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# Antifungal Activity of Allium Sativum L. and Mentha piperita L. against C.albicans from Clinical Samples

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## Abstract

Candida species are one of the most fatal fungal species which has detrimental effects on humans. Antifungal agents against these fungal species are slowly restricting their effect due to development of resistant species. Nowadays, antifungal agents of plant or microbial origin are gaining pace due to their easy development methods, less side effects and less complex metabolites produced y them. This study also focuses on two plant sources whose antifungal potency has been reported earlier against some fungal species. *Allium sativum* L bulbs and *Mentha piperita* L. leaves were extracted with ethanol and their active constituents were studied using GC-MS analysis. Antifungal activity of constituents and their time kill kinetic study have been done to evaluate their efficacy as antigungal agents. The results showed that *Mentha piperita* L. extract activity was strongest against *C.albicans*with ZOI of 25.9±1.31mm quite comparable ZOI (34.9±0.36mm) of the positive control. This was followed by activity against *C.glabrata*(23.97±0.06). *Allium sativum* L. extract showed higher activity against *C.albicans* (23.8 ±1.31mm) and lesser effective against *C.glabrata* (20.57±0.67mm).MICs for *Allium sativum* L. and *Mentha piperita* L. extracts were determined to be 6.25 mg/ml and MFC 12.5 mg/ml in all cases.

**Keywords:** GC-MS profiling, CLSI, MFC, Time-kill curve, colony forming unit, bioactive, retention time

## 1. Introduction

Candida species are one of the major group of fungus which affects human extensively, the category of infections it causes are collectively named as Candidiasis. Immunocompromised individuals are majorly affected in these cases **(Ferreira *et al.*, 2009)**. In recent times, it is seen that fungal infections are majorly caused due to candida and non-albicans Candida (NAC) besides other yeast of this category. The issue also grabs attention due to gradually developing resistant species among this category **(Li *et al.*, 2015).** Researchers are high to develop products of plant origin that could be used to inhibit their growth and thus slow down their speedy spread.Candida albicans are the prevailing species in causing candiasis among other Non-albicans Candida (NAC) species which hold 25%, 8%, 7%, and 4% by *C. tropicalis, C. glabrata, C. parapsilosis* and *C. krusei* respectively **(Mukherjee and Chandra, 2015).** There are several factors in these species that determine the intensity of the effect on individuals which includes the formation of the germ tube, adherence capacity of the pathogen, variation in phenotypic properties, formation of biofilm, and secretion of lytic enzymes **(Deorukhkar *et al.*, 2014).**Thus these factors ultimately define their capacity of pathogenesis.

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Five well-known categories of compounds which are known to possess antifungal properties are polyenes, allylamines, azoles, fluoropyrimidines, and echinocandins **(Rabes *et al.*, 2015)**. The increasing drug resistances against common antifungal agents like fluconazole and voriconazole due to excessive usage have raised concerns among researchers to find better antifungal agents. Plant sources are seen as better alternatives, owing to their cheap source and fewer side effects. Therefore, they have replaced the chemically synthesized compounds almost completely which is evident from the fact that among the marketed antibiotics that are used for clinical purposes, about 80% of them are derived from natural sources.

In this study, *Allium sativum* L. and *Mentha piperita* L. have been focused to identify their antifungal activity on Candida species, majorly emphasizing on *Candida albicans*. Both the plants are of utmost importance from a medicinal and economical point of view. Therefore, they are thoroughly studied in literature and it is reported to possess many properties like antiviral, antibacterial, and antifungal **(Mimica-Dukić *et al.*, 2003; Iscan *et al.*, 2002; Singh *et al.*, 2015).** The present scenario presents a minacious picture where Candidiasis numbers are increasing at an alarming rate, the reason being the incidence of immunosuppressive diseases among people. As the usage of existing drugs increased heavily, they are soon acquiring a resistant label. As plants are the most reliable sources to obtain bioactive components, they play a central role in most studies in recent times. We in this study, aim to determine more such active components from these well-known plants that could possess better potential to control *Candida albicans* and Non-Albicans Candida (NAC) species.

## 2. Material and Methodology

### 2.1 Strains and media

The strains used in the study were *Candida albicans* and *Candida glabrata*. The isolates were obtained from clinical samples, identified and characterized using molecular method of Amplification and sequencing ITS region of the fungus in our earlier work. The obtained sequence were submitted to NCBI/DDBJ and can be accessed from official website (www.ncbi.nlm.nih.gov) for *Candida albicans* and *Candida glabrata*. All the media and chemicals used in the study were obtained from HiMedia Laboratories Pvt. Ltd.

