitro* efficacy of the rhizome extract of *Curcuma longa* L. from district Udalguri, Assam, India against white muscardine fungus *Beauveria bassiana*

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Abstract

The purpose of the study was to evaluate the antifungal efficacy of different solvent-mediated rhizome extracts of *Curcuma longa* (turmeric) against the entomopathogenic fungus *Beauveria bassiana,* the causative agent of white muscardine disease in silkworm *in vitro*. Three different concentrations- 1%, 5% and 10% were prepared and tested against the fungus by agar well diffusion method. The methanolic extract was subjected to GC-MS analysis for detection of possible bioactive constituents as the extract exhibited maximum inhibition against *Beauveria bassiana*. The study revealed that the plant extract is effective in inhibiting the fungus and methanolic extract has shown a significantly greater zone of inhibition compared to the ethanolic and aqueous extracts. 10% methanolic extract showed maximum inhibition zone (72.95 %) followed by 10% ethanolic extract (61.53%) and 5% methanolic extract (58.23%) as compared to the standard drug clotrimazole (1% w/w). The GC-MS study detected the presence of important phytoconstituents such as ar-turmerone, α-turmerone, curlone, ß-Sesquiphellandrene, p-ethylguaiacol, dehydrozingerone, 4-vinylguaiacol, and zingiberene. Based on our findings, it can be concluded that the rhizome extract of turmeric has fungicidal properties against the fungus *B. bassiana* that can be the result of the synergistic action of potential bioactive constituents as detected in the GC-MS study.

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Introduction

Medicinal plants have been extensively used against various ailments as a traditional practice from time immemorial as they are the potent sources of the array of bioactive compounds. According to Ayurveda, Siddha and Unani system of medicine, about 35,000 plants have medicinal potential (Panchal and Parvez, 2019). *Curcuma longa*, commonly known as turmeric, belongs to the family Zingiberaceae (Order: Zingiberals) is one such medicinal herb with about 80 species. It is a rhizomatous perennial herb and has a bright yellow rhizome due to the presence of the yellow-orange pigment curcumin (Lampe, 1918). Many researchers have successfully investigated its considerable inhibitory effect against many microbes and it is cultivated widely in Asia and several parts of Africa and Australia. India is the largest grower of turmeric and different parts of the plant have been broadly explored in the ancient Ayurveda system due to its immense therapeutic potential (Araujo *et al.,* 2001). Several studies have reported the broad-spectrum antibacterial, antifungal, antioxidant as well as antiviral properties of turmeric which can be accredited to the presence of active compounds in it (Ashraf and Sultan, 2017).

A group of hydrophobic polyphenolic compounds called curcuminoids is the principal element of turmeric which has now been emerging as a promising potential source of new drugs against different forms of disorders including cancer (Giordano and Tommonaro, 2019). Researchers also extensively documented a wide range of therapeutic activities of the non-curcuminoids as well (Nair *et al.,* 2019). In humans, curcumin is comparatively safe and well-tolerated with no significant toxicity even at doses up to 8 g/day (Gupta *et al.,* 2012). Studies also demonstrated that curcumin targets the replication of SARS-coronavirus by directly binding with the receptor-binding domain of spike protein, blocking it and subsequently obstructing the entry of the viral particles into the host cells (Soni *et al.,* 2020). Therefore it can be hypothesized that curcumin could be efficient even against highly pathogenic pandemic SARS-CoV-2 (COVID-19) infection (Soni *et al.,* 2020).

This information regarding the therapeutic potential of *Curcuma longa* prompted the current study with an aim to study the efficiency of C. longa against the entomopathogenic fungus *Beauveria bassiana* which causes the dreaded muscardine disease in silkworms mostly during the winter and rainy seasons when the humidity is high (Jayaramaiah *et al.,* 1986). In Assam, about 14-40% loss in Muga silkworm is caused by muscardine disease in every crop (Chakravorty *et al.,* 2007). The disease, when it occurs leads to the complete destruction of the crop that causes massive economic loss to the silk industry in India. The key objective of the present study is, therefore, to evaluate the antifungal efficacy of aqueous, ethanolic and methanolic *C. longa* rhizome extract at three different concentrations (1%, 5%, 10%) *in vitro* against the fungus *B. bassiana* and qualitative phytochemical screening of the maximum effective solvent-mediated extract for possible bioactive compounds by using reliable analytical detection technique GC-MS.

