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Research Article

Antimicrobial activity of *Ferula gummosa* and *Artemisia sieber* essential oils in gaseous phase

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Abstract

Bacterial infections treatment and eradication are mainly based on exploration of new chemical antibiotics. Several clinical side effect and most importantly, emerging resistant bacteria is leading to an urgent need for uncovering alternative or complementary treatments against bacterial infections. Herbal essential oils (HEOs) extracted from *Ferula gummosa* and *Artemisia sieberi* have presented promising results for their significant inhibitory potential. The aim of this study was to compare the antibacterial activity of essential oil both in liquid and in vapor phases, against a panel of bacteria such as *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Shigella dysenteriae*, *Klebsiella pneumonia*, *Salmonella typhi* and *Pseudomonas aeruginosa*. The inhibitory potential of HEOs were determined with in-vitro disc volatilization assay (DVA)in the non-contact technique and Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) with direct contact broth dilution assays (BDA). The results showed that *S. dysenteriae*, *E. coli*, *B. cereus* and *S. typhi* were similarly sensitive to *F. gummosa* HEO. On the other hand, *E. coli*, *S. aureus* and *B. cereus* showed an elevated sensitivity to *A. sieberi* HEO. Moreover, a significant enhancement in the bactericidal action was observed by using *F. gummosa* HEO as compared to those extracted from *A. sieberi* in DVA. In general, it has been observed that gram-positive bacteria were more sensitive than gram-negative strains against *A. sieberi* HEO. Evaluated microorganisms exhibited different sensitivity to tested HEOs in gaseous phase, but overall bactericidal efficiency was elevated by HEOs implementation in the vapor phase.

Keywords: Antimicrobial activity; Essential oils; Vapour phase; Non-contact assay; Inhibitory potential

Introduction

Natural resources originated from local herbs have become increasingly popular during the recent decades. The idea of exploiting secondary metabolites of plants and screening their medical potential is belonged to the traditional medicine [1]. Global antibiotic

resistance and newly emerged infections has resulted in constantly-increasing insatiable global appetite for the development and introduction of herbal antibacterial compounds. These biomolecules could also be used for designing new drugs [2,3]. Moreover, the Federal Drug and Food Administration (FDA) considered the use of herbal essential oils (HEOs) as General Recognized as Safe (GRAS) and further studies demonstrate the cytotoxicity of the HEOs are scarce [4].

Initial screening of these biomolecules is substantial subject in order to reduce the global concerns about multi-drug resistant bacteria. Various in vitro inhibitory assay of herbal metabolites has been broadly investigated and demonstrated against different bacteria by direct contact; Different procedures such as gel diffusion as well as dilution techniques are successfully investigated on gram negative and gram-positive bacterial species [5-7]. However, due to hydrophobic and volatility characteristics of the HEOs and other aromatic secondary metabolites, the direct-contact assays face many problems; the challenges namely low water solubility, affinity to the media, micelles formation and consequently loss their potency in order to attachment to microorganisms [8] limit the application of the herbal antibacterial substances [9]. To overcome the diminished activity of the HEOs in aqueous media, addition of detergents, emulsifiers or solvents such as Tween 80, DMSO and methanol might alter the activity of the HEOs [9]. Moreover, direct supplementation of HEOs and their preservatives as food additives causes a sensory impact resulting to altering the natural odor and taste [10]. Hence, alongside to the classical screening methods to determine the antibacterial activity of natural compounds, another complementary methods should also be used [11]. It is worth noting that volatile nature of secondary compounds in a vapour phase (VP) had more inhibitory effect in comparison with direct contact methods [12,13].

The results of the previously published studies [14,15] confirmed that HEOs extracted from different medicinal herbs inhibited more efficiency against pathogens in the VP. This higher inhibitory effect was achieved at relatively lower concentrations comparing to the direct contact, therefore the challenges of sensory alterations can be reduced [8,16]. Non-contact antibacterial potential of HEOs not only has broad application in different fields [17], but also could be one of the fastest screening method for large quantities of HEOs with higher throughput for different bacteria [9]. Taking into consideration all above mentioned facts, this study focuses on HEOs obtained from two local medicinal herbs, i.e. *Artemisia sciberi* and *Ferula gummosa*. The inhibitory potential on a set of bacterial species was investigated during the direct contact as well as VP.