### 2.2Extraction of *Allium sativum* L. and *Mentha piperita* L.

Extraction of the active constituents frombulbs of *Allium sativum* Land leaves of *Mentha piperita* L. were done using ethanol, cold water and hot water. For this leaves of *Mentha piperita*L.were collected from local area, both parts were rinsed with distilled water thoroughly to remove dust paticles and other contaminants. Leaves were then dried in shade, ground to powder using electric blender white in case of garlic, bulbs were freshly peeled and then powdered. These powders were then separately extracted with ethanol, hot water and cold water using conventional method. Each powdered sample was weighed 10gm and mixed with the solvents in 3 separate flasks. These flasks were then placed on shaker for 72hours. After completion of incubation period, the extracts were

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filtered using whatman filter paper no. 1 and filtrate was collected in separate tubes. The extracts were then dried using rotary evaporator (ZEXTER Pvt. Ltd., India) and dried powder obtained were stored at 4˚C for further processing.

### 2.3 Thin-Layer Chromatography (TLC)

The dried extracts were tested for their phytochemical components using Thin Layer Chromatography following method given by **Marica *et al.* (2010)**. Samples of *Allium sativum* L and *Mentha piperita* L extracts were prepared in separate solvents (described in section 3.1). The mixture was placed on the silica gel coated plates (pre-activated at 105˚C) leaving 1cm distance from base. The sample was allowed to absorb on the surface. The plates were then placed in chamber saturated with the solvent system (described in section 3.1). After completion of the process, plates were developed and visualized under UV light. Rf were calculated for the observed spots. These extracts were then further analyzed using GC-MS profiling.

### 2.4 GC-MS Profiling of the extract

The extracts were analyzed for their using GC-MS Analysis equipment (Thermo Scientific Co.) Thermo GC-TRACE ultra ver.: 5.0, Thermo MS DSQ II.

Experimental set up conditions for GC-MS system

- TR 5-MS capillary standard polar column, dimension: 30Mts, ID: 0.25 mm
- Film thickness: 0.25µm.
- Flow rate of mobile phase (carrier gas: He): 5.0 ml/min.
- Temperature programme (oven temperature): 40°C- 350°C at 5°C/min
- Injection volume: 1 µl.
- Solvent used for sample preparation:Methanol
- Range: 50-650 m/z

Results obtained were compared from Wiley Spectral Library Search Programme available in the system.

### 2.5 Antifungal Activity against characterized candida isolates

Extract were assessed for their antifungal activity against *C.albican* and *C.glabrata* using Agar well diffusion method standardized by the Clinical and Laboratory Standards Institute **(NCCLS/ CLSI, 1997; Fothergill, 2011).**

### 2.6 Minimum inhibitory and fungicidal concentrations of the extracts

MIC for the extracts was determined using standard broth micro-dilution method recommended by the Clinical and Laboratory Standards Institute/The National Committee for Clinical Laboratory Standards **(CLSI/ NCCLS, 2002).** MFC was evaluated from MIC concentrations which

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showed no visible growth onto plates. Results for MFC were mentioned as no growth concentration on plate.

### 2.7 Time-Kill Activity

Time Kill kinetics assay was performed by first preparing concentration of MIC, twice MIC and four times MIC for the extracts (ethanolic extracts of *Allium sativum* L. bulbs and *Mentha piperita* ). Innoculum size of $1.0x10^6$was added to the extracts and incubated at 37°C. 1 ml of media was collected from each tube at regular intervals of 0, 5, 10, 15, 20, 25, 30, 35, 40 hours. These were then spread on Nutrient Agar media and plates were incubated at 25-27°C. CFU was recorded for each case and graph was plotted between $Log_{10}$CFU/ml vs Time.

### 2.8 Statistical Analysis

All the experiments were performed in triplicates, and the results' statistical significance was expressed with the help of IBM SPSS Statistics 20.

## 3. Result & Discussion

### 3.1 Chemical constituents in the Extracts

**3.1.1 Screening of components using TLC:**Identification of chemical constituents inethanolic and aqueous extracts of *Allium sativum* L. bulbs and *Mentha piperita* L. leaves were primarily done based on the thin layer chromatography summarized in the table (table 1) and illustrated in the figure (figure 1).

