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CHEMICAL COMPOSITION AND ANTIFUNGAL ACTIVITY OF ESSENTIAL OILS FROM SOME MEDICINAL PLANTS OF IRAN

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ABSTRACT: Infectious diseases are the second leading cause of death worldwide. The use and search for drugs and dietary supplements derived from plants have accelerated in recent years. The aim of this study was to evaluate the antifungal activities of the essential oils derived from some species of medicinal plants: *Stachys pubescens, Coriandrum sativum, Cinnamomum zelanicum* and *Bupleurum falcatum* against *Fusarium oxysporum, Aspergillus flavus* and *Alternaria alternate*. The essential oils were used to evaluate their MIC and MFC compared to the Amphotericin B as a reference antibiotic. Also the essential oils were analyzed by GC/MS. Results from the antifungal testing indicated that *B. falcatum, S. pubescens* and *C. zelanicum* essential oils showed high activities and inhibited the growth all of the selected fungi. While the essential oil of *C. sativum* displayed the moderate potential activity. The number of 6, 9, 15 and 22 components was identified in *C. sativum, C. zelanicum, B. falcatum* and *S. pubescens* respectively. These oils exhibited a valued potency, against the fungi. With consider of the result and a point of view from an inexpensive and fewer side effects source of natural antimicrobial substances, they have potential benefits of using in pathogenic systems.

INTRODUCTION: Attention and use of herbal medicinal in the world and especially in Asian countries, contributes significantly to primary health care. Researchers and pharmaceutical industries are considering medicinal plants as a good choice, because the medicinal plants as natural resources have ordinarily fewer side effects [1, 2]. Nowadays, about 25 percent of the drugs are prescribed worldwide come from plants and 252 of them are considered as basic and essential by the World Health Organization (WHO).

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The WHO considers phytotherapy in its health programs and suggests basic procedures for the validation of drugs in developing countries. Infectious diseases are the second leading cause of death worldwide [3]. From the time of the ancient Iranian, the plants were considered to protect against diseases. In Iran, many plants are used in the form of oils and crude extracts, infusion or plaster to treat common infections without any scientific evidence of their efficacies. Iran has a very honorable past in traditional medicine, which goes back to the time of Babylonian - Assyrian civilization. Pharmacological studies carried out on essential oils of some aromatic plants' species that were obtained in central regions of Iran. The essential oils have shown antimicrobial activity which is coherent with the use of these plants in folk medicine that show in the following.

One of the most significant ancient heritages is sophisticated experience of people who have tried over millennia to find useful plants for health improvement, with each generation adding its own experience to this tradition [4]. Based on literature search 18% of the species are used for medicinal purposes in Iran [1]. Treatment of infections continues to be a problem in modern time because of side effects of some drugs and growing resistance to antimicrobial agents. Therefore, investigation for novel, safer and more effective antimicrobials are a pressing need. Herbal medicines have received much attention as a source of new antimicrobial with low side effect and significant activity [3, 5]. The essential oil yield and their constituents in plants are related to genetic [6], climate, elevation and topography and genotype (G) [7, 8], growing conditions (E) and their interaction (G x E) [9].

Recent studies have shown that these plant species have biological activity [10-23]. The antifungal activity of *S. pubescens* and *B. falcatum* essential oils has not determined yet and it carried out for the first time in this study. The common use of antibiotics in medicine is playing an important role in the appearance of fungi resistant [24]. Drug resistance has a high metabolic value [65], in pathogens for which this perception is related [25]. It is interesting to determine whether their traditional uses are supported by actual pharmacological effects or merely based on folklore. In the present study, four medicinal plants which are widely used in the folk medicine were selected in our region. All of them have been used by people in folk medicine in the different geographical area [2, 3, 26].

The aim of this study was to evaluate the antifungal potential of the essential oils derived from *Coriandrum sativum, Cinnamomum zelanicum, S. pubescens* and *B. falcatum* against standard fungal strains. For this reason, we harvested the four selected plants that have wild growth in the central part of Iran. The selected strains; *Fusarium oxysporum* (PTCC: 5115), *Aspergillus flavus* (PTCC: 5006) and *Alternaria alternata* (PTCC: 5224) purchased from Iranian Research Organization for Science and Technology (IROST). The antifungal potential was performed by Broth Microdilution method (BMD) to determine the Minimum Inhibitory Concentration (MICs) and minimum fungicidal Concentration (MFCs).

