Environmental metabolomics approaches to identify and enhance secondary compounds in medicinal plants for bio-based plant protection

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1. Reviewer: Dr. Torsten Meiners

2. Reviewer: Prof. Dr. Matthias F. Melzig

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To my wonderful family

This thesis is based on the following manuscripts:

I. Karimi, A., Krähmer, A., Herwig, N., Hadian, J., Schulz, H., & Meiners, T. (2020). Metabolomics approaches for analyzing effects of geographic and environmental factors on the variation of root essential oils of *Ferula assa-foetida* L. Journal of Agricultural and Food Chemistry, 68(37), 9940-9952. DOI: https://doi.org/10.1021/acs.jafc.0c03681.

II. Karimi, A., Krähmer, A., Herwig, N., Schulz, H., Hadian, J., & Meiners, T. (2020). Variation of secondary metabolite profile of *Zataria multiflora* Boiss. populations linked to geographic, climatic, and edaphic factors. Frontiers in Plant Science, 11, 696. DOI: https://doi.org/10.3389/fpls.2020.00969.

III. Karimi, A., Meiners, T., & Böttcher, C. (in preparation). Comprehensive metabolite profiling of *Zataria multiflora* Boiss. by HPLC-ESI-QTOF-MS and bioassays reveal candidate compounds for biobased protection against five important fungal pathogenes.

IV. Karimi, A., & Meiners, T. (2021). Antifungal activity of *Zataria multiflora* Boiss. essential oils and changes in volatile compound composition under abiotic stress conditions. Industrial Crops and Products, 171, 113888. DOI: https://doi.org/10.1016/j.indcrop.2021.113888.

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General introduction and thesis outline

One of the major challenges facing agriculture is the increased pressure of pests and diseases upon crops. Meanwhile, there is reduced availability of synthetic chemical crop protection agents due to resistance developments, negative environmental and human health impacts, and tighter regulatory restrictions (Lamichhane et al., 2016). To cope with these challenges, secondary plant constituents from medicinal and aromatic plants can be harnessed for biological crop protection of agricultural plants. The use of plant extracts such as essential oils (EOs), hydroalcoholic extracts, resins and tannins as botanical pesticides have numerous advantages, including the high effectiveness against a wide range of diseases and pests of agricultural and medicinal importance as well as multiple mechanisms of action. Due to the large number of active ingredients in each blend, the development of resistance is less likely. Furthermore, there is often a low toxicity against non-target organisms (Burketova et al., 2015).

Secondary plant metabolites play important roles in direct and indirect plant defenses against insect pests and pathogens, are responsible for the specific flavor and scent of aromatic plants, and contribute to the nutritional value of edible crops. These characteristics, together with their diverse biological activities (Bakkali et al., 2008) have attracted high interest from industry. For example, EOs are produced in 17,500 aromatic species of higher plants belonging mostly to a few families including the Apiaceae, Myrtaceae, Lauraceae, Lamiaceae, and Asteraceae. However, only a small proportion (approximately 300 species) has found use in commercial applications (Tripathi et al., 2009), whereas they contain a wide range of volatile molecules that have multiple biological activities (Raveau et al., 2020). Also in recent years, many studies are published indicating great prospects for secondary metabolites as active ingredients in the production of natural plant protection products. Nevertheless, only very few commercial products based on these metabolites or the identified active principles have been marketed and the number of newly introduced products remains minimal (Isman, 2020). One reason for this is the incoherent and usually low content of active substances in these plant extracts. Besides, some promising secondary metabolites originate from plants whose cultivation is expensive or disadvantageous due to low yields. Not even plants that are currently grown for commercial production of secondary metabolites can be cultivated easily. The reason can be genetic and phenotypic variability among herbs, which cause secondary compounds variability and instability and/or presence of toxic components in plants. On the other hand, the plant secondary metabolites can vary between different populations, developmental stages and rearing conditions. However, the interactions between these different factors are not well understood.

Plants are genotypically and/or phenotypically adapted to different geographic, climatic and edaphic conditions worldwide, which are shaping the production and accumulation processes of secondary metabolites in plants and probably their biological activity that is reflected in their secondary metabolite profile. Environmental factors, edaphic conditions, geographic regions, collection season, harvest time, genotype, and ecotype influence the quantitative and qualitative composition of secondary metabolite of medicinal plants, which cause different chemotypes (Figure 1). Chemotypes are subspecies of plant populations that contain different secondary metabolites or the same secondary metabolites in different quantities (Keefover-Ring et al., 2009). For example, climatic conditions, altitude, soil properties, and irrigation affect the phytochemical composition and antioxidant activity of EOs in *Matricaria chamomilla* L. causing different chemotypes (Formisano et al., 2015). The proportions of monoterpenes depend on temperature and circadian rhythm and vary according to the plants phenological phase. Finally, soil acidity and climate (heat, photoperiod, and humidity) directly affect the secondary metabolism of the plant (Akula & Ravishankar, 2011) and thus the secondary metabolites.