Materials and methods

Antifungal susceptibility test

Collection and preparation of plant extract: The rhizomes of *Curcuma longa* were collected from local farmers in Tangla, Udalguri district, Assam, India and was identified by using the standard key. The collected rhizomes were washed under tap water, rewashed with distilled water and sliced into small pieces and shade dried at room temperature for 15 days. The dried rhizomes were then grounded to fine powder by using an electric mixer grinder and kept in an airtight container.

For the preparation of aqueous extract, 10 g of the powdered turmeric was dissolved in 100 ml of sterile distilled water. For ethanolic and methanolic extracts preparation, 10 g dried powder was soaked in 100 ml of 80% each ethanol and methanol, respectively. All the samples were then left to stand at room temperature for about 72 hours. Each sample was

then filtered through Whatman filter paper no. 1 and concentrated using a rotary evaporator (make: Equitron, model: EV11) at 45° C under reduced pressure and stored at 4° C for further use.

Procurement and maintenance of test organism: The fungal pathogen *B. bassiana* (MTCC No 984) was obtained from Microbial Type Culture and Gene Bank (MTCC), Chandigarh, India. The culture was grown on PDA media under aseptic condition and the Petri plates were incubated at 25°±2° C in BOD incubator for 7 days. . The PDA media went through autoclave sterilization at 121° C for 15 minutes at 15 lbs before pouring it into Petri dishes. Chloramphenicol (0.25 g/lt) is used as an antibiotic to prevent any unwanted growth of bacteria that can contaminate the media (Ramos *et al.*, 2020). The pure culture was stored on PDA slants under a deep freezer refrigerator (make: Celfrost, model: CF210) at -20° C (Nakasone *et al.*, 2004).

Agar well diffusion assay: The antifungal activity of the extracts was screened in vitro by the agar well diffusion method at three different concentrations- 1%, 5% and 10% as described by Devi (2015) with little modifications. The molten autoclaved agar media (cooled down to 45° C) was dispensed into sterilized plates and left for solidification. The solidified agar plates were then inoculated by dispersing 0.1 ml of fungal inoculum from PDA broth culture by using a sterile cotton swab over the entire agar surface. Wells of approximately 6 mm diameter were cut out by using a sterile cork borer and 70 µl of different concentrations of the plant extract were then dispensed into the wells with the help of a micropipette. Solvents without the plant extract were used as negative control and clotrimazole (1% w/w) was used as the positive control. All the processes were carried out aseptically under laminar flow. The plates were then incubated at 28° C for 5 days in an upright position under a BOD incubator. The antifungal activity was assessed by measuring the diameter of the zones of inhibition (including the diameter of the well) using a vernier caliper scale and compared to that of the standard (clotrimazole). The assay was repeated five times to lessen errors.

Gas chromatography-mass spectrometry study

The GC-MS study of the methanolic extract of turmeric was performed at Guwahati Biotech Park, Guwahati, India. The GC-MS instrument used was Perkin Elmer (USA), model Clarus 680 and Clarus 600C MS comprising a liquid auto-sampler. The capillary column used was 'Elite-5MS' having dimensions- length-60m, ID-0.25 mm and film thickness-0.25 µm. The stationary phase used was 5% diphenyl and 95% dimethyl polysiloxane.

GC-protocol: 2 µl of injection volume of the sample was employed in splitless mode with helium gas used as carrier gas (i.e., mobile phase) at a flow rate of 1ml/min. The injector and ion-source temperature was 280°C and 180°C, respectively. The oven temperature was programmed at 60°C (for 1 min) with an increase to 200°C for 3 minutes at the rate 7° C/min and then again increased to 300°C at the rate of 10° C/min for 5 minutes. During the process, solvent delay was kept for 8 minutes and the total run time was approximately 39 minutes.

MS protocol: Mass-spectra were taken in Electron Impact positive (EI+) mode at 70 eV. A solvent delay of 8 minutes was there for MS scan. M/z range is 50-600 amu. Identification of peaks: Interpretation of the peaks of GC chromatogram was done by library search of the mass spectrum of corresponding peaks using the database software of National Institute Standard and Technology-2014 (NIST-2014).