*Allium sativum* L.extracts were run on silica gel with solvent system of toluene and ethyl acetate in a proportion of 76:24, 50:50 and 24:76. The plates were developed in 1% vanillin in glacial acetic acid and images of the plates were visualized in presence of white light and UV light. The spots which appeared more prominent in this case were yellow, green and brown in colour. Distances covered by the spots were measured and Rf values were calculated. The Rf values corresponding to the spots are summarized in the table (table 1). The table shows presence of various compounds in the extract, majorly sulphur compounds were extracted in ethanolic extracts, defined by presence of spot with Rf of 0.45, referring to allicin and also compounds with very low Rf of 0.062 referring to cepaenes and azoens. Less number of spots were visible in case of hot and cold water extracts.

The result were found similar to **Libbie*et al.* (2008)**work where identification of peppermint leaves extracts constituents were based on TLC and spots' appearance showed similar pattern as in observed in this study.**Liliana*et al.* (2004)** work revealed similar pattern for garlic extracts in their work for TLC.

In case of *Mentha piperita* L. extract, constituents were separated on silica gel using developing solvent system of toluene and ethyl acetate in proportion of 76:24, 50:50 and 24:76. Samples were

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prepared for TLC by extracting powdered leaves with toluene (at 10%). The slurry was then centrifuged and liquid portion were spotted onto silica plates. The reagent used for development of the plate was 1% vanillin in mixture of $H_2SO_4$methanol (40:10). The spots which prominently appeared in this case were of menthol (Rf= 0.4), 1,8 Cineole (Rf= 0.54) and Piperitenone oxide (Rf= 0.6) and also common pigment of chlorophyll (Rf= 0.4) were observed in its extract. In this case also, ethanolic extract proved better solvent as compared to water;more number of compounds were identified using ethanolic extract. Therefore ethanolic extracts of both plants were used in GC-MS analysis for further identification of compounds.

### 3.1.2 GC-MS Analysis of Extracts

A profile of bioactive constituents in *Allium sativum* L. and *Mentha piperita* L. ethanolic extractswere assessed based on GC-MS results. The chromatograms of GC-MS for *Allium sativum* L. (fig 1) and *Mentha piperita* L. (fig 2) are reveals the set of compounds extracted using ethanol (75%). Both aqueous (cold and hot water)extracts were screened for active constituents using TLC, as observed in the results they showed feeble appearance of spots and less effective identification, therefore not analyzed using GC-MS. Ethanolic extract showed better identification and more number phytoactive components, therefore its further analysis was done using GC-MS profiling. Some of the major identified components in *Mentha piperita* L. ethanolic extracts were á-Terpinene (RT: 3.666 min), L-Menthone (RT: 11.156 min), Menthol (RT: 15.780 min), Piperitone oxide (RT: 24.389 min) and Thymol (RT: 30.288 min). Many studied have been conducted earlier and reported the antimicrobial activity of active constituents of Mentha *piperita* L. extracts viz., menthol, menthone and limonene, etc. **Deans and Baratta (1998) and Flemming (1998)** in their work have reported such activity.

In case of*Allium sativum* L. ethanolic extracts, identified compounds were 1-propenyl methyl disulphide (RT: 7.442 min), Trisulfide, allyl trisulfide (RT:24.58 min). Propanone (RT: 12.08 min) while other general acidic components which provide it acidic nature were Acetaldehyde (RT:12.54 min), Acetic acid (RT: 16.51 min), Propanoic acid (RT:16.76 min). These are compounds which are obtained due to degradation of alline **(Mochizuki *et al.*, 1995; Block, 1985; Stoll and Seebeck, 2006).** They impart medically important properties like antioxidant and anticancer to the plant **(Park *et al.*, 2015; Lawson and Wang, 2003; Sparnins *et al.*, 1986; Fukao *et al.*, 2004; Rattanachaikunsopon and Phumkhachorn, 2008; Enders and Balensiefer, 2004; Son and Lee, 2010; Wu *et al.*, 2001)**

