Journal of Infection and Public Health 14 (2021) 1854–1863

Contents lists available at ScienceDirect

Journal of Infection and Public Health

journal homepage: http://www.elsevier.com/locate/jiph

Impact and fungitoxic spectrum of *Trachyspermum ammi* against *Candida albicans*, an opportunistic pathogenic fungus commonly found in human gut that causes Candidiasis infection

Abirami Arasu[a,**], Vaahini Pingley[a], Nagaram Prabha[a], O.V. Ravikumar[a], Kalidoss Annathurai[a], Sudharsan Kasirajan[a], Akshaya Govindasamy[a], Mona S. Alwahibi[b], Mohamed S. Elshikh[b], Mohamed R. Abdel Gawwad[c], Jesu Arockiaraj[d,e,*]

[a] *Department of Microbiology, SRM Arts and Science College, Kattankulathur, 603 203, Chennai, Tamil Nadu, India*
[b] *Department of Botany and Microbiology, College of Science, King Saud University, P.O. Box 22452, Riyadh 11495, Saudi Arabia*
[c] *Department of Genetics and Bioengineering, Faculty of Engineering and Natural Sciences, International University of Sarajevo, Bosnia and Herzegovina*
[d] *SRM Research Institute, SRM Institute of Science and Technology, Kattankulathur 603 203, Chennai, Tamil Nadu, India*
[e] *Department of Biotechnology, College of Science and Humanities, SRM Institute of Science and Technology, Kattankulathur 603 203, Chennai, Tamil Nadu, India*

ARTICLE INFO

Article history:
Received 29 July 2021
Received in revised form
20 September 2021
Accepted 30 September 2021

Keywords:
Trachyspermum ammi
Candida albicans
Candidiasis
GC–MS
Flowcytometry
Cell reduction assay

ABSTRACT

Background: Trachyspermum ammi (commonly known as Ajwain), a medicinal plant of the Apiaceae family is scientifically acknowledged to harbor potential bioactive compounds for the treatment of gastrointestinal issues, loss of appetite, bronchial difficulties, cough, inflammation, diarrhoea, headache, hypertension, stomach discomfort, bronchitis and influenza. *Candida albicans* is generally a commensal fungus found in the gastrointestinal and genitourinary systems.
Objective: This study was focused on secondary metabolites of *T. ammi* and its effects towards candidiasis infection as caused by *C. albicans.*
Methods: Phytochemical components of *T. ammi* as a crude extract were extracted through maceration method using three polar (ethanol, methanol and water) and two non-polar (chloroform and diethyl ether) solvents and subjected to 14 phytochemical tests. Further, the crude extract of *T. ammi* was analyzed over gas chromatography–mass spectroscopy (GC–MS) and Fourier transform infrared spectroscopy (FTIR). Evaluation of antimicrobial property of the extract was carried out by minimum fungal concentration (MFC) and minimal inhibitory concentration (MIC). In addition, cell reduction assay was performed using flowcytometry to confirm the antifungal effect of Ajwain crude extract.
Results: The aqueous extract showed high presence of phytochemicals including alkaloids, carbohydrates, flavonoids, resins, steroids, tannins, inorganic acids, organic acids, phenolic compounds, amino acids, protein and coumarins. GCM—S analysis revealed seven bioactive compounds, in which thymol was detected in significant amount in the chromatogram. FTIR performance showed the presence of various stretching vibration including O—H, C—H, C═C, C—O, C—N and C—O—C. However, the MFC and MIC of Ajwain extracts using different solvent showed that the methanolic extract possesses maximum antifungal efficacy at 250 μg/ml and 15.6 μg/ml, respectively. In addition, cell reduction assay exhibited significant cell reduction in Ajwain methanolic extract compared to the other crude extracts used in the study.
Conclusion: Overall, the findings revealed that Ajwain methanolic crude extract has antifungal activity against *C. albicans*; however, that further needs to be established at molecular level.

Introduction

In non-industrialized civilizations, medicinal plants are extensively utilized, mostly because they are easily available and less expensive than contemporary medications. Since prehistoric times,

* Corresponding author at: SRM Research Institute, SRM Institute of Science and Technology, Kattankulathur 603 203, Chennai, Tamil Nadu, India.
** Corresponding author.
E-mail addresses: abirami@srmasc.ac.in (A. Arasu), jesuaraj@hotmail.com, jesuaroa@srmist.edu.in (J. Arockiaraj).

https://doi.org/10.1016/j.jiph.2021.09.027

therapeutic plants, often known as medicinal herbs, have been identified and employed in traditional healing practices. Plants generate hundreds of chemical compounds for defense against insects, fungus, illnesses and herbivorous animals, among other things. Herbs have yielded a slew of phytochemical substances with promising characteristics and proven biological action. The consequences of utilizing a complete plant as medicine, however, remain unknown because a single plant includes a vast range of phytochemical substances. Furthermore, the phytochemical composition and pharmacological effects of many therapeutic plants, if any, are still being determined via rigorous scientific study [1]. *Trachyspermum ammi* (commonly known as Ajwain), a medicinally essential seed spice from the Apiaceae family, is highly prized. The seeds have good aphrodisiac qualities, while the roots are diuretic. Ajwain oil, which is 2–4.4% brown in hue, is found in the seeds. The major ingredient in this oil is thymol, which is used to treat gastrointestinal issues, appetite loss and bronchial difficulties. *T. ammi* seeds have been used traditionally to treat cough, inflammation, diarrhoea, headache, hypertension, stomach discomfort, bronchitis and influenza [2].

Fungi have become major sources of human diseases. Candidiasis is the most common of the disseminated mycoses. *Candida albicans* is the fungus that causes the most opportunistic and invasive fungal infections of any other fungus [3]. *C. albicans* is generally a commensal fungus found in the gastrointestinal and genitourinary systems. Candidiasis, often known as yeast infection is caused by any *Candida* species, the most common of which being *C. albicans*. More than twenty types of *Candida* can cause infection with *C. albicans* being the most common. *C. albicans* causes severe fungal infections leading to a range of life-threatening diseases caused by invasive infections to non-life-threatening mucocutaneous diseases [4]. Individuals at risk for invasive candidiasis include low birth weight babies, people recovering from surgery, HIV, cancer and people admitted to an intensive care unit and those with an otherwise compromised immune systems. In fact, the fourth most common nosocomial blood stream pathogens are *Candida* species which was reported by national nosocomial infections surveillance (NNIS); also, lack of antifungal therapies and inappropriate diagnostic procedure leads to high mortality rate every year [5]. The *Candida* species that survive on skin and on environmental surfaces may be endogenous or exogenous [6–8] where in invasive fungal infections are most commonly caused by this species.

T. ammi, on the other hand, is still being investigated for its antifungal properties. Previous researches have documented the information on the efficiency of *T. ammi* and the chemical structure of its derivative; however, antifungal efficacy of this particular medicinal herbal derivative has yet to be demonstrated. Hence, this investigation evaluated the phytochemical derivatives of Ajwain and its *in vitro* antifungal activity against a healthcare-related opportunistic pathogen with emphasis on *C. albicans*.