Statistical analysis

The data were subjected to a one-way analysis of variance (ANOVA) for the mean comparison by using IBM SPSS 25.0 version.

The results were presented in the form of mean±SEM of five independent experiments. Posthoc tukey's HSD (honestly significant difference) test was done for multiple comparisons between the groups. Differences between means were considered significant at $P < 0.05$.

Results

Antifungal activity

The results of the *in vitro* antifungal evaluation of the plant extracts are depicted in Fig. 1 and Fig 2. The study revealed that the extract is growth inhibitory against *B. bassiana*. It is evident from fig. 1 that the maximum antifungal activity was obtained at 10% methanolic extract with a diameter of zone of inhibition of 22.06±.66 mm, followed by ethanolic extract at 10% concentration (18.90±.6 mm).

Table 1. GC-MS analysis of methanolic rhizome extract of *C. longa*. RT- retention time, MW- molecular weight, MF- molecular formula.

Peak no	Compound name	RT	Peak area%	MW	MF	Compound type
1	Phenol,2 methoxy-o-guaiacol	12.92	.57	124	$C_7H_8O_2$	Phenolic
2	2-Methoxy-4-vinylphenol 4-vinylguaiacol	17.61	1.22	150	$C_9H_{10}O_2$	Phenolic
3	5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-z)-	19.95	.20	318	$C_{21}H_{34}O_2$	Fatty acid
4	Benzene, 1-methyl-4-(1-methylethenyl)-p-cymenene	20.79	.86	132	$C_{10}H_{12}$	Monoterpenoid
5	1,3-Cyclohexadiene, 5-(1,5-dimethyl-4-hexenyl)-2-methyl-, [s-(r*,s*)]-zingiberene	21.02	.93	204	$C_{15}H_{24}$	Sesquiterpene
6	Cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-methylene-, [s-(r*,s*)]-ß-sesquiphellandrene	21.59	1.74	204	$C_{15}H_{24}$	Sesquiterpene
7	Glutaric acid, 3-methylbut-2-yl 1-phenylpropyl ester	22.57	.21	318	$C_{19}H_{28}O_4$	Fatty acid
8	Ar-tumerone	24.41	22.67	216	$C_{15}H_{20}O$	Sesquiterpene
9	α-Turmerone	24.53	17.52	218	$C_{15}H_{22}O$	Sesquiterpene
10	Phenol, 4-ethyl-2-methoxy-p-ethylguaiacol	24.88	1.42	152	$C_9H_{12}O_2$	Phenolic
11	Curlone	25.24	13.71	218	$C_{15}H_{22}O$	Sesquiterpene
12	(6r,7r)-Bisabolone	26.05	.78	220	$C_{15}H_{24}O$	Sesquiterpene
13	(z)-2,6-Dimethylocta-2,5,7-trien-4-one ocimenone	26.91	.38	150	$C_{10}H_{14}O$	Monoterpene
14	3-Buten-2-one, 4-(4-hydroxy-3-methoxyphenyl)-dehydrozingerone	27.22	1.28	192	$C_{11}H_{12}O_3$	Phenol
15	Santolina triene	27.34	.56	136	$C_{10}H_{16}$	Hydrocarbon
16	1-Cyclohexene-1-carboxaldehyde, 4-(1-methylethenyl)-phellandral	27.98	.13	150	$C_{10}H_{16}O$	Monoterpenoid
17	(f)-.Gamma.-atlantone	29.09	.29	232	$C_{15}H_{22}O$	Sesquiterpene
18	Prop-2-ynyl (e)-2-methylbut-2-enoate	29.97	.54	138	$C_8H_{10}O_2$	Alkaloid

The test organism was found to be least susceptible to the aqueous extract with no inhibition zone at 1% concentration. The negative control showed no antifungal activity (Fig. 3). Statistically, there are significant differences ($p < 0.05$) within the groups of different concentrations for both alcoholic and aqueous extracts (Fig. 1). When compared to the positive control (clotrimazole), all the groups are significantly different with $P < 0.05$. According to the result of the percent of inhibition, the maximum inhibition rate was observed for 10% methanolic extract 74.22 % (Fig 2). All the groups of extracts however showed significantly lower inhibition compared to the positive control (clotrimazole).

