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Inhibitory Effect of *Helichrysum arenarium* Essential Oil on the Growth of Food Contaminated Microorganisms

Ali Mohamadi Sani

***Abstract*—**The aim of this study was to determine the antimicrobial effect of *Helichrysum arenarium* L. essential oil in "in-vitro" condition on the growth of seven microbial species including *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus, Saccharomyces cereviciae, Candida albicans, Aspergillus flavus* and *Aspergillus parasiticus* using micro-dilution method. The minimum inhibitory concentration (MIC) and minimum bactericidal or fungicidal concentration (MBC, MFC) were determined for the essential oil at ten concentrations. Finally, the sensitivity of tested microbes to essential oil of *H. arenarium* was investigated. Results showed that *Bacillus subtilis* (MIC=781.25 and MBC=6250 µg/ml) was more resistance than two other bacterial species. Among the tested yeasts, *Saccharomyces cereviciae* (MIC=97.65 and MFC=781.25 µg/ml) was more sensitive than *Candida albicans* while among the fungal species, growth of *Aspergillus parasiticus* inhibited at lower concentration of oil than the *Aspergillus flavus*. The extracted essential oil exhibited the same MIC value in the liquid medium against all fungal strains (48.82 µg/ml), while different activity against *A. flavus* and *A. parasiticus* was observed in this medium with MFC values of 6250 and 390.625µg/ml, respectively. The results of the present study indicated that *Helichrysum arenarium* L essential oil had significant ($P<0.05$) antimicrobial activity; therefore, it can be used as a natural preservation to increase the shelf life of food products.

Keywords*—Helichrysum arenarium*, Antimicrobial agent, Essential oil, MIC.

I. Introduction

FOOD borne disease mediated by pathogenic microorganisms or microbial toxins is an important global public health problem because they take a huge toll on human health and mortality [1]. It has been estimated that as many as 30% of people in the industrialized countries suffer from food borne diseases each year caused by microbes [2]. Food additives have been used for centuries in the food processing practices for several purposes including the prevention of microbial growth and increase in the food shelf lives [3]. Due to the excessive use of food preservatives which some of them are doubtful to be carcinogenic and teratogenic and also increasing consumer demand to natural foods with a long shelf life and without chemical preservatives, food producers trend to replace chemical preservatives with natural forms such as oils and herbal extracts as antibacterial additives [4]-[6]. In the recent years, efforts have been devoted to find new antimicrobial materials from natural resources for food preservation [7]. Reports indicated that many extracts and essential oils of edible plants had properties to prevent against a wide range of fungal contamination of foods [4], [7]-[12].

Essential oils are liquid, volatile, natural, limpid and rarely coloured, lipid soluble and soluble in organic solvents with a generally lower density than that of water and formed by aromatic plants as secondary metabolites [12]. They have been used in many cases because of their natural properties such as antifungal, antibacterial and insecticidal activities. An estimated 3000 Essential oils are known, of which about 300 are commercially important especially for the pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries [13], [14].

Helichrysum arenarium L. (popularly known as everlasting, immortal flower or fadeless flower) belongs to asteraceae family [15]. It is a perennial herbaceous plant of height 15–40 cm that flowers in June- August [16] with yellow to reddish-orange or even brown inflorescences of various colour intensity [17] and broadly distributed in Europe, western Siberia, and central Asia [16], [18]. *Helichrysum arenarium* are used for the treatment of kidney stones, uro-genital disorders, stomach pain, jaundice, diarrhea, asthma [15], gall-bladder and gastric disorders, cystitis, and arthritis [17]. For coughs and colds, a tea is prepared or the leaves are boiled in milk. For pain relief, leaves are burned and the smoke is inhaled. Leaves are widely used on wounds to prevent infection [19].

The aims of the present study were to evaluate the potential antimicrobial activities of essential oil of *Helichrysum arenarium* L collected from Iran on the growth of some bacteria, yeasts and fungi.

II. Materials and Methods

A. Plant Material and Extraction of Essential Oil

Aerial parts of the *Helichrysum arenarium* L. plant were collected in 2011 from Khorasan Razavi Province (the northeast of Iran). The plant confirmed by Medicinal Plants Institute, Ferdowsi University, Mashhad, Iran. The essential oil of aerial parts of the *H. arenarium* L. was extracted with water steam distillation using a clevenger apparatus according to the method of British Pharmacopoeia. The distilled essential oils were dried with anhydrous sodium sulfate and stored in the sterilized vial at 4°C until use [4], [5], [20].