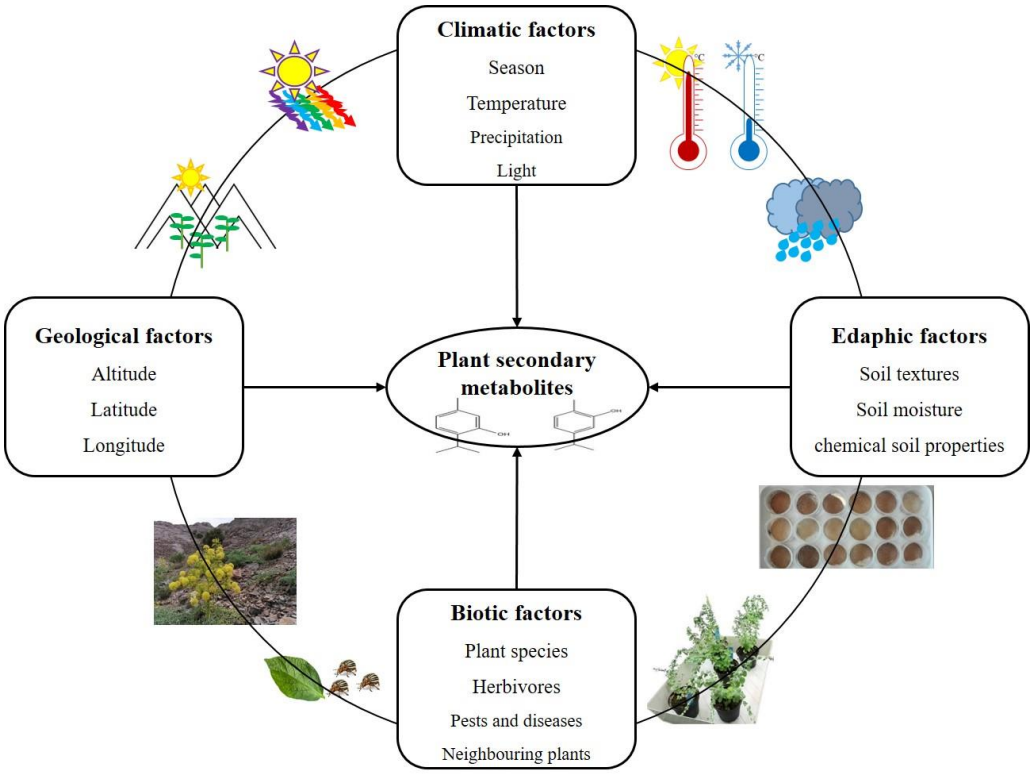

Figure 1. Overview of the effect of environmental factors on medicinal plants.

Environmental metabolomics approach in plant research

Environmental metabolomics is an approach that studies the interactions between living organisms and their environments at the metabolic level. It has been used in the plant sciences for investigating metabolic fingerprints and/or profiles exposed to environmental stress (Stierlin et al., 2020; Sampaio et al., 2016). The demand for investigating more deeply the metabolomes of plants in responses to their natural environments have turned the attentions towards approaches that offer higher sensitivity, particularly mass spectrometry [e.g. Gas chromatography (GC), liquid chromatography (LC) and vibrational spectroscopy techniques] (Viant and Sommer, 2013). The application of the environmental metabolomics approach in plant science allows the detection of a wide range of metabolites and expanding the development and acceleration of the discovery of bioactive metabolites (Figure 2). Metabolomics aims to provide quantitative comparisons of metabolite concentrations between plant species to reveal the relationship between plant diversity as well as the effects of environmental factors on the metabolites (Tugizimana et al., 2018).

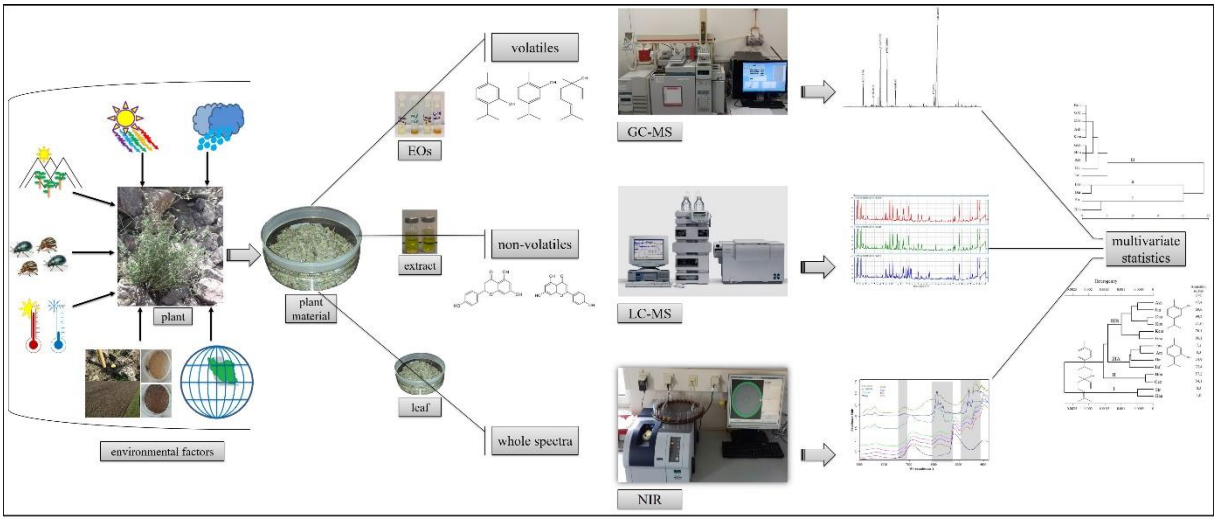

Figure 2. Schematic representation of the environmental metabolomics approach in plant science.

Currently, MS based methods such as GC−MS and LC−MS as well as nuclear magnetic resonance (NMR) are popular for metabolite detection. GC−MS and LC−MS are well established methods to analyze volatile and non-volatile compounds for providing and identifying fingerprints of biological samples (Viant and Sommer, 2013). GC−MS and LC−MS have several advantages such as ease of use, excellent separation capability, selectivity, sensitivity and reproducibility (Beale et al., 2018; Baniasadi et al., 2014). Another advantage of GC−MS is the availability of several mass spectral databases (Beale et al., 2018). Vibrational spectroscopy methods such as near infrared (NIR) are useful tools due to distinct advantages of being nondestructive, no sample preparation and low cost analysis as well as portability to examine the plant secondary metabolites leading to better quality control for food and pharmaceutical industries (Correia et al., 2018).