The result reflected an increase in the antifungal effects of turmeric with the increase of concentrations against the tested fungus.

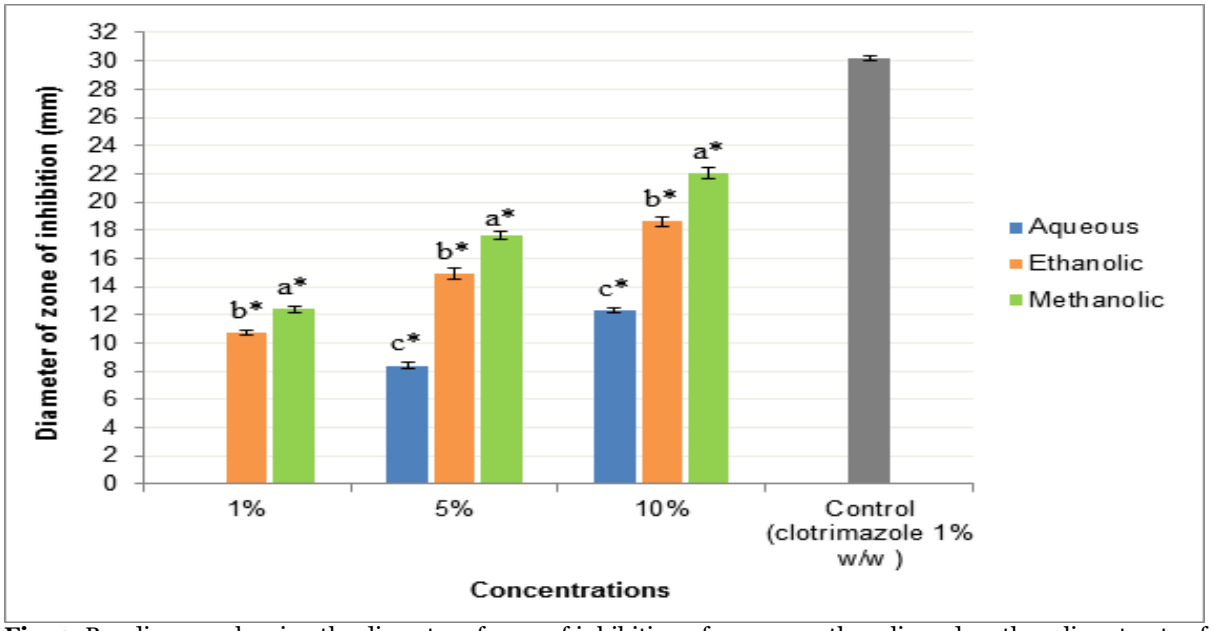

Fig. 1. Bar diagram showing the diameter of zone of inhibition of aqueous, ethanolic and methanolic extracts of turmeric at 1%, 5% and 10% concentration against *B. bassiana*. Data are expressed in mean±SEM. n=5. 1% aqueous extract showed no inhibition zone. Different small letters indicate significant differences within groups of each concentration (P <0.05). * represents significant differences compared to control (P < 0.05).