B. Organisms and Inoculation Conditions

A total of three bacteria (*Bacillus subtilis* ATCC 6633, *Escherichia coli* 0157 NTCC 12900, *Staphylococcus aureus* ATCC 6538), two yeasts (*Saccharomyces cereviciae* 5052 PTCC, *Candida albicans* ATCC 10231) and two fungi species

Ali Mohamadi Sani is with the Department of Food Science and Technology, Quchan Branch, Islamic Azad University, Quchan, Iran (phone: +98-9155120457; e-mail: msani@yahoo.com).

(*Aspergillus flavus* PTCC 5006 and *Aspergillus parasiticus* PTCC 5018) were used in the present study that obtained from Persian Type Culture Collection (PTCC), Iran.

To prepare microbial suspension, bacterial species were cultivated on nutrient agar (Merck, Germany) slant at 37oC for 24 h while yeasts and fungal species were cultivated on PDA (Merck, Germany) slants and incubated at 25oC for 48 h. Finally, suspensions were adjusted to 0.5 McFarland standard turbidity [21]-[25]. The yeasts and fungal suspensions were adjusted to make a conidial or spores concentrations of 106 cell or spore/ml via counting with a hemacytometer [10], [20], [26], [27]. Bacterial suspensions were standardized to concentrations of 1.5×108 CFU/ml [21], [23].

C. Minimum Inhibitory Concentration (MIC) Test

H.arenarium essential oil dissolved at 5% dimethyl sulfoxide (Aplichem, Germany) and Then, it diluted to the highest concentration (50000 µg/ml), and then serial twofold dilutions were made in a concentration range from 48.82 to 25000 µg/ml.

MIC values of essential oil against microbial strains were determined based on a microwell dilution method. Ninety five µl of Mullerhinton broth (Merck, Germany) was dispended in to each 96 wells. 100 µl of stock solution of *H.arenarium e*ssential oil was added in to the first wells. Then 100 µl from their serial dilutions was transferred in to other consecutive wells except the well number 11 as positive control. Then 5 µl of the microbial suspension was added to each well except well number 12 as negative control. Contents of each well were mixed on a plate shaker at 300 rpm for 20 s and then incubated at 25oc for 48 h for yeasts and fungi and 37oc for 24 h for bacterial strains. Microbial growth was determined by detecting the absorbance at 630 nm using the ELX808 Elisa reader (Biotek Instrument Inc, USA). The MIC of essential oil was taken as the lowest concentration that showed no growth [21], [23], [24], [28], [29].

D. Minimum Fungicidal Concentration (MFC) Test

The minimum fungicidal or bactericidal concentrations (MFC and MBC) were determined with sub-culturing 10µl aliquot from all MIC wells showing no visible growth on the mullerhinton agar plates [10], [30].

E. Statistical Analyses

All data obtained from the trial were analyzed as a completely randomized design using the procedure of the general linear model of SPSS 19 software (SPSS Inc., Chicago, IL, USA). The mean values were compared using Duncan's new multiple range test at 5% probability level of significance.

III. Results and Discussion

A. The Effect of Essential Oil of Helichrysum arenarium L. on Microbial Species

Antimicrobial activity of essential oil of *Helichrysum arenarium* L. was determined via the microwell dilution method at 10 concentrations against three bacteria, two yeasts and two fungi species. The results of *in vitro* antimicrobial activity assay showed that the essential oil possessed broad antimicrobial activity against the microorganisms tested.

The antimicrobial effects of essential oil against seven microorganisms were shown in Table I. Results obtained from the microdilution method, followed by measurements of MIC and MBC indicated that essential oil of *H. arenarium* L. exhibited significant ($P< 0.05$) antibacterial activity against tested bacteria and its effect on *S. aureus* and *E. coli* was more than *B. subtilis*. Among the tested yeasts and fungi, the most sensitive yeast was *S. cereviciae* while resistant of *A. flavus* was more than *A. parasiticus*.

The antimicrobial activities of different Helichrysum species have been studied by different researchers [31]-[38], but there is not enough data about the antimicrobial activity of essential oil of the *Helichrysum arenarium*.