MATERIALS AND METHODS:

Collection of plant materials and essential oil extraction: The plants were collected from their wild habitat in Semnan region, the central part of Iran between April and June 2011 which are shown Geographical and environmental conditions in **Table 1**. All plants were identified by experts of the University of Applied Science and Technology Education Center (UAST), Semnan, Iran. The flowers of *B. falcatum* and the leaves of *S. pubescens, C. zelanicum* and *C. sativum* were collected to determine their antifungal activity.

A voucher specimen for each plant has been deposited in the herbarium of this center. Air-drying of plant material was performed in a shady place at room temperature for 4 days. Grinding and dried aerial parts of plants (100 g) were subjected to hydro-distillation for 3 hr, using a Clevenger-type apparatus. The distillated oils were dried over anhydrous sodium sulfate and stored in tightly closed dark vials at 4°C until analysis.

Gas chromatography/ mass spectrometry (GC/MS) analysis: The oil was analyzed by GC and GC/MS. GC analysis was carried out on a Perkin-Elmer 8500 gas chromatograph with a flame ionization detector (FID) detector and a fused silica capillary column DB-5MS (30 m × 0.25 mm i.d., 0.25 µm film thickness). The oven temperature was programmed at 60°C (4 min), and then rising to 300°C at 4°C/min. Other operating conditions were as follows: Helium was used as the carrier gas at a flow rate of 1 ml/min. The injector and detector temperature were kept at 250°C and 300°C, respectively. Volume of injected samples was 0.5µl.

The split ratio was 1:50. The MS operating parameters were as follows: ionization potential, 70 ev; ionization current, 2 A; inlet and ionization source temperatures were 320 and 300°C, respectively; resolution, 1000; scan rate was 0.34 s per scan. Identification of components in the oil was based on GC retention indices relative to *n*-alkanes and computer matching with the Wiley 275 L library as well as by comparison of the fragmentation patterns of mass spectra with those reported in the literature [27, 28, 29]. The relative percentage of the oil constituents was calculated from the GC peak areas (**Table 3**).

Microorganisms, inoculums and antifungal assay:

- **Microorganisms and cultural methods:** In the present study, three standard fungal strains: *Fusarium oxysporum* (PTCC: 5115), *Aspergillus flavus* (PTCC: 5006) and *Alternaria alternata* (PTCC: 5224) were obtained from IROST Center in 2011. The tested organisms were selected according to their ease of availability and pathogenicity to human, animals and plants. The isolates of organisms were subculture once onto potato dextrose agar (PDA) (Merck, Darmstadt, Germany) and incubated for 48 to 72 hr at 35°C.

- **Inocula preparation:** Inocula were prepared by growing the fungi on PDA for 48 to 72 hr at 35°C and then until 7th day at 25°C as described by the reference method M38-A2 recommended by NCCLS guidelines [30]. The Inocula were prepared by flooded colonies with approximately 5 ml of sterile 0.85% saline. Tween 20 (0.01ml) was added to facilitate the preparation of *A. flavus* inocula. The resulting mixture is transferred to a sterile tube. After the settling of the larger and heavy particles for 4 to 5 minutes, the upper homogeneous suspension is transferred to a sterile tube and mixed with a vortex mixer for 15 seconds. These suspensions were diluted 1:50 in the RPMI medium. The suspensions were mixed for 15 second to ensure homogeneity and subsequently diluted to adjust the turbidity of a 0.5 McFarland standard (0.4 × 10 4 to 5 × 10 4 CFU /ml). This density of Inocula were read using a spectrophotometer (UV-VIS 1650 Shimadzu, Japan) and matched to an optical density (OD) for each strain.

- **Assay for antifungal activity:** The MICs of each essential oil was determined using broth microdilution method as described by the NCCLS guidelines for fungal strains 30, in flat-bottomed 96-well (Cellstar, Greiner Bio-One, Germany) clear plastic tissue-cultured plates composed of eight series identified from A to H, each one with twelve wells. The RPMI 1640 medium with glutamine and morpholine propanesulfonic acid (MOPS) buffered to pH 7.0, 0.165 mol/l was used.