Materials and Methods

Isolation and identification of C. albicans

C. albicans was collected from AG Hospital, Chennai. The collected swabs were inoculated in Sabouraud dextrose agar (SDA) plate and CHROM agar (Hi-media) and incubated for 24 h at 30 °C. CHROM agar was utilized to isolate and distinguish *C. albicans* from other *Candida* species based on color and morphological observation. Moreover, the species can also be identified based on the colony color as green colonies represent *C. albicans*, creamy colonies represent *C. glabrata* and pink colonies with a whitish border represents *C. krusei*; in order to identify *C. albicans*, further examinations were carried out.

Gram staining method

The obtained *Candida* culture was examined for further identification on its morphology by conventional Gram staining technique.

Germ tube test

In an Eppendorf tube, 0.5–1 ml of serum was collected. Using an inoculation loop, the fungal isolate was gradually emulsified in the serum and incubated at 37 °C for 2–4 h. Then, a drop of serum is applied to a grease-free glass slide with a cover slip over it; and observed under a microscope [9] and identification of *C. albicans* was carried out based on the morphology.

Chlamydoconidia production test

The Chlamydoconidia identification was performed using rice agar medium. The culture was previously grown in Sabouraud dextrose agar (SDA) and seeded as 3 parallel streaks on a rectangular piece of rice agar placed between the slides and incubated in a wet chamber at 30 °C for 72 h. After the incubation, cover slip was kept over the slides and was viewed under a microscope. *C. albicans* was recognized and the production of chlamydospore [10] on the rice agar media is to be observed.

Sugar fermentation test

Peptone water was prepared and sterilized at 121 °C for 15 min. The sugar solution such as glucose, maltose, sucrose and lactose were prepared individually using sterile peptone water. Sugar solutions were poured into sterile test tubes, Durham tubes were inserted into the test tubes without bubbles. The test tubes with sugar solutions were steam sterilized for 15 min. Fungal colonies were inoculated into each sugar tubes using an inoculation loop and incubated for 24 h at 37 °C [11].

Solvent extraction

Ajwain seeds were extracted using polar and non-polar solvents, including (polar solvents: ethanol, methanol and water and non-polar solvents: chloroform and diethyl ether). In brief, Ajwain seeds were gathered, cleaned and dried in the open air. Mortar and pestle are being used to grind the seeds into a coarse powder. The coarsely crushed seeds (15 g) were steeped in 100 ml of the above-mentioned solvents overnight at room temperature. Whatman filter paper or muslin cloths were used to filter the soaked solvent. The filtered solvent was poured into an evaporating dish and placed in a sand bath. Semi-solid extract was obtained as the solvent evaporated and placed in a separate Eppendorf tube. For further usage, the crude extract was dissolved with dimethyl sulphoxide (DMSO) [12].

Phytochemical analysis

Traditional phytochemical tests on different Ajwain extracts as prepared were carried out to ascertain the phytoconstituents. Phytochemical analysis has been carried out to identify alkaloids, carbohydrates, flavonoids, glycosides, resins, steroids, tannins, starch, inorganic acids, phenolic compounds, amino acids, protein, coumarins and phlobatannins. The phytochemical assay was approached using [13] modified methodology of Hossein Mostafavi.

Gas chromatography–mass spectroscopy analysis

The sample was injected into an Agilent technologies 5975 C model HP-5 column (30 m, 0.25 mm, 0.25 μm) for GC–MS analysis. We utilized the following chromatographic conditions: the injector was set to 250 °C and the column oven temperature was set to 60–250 °C at a rate of 5 °C/min injection mode, using helium as the

carrier gas at a flow rate of 1 ml/min. The following MS settings were used: 70 eV ionization voltages, 250 °C ion source temperature, 250 °C interface temperature and 30–600 mass range [1,14].

Fourier-transform infrared spectroscopy analysis

To detect the sorts of chemical bonds (functional groups) found in compounds or extract, we applied Fourier transform infrared spectrometer (FTIR) analysis. Hence, a solidified semi-solid plant extract was poured in a separate Eppendorf tube and maintained at −20 °C overnight. The ice that developed during freezing was removed by sublimation under vacuum below 200 ml and the temperature was kept at −45 °C to −20 °C for 3 h. Secondary drying, in which the most of the remaining water in the sample was absorbed by raising the temperature and low pressure provides the dry powder that is suitable for FTIR analysis. The dried extract powder was encapsulated in 100 mg of KBr pellet and made transparent sample discs. An FTIR spectroscope with a scan range of 400 to 4000 cm^{-1} and a resolution of 4 cm^{-1} was used to examine the powdered extract sample. The data were generated from the peak or possible functional groups obtained from the extract powder [15].

Antifungal activity of T. ammi

Minimum fungal concentration (MFC)

To assess the antifungal activity, a minimum fungal concentration was determined using the standard agar well-diffusion method as suggested by the National Committee of Clinical and Laboratory Standards (NCCLS, 2007) of CLSI M44-A2 method.

Preparation of fungal inoculum

Sabouraud dextrose broth (peptone, dextrose) was prepared and a loop of fungal culture was inoculated and incubated for 24 h at 35 °C. The next day, 2–3 ml of 24 h incubated fungal inoculum was inoculated in new Sabouraud dextrose broth and then cultured nearly 4 h before being utilized for further study.

Agar well diffusion method

Agar well diffusion was conducted using Muller–Hinton agar medium supplemented with 0.2% glucose and 0.5 g/ml methylene blue, according to the CLSI M44-A2 guidelines [14]. The pH of the medium was maintained between 6.5 and 7.5. Using a sterile gel puncture, 3 wells (each in 4 mm diameter and roughly 2 cm apart) were cut in each plate. At a concentration of 1 mg/ml, a stock solution of Ajwain solvent extract was prepared. Fluconazole, a common antifungal drug was used as a positive control. The Ajwain solvent extracts have been taken for the assay at different quantities (50 μl, 100 μl, 150 μl, 200 μl and 250 μl) and poured into the wells using a sterile micropipette. The plates were incubated for 24 h at 35 °C, and the diameter of the inhibitory zone was measured in millimeters.

Minimum inhibitory concentration (MIC)

The efficacies of the Ajwain extracts from different solvents were evaluated using the spot inoculation technique to determine minimum inhibitory concentrations against the test fungal pathogen [16].

Preparation of fungal inoculum

Fungal colonies were inoculated into Sabouraud dextrose broth and cultivated at 35 °C for 24 h. The 24 h broth culture was inoculated in fresh Sabouraud dextrose broth for the following phase, and the broth was incubated at 35 °C for 4 h before being used in the procedure.