Materials and methods

Plant material

The oleo-gum-resin of *Ferula gummosa*, and the whole plant of *Artemisia sieberi* were collected from the 2 years old plants growing in Mehdishahr, Semnan, province, Iran (N 35° 42´, E 53° 21´, 1630 m a.s.l.) in June 2019, during the period of plant harvesting. In the case of *A. sieberi*, the leaves were air-dried in the shade at room temperature, powdered with electric mills, protected from the direct light. The oleo-gum-resin of *F. gummosa* was harvested from natural scraps were made on the healthy shoots and exudates were transferred to the laboratory by stainless steel containers. The collected oleo-gum-resin was dried and crushed into a soft powder and stored in refrigerator (6^{o}C) in a dark container.

Preparation of HEOs

Air dried leafage of *A. sieberi* were powdered and subjected to hydro distillation for 4 h using a Clevenger-type apparatus (Ashke shishe, Iran) as previously reported [18]; 173 g of the dried weight biomass were placed in a

flask (1L) and 600 ml distilled water were also added. The mixture was boiled for 4 h, then the oil was decanted, dehydrated with anhydrous sodium sulphate and collected in amber vials that were kept at −4 °C till their biological activity assay. The oil yield was estimate on dry matter (v/w). The oleo-gum-resin of *F. gummosa* (15g) was inserted in a bag with large pores, then placed in a flask (1L) with 700 ml distilled water. The process of hydro distillation was performed as described above for 3 h.

Microorganisms and growth conditions

The tests were performed against 7 bacterial strains. Gram-positive bacteria were *Staphylococcus aureus* ATCC 25923, and *Bacillus cereus* PTCC1015. Gram-negative bacteria were *Escherichia coli* ATCC25922, *Shigella dysenteriae* ATCC12678, *Klebsiella pneumonia* ATCC13883, *Salmonella typhi* PTCC1609 and *Pseudomonas aeruginosa* ATCC27853. All bacterial strains were grown in Mueller-Hinton (MH) agar and Nutrient broth and Nutrient agar (Merck, Darmstadt, Germany). Stock cultures of bacterial strains were grown in Nutrient broth at 37 °C, 160 rpm for 18 h. The culture were subjected to adjust the turbidity of the inoculum concentration using the 0.5 McFarland standard (1.5×10^8 CFU/ml) solution. The cells were suspended in sterile Nutrient broth to reach $5x10^5$ CFU/ml for the antimicrobial tests.

Antimicrobial assay

The major task of the present study was to evaluate the antibacterial activity of the extracted HEOs in VP; so disk volatilization method was conducted and the results compared by direct-contact observations in liquid phase.

Non-contact antimicrobial assay by disc volatilization assay

In vitro antimicrobial activities were determined by non-contact method based on the method previously published [16]. The tests were performed in 60 mm petri dish (PD). MH agar was used as growth media and inoculated by spreading 50 µL of the prepared suspensions containing approximately $5x10^5$ CFU/ml of different bacterial strains. Sterilized paper discs (6.4 mm in diameter) were soaked in 350 µL pure HEOs of *A. sieberi* and *F. gummosa* for 30 min and placed in the inside surface of the lid of the PD. Finally, the inoculated PD was closed with its lid containing impregnated disc and hermetically sealed with parafilm to prevent leakage of HEO vapor. Positive control was left by untreated discs. The Petri dishes were incubated at 37 °C and the bacterial cells were exposed to the HEOs vapors for 18 h. After incubation, survival count was determined and the results were compared by its control. All tests were carried out in triplicate.