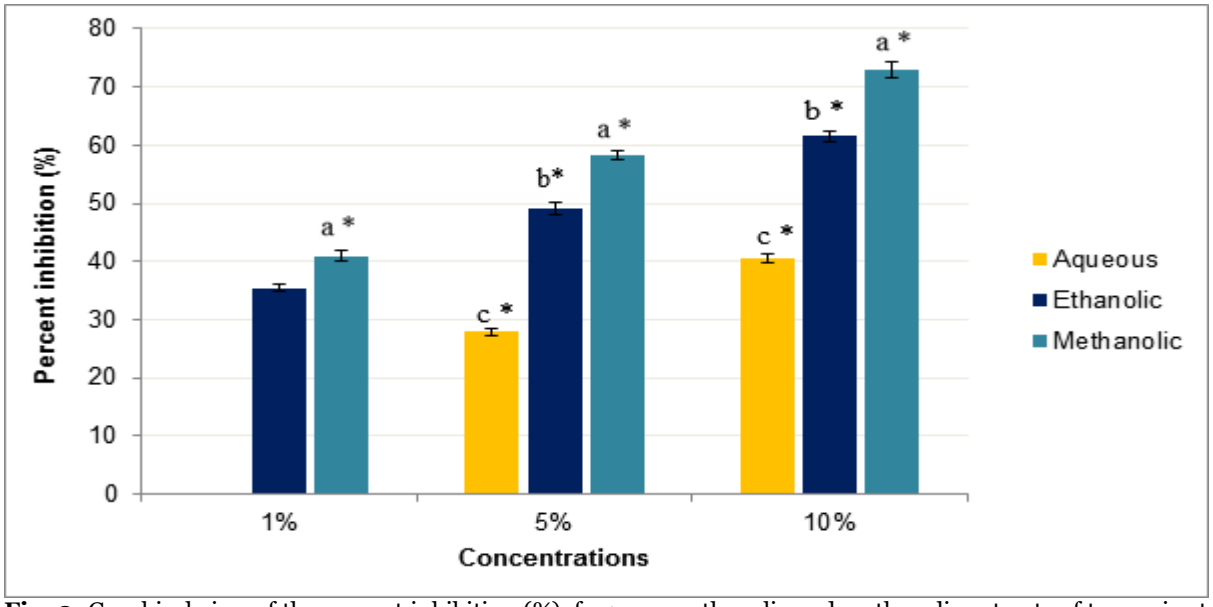

Fig. 2. Graphical view of the percent inhibition (%)of aqueous, ethanolic and methanolic extracts of turmeric at 1%, 5% and 10% concentration against *B. bassiana* compared to a positive control (clotrimazole 1% w/w). Inhibition by clotrimazole is accounted as 100%. Different small letters indicate significant differences within groups of each concentration (P < 0.05). * represents significant differences compared to positive control (P < 0.05).

GC-MS study

The GC-MS study was done on methanolic extract of *C. longa* for detection of volatile non-polar compounds as it has shown maximum inhibition against the fungus. The GC-MS chromatogram is shown in Fig. 4 and several peaks were detected some of which were major peaks and others were minor.

A total of 18 important volatile bioactive compounds were detected and identified.

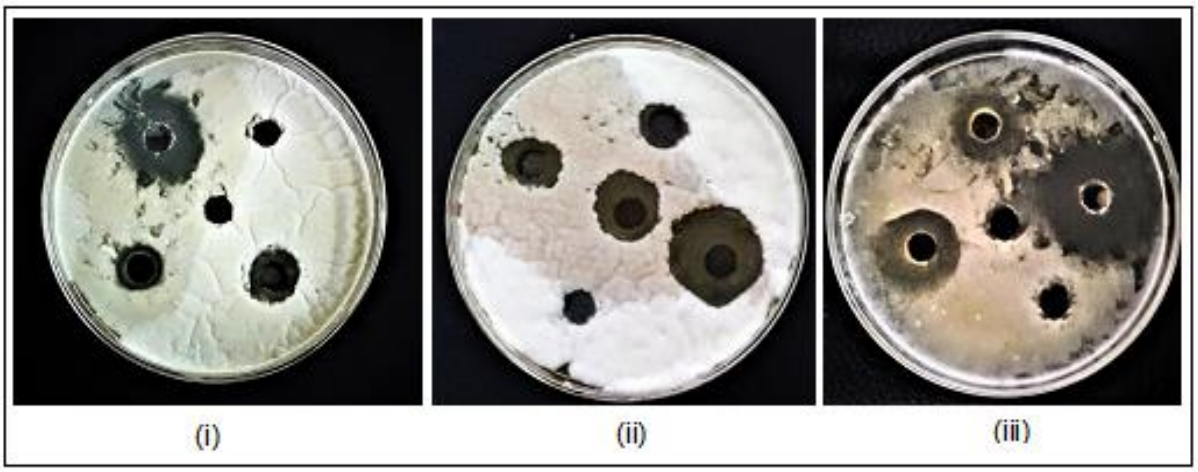

Fig. 3. Antifungal effect by *C. longa* rhizome extract against *B. bassiana* at 1%, 5% and 10% concentration**.** i) Aqueous extract ii) ethanolic extract iii) methanolic extract.

The major volatile compounds present are ar-turmerone (22.67%), α-turmerone (17.52), curlone (13.71), ß-Sesquiphellandrene (1.74%), p-ethylguaiacol (1.42%), dehydrozingerone (1.28%), 4-vinylguaiacol (1.22%) and zingiberene (.93%) along with other minor constituents. No curcumin was detected in the analysis. The phytoconstituents were depicted in Table 1 with their molecular weight (MW), molecular formula (MF), compound nature, retention time (RT) and peak area %. The peak area indicates the concentration of the compound present.