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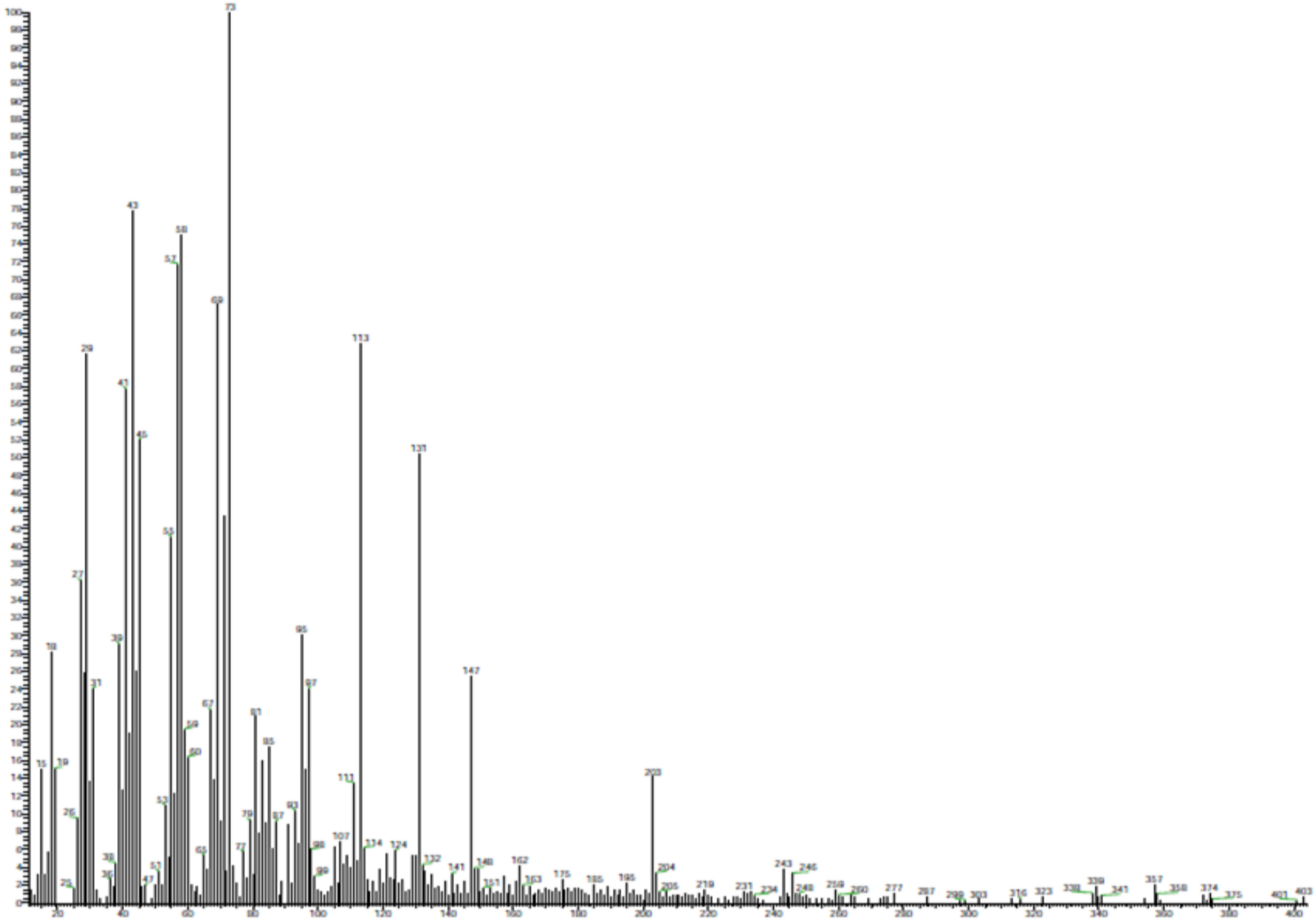

Fig 1: A typical GC-MS Chromatogram of *Allium sativum* L. bulbs ethanolic extracts