The secondary compounds of plants have multiple ecological functions, which contribute to the diversity of metabolites because of different environmental effects. Moreover, environmental factors might constrain or enhance the production of specific compounds (Karimi et al., 2020a; 2020b). Understanding how environmental factors are influencing, for example, EO content and composition of plant species and elucidating how the resulting variation in EO chemical components causes the distribution of plant species and the abundance of populations in natural habitats are of high importance for predicting and selecting plant resources rich in specific compounds. Environmental and edaphic factors can be strongly influential in promoting the biosynthesis and accumulation of EOs and also help to identify individual aromatic plants with specific constituents (Karimi et al., 2020a; 2020b) for food and pharmaceutical industries or agricultural uses. For example, phenolic compounds play important roles in the plant tissues to adapte plants in respond to environmental stressors (Agati and Tattini, 2010). In addition, metabolomics has been used to reveal biotic interactions such as chemical defence of plants to herbivory (Austel et al., 2021).

Due to the fact that different constituents may be considered for different agricultural or pharmaceutical applications, it is necessary to identify populations with high amount of specific compounds. The metabolomics approach is used in this study to identify secondary metabolites in medicinal and aromatic plants to increase their yield in order to use the extracts and constituents as biological pesticides. The subsidiary objective is to get a better understanding of mechanisms and processes at the level of medicinal plant and secondary compound production and to apply this understanding to improving individual agricultural processes. I used analytical screening methods such as GC−MS, LC−MS and NIR spectroscopy, which are the most common analytical technologies in metabolomics investigation, to obtain individual metabolite profiles. This approach has the potential to identify the most critical biochemical differences between plant populations. Furthermore, I performed invitro and invivo bioassays to determine the effect of the extracts and compounds

(volatiles and non-volatiles) against phytopathogenic fungi to correlate the bioassay data with the metabolome data.

Selection of plant species

Iran harbors about 8,000 different plant species, 1700 of them are endemic and 1,200 possess an economical relevance, amongst them many medicinal plants. Iran has a huge agricultural potential since its many different climate zones (11 out of 13 worldwide) provide an ideal habitat for each plant and for providing optimal environmental conditions for producing metabolites valuable for agricultural or medicinal purposes (Nikbakht et al., 2008). The selection criteria should guarantee the highest chances of achieving high yields and better biological efficacy of EOs and certain secondary plant metabolites and of detecting novel compounds with novel activities. Thus, the selected plants should have first of all 1) a high genetic and chemical (e.g., EO compounds) diversity within the species; 2) a wide geographical distribution (with a range of abiotic and biotic factors acting on the plant); and 3) novelty features (e.g. expected from endemic and rarely exploited species).

It would furthermore be advantageous if the selected plants are perennials; occur naturally in large numbers and/or are amenable to biotechnological procedures like tissue culturing; show resistance to pests, diseases, competing weeds or herbicides; and contain active ingredients that are effective already at low concentrations. Most promising candidates (with main compounds indicated) were *Satureja khuzistanica*, *Zataria multiflora* (carvacrol, thymol) (Farzaneh et al., 2015; Hadian et al., 2011), *Thymus daenensis* (thymol, rosmarinic acid) (Sajjadi & Khatamsaz, 2003), *Kelussia odoratissima* (ligustilide) (Raeisi et al., 2015), *Artemisia* species (artemisia ketones, camphor) (Hadian et al., 2007), *Solidago* species (rutin, astragalin, rosmarinic acid), *Allium hirtifolium* (allicin) (Ghahremani-majd et al., 2012), *Ferulago angulata*, *Scrophularia* species (terpenoids) (Pasdaran et al., 2012), *Ferula assa-foetida* (coumarins, sulphur-containing compound) (Iranshahy & Iranshahi, 2011). Using antifungal assays with *Fusarium graminearum,* two medicinal plant species including *Zataria multiflora* Boiss. and *Ferula assa-foetida* L. which exhibited strong antifungal activities on the basis of EO and hydroalcoholic extracts comparing to the other species, were selected for collection and further studies (Figure S1 & S2).

Zataria multiflora Boiss.

Z. multiflora is an aromatic and perennial shrub belonging to Lamiaceae family and growing wild in Iran, Pakistan, and Afghanistan. This aromatic plant is known by the Persian name of Avishan Shirazi, which is also entitled Sattar or Zattar, meaning thyme (Figure 3). *Z.*

multiflora is used in traditional folk remedies for its antiseptic, analgesic, carminative, anthelmintic, and antidiarrheal properties, and it is also a condiment (Khazdair et al., 2017; Iranian Herbal Pharmacopoeia Committee, 2002). Currently, some pharmaceutical forms of this plant, such as syrups, oral drops, soft capsules, and vaginal creams are produced (Mahboubi, 2019; Sajed et al., 2013). In addition, its aerial parts are used in the pharmaceutical and food industries (Sajed et al., 2013). The EO of *Z. multiflora* is rich in phenolic oxygenated monoterpenes. The main chemical constituents are carvacrol, thymol, linalool, and *p*-cymene (Hadian et al., 2011). Although there are some studies based on *Z. multiflora* EO constituents (Niczad et al., 2019), there is hardly any information on the environmental factors affecting EO content and composition. *Z. multiflora* is not only harvested for local markets but is also one of the valuable species for industry, so this plant is under severe threat from overharvesting. Thus, a deep perception of its phytochemical and environmental characteristics in its natural habitats is crucial to foretell its behavior under man-made cultivation.