Determination of minimum inhibitory concentration (MIC)

The direct-contact broth dilution assays were performed based on the recommendations of the Manual of Clinical Microbiology associated with modifications published before [19]. Twofold serial dilutions of HEOs were prepared from 40 to 0.078% in sterile Nutrient broth. The medium was divided within test tubes which were further inoculated with 20 µl of an overnight bacterial culture (~$5x10^8$ CFU/ml). All the tubes were incubated in triplicate for each concentration. The turbidity of the tubes (bacterial growth) was observed after 18 h incubation at 37 °C to determine minimum inhibitory concentrations (MIC). Then the tubes without turbidity plated out on nutrient agar and incubated for 24 h. The number of bacterial colonies was compared with the controls and then the values of the minimum bactericidal concentrations (MBC) were also determined.

The MBC is the lowest concentration of the HEOs required to kill the cells. The MIC values were determined as the minimal

concentration of HEOs that could completely inhibit visible growth of bacterial strains in comparison with the control after exposure to HEOs. The control tube of the bacterial growth contained no HEOs. Nutrient broth containing mentioned HEO was observed as pollution control.

Results

Antimicrobial assay

HEOs extracted from *A. sieberi* and *F. gummosa* were evaluated for their antimicrobial potential by Broth macro dilution and non-contact methods against seven bacterial strains.

Broth macro dilution assay (BDA)

In this method antimicrobial potential of HEOs was studied in liquid phase. MIC and MBC values of HEOs were summarized in Tables 1. MIC values of the studied HEOs are expressed in percent. Both of *A. sieberi* and *F. gummosa* HEOs presented inhibition potent against all of the bacterial strains. *A. sieberi* had more potent inhibition activity than *F. gummosa.*. The most sensitive strain to *A. sieberi* was *S. aureus* (MIC: 5%) while *F. gummosa* produced the highest MIC value against this pathogen (MIC: 40%). In the case of *P. aeruginosa*, equal MIC values were observed for Both of the HEOs (40%). The results showed that *S. dysenteriae, E. coli, B. cereus* and *S. typhi* were similarly sensitive to *F. gummosa* HEO. On the other hand, *E. coli, S. aureus* and *B. cereus* showed an elevated sensitivity to *A. sieberi* HEO. Moreover, the results depicted that, HEO extracted from *F. gummosa* had equal MIC and MBC values for all tested bacteria. In general, it has been observed that gram positive bacteria were more sensitive than gram negative strains against *A. sieberi* HEO.

MBC	MIC	Microorganisms	Essential oil
40	40	*S. aureus*	*Ferula gummosa*
20	20	*B. cereus*	
20	20	*E. coli*	
20	20	*S. dysenteriae*	
40	40	*P. aeruginosa*	
20	20	*S. typhi*	
20	5	*S. aureus*	*Artemisia sieberi*
20	10	*B. cereus*	
20	10	*E. coli*	
20	20	*S. dysenteriae*	
40	40	*P. aeruginosa*	
-	20	*K. pneumoniae*	
(-) It had no bactericidal effect; MIC minimum inhibitory concentration; MBC minimum bactericidal concentration (percent).			

Table 1: Antibacterial activity of HEOs against some bacterial strains by broth macro dilution.

Disc volatilization assay (DVA)

Comparative investigations of antibacterial potential of the HEOs were also conducted in the VP (Table 2). HEO obtained from *A. sieberi* had inhibitory potential with reduce in colony number against studied gram positive bacteria. 0n the contrary, this HEOs had no inhibitory effect against gram negative strains such as *E. coli* in VP.

Artemisia sieberi	*Ferula gummosa*	Microorganism
18.18±2	73.80 ± 1	*S. aureus*
28.57±1.53	90±0.58	*B. cereus*
-	33.23±1.53	*E. coli*
-	52.85±1	*S. dysenteriae*
-	28.28±0.58	*P. aeruginosa*
-	26.57±2	*S. typhi*
(-) It had no inhibitory potential; Inhibitory potential of HEOs were measured based on percentages of decreased colony formation comparing to the control		

Table 2: Antibacterial activity of studied HEOs against gram positive/negative bacterial strains by Disc volatilization assay.