The chemical structures of the compounds were retrieved from the web search of structural databases of National Institute Standard and Technology (NIST) and are presented in Fig. 5.

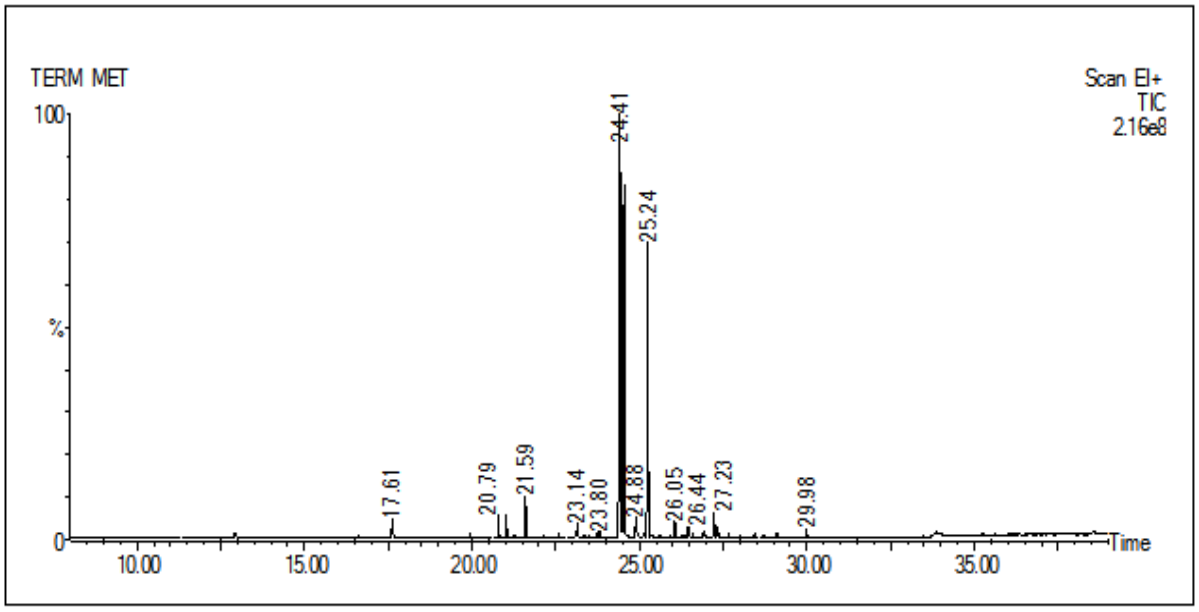

Fig. 4. GC-MS chromatogram of methanolic rhizome extract of *C. longa*.

Discussion

The present study has demonstrated that *C. longa* has antimycotic activity against *B. bassiana* which is in accordance with the findings of previous in vitro experiments conducted by several workers against *Fusarium solani* (Chowdhury *et al.,* 2008), *Helminthosporium oryzae* (Chowdhury *et al.,* 2008), *Cryptococcus neoformans* (Ungphaiboon *et al.,* 2005), *Aspergillus niger* (Kapoor, 1997), *Penicillium digitatum* (Kapoor, 1997), *Trichophyton rubrum*

(Prastiyanto *et al.,* 2021), *Aspergillus fumigatus* (Shanmugam and Bhavani, 2014), *Rhizoctonia solani* (Saju *et al.,* 1998), *Phytophthora infestans* (Kim *et al.,* 2003), *Puccinia recondite* (Kim *et al.,* 2003), *Trichophyton rubrum* (Sharma and Sharma, 2011) *and Microsporum Gypsum* (Sharma and Sharma, 2011). However, according to the results, the fungus is most sensitive to the standard antifungal drug clotrimazole. In the present investigation, it was observed that the alcoholic rhizome extract of turmeric is more effective, whereas the aqueous extract exhibited moderate effect against *B. bassiana* which is in conformity with the results of Shanmugam and Bhavani (2014) and Gul and Bakht (2015).