Broth dilution method

In 96 well microtiter plates, the broth dilution technique was carried out (M27-A3). Using a sterile micropipette, 100 μl of fungal inoculum was applied into each well. Each solvent extract's concentration (250, 125, 62.5, 31.25, 15.6 and 7.81 μg/ml) was diluted into two-fold before being added to each well. Fluconazole was maintained as positive control. Microtiter plates were sealed with lead and incubated for 24 h at 35 °C. Following incubation, treated culture broth was evaluated at 560 nm by Spectrophotometer for OD values. The MIC was measured based on the growth in least concentration.

Cell reduction assay by flowcytometry

Sabouraud dextrose broth was prepared; loopful of fungal culture was inoculated and incubated for 24 h at 35 °C. Seven Eppendorf tubes were taken; 1 ml of culture broth was transferred into each tube. Ajwain extract (250 μl) from all solvents were added in each Eppendorf tubes, whereas control tube was kept separately without adding any extracts and incubated for 24 h. The treated and the untreated samples were transferred in flowcytometry tubes. The tubes were then allowed to run in the flowcytometry machine for 20 s and the results were recorded [17].

Results and Discussion

Candidiasis has become a significant problem across the world. Candida infections are associated with multitude illnesses, including AIDS, neutropenic, metabolic conditions like diabetes mellitus and as well as the overuse of broad-spectrum antibiotics. Although there are several drugs available to treat such infections, we also really need a potential candidate that is successful in controlling Candidiasis. Therefore, this study emphasizes the antifungal properties of Ajwain crude extract against *C. albicans*, which causes candidiasis infection. The results of the present study have proved that *C. albicans* was completely controlled by Ajwain extract.

C. albicans *collection and identification*

CHROM agar is a fast analysis to look at different *Candida* species. It makes easier to discover and identify *Candida* species even in mixed cultures within 24–48 h. In the study, in CHROM agar media, apple green colony development was observed, this implies the presence of *C. albicans*. Gram's staining was performed on the collected candidiasis culture which was also revealing the presence of yeast-like budding cells while observing under the microscope. Germ tube test indicated the formation of short hyphae (filamentous) extension arising laterally from a yeast cell (pseudo hyphae) with no constriction at the point of origin, identifying the organism as *Candida* species (Fig. 1). Whereas, it was observed [18] that the formation in germ tube was identified as *C. albicans.* In addition, it was identified as [19] elongated daughter cells from the round mother cell without constriction at their origin and is referred to as true germ tubes while constricted hyphae as pseudo hyphae.

Chlamydoconidia production test was carried out in Rice agar medium where the thick-walled resting spores are identified as a Chlamydoconidia and referred as *C. albicans* (Fig. 1). A previous study [20] performed Chlamydoconidia test and observed the formation of rounded spores with double walled isolates identified as Chlamydoconidia. Earlier researcher [21] identified Chlamydoconidia formation using Corn Meal Agar supplemented with milk which enhances the formation of Chlamydoconidia production.

A sugar fermentation test was performed to examine *C. albicans* sugar utilization pattern. Candida utilized three sugars: glucose, maltose and sucrose, whereas lactose has not been utilized (E-suppl. Table 1). *C. albicans* normally requires the lactase enzyme

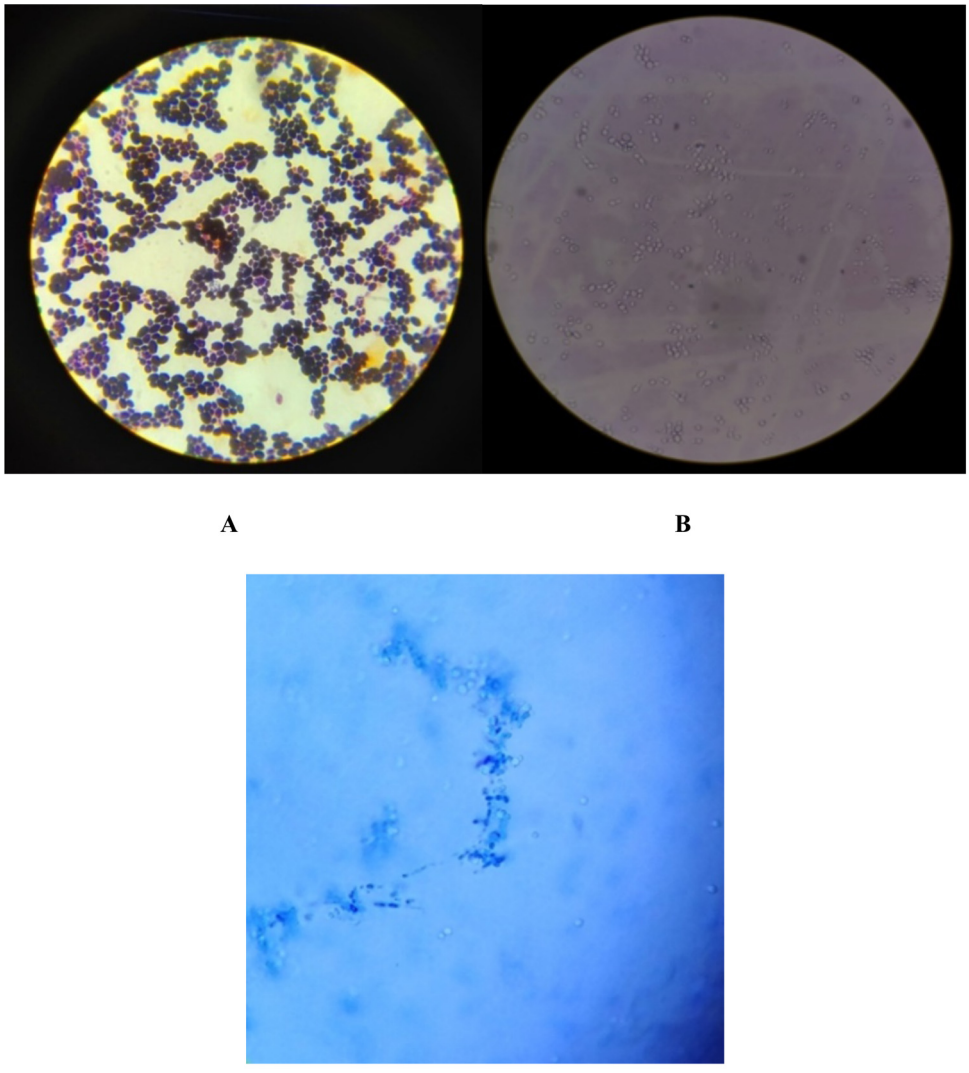


Fig. 1. Microscopic observation of *Candida* species. **A:** Yeast cell identification by Gram staining technique; **B:** pseudohyphae observation by Germ tube test and **C:** chlamydoconidia spore identification by chlamydoconidia production test.

to breakdown lactose; however, this enzyme is lacking in *C. albicans*, preventing it from using lactose sugar. Previous study [22] performed sugar fermentation test where glucose, maltose and sucrose were utilized by *Candida*; lactose was not utilized. The germ tube test, chlamydospore production and sugar fermentation assays were used to conventionally speciate *C. albicans* isolates, though they are time consuming and labor-intensive process. Based on these investigations, *C. albicans* was identified and confirmed, the same culture was used for further activity analyses.