Inhibitory potential of *F. gummosa* HEO was considerable. , The most antibacterial effects were observed against gram positive bacterial species; Colony number of *S. aureus* and *B. cereus* reduced by 73.80% and 90% in comparison with the control, respectively. Antibacterial activity of *Ferula* HEOs was also considerable against gram negative bacteria such as *E. coli, S. dysenteriae, S. typhi* and *P. aeruginosa* compared to the control by 33.32%, 52.85%, 26.57% and28.28%, reduction, respectively. *S. typhi* was in general less susceptible to *Ferula* HEOs compared to *B. cereus* as only in one case without MIC records.

Discussion

In this study indirect antimicrobial evaluation was performed to combat against the high hydrophobicity and irreversible changes in HEOs in aqueous culture medium,. The physicochemical properties of extracted aroma antibiotics face with sudden changes in response to differences in polarity of the solvents [7]. This phenomenon might also alter the inhibitory potential of aroma antibiotic substances [9,20]. Therefore, an effective technique is applied herein, to overcome the shortcomings caused by the contact method. Disc volatilization of HEOs in VP, potentially enhance the inhibitory potential of aromatic and antibiotic properties of HEOs extracted from *F. gummosa* and *A. sciebri.* Therefor both of herbal aroma antibiotic and development of non-contact assay could be promising approaches to support the treatment of bacterial infections [11].

The inhibitory potential of HEOs in liquid phase was also determined with direct contact method; however, the inhibitory effect of HEOs determined with non-contact disc volatilization assay were significantly higher. According to the result of the non-contact and direct contact antibacterial assays, *A. sciebri* and *F. gummosa* HEOs showed inhibitory potential in both vapor and liquid phase. According to our results in liquid phase, *A. sciebri* HEO had inhibitory potential against both Gram-positive and Gram negative bacteria, which was parallel to previous publication [21]. In liquid system, *A sciebri* HEOs was the most effective against gram positive bacteria, which was in accordance with previously published results [22]. In the liquid phase, *F. gummosa* HEO, showed similar inhibitory potential for all tested bacteria, in contrast with our observations, [23] reported that, gram positive bacteria were more sensitive than the gram-negative bacteria. MIC of *F. gummosa* HEOs was equivalent to MBC for the all tested bacteria, which indicates the bactericidal property [23]. Although, differences probably caused by the compositions of the oleo gum resin oils and the type of species might impose some variation reported in different experiments [11,24].

In the VP, Gram-positive bacteria were more sensitive to the herbal aroma antibiotic treatment compared to Gram-negative

bacteria. Our observations were in accordance with previous reports [25], Minor difference is probably caused by the compositions of the herbal secondary metabolite and tested bacteria in the experiments [26,11]. Gram-negative bacteria are generally less susceptible to EOs than Gram-positive bacteria because the outer membrane of Gram-negative bacteria contains hydrophilic lipopolysaccharides that create a barrier against macromolecules and hydrophobic compounds, providing a higher tolerance of hydrophobic antimicrobial compounds such as those present in EOs [27].

Herbal essential oils possess various number of chemical metabolites especially monoterpenes, which inhibit the growth and proliferation of wide variety of pathogenic microorganisms. HEOs have high level of phenolic compounds, aldehydes, and alcohols, such as carvacrol, eugenol, and thymol, which show significant antimicrobial potential against pathogens [28]. Carvacrol is one of the constituents of Artemisia EO, increase membrane fluidity and cause the leakage of protons and potassium ions, resulting in a collapse of membrane potential and inhibition of ATP synthesis [12].