Figure 3. *Zataria multiflora* in the mountains near Jandaq, Iran.

Ferula assa-foetida L.

F. assa-foetida is a perennial, monoecious, and herbaceous species with an unpleasant odor (Figure 4) (Iranshahy & Iranshahi, 2011). It belongs to the Apiaceae (Umbelliferae) family, which is native to central Asia and grows wildly in Iran and Afghanistan. *F. assa-foetida* is commonly known by the Persian names of “anghuzeh” and “koma”, while there are various names of *F. assa-foetida* in different languages. The plant grows up to approximately 2 m and

is the main source of oleo-gum resin (OGR) which is called asafetida and exudes from the roots with two forms of taste (bitter and sweet) (Moghaddam & Farhadi, 2015). Asafetida has been used not only as medicine in Iranian folk medicine but also as a spice, particularly in India (Iranshahy & Iranshahi, 2011). It is traditionally used to treat a vast range of diseases, such as microbial infections, epilepsy, diabetes, earache, stomachache, hysteria, nervous disorders, flatulence, weak digestion, asthma, influenza, and bronchitis (Latifi et al., 2019; Mahendra & Bisht, 2012). It is also used against insects (Muturi et al., 2018). The OGR consists mainly of three main fractions, including resin (40−64 %), gum (25 %), and EO (10−17 %). Considerable variation in EO content and components of *F. assa-foetida* has been reported especially for OGR constituents. These variations might be due to the varying geographical, climatic, and soil conditions, season of collection, phenological stage, genetic constitution, and plant part harvested (Jafari et al., 2019). The major EO constituents of *F. assa-foetida* are sulphurous compounds such as propenyl secbutyl disulfide, *Z*-1-propenyl *sec*-butyl disulfide, and *E*-1-propenyl *sec*-butyl disulfide. Besides, it contains other components including α-pinene, β-pinene, myrcene, limonene, β-selinene, γ-eudesmol, and α-eudesmol.

Figure 4. *Ferula assa-foetida* in the mountains near Yasuj, Iran.

Thesis outline

Using different chemical analysis techniques and various bioassays for the evaluation of genetic raw materials from different climatic origins in Iran and controlling/mimicking the

challenging environmental conditions when culturing the selected species is an innovative and comprehensive way in the research on medicinal and aromatic plants. It will enable the choice of suitable chemotypes with high yields or better biological efficacy and will open new prospects for the sustainable production and practical employment of EOs and secondary plant metabolites for various applications in the field of bioeconomy. Hence, this thesis investigated metabolomics approaches to identify secondary plant metabolites as active structures/compounds against agricultural plant infestations; enhance the content of biologically and pharmacologically active substances by selecting genetic raw materials from extreme environments; and enhance the production of these metabolites by mimicking extreme environmental conditions during cultivation. The main objective was to obtain plant extracts which can be used as bio-based plant protection products. Particularly, the following questions were examined:

(1) Can a metabolomics approach help to identify regions for sampling plant material with the desired chemical profile?
(2) Are there differences between medicinal plant populations in chemotype and chemical profile?
(3) Can vibrational spectroscopy methods like NIR be used to identify chemotypes in medicinal and aromatic plants?
(4) Do environmental factors (e.g., geographic, edaphic and climatic factors) affect chemotype, chemical profile and compounds content?
(5) Do plant extracts such as essential oils (volatile compounds) or hydroalcoholic extracts (non-volatile compounds) from different populations differ in their biological activities against pathogens and pests?
(6) Which compounds are responsible for activity against pathogens and pests?
(7) Can we enhance the production of bioactive compounds by mimicking extreme environmental conditions?

In *chapter 2* the populations of *F. assa-foetida* were characterized according to the content of essential oil compounds and chemotype by GC−MS and rapid analytical method (NIRS). Furthermore, the relationship between the production of essential oils in *F. assa-foetida* roots and geographical, climatic and edaphic factors were evaluated in order to predict the distribution and the content of specific compounds in the different populations of this species with respect to environmental conditions (Question 1-4).

Chapter 3 investigated whether different environmental factors affect *Z. multiflora* populations with respect to essential oil production, chemotype as well as the content of specific compounds. In addition, it evaluated whether geographic, edaphic and climatic information can predict the presence of specific compounds. Furthermore, rapid analytical methods based on NIRS coupled with GC−FID/GC−MS methods were tested for characterization and classification of metabolite profiles of *Z. multiflora* populations (Question 1-4).

In *chapter 4* metabolite fingerprinting of *Z. multiflora* populations were investigated by HPLC-ESI-QTOF-MS with respect to non-volatile compounds of hydroalcoholic extracts. The antifungal activity of *Z. multiflora* extracts and fractions were evaluated against pre- and postharvest fungal pathogens to examine chemotypes and/or populations with strong antifungal activity. Furthermore, correlation analysis were performed in order to identify bioactive compounds in extracts and fractions (Question 2, 5, 6).

Chapter 5 elucidates the question whether essential oils of *Z. multiflora* populations differ in biological activities against pre- and postharvest fungal pathogens and whether a relationship exists between antifungal activities of *Z. multiflora* essential oil, the content of essential oil constituents and total leaf elemental concentration. The chemical profile responses of two main *Z. multiflora* chemotypes to the abiotic stresses heat and drought and to UV-A radiation intensity were examined in order to enhance the production of bioactive compounds (Question 5-7).