Stock solutions of the essential oils and the positive control drug, Amphotericin B (Sigma-Aldrich Co.) were prepared in dimethyl sulphoxide (DMSO) and further diluted (1:50) in the RPMI medium. Amphotericin B was used with concentrations ranging from 16 to 0.125µg/ml. The final concentration of oils was prepared from 8 to 0.125µg/ml. The final solvent concentration (1%) DMSO, without oil or the standard drug was used in the test as a negative control. A growth control well together with the RPMI medium without antifungal agents was used for each fungi tested (RPMI + fungi) and the tests were performed simultaneously for sterility control (RPMI + oil). The diluted oils solutions plus RPMI were transferred in 96-wells plates (100µl/well). Briefly, MIC parameters were determined in triplicate by inoculating 0.1 ml of fungal suspensions. The 96-well microtiter plates were used for twofold serial dilution with the RPMI. The final Volume of each well was 200µl/well. The cultures were incubated at 35°C for 72 hr. The minimum inhibitory concentration (MIC) was the lowest concentration of oil that completely inhibited the growth of microorganisms in the microdilution wells, as can be detected by the unaided eye.

In this study, the MICs were recorded by spectrophotometrically at 530 nm using SpectraMaxplus384 after 72 hrs incubation. The minimum fungicidal concentrations (MFCs) were determined by sub-culturing the negative wells on a Sabouraud Dextrose Agar (Oxoid) plates. MFC was defined as the lowest concentration resulting in no growth on subculture. For determined of MFC, after the MIC for each strain was determined, the microtiter plates were shaken and 20 µl suspensions from each well showing complete inhibition (100% or an optically clear well) and from the growth control (drug-free medium) was subcultured onto Sabouraud Dextrose agar plates. The plates were incubated at between 28 and 30°C until growth was seen in the growth control subculture. The MFC was the lowest drug concentration that resulted in either no growth or fewer than three colonies after 48 h (99.9% killing).

- **Statistical analysis:** Comparison of data was performed using the one way ANOVA or the unpaired Student's t-test and is presented as mean ± standard deviation. Comparison of MIC and MFC values, tests were made in triplicate for quantification. Values of $p < 0.05$ were considered significant.

RESULTS: All essential oils showed effective antifungal activities on the selected saprophytic, pathogenic and phytopathogenic fungi. Antifungal activities of essential oils were investigated by broth macrodilution method. The MICs and MFCs of the selected oils on the fungi are shown in **Table 2**. The results showed that essential oil of the plants were active against all the pathogenic fungi species with different degree in the following range of concentrations:

Essential oil of *S. pubescens* and *C. zelanicum* had a best antifungal effect and its MIC values was between 0.5 to 1µg/ml. *B. falcatum* is second degree with MIC values between 0.5 to 2µg/ml. while, *C. sativum* had a lowest antifungal effect comparison to above essences and its MIC values was 2 to 4µg/ml. " Amphotericin B" used as a positive control as well as DMSO as a negative control which did not show any inhibition against the pathogens fungi. The MIC range of standard antibiotics "Amphotericin B" was 0.5 - 2µg/ml. Even at low concentrations, the plant's species showed antifungal activity more or nearly equal to the commercial fungicidal agent (Amphotericin B). *A. alternata* with the highest MIC and MFC was persistent strain but *A. flavus* and *F. oxysporum* were sensitive with the lowest MIC and MFC against the oils.

In comparison to the Amphotericin B, these data showed that antifungal activity of *S. pubescens* and *C. zelanicum* had the highest activity; *B. falcatum* had the lower activity but with the lowest different, while the different properties of *C. sativum* was more. All of the oils had a potent antifungal activity except *C. sativum* that had not antifungal activity against *A. alternata*. The weakest activity was observed against *A. alternata* with the highest MIC and MFC and *A. flavus* was displayed more sensitivity against oils and did not show any resistance.

The results of the chemical analyses using GC/MS of the essential oils were listed in Table 3. The number of 6, 9, 15 and 22 components was identified in *C. sativum, C. zelanicum, B. falcatum* and *S. pubescens* respectively. Also, analysis of data with creditable library shows that, the main components of *S. pubescens* were: Germacrene (22.40%), δ-Cadinene (19.70%), 2,6-Octadien (13.60%), Linalool (9.70%); and in *B. falcatum* were: Torilenol (39.10%), Spathulenol(19.6) and α-Cubebene (8.10%), in *C. zelanicum* were: 2-Propenal (54.28), Cinannamaldehyde (18.57) and 2-Propenoic acid (16.24) and *C. sativum* was include: Linalool (91.54), Geranyl acetate (2.60) and Benzene (1.96).