Solvent extraction of Ajwain seed

Ajwain seed was extracted using a variety of solvents such as aqueous, ethanol, methanol, chloroform and diethyl ether. In a sand bath, a liquid solvent extraction was done using the hot extraction method, yielding a semi solid extraction. Earlier researchers [23] performed solvent extraction of Ajwain using different solvents to study the antifungal activity on *C. albicans* and *Aspergillus clavatus.* Moreover, former researcher performed [24] ethanol and methanol solvent extraction of Ajwain by maceration method.

In this study, fourteen phytochemical tests were carried out for various solvents including ethanol, methanol, aqueous, chloroform and diethyl ether (Table 1). Aqueous extraction of Ajwain revealed more than ten phytochemical components among all solvents. As a result, aqueous extraction yields more components from Ajwain than any other solvent in the study. In previous study phytochemical tests were carried out using different solvents; for example, in an earlier work [25] phytochemical screening of *T. ammi* was evaluated for total phenolic and flavonoids compound presence in Ajwain extract; also, another qualitative phytochemical analysis and phytoconstituents of Ajwain [26] was carried out.

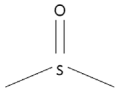
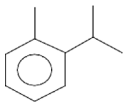
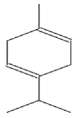
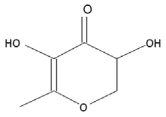
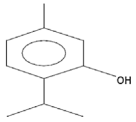
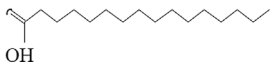
Gas chromatography and mass spectroscopy analysis

Gas chromatography and mass spectroscopy (GC–MS) analysis of Ajwain extract revealed the presence of seven bioactive compounds (Table 2), which was confirmed based on the peak obtained, retention time, molecular weight and molecular formula. The analysis detected various bioactive compounds including dimethyl sulphoxide, *o*-cymene, γ-terpinene, 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl, thymol, *n*-hexadecanoic acid and cis-vaccenic acid with retention time 5.402, 8.290, 8.920, 11.458, 13.640, 23.152 and 25.283, respectively. The extract showed three highest peaks based on the retention time, in which

Table 1
Phytochemical constituents of Ajwain solvent extracts. Eleven phytochemical components identified from Ajwain aqueous extract. Ethanol, Methanolic and Diethyl ether extracts showed seven phytochemical constituents. Six phytochemical substances recognized from chloroform extract.

S. no	Phytoconstituents	Aqueous	Ethanol	Methanol	Chloroform	Diethyl ether
1.	**Alkaloids:** Mayer's test	+	–	–	–	–
2.	**Carbohydrates:** Benedict's test	+	+	–	–	+
3.	**Flavonoids:** Ferric chloride test	+	+	+	+	–
4.	**Glycosides:** Borntrager's test	–	–	–	–	–
5.	Resins	+	+	+	+	+
6.	**Steroids:** Liebermann–Burchard Test	+	+	+	–	+
7.	**Tanins:** Lead acetate test	+	+	+	+	+
8.	Starch	–	–	–	–	–
9.	**Inorganic acids:** Sulphate test	+	–	+	–	+
10.	**Organic acids:** Malic acid test	–	–	–	+	–
11.	**Phenolic compounds:** Lead acetate test	+	+	+	+	+
12.	**Amino acids:** Millons test	+	–	–	–	–
13.	**Protein:** Biuret test	+	–	–	–	–
14.	Coumarins	+	+	+	+	+
15.	Phlobatannins	–	–	–	–	–
16.	Anthraquinones	–	–	–	–	–

Table 2
Results of gas chromatography–mass spectrometry analysis (Ajwain crude extract). It includes the spectral fingerprints of bioactive compounds, molecular weight, structure and compound names.

S. no	RT	Name of the compound	Structure	Mol. wt g/mol	Mol. formula
1.	5.402	Dimethyl sulphoxide		78.013	C_2H_6OS
2.	8.290	*o*-Cymene		134.109	$C_{10}H_{14}$
3.	8.920	γ-Terpinene		136.125	$C_{10}H_{16}$
4.	11.458	4*H*-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl		144.042	$C_6H_8O_4$
5.	13.640	Thymol		150.104	$C_{10}H_{14}O$
6.	23.152	*n*-Hexadecanoic acid		256.240	$C_{16}H_{32}O_2$
7.	25.283	cis-Vaccenic acid		282.255	$C_{18}H_{34}O_2$

Thymol showed the maximum and highest peak in chromatogram (Fig. 2).

For more than 40 years, volatile chemicals have been intensively explored, with numerous researchers focusing on them. So far, over 245 volatile components have been found in Ajwain's volatiles, although only a few have been known to possess antifungal action. The volatile compounds identified from Ajwain are shown in Table 3 and its chromatogram was showed in Fig. 2. Earlier research reported seven volatile compounds from Ajwain [27]. In this study, GC–MS results showed the presence of a mixture four main components including Thymol (71.06%), *o*-cymene (3.37%), γ-terpinene (3.826%) and 2-methyl-5-(1methylethyl)-phenol (0.512%); altogether it was 99.97% of the total oil. All these main components were found in phenolic components of Ajwain; in other words, the major component, thymol was together with other small amounts of volatile compounds. Another study [28] reported γ-terpinene (8.07%), *o*-cymene (3.73%) and thymol (66.41%) as major constituents from Ajwain through GC–MS analysis. Similarly, seven major compounds from Ajwain extract were identified previously [29] which is in accordance with our study. In this study, it was observed that Thymol was present in higher concentration in Ajwain, the other components namely Terpinene and cymene were present relatively in small quantities.