Chapter 6 discusses and relates the findings of this study on *F. assa-foetida* and *Z. multiflora* and the differentiation of their chemotypes to the recent knowledge on environmental metabolomics and the effects of environments on plant metabolites. Moreover, antifungal activity of EOs and hydroalcoholic extracts of *Z. multiflora* were investigated to find an effective alternative to synthetic fungicides. Finally, the effects of heat and drought stresses, and ultraviolet light intensity (UV-A radiation) on the relative content of specific volatile compounds were elucidated.

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Appendix. Supplementary data

To select plant species, the antifungal activity of hydroalcoholic extracts and essential oils of seven medicinal plants including *Zataria multiflora* Boiss., *Satureja macrosiphon*, *Satureja khuzistanica*, *Oliveria decumbens* Vent., *Kelussia odoratissima* Mozaff., *Ferula assa-foetida* L., and *Ferulago angulata* were examined against *Fusarium graminearum*.

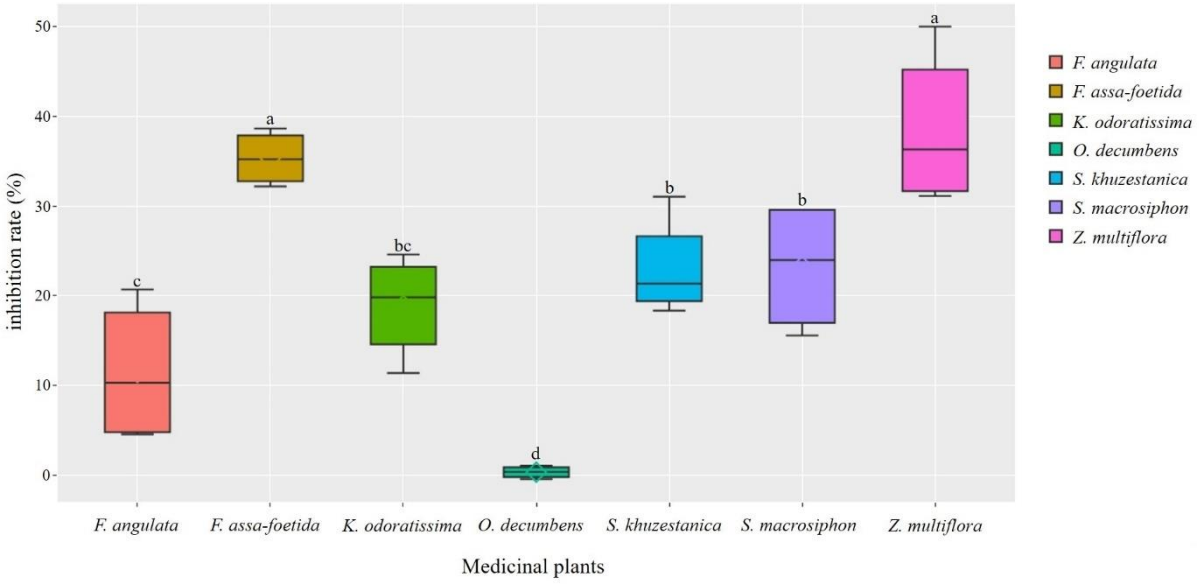

Figure S1. Box plot graphics representing the median, 25-75 % quartiles, min and max (%) of 3 replicates of the antifungal activity (%) of hydroalcoholic extracts of seven native medicinal plants from Iran against mycelial growth of *Fusarium graminearum*. Different lowercase letters indicate significant differences among the means [+ SD] of antifungal activity by LSD tests ($p < 0.05$).

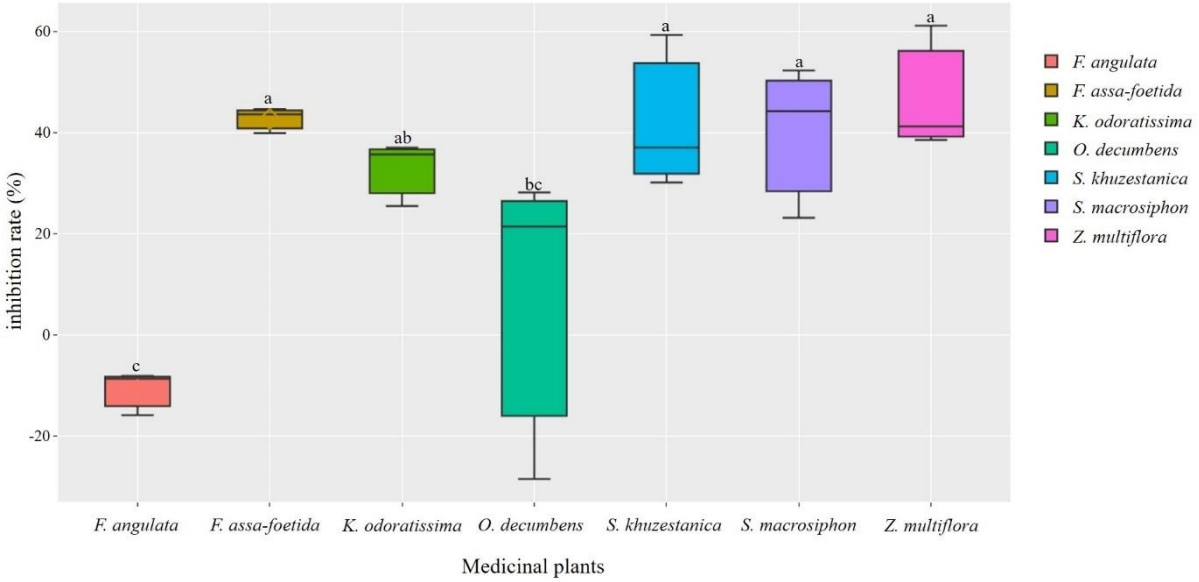

Figure S2. Box plot graphics representing the median, 25-75 % quartiles, min and max (%) of 3 replicates of the antifungal activity (%) of essential oils of seven native medicinal plants from Iran against mycelial growth of *Fusarium graminearum*. Different lowercase letters indicate significant differences among the means [+ SD] of antifungal activity by LSD tests ($p < 0.05$).