The results of this study showed that essential oils of plants have a very broad spectrum of antifungal activities. Concerning the antifungal properties of *C. sativum* and *C. zelanicum*, other studies confirmed the inhibition effects of these plants on the some microorganisms such as; *Staphylococcus aureus, Bacillus* spp, *Escherichia coli, Salmonella typhi, Klebsiella pneumonia, Proteus mirabilis, Pseudomonas aeruginosae, Candida albicans* [15-18,22]. Results in this study were confirmed the antifungal potency of these plants, especially about the essential oil of *S. pubescens* and *B. falcatum*, that evaluated for the first time in the world. In this assay the composition of the plants were determined by GC/MS which were different from other regions in the world.

Concerning the *S. pubescens* many studies have not been conducted so far; Iranian researcher reported; (*Z*)-β-Ocimene, Germacrene D and Bicyclogermacrene as main components [31]. In the previous study related to the chemical composition of *B. falcatum*, α-pinene reported as main component which are not similar to our result [32]. In previous study on *C. sativum* showed that the major constituents of the essential oil were 2E-decenal (15.9%), decanal (14.3%), 2E-decen-1-ol (14.2%) and n-decanol (13.6%) [33]. In a one study in concerning *C. zeylanicum* the cinnamaldehyde (37.6%), cinnamyl acetate (23.7%), cinnamyl benzoate (16.4%), and in the another study ; terpene hydrocarbons (78%) and oxygenated terpenoids (9%). α-Bergamotene (27.38%) and α-copaene (23.05%) were found as the major compounds [34, 35].

It was suggested these differences in components could be due to the variety of the ecotype system that reported by other scientists and references [2, 7]. Since the essential oils are complex mixtures of several compounds, it is difficult to attribute their biological activity to a particular constituent. Usually, major compounds are the ones responsible for the antimicrobial activity of the essential oils. However, some studies showed that minor components may have a crucial role in the biological activity of the oils [36]. According to the results obtained, it is difficult to attribute the antifungal activities of *Stachys pubescens* and *Bupleurum falcatum* essential oils, characterized by a complex mixture, to a single or particular constituent, because there is not a significant component with high amount in its chemical compositions. Hence, suggested that the whole essential oils have a greater antifungal activity than a single constituent or mixture of all the major components. But, concerning the *C. sativum* and *C. zelanicum*, there was the significant major component in their chemical compositions, linalool (91.54%) in *C. sativum* and 2-Propenal (54/28%) in *C. zelanicum*. Therefore, suggested that these components can played the considerable role in biological activity of these plants.

TABLE 1: GEOGRAPHICAL AND ENVIRONMENTAL CONDITIONS

No .S.	plant	Region	Altitude (m asl[1])	latitude	Longitude
1	*Cinnamomum zelanicum*	Fooladmahaleh, Semnan	2167	35.432639	53.256048
2	*Bupleurum falcatum*	Semnan Fullad mahaleh	2650	35.78527	53.28145
3	*Coriandrum sativum*	Parvar, Semnan	2632	35.63543	53.32424
4	*Stachys pubescens*	Shahrood, Semnan	2315	36.32415	54.35316

TABLE 2: MIC AND MFC (µg/ml) VALUES FOR ESSENTIAL OIL OF PLANTS

Microorganisms	Amphotericin B		C.Z		S.P		B.F		C.S	
	MIC[a]	MFC[a]	MIC[a]	MFC[a]	MIC[a]	MFC[a]	MIC[a]	MFC[a]	MIC	MFC
As.flavus	1	2	0.5	0/5	0/5	0/5	0/5	1	2	2
Al. alternata	2	4	1	2	1	2	2	4	-	-
F. oxysporum	0.5	1	0/5	1	1	1	2	2	4	4

MIC = Minimum Inhibitory Concentration; MFC= Minimum Fungicidal Concentration. S.P*: Stachys pubescens*, C.Z*: Cinnamomum zelanicum*, C.S*: Coriandrum sativum*, B.F*: Bupleurum falcatum*, As= *Aspergillus* , Al= *Alternaria* , F= *Fusarium*. "-" No growth inhibition