Statistical analysis of the results also exhibited that the methanolic rhizome extract was found to be more effective in resisting the growth of the fungus.

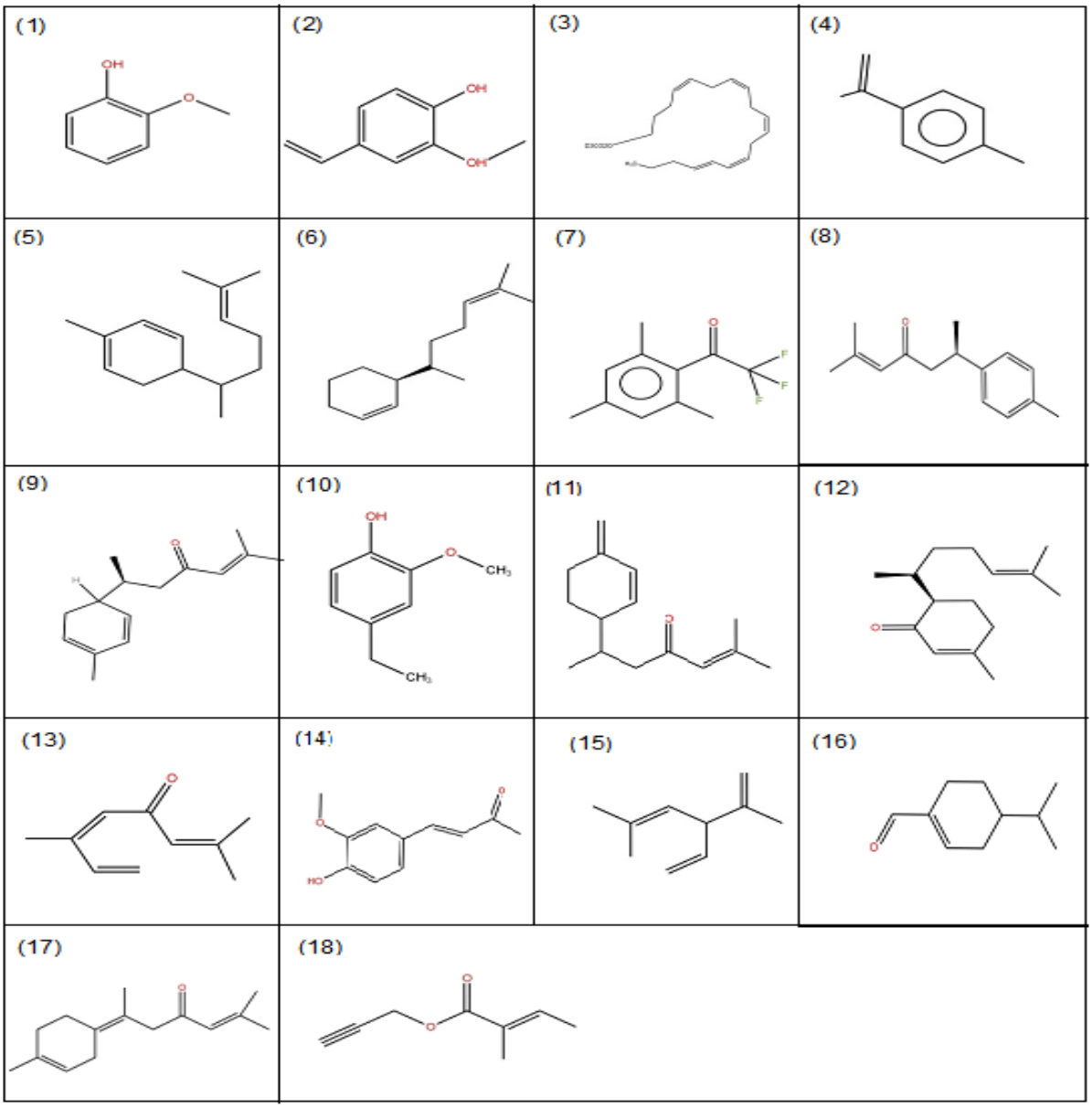

Fig. 5. Molecular structures of the compounds from *Curcuma longa* L. detected in GC-MS analysis. Details of the compounds are given in Table 1. (1)o-Guaiacol, (2)4-vinylguaiacol, (3)5,8,11,14-eicosatetraenoic acid, methyl ester, (all-z)- benzene, (4)p-cymenene, (5)zingiberene, (6)ß-sesquiphellandrene, (7)ethanone, 2,2,2-trifluoro-1-(2,4,6-trimethylphenyl)-, (8)ar-tumerone, (9)α-turmerone, (10)p-ethylguaiacol, (11)curlone, (12)(6r,7r)-bisabolone, (13)ocimenone, (14) dehydrozingerone, (15)santolina triene, (16)phellandral, (17)(f)-.gamma.-atlantone, (18)prop-2-ynyl (e)-2-methylbut-2-enoate.