Metabolomics approaches for analyzing effects of geographic and environmental factors on the variation of root essential oils of *Ferula assa-foetida* L. https://doi.org/10.1021/acs.jafc.0c03681

Abstract

Environmental factors shape the production and accumulation processes of plant secondary metabolites in medical and aromatic plants, and thus, their pharmacological and biological activity. Using an environmental metabolomics approach, we determined chemotypes and specific compounds on the basis of essential oils (EOs) from roots of 10 Iranian *Ferula assa-foetida* L. populations, and related them to geographical, climate and edaphic data. GC−MS revealed three distinct chemotypes characterized by (I) monoterpenes and *Z*-1-propenyl *sec*-butyl disulfide; (II) eudesmane sesquiterpenoids and *α*-agarofuran; (III) *Z*- and *E*-1-propenyl *sec*-butyl disulfide. NIRS measurements indicated a similar, but less distinct pattern. Structural equation models showed that EO constituents and content were directly influenced by edaphic factors (texture, pH, iron, potassium and aluminum content) and temperature, and predominantly indirectly by latitude, longitude and altitude. Predicting EO constituents or chemotypes by geographical, climate and soil factors can be used in *F. assa-foetida* to select populations with specific EO characteristics.

Keywords: environmental metabolomics, phytochemical diversity, chemometrics, soil chemistry, structural equation model, *Ferula assa-foetida* L.

1. Introduction

Today, agriculture is facing huge challenges. One of these challenges is the increased pressure of pests and diseases and the reduced availability of synthetic chemical plant protection agents due to resistance developments, negative effects on the environment and human health, and increased regulatory restrictions (Lamichhane et al., 2016). To cope with these challenges, secondary compounds from medical and aromatic plants might be harnessed for biological protection of agricultural plants. One option to increase the efficacy of this approach is to identify plant populations with high amounts of specific compounds, e.g. by environmental metabolomics, an interesting approach (Bundy et al., 2009) that has been used in the plant sciences in investigating the effects of environmental factors on plant metabolism (Stierlin et al., 2020; Sampaio et al., 2016), in food science to detect the origin food products (Ghisoni et al., 2019), as well as in animal science to study well-being (Wei et al., 2018).

Essential oils (EOs), which are naturally produced by aromatic plants, contain a wide range of volatile molecules, including mostly secondary metabolites that have several biological activities (Raveau et al., 2020). Plants are adapted genotypically and/or phenotypically to the different geographical, climatic and edaphic conditions across the world which are shaping the production and accumulation processes of secondary metabolites in plants and probably their biological activity that is reflected in their EO profile (Karimi et al., 2020; Albert et al., 2009). Understanding how environmental factors are influencing, e.g., EO content and composition of plant species as well as elucidating how the resulting variation in EO chemical components causes the distribution of plant species and the abundance of populations in natural habitats is of high importance for predicting and selecting plant resources rich in specific compounds. Environmental and edaphic factors can be strongly influential in promoting the biosynthesis and accumulation of EOs and also help to identify individual aromatic plants with specific constituents for food and pharmaceutical industries or agricultural uses.

Ferula assa-foetida L. is a perennial, monoecious and herbaceous species with an unpleasant odor (Iranshahy & Iranshahi, 2011). It belongs to the Apiaceae (Umbelliferae) family, which is native to central Asia and grows wildly in Iran and Afghanistan. *F. assa-foetida* is commonly known by the Persian names of “anghuzeh” and “koma” while there are various names of *F. assa-foetida* in different languages (Iranshahy & Iranshahi, 2011; Ross, 2005). The plant grows up to approximately 2 m, and is the main source of oleo-gum-resin (OGR) which is called asafetida and exudes from the roots with two forms of taste (bitter and sweet) (Jafari et al., 2019; Moghaddam & Farhadi, 2015). Asafetida has been used not only as medicine in Iranian folk medicine but also as a spice, particularly in India (Iranshahy & Iranshahi, 2011). It is traditionally used to treat a vast range of diseases, such as microbial

infections, epilepsy, diabetes, earache, stomachache, hysteria, nervous disorders, flatulence, weak digestion, asthma, influenza and bronchitis (Latifi et al., 2019; Mahendra & Bisht, 2012). It is also used against insects (Muturi et al., 2018; Poorjavad et al., 2014). The OGR consists mainly of three main fractions, including resin (40-64 %), gum (25 %) and essential oil (10-17 %) (Takeoka, 2001). Considerable variation in EO content and components of *F. assa-foetida* have been reported especially for OGR constituents. These variations might be due to varying geographical, climatic and soil conditions, season of collection, phenological stage, genetic constitution and plant part harvested (Jafari et al., 2019; Mirzaei & Hasanloo, 2012). The major EO constituents of *F. assa-foetida* are sulphurous compounds such as propenyl *sec*-butyl disulfide, *Z*-1-propenyl *sec*-butyl disulfide and *E*-1-propenyl *sec*-butyl disulfide (Moghaddam & Farhadi, 2015; Kanani et al., 2011; Khajeh et al., 2005). Besides, it contains other components including *α*-pinene, *β*-pinene, myrcene, limonene, *β*-selinene, *γ*-eudesmol and *α*-eudesmol (Zomorodian et al., 2018; Kavoosi & Rowshan, 2013). Some important EO constituents of *F. assa-foetida* and their biological and pharmacological activities are listed in Table 1. Since for different usages different compounds might come into focus, it is necessary to identify populations with a high content of a certain compound.