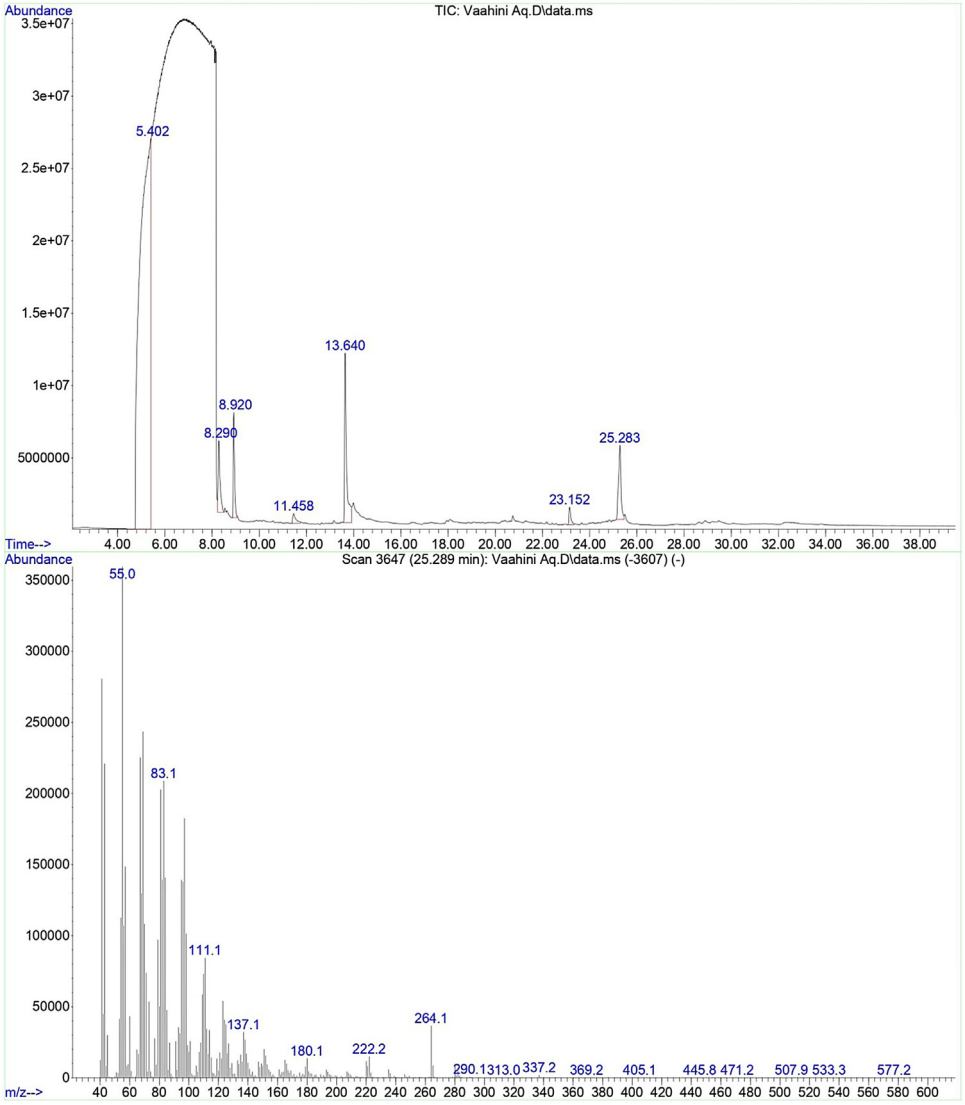


Fig. 2. Gas-chromatography and mass-spectrometry analysis of Ajwain crude aqueous extract in chromatogram. The peaks in the GC–MS spectra of Ajwain extract show the presence of various secondary metabolites (or bio active compounds).

FTIR analysis

Ajwain extract showed characteristic absorption band at 3434.27 cm^{-1} for O—H stretching vibration in the presence of alcohols and phenols, 2925.26 cm^{-1} for C—H stretching vibration in the presence of alkenes, 2855.68 cm^{-1} for C—H stretching, 1610.09 cm^{-1} for —C=C— stretching vibration in the presence of alkenes, 1414.22 cm^{-1} for —C=C— stretching, 1317.22 cm^{-1} for C—O stretching vibration in the presence of alcohols, carboxylic acids, esters and ethers, 1190.66 cm^{-1} for C—N stretching vibration in the presence of aliphatic amines, 1086.77 cm^{-1} for C—N stretching, 953.37 cm^{-1} for C—O—C stretching in the presence of proteins/ polysaccharides and 874.60 cm^{-1} for bending of benzene ring. The results indicates that the extract is surrounded by proteins and metabolites such as alkaloids, terpenoids that have functional groups of amines, alcohols, ketones, aldehydes and carboxylic acids which are the major components responsible for antifungal activity (Table 3 and Fig. 3).

Ajwain extracts shows ten functional groups based on adsorption range (Table 3) and based on the chromatogram indication (Fig. 3). Studies reported [30] the functional groups of carboxylic acids, amines, amides, sulphur derivatives, polysaccharides, organic hydrocarbons and halogens that are responsible for various

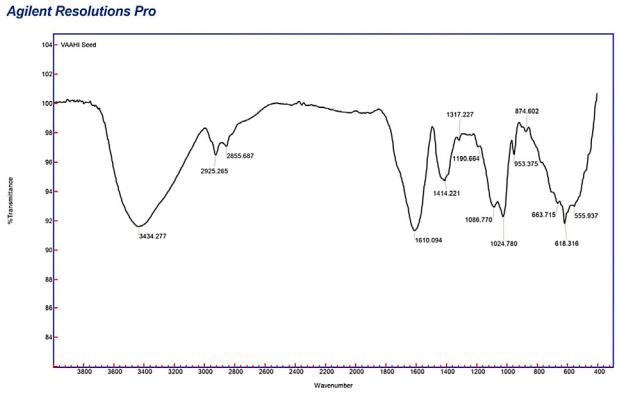
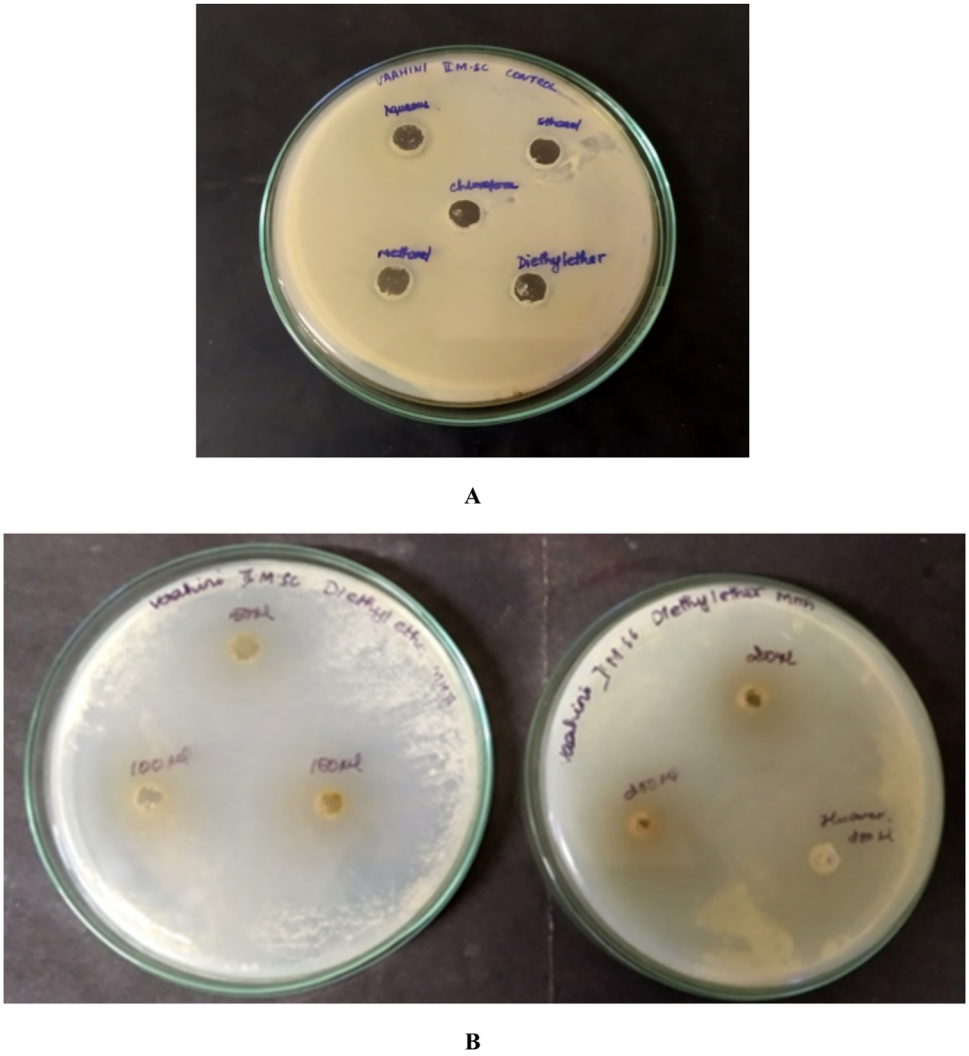


