DOI: https://doi.org/10.54393/pbmj.v4i2.153

PAKISTAN BIOMEDICAL JOURNAL
https://www.pakistanbmj.com/journal/index.php/pbmj/index
Volume 4, Issue 2 (Jul-Dec 2021)

Original Article

Effect of Trachyspermum ammi Essential oil, fractions and its derivatives on resistant fungal Strains

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ARTICLE INFO

Key Words:
Antifungal, antimicrobial, essential oil, ajwain, resistant fungi.

How to Cite:
Iram, N., Hanif, M. A. ., Bhatti, H. N. ., & Shahid, M. (2021). Effect of Trachyspermum ammi essential oil, fractions and its derivatives on resistant fungal Strains. Pakistan BioMedical Journal, 4(2). https://doi.org/10.54393/pbmj.v4i2.153

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ABSTRACT

Persistent antimicrobial drugs treatment has resulted in antimicrobial resistance in fungi. There is always a gap for newer antifungal agent. As fungi are associated with multiple health risks in humans and many diseases in crops as well. **Objective:** To find alternate natural antimicrobial agent as compared to the synthetic one. **Method:** Essential oil of Trachyspermumammi was isolated, fractionated, and subjected to GC-MS analysis. Components from fractions were derivatized to check their antimicrobial potential against fungal resistant strains. **Results:** Analysis showed γ -terpinene (39%), α-phellandrene (1.3%), α-pinene (0.5%), Sabinene (0.15%), β-pinene (4.40%), β-myrcene (1.14%), O-cymene (15.78%), p-cymefne (38.78%), and other components were less than 1%. Fractional components were derivatised and their antifungal action was studied. **Conclusion:** Ajwain oil components found to be good against resistant fungal strains. While some derivatives showed more and some less antimicrobial action.

INTRODUCTION

To control diseases in animal and plants we need antimicrobial agents. These drugs throttle the growth and development of various micro-organisms. Synthetic antimicrobial drugs are employed against multiple strains of bacteria, fungi, and other microbes. Specially, thiazide and sulfonyl ureases are based upon sulfonamide [1]. Various new drugs have been introduced in the market but have not gained popularity as a sole controller of pathogens as they have developed multidrug resistance [2]. Antimicrobial resistance comes out when microbes build up the capacity to subjugate the medicines intended to annihilate them. That implies the micro-organisms do not die but keep on developing immunity against various chemicals [3]. Consequently, diseases that are caused by resistant bacterial and fungal strains are often incurable. Some strains of bacteria and fungus produce mycotoxins in target substances and can cause death in animals and humans on the consumptions of infected substances [4]. Numerous antimicrobial compound which has been developed so far, are not able to manage mycotoxins and are not very much effective [5]. Over the last 20 years, fungal strains like *Aspergillus spp.* have increased their resistance to these chemicals due to the persistent use of antifungal agents[6]. Resistant strains of fungi have come to light in Denmark, where azole components were the major drugs used against fungi[7]. Recent trend is the use of plant-based products for disease control both in animals and in plants. Components like, thymol, eugenol, carvacrol, anethole, estragole, etc. are isolated from different parts of the plants and are used individually to fight against disease-causing pathogens.

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More recently, derivatives of these components are used for pathogenic control. Particularly resistant bacteria and fungi are the main targets of the researcher. New strategies are being developed to control phytopathogens[8]. Several new antimicrobial compounds have been developed, and resistant isolates of bacteria and fungi have been recognized. Therefore, pursuing safe and eco-friendly antimicrobial compounds is always a hot topic, and the research into botanical antimicrobial agents is an important aspect to prevent the emergence of resistant pathogenic micro-organisms[9].

METHODS

Plant family Apiaceae has a strong potential to inhibit or kill fungal strains. Fungal strains utilized in our research work were Candida krusei(C. krusei), Fusariumsolani(F. solani), Alternariaalternata(A. alternate), Aspergillusniger(A. niger), Mucormucedo(M. mucedo), Botryodiploidiatheobrme(B. theobrme), Aspergillusflavus (A. flavus) and Alternariasolani(A. solani).

Essential oil isolation: Isolation of the essential oil from dried seeds of the plants was carried out by hydro-distillation method [10]. Isolated essential oil, dried with anhydrous sodium sulfate, stored in sealed glass bottles at -4 °C until analyzed. Fractionation was done to separate major components from minor components. All the essential oils and their fractions extracted from ajwain were subjected to GC-MS analysis for complete characterization [11]. Microorganisms (fungal strains) were selected based on their ability to show multidrug resistance. The pure strains were obtained from the Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan and Instt. Of Soil and Environmental Sciences, Agriculture University, Faisalabad, Pakistan. In nutrient agar and (PDA) potato dextrose agar medium, the fungal and bacterial cultures were maintained overnight at(30±1°C)[12].

Derivatization: Ajwain oil fraction rich in thymol was derivatized by addition of formaldehyde in the presence of strong alkali using the method of Mastelic *et al.*, 2008 with some minor changes. The prepared derivative was hydroxymethylation product of thymol fraction[13].