Table 1. Major chemical constituents of essential oil of *Ferula assa-foetida* and its biological and pharmacological activities.

main compound	chemical class	biological and pharmacological activities of EO	references
α-pinene	monoterpene	antimicrobial and antioxidant activity	Kavoosi & Rowshan, 2013
camphene	monoterpene		
myrcene	monoterpene	antimicrobial activity	Zomorodian et al., 2018
limonene	monoterpene		
Z-1-propenyl *sec*-butyl disulfide	sulphur-containing compound	antiprotozoa activity	Iranshahy & Iranshahi, 2011
E-1-propenyl *sec*-butyl disulfide	sulphur-containing compound	treat weak digestion and intestinal parasites	Takeoka, 2001
β-selinene	eudesmane sesquiterpenoids		
5-*epi*-7-*epi*-*α*-eudesmol	eudesmane sesquiterpenoids	antidiabetic and antihyperlipidemic	Latifi et al., 2019
γ-eudesmol	eudesmane sesquiterpenoids		
dihydro-eudesmol	eudesmane sesquiterpenoids	digestive enzyme and antispasmodic activity	Poorjavad et al., 2014
7-*epi*-*α*-eudesmol	eudesmane sesquiterpenoids		

While EO can be determined accurately by different gas chromatography (GC) detectors like flame ionization (FID) and mass spectrometry (MS), in many cases a rapid determination on raw materials is desirable. Sensitive and reliable analytical methods such as near-infrared (NIR), attenuated total reflectance-Fourier transform infrared (ATR−FTIR) and Raman spectroscopy have distinct advantages of being time-saving and non-destructive. They are rapid since no sample preparation is needed and have been useful tools to determine the

chemical fingerprint and secondary metabolites of plants for the last two decades (Bittner et al., 2017; Gudi et al., 2014).

In the present study we focused on the EOs of the roots of *F. assa-foetida* L., a plant where EOs are not the main/only active secondary metabolites. The aim was to evaluate the relationship between the production of EOs in *F. assa-foetida* roots and geographical, climatic and edaphic factors in order to predict the distribution and the content of specific EO compounds in the different populations of this species with respect to environmental conditions. Therefore, ten *F. assa-foetida* populations from different locations in Iran were characterized according to the chemotype and content of EOs by GC−MS and rapid analytical method (NIRS). EO content and composition were related to environmental factors using Pearson correlations and structural equation models (SEMs).

2. Materials and Methods

2.1. Plant materials

To evaluate EO content and composition of *F. assa-foetida* and to correlate it with geographic, climatic and edaphic conditions, its roots were collected in July 2018 across its major natural habitats from the center to the south of Iran. The sampling regions were located in Kohgiluyeh and Boyer-Ahmad (Yas population), Isfahan (Aze and Jan populations), Kerman (Hot, Qal and Kha populations), Yazd (Ash and Taf populations), Fars (Ney population) and South Khorasan (Tab population) provinces (Figure 1). For each population, 8 to 10 individual plants were collected with a minimum distance of 100 m from each other. Voucher specimens (No. MPH-1254) were deposited in the Herbarium of Medicinal Plants and Drugs Research Institute (MPH), Shahid Beheshti University, Tehran, Iran. Geographical data and altitude for each sampling area were recorded using GPS (Garmin, Oregon® 600 Series, USA). In addition, climate data for five years were taken from meteorological stations closest to the habitats (Table 2).

2.2. Soil analysis

Soil samples were taken from the surface layer (0 to 30 cm depth) from five randomly selected plots in each sampling site. The five soil samples were mixed and dried at room temperature (20-25 °C). The soil samples were sieved through a 2 mm filter to ascertain soil chemical features such as the amount of nutrients [phosphor (P), potassium (K), Calcium (Ca), magnesium (Mg), manganese (Mn), sulfur (S), zinc (Zn), sodium (Na), iron (Fe) and aluminum (Al)], soil texture (percentage content of sand, silt and clay; according to DIN 19683-2, 1997), cation-exchange capacity (CEC; DIN ISO 11260, 1997), carbon and nitrogen

content (C, N; DIN ISO 10694, 1998), pH-value (A 5.1.1, VDLUFA, 1991) and organic matter (OM; DIN 19684-3, 1998). The total heavy metal and nutrient contents of soil samples were determined after pressure dissolution with 69 % supra pure nitric acid (according to A2.4.3.1, VDLUFA, 1991) by ICP-AES (iCAP™ 7600 Duo, Thermo Fischer Scientific). Contents of total carbon and total nitrogen were determined with CNS elemental analyzer (Vario EL Cube, Elementar Analysesysteme GmbH). Pedological base parameters (soil particle size, pH-value, C/N) were collected for characterization. The particle size determination of soil texture was carried out according to DIN 19683-2 (1997).