TABLE 3: CHEMICAL ANALYSES CONSTITUENTS OF ESSENTIAL OILS

S. No.	Compound	RI	*C. sativum*	*C. zelanicum*	*B. falcatum*	*S. pubescens*
1	α-Pinene	917	1.22	-	3.50	-
2	β-Pinene	921	-	-	-	1.80
3	Limonene	931	-	-	-	6.30
4	Benzaldehyde	980	-	4.50	-	-
5	Benzene	986	1.96	0.56	-	0.90
6	Phenylmethanal	995	-	2.50	-	-
7	Pinocarvone	1009	-	-	4	-
8	1,4-Cyclohexadiene	1012	-	-	-	0.40
9	α-Terpinene	1016	1.40	-	-	2.70
10	α-Cubebene	1035	-	-	8.10	-
11	γ-terpinene	1046	-	-	-	1.20
12	Myrcene	1051	-	-	-	0.90
13	Cyclopropane	1058	0.24	-	-	-
14	Acetophenone	1062	-	0.73	-	-
15	Pinocamphone	1082	-	-	1.70	-
16	Linalool	1098	91.54	-	-	9.70
17	Heptanal	1107	-	-	4.20	-
18	2-Propenal	1130	-	54.28	-	-
19	cis-Verbenol	1139	-	-	1.50	-
20	Myrtenal	1142	-	-	2.90	-
21	Geranyl acetat	1148	2.60	-	-	-
22	Cinannamaldehyde	1156	-	18.57	-	-
23	Linalyl acetate	1165	-	-	-	1.20
24	(E)-β-Ocimene	1171	-	-	-	2.80
25	Thyopsene	1179	-	-	3.10	-

26	trans-Pinocarveol	1289	-	-	4.10	-
27	2-Propenoic acid	1290	-	16.24	-	-
28	3-Cyclohexen-1-ol	1299	-	-	-	1.50
29	Ortho mettoxy aldehyde	1351	-	0.79	-	-
30	Trans-Verbenol	1375	-	-	0.30	-
31	Cuparene	1377	-	-	2.80	-
32	2,6-Octadien	1388	-	-	-	13.60
33	Torilenol	1396	-	-	39.10	-
34	Octen-1-ol acetate	1415	-	-	-	1.60
35	Spathulenol	1427	-	-	19.60	0.80
36	δ□-Elemene	1435	-	-	-	5.40
37	β-Bourbonene	1442	-	-	-	0.20
38	δ-Cadinene	1454	-	-	-	19.70
39	Benzeneacetic acid	1458	-	0.82	-	-
40	Thyopsene	1460	-	-	3.10	-
41	Naphthalene	1467	-	-	-	1.20
42	α-Calacorene	1473	-	-	2.40	-
43	β-Gurjunene	1486	-	-	-	0.30
44	Bicyclogermacrene	1495	-	-	-	1.80
45	Caryophyllene oxide	1502	-	-	-	1.30
46	Pentacosane	1519	-	-	1.50	-
47	Germacrene	1532	-	-	-	22.40
	Total		98.02	98.99	98.8	97.7

DISCUSSION: Our result confirmed that a variety of fungi such as saprophytes, yeasts, dermatophytes and other phytopathogenic fungi are affected by our selected plants. These plants could safely be used as organic preservatives to replace synthetic fungicides in the prevention and cure of some human and animal mycoses disease as well as food industrial preservatives. These data, together with high yield and non-toxicity [37], justify their usage for medical purposes. However, the mechanism of inhibitory effects of these plant's oils against infectious fungi is still unclear.

Further investigations regarding the *in vitro* and *in vivo* should be conducted in order to clear mechanisms pathway and develop such products. These data suggest that the essential oils of *C. sativum, C. zelanicum, B. falcatum* and *S. pubescens* could be a possible source to obtain new and effective herbal medicines to treat infections caused by multi-drug resistant strains of microorganisms and also in the search for novel antifungal agents with the potential application of some major or minor constituents alone, mixed of presented essences or in combination with antibiotics for the treatment and prevention of pathologies associated with multiresistant fungi. Finally, it was suggested it would be useful to carry out more study on the synergistic effects plants oil in combination with synthetic antibiotics such as Amphotericin B. In conclusion, it was suggested the combination of plants oil and Amphotericin B may be reduce the efficacious dose and then decrease the side-effects of Amphotericin B for the treatment of the fungi especially *Aspergillus* sp., *F. oxysporum* and *A. alternata* which have spread to other parts of the world.

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