Albayrak et al. (2010) showed that methanolic extract of *H. arenarium* can inhibited growth of *E. coli* and *S. aureus* While they did not find a significant influence on *B. subtilis* [34]. Also antibacterial activity of methanolic extract of two subspecies of *H. arenarium* on *B. cereus* and *S. aureus* was demonstrated by Albayrak et al. (2010) but any antibacterial activity was specificated for *B. subtilis* [33]. Aslan et al in their research showed that petroleum ether and ethanol extracts of *H. arenarium* have antibacterial activity on *S. aureus* but have no effect on *E. coli* [31].

According to Table I essential oil of *H. arenarium* has significant effects on reducing and eliminating yeasts and its effects on *S. cereviciae* is more than *C. albicans*.

Antifungal activities of extracts of *Helichrysum compactum* and *Helichrysum chasmolycicum* have been reported by Suzgec-Selcuk [36], [37].

Results of this study are different from albayrak research on ethanolic extract of two subspecies of *H. arenarium* at 100000, 50000, 25000 and 10000 µg/ml concentrations [33]. They reported that ethanolic extract of this plant had no antifungal effect on *S. cereviciae* and *C. albicans*.

The results showed that essential oil of *H.arenarium* was effective on growth and activity of selected fungi and its effect on *Aspergillus parasiticus* was more than *Aspergillus flavus*. Many researchers have been proved antibacterial and antifungal effect of essential oil and extract of different species of helichrysum [32], [35]-[38].

The differences observed in activity of essential oil or extraction products in other researches [31], [33] may be due to their different quality and quantity of active compounds in essential oil and extract. It is clear that essential oil of plant is more active than its extract [21] furthermore, the extraction product can vary in quality, quantity and in composition according to climate, soil composition, plant organ, age and vegetative cycle stage [13], [14], [39].

TABLE I
MINIMUM INHIBITORY CONCENTRATION (µG/ML) AND MINIMUM FUNGICIDAL OR BACTERICIDAL CONCENTRATION (µG/ML) OF ESSENTIAL OIL OF *HELICHRYSUM ARENARIUM*

Microorganisms	MIC (µg/ml)	MBC or MFC (µg/ml)
Bacillus subtilis	781.25	6250
Escherichia coli	97.65	1562.5
Staphylococcus aureus	97.65	1562.5
Saccharomyces cereviciae	97.65	781.25
Candida albicans	195.31	3125
Aspergillus flavus	48.82	6250
Aspergillus parasiticus	48.82	390.625

The values in the table are an average of 3 experiments.

It was found that the characteristic secondary metabolites of *Helichrysum arenarium* are isoprenoids (mono-, sesqui-, di-, and triterpenoids) and phenolic compounds (aromatic phenols and acids, coumarins, phthalides, flavonoids, etc.) [40].

The phytochemical investigations on divergent Helichrysum species indicated that this genus is quite rich in flavonoid content [31] and researches reported that that total flavonoids content for various subspecies of *H. arenariun* was 1-1.95 percent [31] which is lower than the other most Helichrysum species.

Hydrophobicity characteristic of essential oils and their components enables them to partition in the lipids of the bacterial cell membrane, disturbing the structures and rendering them more permeable therefore Leakage of ions and other cell contents occur [2]. Essential oils can also affect fungal morphogenesis and growth. Cell of fungal that treated by oils became small and their cell wall was disrupted and became very thin, rough and villiform and in some old hyphae the cell wall seemed to disappear. Interference of essential oil components with enzymatic reactions involved in cell wall synthesis are the reason of antifungal activity of essential oils [10].

B. Comparison Effect of Essential Oil of H. arenarium on the Growth of Tested Microorganisms

Comparison results of ten essential oil concentrations on the growth of *B. subtilis*, *E. coli* and *S. aureus* were showed in Fig. 1.

According to Fig. 1 the inhibitory effect of the essential oil on the growth of all microbial species increased significantly ($P<0.05$) as oil concentration increased.

Growth of *B. subtilis* in two initial concentrations of 25000 and 12500 µg/ml was the same (0.190 and 0.198 cfu/ml respectively) and was lower than the growth of *E. coli* (0.211 and 0.221 cfu/ml) and *S. aureus* (0.246 and 0.264 cfu/ml) but at lower concentrations, growth of *B. subtilis* is more than two other bacteria.