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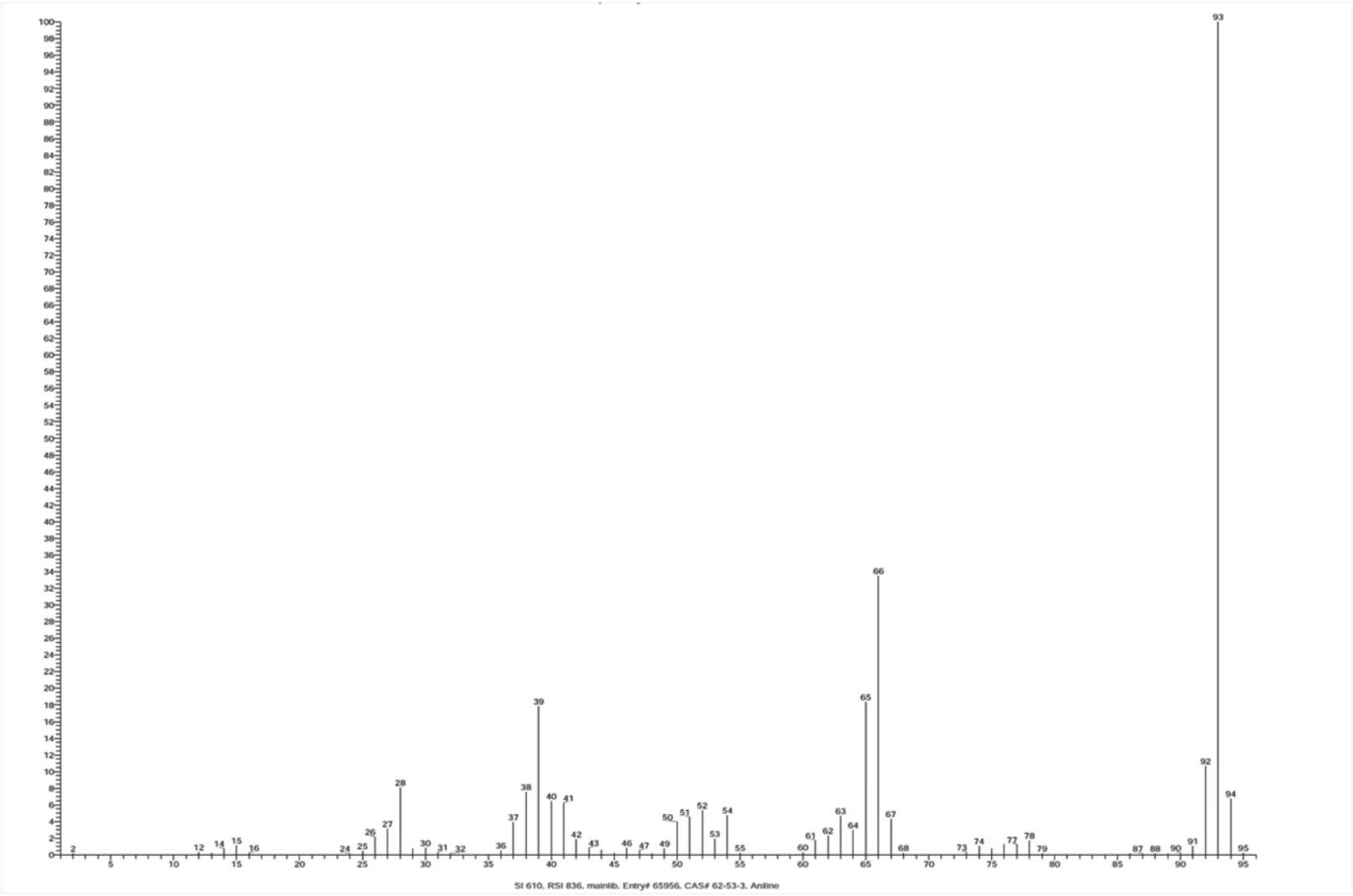


Fig 2: A typical GC-MS Chromatogram of *Mentha piperita* L. leaves ethanolic extracts

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*Table 3: GC-MS profile representing chemical composition of the Allium sativum L. bulbs ethanolic extracts*

| S.No. | Peak No. | Compound name | Retention time (RT) |
|---|---|---|---|
| *Mentha piperita* L. leaves ethanolic extract | | | |
| 1 | Peak 2 | á-Terpinene | 3.666 |
| 2 | Peak 12 | L-Menthone | 11.156 |
| 3 | Peak 14 | neo-Menthol acetate | 13.351 |
| 4 | Peak 15 | neo-Menthol | 15.338 |
| 5 | Peak 16 | 5-Methyl-2-(1-methylethylidene)-cyclohexanone | 15.727 |
| 6 | Peak 16 | Menthol | 15.780 |
| 7 | peak 24 | Piperitone oxide | 24.389 |
| 8 | peak 30 | Thymol | 30.288 |
| 9 | Peak 40 | Methyl linolenate | 40.624 |
| 10 | Peak 41 | 3,3-Isopropyledenebis(1,5,8,11-tetraoxacyclotridecane) | 41.808 |
| 11 | Peak 43 | Octadecanoic acid | 43.507 |
| *Allium sativum* L. bulbs ethanolic extracts | | | |
| 1 | Peak 4 | 1-propenyl methyl disulphide | 7.442 |
| 2 | Peak 7 | Propanone | 12.08 2 |
| 3 | Peak 8 | Acetaldehyde | 12.54 |
| 4 | Peak 11 | Acetic acid | 16.51 |
| 5 | Peak 12 | Propanoic acid | 16.76 |
| 6 | Peak 25 | Trisulfide, allyl trisulfide | 24.58 |
| 7 | Peak 35 | 1,3-Dihydroxyacetone dimer | 30.46 |