Hydrophobic character of HEOs is due to its potential to change in the cell wall lipid structure and inner membrane, disturbing cellular structures leading to protein denaturation and increasing cell membrane permeability, which is associated with ions leak out and decrease of membrane potential, collapsing the proton pump and depleting the ATP pool and eventually microbial death [16]. Cell membrane disruption followed by reduction in PH , and consequently control of DNA transcription, protein synthesis and enzyme activity would lost [12]. Some of HEOs or their components possess an anti-plasmid effect; that means prevent from emergence of antibiotic resistant strains since extra-chromosomal DNA sequences cannot be shared among pathogens through the plasmid [16].

Wild aromatic medicinal plants specially *F. gummosa* and *A. sieberi* are introduced as renewable and naturalmajor source of new antibacterial compounds. In many researches, which have been shown that the α-pinene, β-pinene, menthol, myrthenole, and benzene are most anti pathogenic fractions of *F. gummosa* and *A. sieberi,* which were evaluated. Oily constituents of *F. gummosa*, thujone, terpinolene, camphor, α-humulene, camphene, β-caryophyllene, cadinene, verbenone, 1,8-cineole and camphor have good antibacterial activity against Gram-positive and Gram-negative bacteria [4]. So many investigations have mentioned that the antibacterial potential of *F. gummosa* can be depended to their terpenoids and flavonoids in EO extracted [29,30]*P. aeruginosa* is an opportunistic pathogen of immunocompromised hosts by high resistance to antimicrobials compounds has successfully inhibited by *F. gummosa* HEO. Although numerous investigation were unable to control this pathogen in direct-contact assay by *F. gummosa* HEO [31,32,33]. *S. dysenteriae* infections are one of the most contagious bacterial diarrhea diseases that can cause widespread epidemics with high mortality [34]. According to the result of the present study, ferula oleo-gum-resin against this bacterium showed the high inhibitory potential to 52.85%.

In the case of E. coli, Ferula HEO could significantly decrease the growth by one-third, contrary to our result, kavoosi et al. reported that F. assafoetida EOs in the contact assay had no inhibitory effect against E. coli [35]. The difference is probably caused by different method of antibacterial assay and differences in preparation of secondary metabolites and their composition.

S. aureus is a leading cause of health care-associated infections world- wide. Despite active surveillance efforts, advances in the prevention of infection and new antibiotics, methicillin-resistant *S. aureus* (MRSA) remains a prominent pathogen associated with high rates of mortality [36]. At the present

study it should be highlighted that aromatic phase of *F. gummosa* HEO showed significant inhibitory effect till 73.80% against this bacteria. According to the obtained results, the antimicrobial activity of this aroma antibiotic compound can be attributed to monoterpene hydrocarbons [23]. Also in the case of B. cereus, *F. gummosa* HEO had considerable potential to 90% infection inhibition.

In conclusion, we must highlight HEO extracted from *F. gummosa* as the most active secondary metabolite in the VP in comparison with *A. sieberi* pure HEO that had moderate activity just against gram positive bacteria. Because of the various bio-compounds presented in HEOs, it seems they have no specific cellular targets [31]. The antimicrobial action of HEOs is determined with their hydrophilic or hydrophobic nature, chemical components, type of microorganism and eventually the method of implementation [32].

Conclusion

The inhibitory efficacy of herbal secondary metabolites against various bacteria by direct-contact antibacterial assay has been extensively demonstrated. However, investigation of HEOs in VP has not been thoroughly understood using newly emerged indirect contact techniques. Hence, based on our results, we suggest that non-contact antibacterial assay provides the best detection for the both properties; volatility and inhibitory potential of aromatic oily liquids based on vapor phase.

Inhibitory potential of HEOs were successfully examined. In conclusion , *F. gummosa* HEO showed dramatic antibacterial efficacy in VP against all tested bacteria and the EOs extracted from this plant could be considered as an alternative or enhancer to the present antibiotics in various industries [37]. in the contrary, *A.sciberi* HEO was more potent inhibitor in liquid phase.. The results showed that HEOs have different properties in liquid or vapor phases which results in diverse biological activity [11]. Comparison between BDA and DVA results confirms that non-contact antibacterial assay could be a promising alternative or complementary method to eliminate the shortcomings caused by contact methods.

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