Antifungal evaluation of essential oils and hydroxymethylated derivative.

Potato dextrose agar solution was made by dissolving the PDA in distilled water. The solution prepared was autoclaved under pressure at 125°C for 20 minutes. After autoclave, the solution mixed well and then poured an appropriate amount of molten agar into sterilized petri dishes. The fungal strains previously selected were added homogeneously to the agar medium. Disks were put on a solid agar medium by slightly pressing, and then 10 µl of essential oils, fractions, derivatives, and standard drugs was added[14]. The treated petri plates have been incubated at room temperature for 48 hours. Lastly, the diameter (mm) of inhibition zone was measured in millimeters[15].

Microdilution assay: Microdilution broth assay was used for the determination of MIC values, as stated by NCCLS (2001) [16]. Essential oils, fractions, and derivatives were solubilized in dimethylsulfoxide (10% DMSO) diluted in culture media for use. Series of dilution concentrations of 0.01 to 30 mg / mL of essential oil in 96 well plates, including one control, was prepared. 160 µL of the fungus nutrient broth and 20 µL of the test solution were applied to the microplates [14]. Normal microorganism suspension was then inoculated in microplates. The MIC values were determined as the highest dilution to demonstrate total inhibition of the strain studied[14, 17].

Results & Discussion: By hydro distillation, the extraction method, maximum essential oil yield, was 2.7±1.8%, which was obtained during four-hour extraction while minimum (1.7±0.21%) was collected during two and half hours of extraction [18].The essential oil obtained by hydro distillation was subjected to short path vacuum fractionation. 60 ml of essential oil after fractionation gave three fractions A1, A2, and A3. The fraction and sub-fractions of essential oil and their components with their boiling point data are given in table 1&2.

Fraction	Temperture (°C)	Oil yield (mL)
A1	33.9- 37.1	32.2±0.98
A2	55-67	10.4±0.23
A3	64-69	8.6±1.14

Table 1: Showing Fractionation yield

Some oil was evaporated during the process, and leftover was a residue of fractionation. Ajwain essential oil that gave a higher yield was subjected to re-fractionation, and three new fractions were obtained.

Fraction	Temperture (°C)	Oil yield (mL)
A4	31- 34.1	12.2±0.07
A5	46-49	8.4±0.04
A6	54-59	6.3±0.34

Table 2: Showing Re-fractions of a major fraction

GC-MS analysis showed gamma-terpinene (39%) as a major component of the ajwain essential oil. -phellandrene 1.3%, -pinene 0.5%, Sabinene 0.15, -pinene 4.40%, .-myrcene 1.14%, -phellandrene 1.01%, O-cymene 15.78%, p-cymene

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38.78, -terpinene 39.5% and other components were less than 1%.