The possible explanation for this could be the potential bioactive compounds contained in the plant extracts responsible for antifungal activity may be relatively solubilized more in methanol than in ethanol and water (Shekhawrat and Prasada, 1991).

Similar results were obtained by Samadi *et al.* (2019) who demonstrated turmeric can reduce the growth of *Candida albicans* with a maximum diameter of zone of inhibition of 21 mm at a 100mg/ml concentration of methanolic extract. Further, it was detected that the inhibition of the fungus by the extract was concentration-dependent which is in agreement with the findings of Okiki *et al.* (2017) and Murugesh *et al.* (2019).

The GC-MS analysis of the methanolic rhizome extract revealed the presence of different monoterpene, sesquiterpene, fatty acid and phenolic compounds. The major bioactive constituents are ar-turmerone (22.67%), α-turmerone (17.52), curlone (13.71), ß-sesquiphellandrene (1.74%), p-ethylguaiacol (1.42%), dehydrozingerone (1.28%), 4-vinylguaiacol (1.22%), zingiberene (.93%) which is in conformity with the results Dosoky *et al.* (2019) and Kumar *et al.* (2022) similar to our study.

The presence of dehydrozingerone, a structural half analogue of curcumin was also reported by Chaterjee *et al.* (2000) in a study of volatile oil constituents of turmeric. The present study also detected the presence of (6R,7R)-bisabolene which in in contradictory to the result of Abdel-Lateef *et al.* (2016) who reported ß-bisabolene from methanolic rhizome extract of turmeric. This variation in the compounds in different experiments could be due to the different geographical locations, soil types, different climates, availability of minerals and numerous other natural factors (Prance *et al.,* 1994).

The results indicated the antifungal activity of the *C. longa* rhizome extract which can be probably attributed to the synergistic effects of these active compounds present in it (Avanco *et al.,* 2017). A number of compounds from turmeric have pharmacological activities, as shown by previous experiments. One study by Jankasem *et al.* (2013) showed that ar-turmerone is quite effective against dermatophytes with MIC of 3.90-7.81 µg/ml. Another study by Lee *et al.* (2003) demonstrated the fungicidal activity of ar-turmerone at 500 ppm against *Phytophthora infestans* and *Erysiphe graminis*. Negi *et al.* (1999) reported that both turmerone and curlone present in turmeric possess high antimicrobial activity against an extensive variety of microbes. However, the antifungal efficacy of the extract is not always firmly associated with the major constituents because the presence of minor components may play role in resisting the growth of the fungus (Girija *et al.,* 2014). This study reported two oxygenated monoterpenes, namely ocimenone and phellandral which can also be accountable for the antifungal potency of the plant as described by Barra *et al.* (2010). ß-sesquiphellandrene is also reported in this study which is likely to be responsible for the antifungal activity (He *et al.,* 2019). Another study described the strong antimicrobial activity of 4-vinylguaiacol by binding with lipoprotein leading to disruption of the bacterial cell wall (Rubab *et al., 2020)*. So it can be hypothesized that 4-vinylguaiacol might possess antifungal activity as well, on which further clinical evidence are required.

Conclusion

Hence, from the results of this *in vitro* study, it can be concluded that the rhizome extract of *C. longa* can be used as a natural source of antifungal compounds against the fungus *B. bassiana*. The antimycotic activity of the plant can be attributed to the presence of mixture of monoterpenes, sesquiterpenes, fatty acids and phenolic compounds as detected in the GC-MS study of the methanolic extract of *C. longa* rhizome. The study also showed that the methanolic extract is more promising than the aqueous and ethanolic extracts in resisting the fungus.

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Competing interests

The authors declare that there are no conflicts of interest.

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