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| | | | |
|---|---|---|---|
| 8 | Peak 36 | 2-Hydroxy-gamma-butyrolactone | 32.12 |
| 9 | Peak 39 | 4H-Pyran-4-one | 33.86 |
| 10 | Peak 47 | Hexadecanoic acid | 44.2 |
| 11 | Peak 51 | Heptadecane | 47.54 |

### 3.2Antifungal activity and MIC/MFC of the extracts

Three extracts of *Allium sativum* L. bulbs and *Mentha piperita* L. leaves prepared in ethanol (EAE), hot water (HWE) and cold water (CWE) were assessed for their antifungal activity against *C.albicans* and *C.glabrata* characterized earlier in this work. Susceptibility of these microorganisms is tabulated in the table given below (table 4).

***Table 4: Antifungal activity of Allium sativum L. and Mentha piperita L. extracts against C.albicans and C.glabrata isolates***

| Extracts | Zone of Inhibition (Mean ± S.D.) | | | |
|---|---|---|---|---|
| | *Allium sativum* L. | | *Mentha piperita* L. | |
| | *C.albicans* | *C.glabrata* | *C.albicans* | *C.glabrata* |
| **EAE** | 23.8 ±1.31 | 20.57±0.67 | 25.9±1.31 | 23.97±0.06 |
| **CWE** | 9.1 ± 0.22 | 9.98±0.57 | 10.07±0.31 | 7.43±1.11 |
| **HWE** | 12.63 ± 0.60 | 14.2±0.60 | 19.83±0.29 | 11.6±0.78 |
| **PC** | 37.53 ± 0.99 | 32.33±0.58 | 34.9±0.36 | 30.03±0.35 |
| **NC** | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |

**EAE: Ethanolic extract; CWE: Cold water extract; HWE: Hot water extract; PC: Positive control (Clotrimazole :10mg/ml); NC: Negative control (DMSO); zone of inhibition are recorded excluding well diameter -6mm; Data are expressed as means±standard deviation independent experiments in triplicates.*

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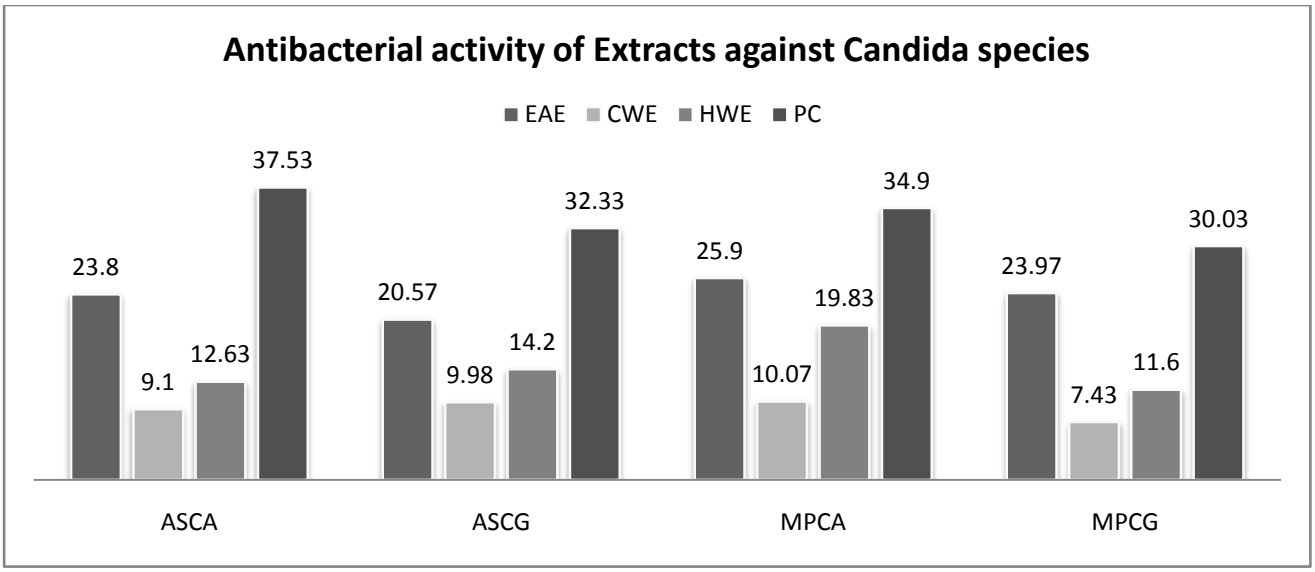