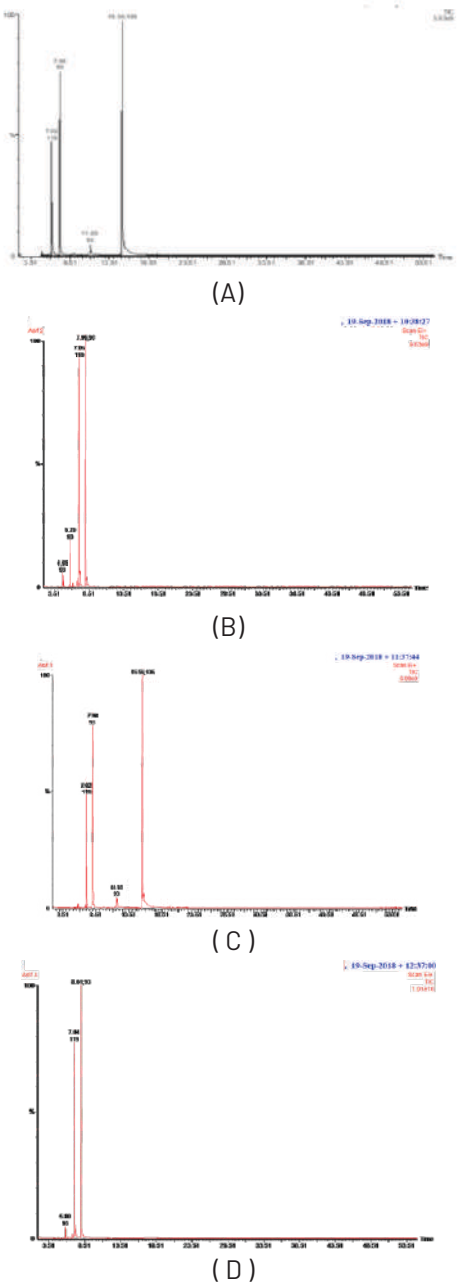

Figure 1: (A-D) GC-MS chromatogram of the ajwain oil, and fraction A1, A2, A3

Fractionation separated major, potentially active antimicrobial and antioxidant chemical constituents from essential oil. The chemical composition of the ajwain oil fraction A1 showed that it has -terpinene 39%, followed by p-cymene 30% and 4%, respectively. Some minor -pineneand p-cymene. Some trace components like -myrcene, -terpinene, and -phellandrene were also found. second fraction of ajwain essential oil (A2) revealed the presence of phenol, 5-methyl-2-(1-methyl ethyl)- 62% as the major component. gamma-terpinene had a 20% share in its chemistry followed by o-cymene, which made 11% of its part. Its minor component was gamma-terpinene 2%, while its trace components included 3-Cyclohexene-1-methanol, alpha, alpha, 4-trimethyl-, (S) -, 3-cyclohexene-1-carboxaldehyde, 1,3,4-tr, Ethanone, 1-(1,4-dimethyl-3-cyclohexane-1-yl) -, 2-cyclohexane-1-ol, 1-methyl-4-(1-methyl, alpha-terpinene and thymol. Each of them was less than 1%. GC/MS analysis of ajwain essential oil fraction (A3) revealed the presence of o-cymene 35% and gamma-terpinene 61%. Bicyclo [3.1.1]-heptane, 6,6-dimethyl-2-methylene-, (1s)- was minor component. Trace components found in this fraction were alpha-phellandrene, beta-myrcene, and alpha-terpinene. Thus, by using fractionation, approximately 61% pure phenolic component was obtained for its use in the derivatization of essential oil. Major components of ajwain essential oil and fractions showed the presence of hydrocarbons and phenolic components. Ajwain essential oil and its fractions were tested for their antifungal activity by assessing inhibition zone as reported by many researchers [19]. The oils become toxic for the fungus by damaging the cell wall and by blocking the energy generation in the cells.The strong antimicrobial action of ajwain essential oil (33mm) against *C.krusei* may be attributed to the presence of thymol, -terpene. -pinene, -pinene, and terpinene. Oxygenated monoterpene & thymol shows its action by penetrating the cell wall. Ajwain oil was almost equally effective for all the fungal strains selected. Strong antifungal action of ajwain was shown against fungi (*A.niger and Falvus*). They show their action by stopping the mycelial growth of the fungus. Ajwain oil fractions A1, A2 and A3 showed maximum MIC values against *M. mucedo*, *A.solani*, and *F.solani*, respectively. It was observed as a result of the current investigation that terpene components of essential oil were mainly responsible for the antifungal action followed by various organic functional groups like carbonyl and alcoholic groups. Significant components of essential oils and fractions of ajwain, fennel and cumin (alpha-terpinene, beta-terpinene, o-cymene, gamma-terpinene, alpha-phellandrene, 1-8-cineole, anethole, estragole, cumin aldehyde, cuminol etc.) are also responsible for antifungal action of the oils.

Hydroxymethylation derivative of Ajwain essential oil

GC-MS analysis result showed that ajwain is rich in alkyl benzene and benzene components. They can be derivatised by several methods. Ajwain oil fraction rich in thymol was derivatized by addition of formaldehyde in the presence of strong alkali using the method of (Mastelic *et al.*, 2008) with some minor changes [13]. The prepared derivative was hydroxymethylation product of thymol fraction. This

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derivative on antibacterial study showed that it had potential against bacterial strains to stop their growth but less effective against the selected microorganisms as compared to the pure essential oil and fractions. It showed maximum zone of inhibition against *Pseudomonas aeruginosa*and minimum against *E.coli*. The findings of our research are in good agreement with the studies carried out previously in which hydroxyl derivatives were investigated against the bacterium *E.coli*. Diameter of inhibition zone value of the prepared derivative is also comparable to the studies previously carried out [20].

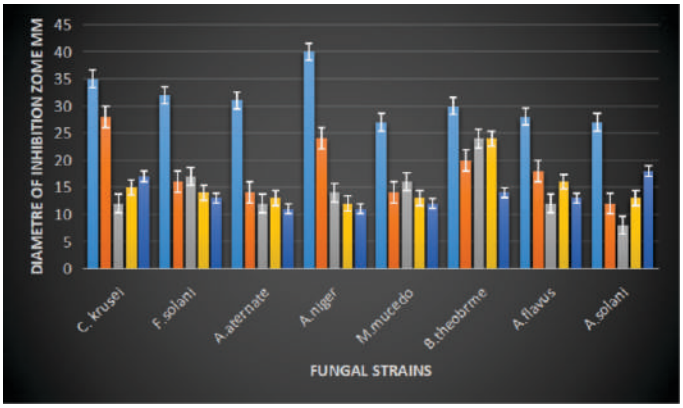

Figure 2: Zone of inhibition of essential oil, fractions and derivative against resistant fungal species.

CONCLUSION

Our study proven the effectiveness of the essential oils and their components for inhibiting the growth of the isolated resistant fungal strains. Ajwain oil showed maximum inhibition due to synergic effects of the components present in oil. Fraction 1, 2 and 3 also showed somewhat comparable action. While derivative proved its effectiveness against C.krusei and F.Solani. So ajwain oil can be used as antifungal agent.

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