Fig. 3. FTIR analysis results of Ajwain crude (aqueous) extract. The different adsorption ranges reflect the functional groups of phytochemicals. The O—H bond stretching is attributed by a broader peak observed at 3434.27 cm^{-1} adsorption range. The O—H functional indicates the presence of alcohols and phenolic compounds.



A

B

Fig. 4. A. Antifungal activity results of various solvent extract. No zone was produced by the solvents. **B.** Antifungal activity of Ajwain methanolic extract. Various concentrations of crude extract inhibited the growth of *C. albicans*. Ajwain methanolic extract at 250 µg/ml concentration showed the largest zone of inhibition against *C. albicans*.

Table 3
Functional group of Ajwain crude extract analyzed by FTIR. Absorption range denotes the specific functional groups of crude extract.

Adsorption range (cm^{-1})	Functional group
3434.27	OH stretching vibration (presence of alcohols and phenols)
2925.66	C—H stretching vibration (presence of alkenes)
2855.68	C—H stretching
1610.09	—C=C— stretching vibration (presence of alkenes)
1414.22	—C=C— stretching
1317.22	C—O stretching vibration (presence of alcohols, carboxylic acid, ester)
1190.66	C—N stretching vibration (presence of aliphatic amines)
1086.77	C—N stretching
953.37	C—O—C stretching vibration (presence of proteins/polysaccharides)
874.60	Bending of benzene ring

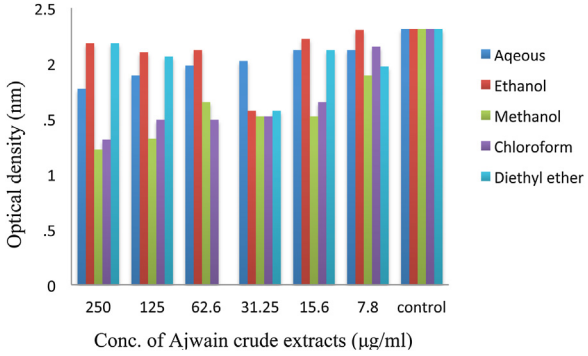


Fig. 5. Minimal inhibitory concentration of solvent extracts of Ajwain. *C. albicans* incubated with various concentration of Ajwain crude extract. Ajwain methanolic extract exhibited antifungal activity even at low concentration.

medicinal properties of Ajwain. Also, it was analyzed [31] that the extracts of Ajwain using FTIR revealed functional group of components including amino acids, amides, amines, carboxylic acid, carbonyl compounds and organic acids. Previously [32], the FTIR analysis reported the presence of various characteristic functional groups such as carboxylic acids, amines, amides, sulphur derivatives and polysaccharides in Ajwain extract.