According to Fig. 2 growth of yeasts decreased with increasing concentration of essential oil and the mount of growth decreased substantially (from 0.064 to 0.196 cfu/ml for *C. albicans* and from 0.055 to 0.177 cfu/ml for *S. cereviciae*) but effect of oil at all concentrations on *S. cereviciae* is more than *C. albicans*.

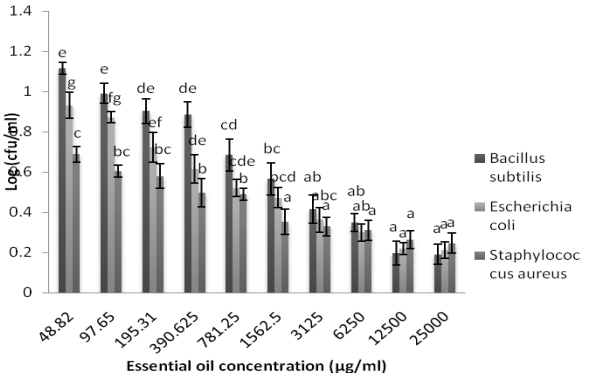

Fig. 1 Effect of different concentration of essential oil of *H. arenarium* on the growth of *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus*. (n=3). Data with the same letter for each essential oil concentrations are not significantly different ($p<0.05$) according to Duncan's multiple range test

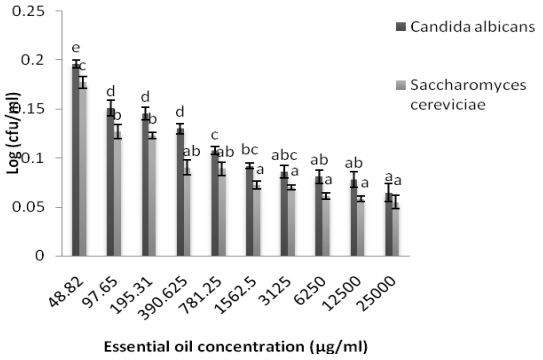

Fig. 2 Effect of different concentration of essential oil of *H. arenarium* on the growth of *Saccharomyces cereviciae* and *Candida albicans*. (n=3) -Data with the same letter for each essential oil concentrations are not significantly different ($p<0.05$) according to Duncan's multiple range test

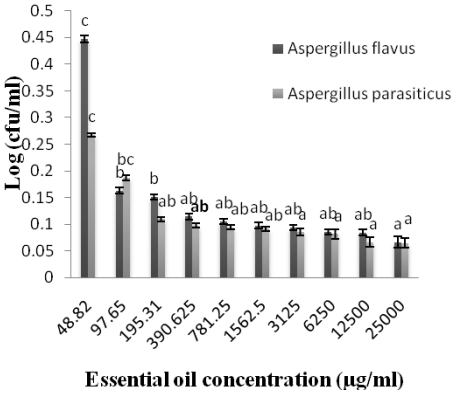

Fig. 3 Effect of different concentration of essential oil of *H. arenarium* on the growth of *Aspergillus flavus* and *Aspergillus parasiticus*. (n=3) -Data with the same letter for each essential oil concentrations are not significantly different ($p<0.05$) according to Duncan's multiple range test

Growth of *A. flavus* at all concentrations is more than *A. parasiticus* except at concentrate of 97.65 µg/ml (Fig. 3). Difference in growth of *A. flavus* (0.447 cfu/ml) and *A. parasiticus* (0.267 cfu/ml) in concentration of 48.82 µg/ml was specified clearly so can be concluded that effect of essential oil of *H. arenarium* on *A. parasiticus* is more than *A. flavus.*

The results obtained in this study clearly indicate that the essential oil of *H. arenarium* could be used to control bacterial and fungal contamination in stored foods.

Considerable pressure from world health organization to use natural preservations in foods and also consumer demand to reduce or eliminate chemically synthesized additives in their foods has led to a renewal of scientific interest in natural substances. This study has demonstrated that essential oil of *H. arenarium*, already used in many parts of the Iran for medical purposes, can also serve as alternative means to control bacterial and fungal contamination in stored foods and improve shelf life, quality and nutritional value of food commodities.

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