*Fig 3: Antibacterial activity of Extracts against Candida species; ASCA: A.sativum against C.albicans; ASCG: A.sativum against C.glabrata; MPCA:Mentha.piperita against C.albicans; MPCG: Mentha.piperita against C.glabrata; EAE: Ethanolic extract; CWE: Cold water extract; HWE: Hot water extract; PC: Positive control (Clotrimazole :10mg/ml)*

The results revealed that both extracts(*Allium sativum* L. and *Mentha piperita* L.) had no significant antifungal activity in aqueous extracts. Whereas ethanolic extracts of both plants had considerably higher inhibitory action against test fungal strains.

Cold water extract showed lowestantifungal activity of 9.1±0.22 and 9.98±0.57mm (*C.albicans* and *C.glabrata*)by *Allium sativum* L. extract while 10.07±0.31mm and 7.43±1.11mm (*C.albicans* and *C.glabrata*) by *Mentha piperita* L. extract. Hot water extracts had intermediate potency with 12.63 ± 0.60 mm and 14.2±0.60 mm(*C.albicans* and *C.glabrata*) by *Allium sativum* L. extract while 19.83±0.29 mm and 11.6±0.78 mm(*C.albicans* and *C.glabrata*) by *Mentha piperita* L. extract. Susceptibility to ethanolic extracts was found comparable to the positive control. *Mentha piperita* L. extract activity was strongest against *C.albicans*with ZOI of 25.9±1.31mm quite comparable ZOI (34.9±0.36mm) of the positive control. This was followed by activity against *C.glabrata*(23.97±0.06). *Allium sativum* L. extract showed higher activity against *C.albicans* (23.8 ±1.31mm) and lesser effective against *C.glabrata* (20.57±0.67mm). Our study was found in accordance to the earlier reported antifungal potency of *Allium sativum* L. and *Mentha piperita* L. extracts. **Abdallah (2017)** reported significant activity of garlic bbulb extract against *C.albicans.* Inhibitory zone observed against this fungus was 28.0±1.0 mm.

MIC and MFC were performed for only ethanolic extracts as aqueous extracts did not show very significant results for both *Allium sativum* L. and *Mentha piperita* L. extracts. It was done to evaluate the minimum concentration at which this extract could inhibit/kill the fungal strains.In case of MIC, each extracts were prepared in concentration ranging from 3.125 mg/ml - 100 mg/ml while positive control (Clotrimazole) concentrations were in range of 0.25 to 128 µg/mL. MICs for

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*Allium sativum* L. and *Mentha piperita* L. extracts were determined to be 6.25 mg/ml and MFC 12.5 mg/ml in all cases. The number of viable cells of C.albicans and C. glabrata ar different point of time were assessed using Time-Kill activity plot between $Log_{10}$CFU of Cells/ml and time intervals (in hours). The plot (shown in fig 4) shows that nubber of viable cells reduce in case of antifungal agents (PC, *Allium sativum* L. and *Mentha piperita* L. extracts) as compared to untreated cells (UC).