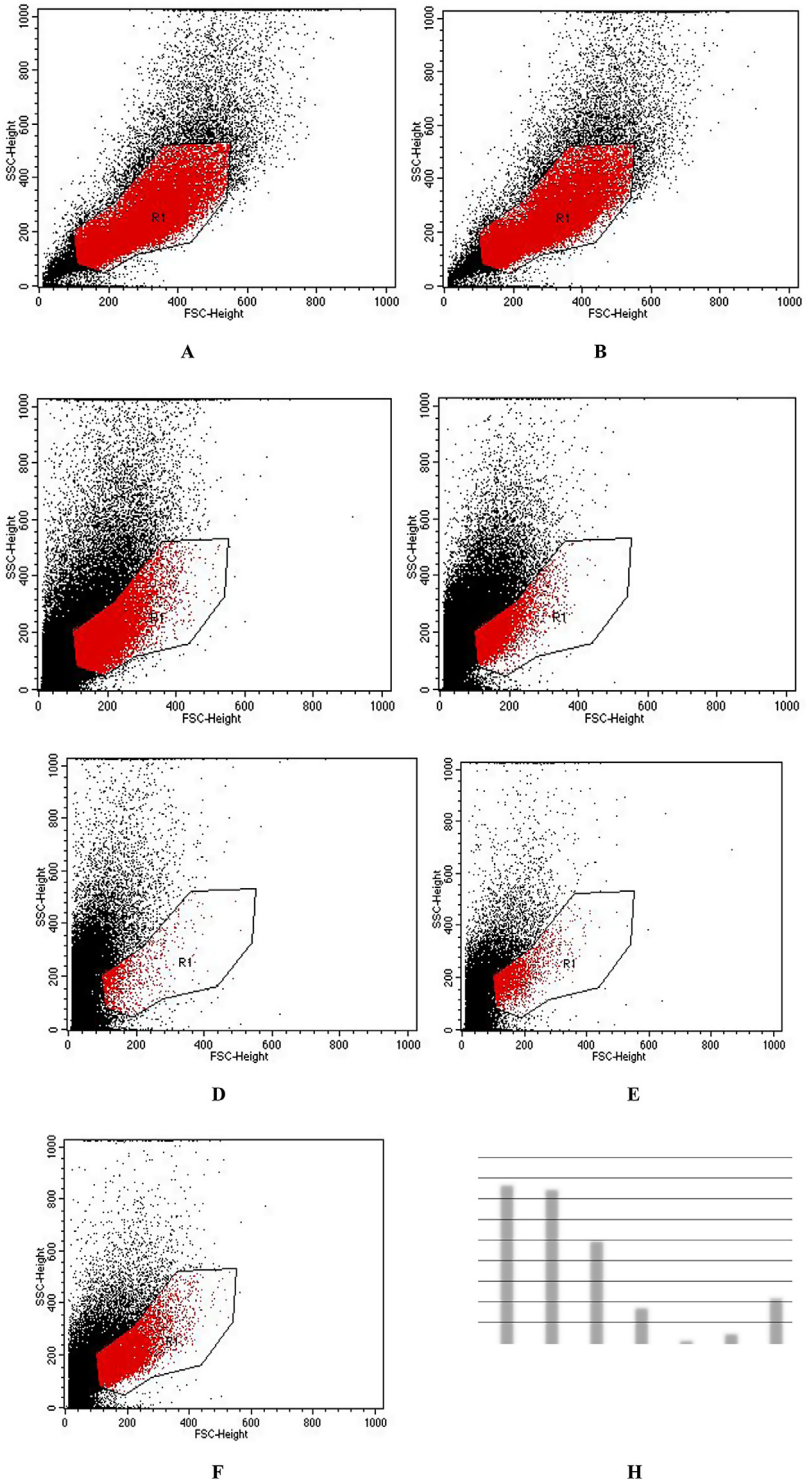


Fig. 6. Cell reduction analysis of *C. albicans* treated with various Ajwain crude extract in Flowcytometry. **A.** Untreated control and **B.** DMSO control samples showed more viable cells. **(C)** aqueous extract, **(D)** ethanolic extract, **(E)** methanolic extract, **(F)** chloroform extract and **(G)** diethyl ether extract. The red dotted regions are showing the viable cell and black dotted regions showing dead cells in the panel. Among the treated samples, methanolic extract highly reduced the viability of the cells **(H)**.

Anti-fungal activity

From the antifungal study, it was observed that methanol extract of Ajwain was found to be effective against *C. albicans* with 27 mm in 250 µl concentration, where aqueous extract shows 25 mm in 250 µl, ethanol extract shows 24 mm in 250 µl, chloroform extract and diethyl ether extract showed 23 mm in 250 µl concentration (E-Suppl. Table 2 and Fig. 4). In the study, Ajwain methanolic extract have significant anti-fungal effect even at lowest concentration, which implies that Ajwain extract has more potential effect against the fungi and the results coincides with earlier demonstration [33] which showed that Ajwain extract was more effective against *Aspergillus niger*, *A. clavatus*, *Epidermophyton flocossum* and *Trichophyton rubrum* with an inhibition zone 23 mm diameter at concentration 1 mg/ml. Another study reported [34] that Ajwain

extract was effective against *C.albicans* with an inhibition zone 21 mm dia.

In previous investigation [35] the ethanolic and methanolic extracts of Ajwain showed an effective zone of inhibition against *C. albicans*, *A. niger*, *A. fumigatus* and *Trichophyton rubrum.*

Minimal inhibitory concentration (MIC)

In the study, a least inhibitory concentration of Ajwain solvent extracts against *C. albicans* was determined using broth dilution technique and determined its MIC values based on the OD values (Fig. 5). The inhibitory action of Ajwain crude extract has been observed at different concentrations (250, 62.5, 31.25, 15.6 and 7.81 μg/ml), however among all, Methanolic extract showed inhibitory efficacy even at low concentration (15.6 μg/ml).

Cell reduction assay by flowcytometry

The culture was comparatively analyzed between Ajwain solvent extract treated or untreated cultures. From the obtained results, we can determine that methanol extract of Ajwain showed effective cell reduction on *C. albicans* (Fig. 6). The aqueous extract showed less cell reduction compared to other solvent extracts of Ajwain. From the Flowcytometry analysis, it is known that the red region indicates the total number of viable cells before or after treatment, whereas black scatter dots indicated the dead cells in the panel. The results of this experiment clearly show that Ajwain methanolic extract acts as a promising antifungal agent. It was exhibited [36] that the yeast viability through flowcytometry using a novel fluorescent probe, FUN-1. Further, in earlier work [16], cell count assay using flowcytometry for yeast cells along with a fluorescent probe was studied. The study also demonstrated [37] yeast cell viability due to treatment or un-treatment through light scattering method on flowcytometry. Altogether, the study indicated the performance of Ajwain extract, especially the methanolic extract against the opportunistic human gut pathogen, *C. albicans.*

Conclusion

The evolution of the potential advantages of bioactive secondary metabolites produced from edible seeds of Ajwain might lead to the development of new antifungal strategies and concepts. Ajwain intake has been linked to a lower incidence of fungal infections suggested in several studies. This research work revealed that Ajwain crude extract had remarkable antifungal efficacy against *C. albicans*. It might be used as a significant alternative medicine in the future unless the bioactive component can be separated from the crude. Thymol is a novel bioactive molecule that might be isolated and developed as an antifungal drug against infectious disease caused by various species, particularly *C. albicans* in future. Ajwain bioactive compounds have been recognized as a safe and successful type of therapeutic agent and could be used by individuals worldwide for a number of ailments. Further studies in this area may lead to promising result for Thymol based anti-fungal, anti-microbial agents and health supplements.

Funding

This work was supported by the Researchers Supporting Project (RSP-2021/173), King Saud University, Riyadh, Saudi Arabia.

Ethical approval

Not required.

Competing interests

The entire author declares that they have no conflict of interest. The authors of this research acknowledge that they are not involved in any financial interest.

Acknowledgments

The authors extend their appreciation to The Researchers Supporting Project (RSP-2021/173), King Saud University, Riyadh, Saudi Arabia.

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.jiph.2021.09.027.

References

[1] Farnoosh yavari *Trachyspermum ammi* (L.) sprague. J Evid Based Complementary Altern Med 2014;20(1), http://dx.doi.org/10.1177/2156587214553302.
[2] Morsi NM. Antimicrobial effect of crude extracts of *Nigella sativa* on multiple antibiotic resistant bacteria. Acta Microbiol Polonica 2000;49(1):63–74.
[3] Elizabeth MJ. Rare and emerging *Candida* species. Curr Fungal Infect Rep 2009;3:152–9.
[4] Achkar Jacqueline M, Fries Bettina C. *Candida* infections of the genitourinary tract. Clin Microbiol Rev 2010;23(April (2)):253–73.
[5] Jarvis WR. Epidemiology of nosocomial fungal infections, with emphasis on *Candida* species. Clin Infect Dis 1995;20(June (6)):1526–30, http://dx.doi.org/10.1093/clinids/20.6.1526. PMID: 7548503.
[6] Vazquez JA, Sobel JD. Candidiasis. In: Kauffman CA, Pappas PG, Sobel JD, Dismukes WE, editors. Essentials of clinical mycology. New York, USA: Springer; 2011. p. 167–206.
[7] Johnson AD, Lohse MB. White-opaque switching in *Candida albicans*. Curr Opin Microbiol 2009;12(6):650–4, http://dx.doi.org/10.1016/j.mib.2009.09.010.
[8] Soll DR, Daniels KJ. MAT, Mating, Switching and Pathogenesis in Candida albicans, Candida dubliniensis and *Candida glabrata*; 2007. p. 225–45.
[9] Fenn JP, Billetdeaux E, Segal H, Skodack-Jones L, Padilla PE, Bale M, et al. Comparison of four methodologies for rapid and cost-effective identification of *Candida glabrata*. J Clin Microbiol 1999;37:33873389.
[10] de Castro Ricardo Dias, de Souza Trícia Murielly Pereira Andrade, Bezerra LouiseMoraisDornelas. Antifungal activity and mode of action of thymol. Altern Med 2015;15:417.
[11] Elizabeth MJ. Rare and emerging *Candida* species. Curr Fungal Infect Rep 2009;3:152–9.
[12] Barabde Gayatri, Qureshi Aqeela AS. Optimization of extraction process and investigation of antioxidant activity, DNA protection potential and antimicrobial activities of *Trachyspermum ammi* seed extract. IOSR J Appl Chem (IOSR-JAC) 2278-5736 2018;11(November (11) Ver. I):45–9.
[13] Mostafavi Hossein, Pezhhanfar Sakha. Qualitative phytochemical analysis of ajwain (*Trachyspermum ammi*) from north-west Iran. Int Res J Pharm 2015;6(9):610–5.
[14] Ramasamy M, Ashok kumar R. Phytochemical screening by FTIR spectroscopic analysis of leaf extracts of selected Indian medicinal plants. Int J Curr Microbiol Appl Sci 2014;3(1):395–406.
[15] Shokri H, Sharifzadeh A, Khosravi AR. Antifungal activity of the *Trachyspermum ammi* on some of the most common fungal pathogens in animals. IJVM 2016;10(3):173–80.
[16] Jorgensen JH, Ferraro MJ. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. Clin Infect Dis 2009;49:1749–55, http://dx.doi.org/10.1086/647952.
[17] Ongena K, Das C, Smith JL, Gil S, Johnston G. Determining cell number during cell culture using the scepter cell counter. J Vis Exp 2010;45:2204, http://dx.doi.org/10.3791/2204.
[18] Roushani Shahriar, Deorukhkar Sachin Chandrakant. Identification of *Candida* species: conventional methods in the era of molecular diagnosis, molecular diagnosis. Ann Microbiol Immunol 2018;1(1):1002.
[19] Jan Abiroo, Bashir Gulnaz, Qadir Rubhana, Fomda Bashir A, Sofia, Yaqoob Hakak Aamir. Modified germ tube test: a rapid test for differentiation of *Candida albicans* from *Candida dubliniensis*. Int J Contemp Med Res 2018;5(March (3)). ISSN (Online): 2393-915X; (Print): 2454-7379 | ICV: 77.83 |.
[20] Marinho Sandra Aparecida, Teixeira Alice Becker, Santos Otávio Silveira, Cazanova Ricardo Flores, Ferreira Carlos Alexandre Sanchez, Cherubini Karen, et al. Identification of candida spp. By phenotypic tests and PCR. Braz J Microbiol 2010;41:286–94.
[21] Benham RW, Pollack JD. The chlamydospores of *Candida albicans*: comparison of three media for their induction. J Lab Clin Med 1957;50(August (2)):313–7. PMID: 13449414.

[22] Umadevi MeenigaSailaja. A study of antifungal drug sensitivity of *Candida* isolated from human immunodeficiency virus infected patients in Chennai, South India. J Oral Maxillofac Pathol 2011;15(2):182–6, http://dx.doi.org/10.4103/0973-029X.84490.
[23] Kumar Hanwant, Sharma LK, Agarwal D, Saxena SN, Kumar Manish, Verma JR, et al. Antibacterial and antifungal activity of ajwain (*Trachyspermum ammi*) in different solvent. J Pharmacogn Phytochem 2018;7(3):2672–4.
[24] Khanavi M, Farjadmand F, Eftekhari M, Hosseinsalari A, Akbarzadeh T, Safavi M, et al. The effect of extraction method on the major constituents and biological effects of*Trachyspermum ammi* L. fruits. Res J Pharmacogn 2018;5(1):55–61.
[25] Bhogineni Srinu, Damodar Katasani, Ramanjaneyulu Bala. Phytochemical screening quantitative estimation of total phenolic, flavonoids and antimicrobial evaluation of *Trachyspermammi*. J Atoms Mol Jamonline 2011;1(1):1–8.
[26] Hossein Mostafavi, Pezhhanfar Sakha. Qualitative phytochemical analysis of ajwain (*Trachyspermum ammi*) from north-west Iran. Int Res J Pharm 2015;6(9):610–5, http://dx.doi.org/10.7897/2230-8407.069119.
[27] Devi Nisha, Jeet Kamal, Narender Thakur, Thakur Raneev. Trachyspermummammi: a comprehensive review. Int Res J Pharm 2012;3(May (5)):6.
[28] Keyvan Pakshir K, Saharkhiz MJ, Kamyab AA, Kazerani NK, Zomorodian K, Rahimi MJ. Chemical compositions and antimicrobial activities of *Ocimum sanctum* L. essential oils at different harvest stages. Jundishapur J Microbiol 2014;8(December (1)):e13720, http://dx.doi.org/10.5812/jjm.13720. PMID: 25763132; PMCID: PMC4344766.
[29] Thangavel Arun, Balakrishnan Senthilkumar, Arumugam Aarthy, Duraisamy Senbagam, Muthusamy Sureshkumar. Phytochemical screening, gas chromatography–mass spectrometry (GC–MS) analysis of phytochemical constituents and anti-bacterial activity of *Aervalanata* (L.) leaves. Afr J Pharm Pharmacol 2014;8(5):126–35.
[30] Ragavendran P, Sophia D, Arul Raj C, Gopalakrishnan VK. Functional group analysis of various extracts of *Aervalanata* (L.,) by FTIR spectrum. Pharmacologyonline 2011;1:358–64.
[31] Thangarajan Starlin, Paramasivam Ragavendran, Raj Chinthamonyarul, Perumal Palanisamy Chella, Gopalakrishnan Velliyur Kanniappan. Element and functional group analysis of *Ichnocarpus frutescens*R.Br. (Apocynaceae). Int J Pharm Pharm Sci 2012;4(Suppl 5). ISSN- 0975-1491.
[32] Muruganantham S, Anbalagan G, Ramamurthy N. FT-IR and SEM-EDS comparative analysis of medicinal plants, Eclipta alba Hassk and Ecliptaprostrata Linn. Romanian J Biophys 2009;19(4):285–94.
[33] Manish Kumara B, Shanthia Nithya, Mahatoa ArunKumar, Sonia Shashank, Rajnikanth PS. Preparation of luliconazole nanocrystals loaded hydrogel for improvementof dissolution and antifungal activity. Heliyon 2019;5:e01688.
[34] de Castro Ricardo Dias, de Souza Trícia Murielly Pereira Andrade, Bezerra Louise Morais Dornelas. Antifungal activity and mode of action of thymol. Altern Med 2015;15:417.
[35] Sharifzadeh A, Shokri H. Antifungal activity of essential oils from Iranian plants against fluconazole-resistant and fluconazole-susceptible *Candida albicans*. Avicenna J Phytomed 2016;6(2):215–22.
[36] Wenisch Christoph, Linnau Ken Floris. Rapid susceptibility testing of fungi by flow cytometry using vital staining. J Clin Microbiol 1997;35(1):5–10.
[37] Oliver JD. The viable but nonculturable state in bacteria. J Microbiol 2005;43:93–100.