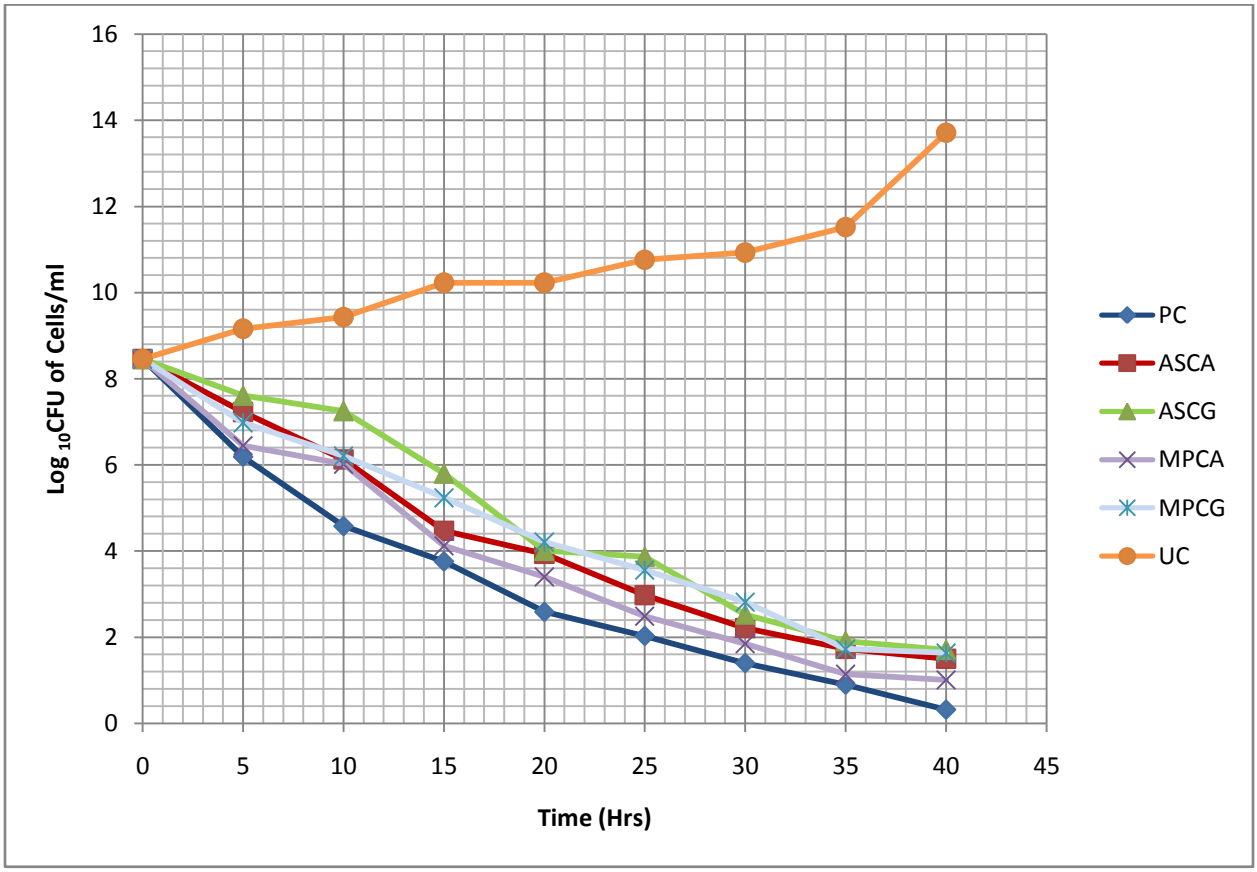


**Fig 4: Time Kill plot of *Allium sativum* L. and *Mentha piperita* L. ethanolic extracts at different time intervals; PC: Positive control; *ASCA: A.sativum against C.albicans; ASCG: A.sativum against C.glabrata; MPCA:Mentha.piperita against C.albicans; MPCG: Mentha.piperita against C.glabrata;UC: untreated control***

Antifungal activity of methanolic and choloroform extracts from *Mentha piperita* L. leaves has been reported by **Wenji *et al.* (2019)** in his work. His results showed that both methanolic and chloroform extracts possess antifungal activity against *C. albicans.*Diba and Alizadeh (2018) in their study revealed antifungal activity of *Allium sativum* L. against *C.tropicalis.*These two plants have been widely studied for their antifungal potential, against various Albicans and Non-Albbican species. Our study aimed to explore their potency against *C.albicans* and *C.glabrata* species.

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## Conclusion

This study dealt with extraction and identification of active constituents from*Allium sativum* L. and *Mentha piperita* L. extracts. The results in the study suggested that ethanol turns out to be a better solvent for extraction of active constituents rather than water. Also, in aqueous extract hot water extracts had significant activity than cold water extract. The preliminary identification based on TLC results showed presence of many constituents and their confirmation was done using GC-MS analysis. Antifungal activities of these two extracts on *C.alicans* and *C.glabrata* showed *Mentha piperita* L. extracts more potent than garlic extract. Many studies have been reported earlier for *C.albicans* inhibition but *C.glabrata* is less studied and not many antifungal agents are yet well known. Since natural products are in high demand owing to their low complexity, less side effects and less harmful metabolites. Therefore, this study provides an insight into beneficial roles of *Allium sativum* L. and *Mentha piperita* as potential natural active agents for future use in field of therapeutics and drugs.

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## Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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