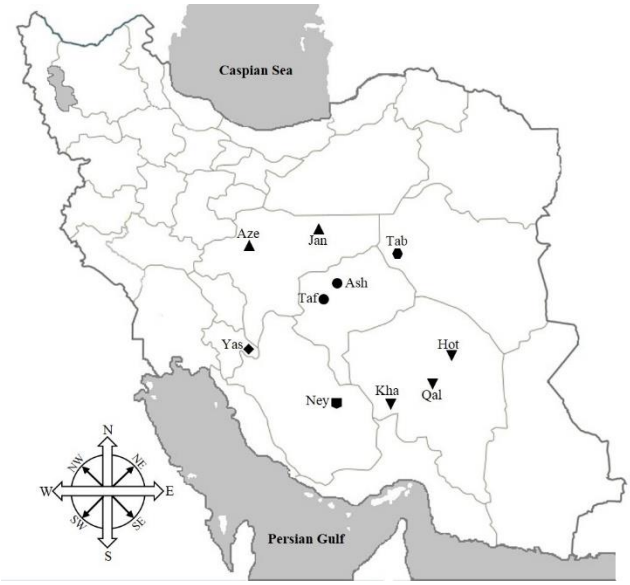

Figure 1. Study regions of *Ferula assa-foetida* populations in Iran.

Table 2. Climatic and geographical information of natural habitats of *Ferula assa-foetida* populations.

Population name	Code	Location	Latitude (N)	Longitude (E)	Altitude (m)	Av. Annual temperature (◦C)	Annual precipitation (mm)
Ashkezar	Ash	Zarband village	31° 48' 26''	54° 00' 19''	2113	21.06 ± 0.36	40.5 ± 14.7
Azeran	Aze	Azeran-Kamoo Road	33° 41' 58''	51° 12' 50''	2861	19.84 ± 0.23	96.1 ± 30.2
Hotkan	Hot	Reyhan Shahr- Hojedk Road	30° 46' 43''	56° 51' 15''	2592	20.20 ± 0.52	107.8 ± 46.7
Jandaq	Jan	Jandaq toward mesr desert	33° 57' 35''	54° 31' 00''	1262	21.46 ± 0.36	55.9 ± 27.9
Khajuei	Kha	Khajuei mountain- Sirjan	29° 08' 24''	55° 39' 23''	2096	18.09 ± 0.38	158.2 ± 53.1
Neyriz	Ney	Gardaneh-ye Layzar-Neyriz	29° 13' 55''	54° 24' 30''	1854	21.26 ± 0.48	209.8 ± 86.6
Qaleasgar	Qal	Qaleasgar village	29° 31' 31''	56° 41' 41''	2652	12.17 ± 0.38	226.7 ± 46.4
Tabas	Tab	Neyaz village	33° 39' 48''	57° 09' 28''	1326	23.70 ± 0.37	46.3 ± 13.5
Taft	Taf	Darreh-ye Gahan mountains	31° 42' 09''	54° 09' 57''	1844	20.26 ± 0.74	49.8 ± 23.9
Yasuj	Yas	Tange sorkh- Yasuj	30° 28' 10''	51° 46' 50''	2199	15.18 ± 0.52	573.2 ± 108.2

2.3. Essential oil isolation

The roots of *F. assa-foetida* were dried at room temperature (20-25 °C) in the shade. In order to extract the EOs, air-dried roots (40 g of each plant) were ground manually and then subjected to hydro-distillation using a clevenger-type apparatus for 3 h according to the method outlined by the British Pharmacopeia (British Pharmacopeia, 1998). The percentage of oil content of the roots was determined based on the dry weight of the plant material. The isolated EOs were dried over anhydrous sodium sulfate and stored at 4 °C in sealed glass vials before GC−FID and GC−MS analysis and further experiments.

2.4. GC−FID and GC−MS analysis

Separation and analysis of *F. assa-foetida* EO components were achieved on GC−FID using an Agilent gas chromatograph 6890N, equipped with a HP-5 column (30 m × 0. 25 mm i.d., with a film thickness of 0.5 µm). The oven temperature was programmed at 50 °C for 2 min, then from 50 to 320 °C at 5 °C min^{-1}, and held at 320 °C for 6 min. Injector and detector temperatures were 250 °C. The carrier gas was hydrogen with a constant flow rate of 1 mL min^{-1}, and one µl of the diluted EOs (1/500 v/v in n-hexane) was injected automatically (Gerstel MPS) in a splitless mode. Nitrogen was used as make-up gas, which was set at a flow of 45 mL min^{-1}.

Mass spectrometry of the oils was carried out using an Agilent MSD 5975B/GC 6890N, equipped with a 30 m × 0.25 mm i.d., 0.5 µm, HP-5MS column. The injector temperature was 250 °C and the temperature programming was: 50 °C, held for 2 min, then raised to 320 °C at 5 °C min^{-1} and held for 6 min. One µl of the diluted EOs (1/500 v/v in n-hexane) was injected automatically (Gerstel MPS) in a splitless mode. Injector and detector temperatures were set at 250 °C. The EI^{+}-MS operating parameters were as follows: ionization energy, 70 eV and ion source temperature 230 °C. The quadrupole mass spectrometer was scanned over 35 to 350 *m/z*. The runtime and solvent delay were set at 60 min and 5 min, respectively (4.45 scans/s). Available standards including *E*-*β*-farnesene, limonene, camphene, myrcene, carvacrol and linalool were purchased from Sigma-Aldrich-Fluka (Germany), and thymol and *α*-pinene from Roth (Germany). 6-Methyl-5-hepten-2-one (Sigma-Aldrich-Fluka, Germany) was used as internal standard and was added to the diluted EOs before the analysis. The oil components were identified by comparison of their mass spectra and retention indices with those of the internal reference mass spectra library (Adams and NIST mass spectral databases), standard constituents and the previously published data. The retention indices of individual components were calculated using a series of n-alkanes (C8-C40) (Sigma-Aldrich-Fluka, Germany) (1/100 in n-Pentan). The relative percentage composition of individual compounds was computed from the GC peak areas obtained without using correction factors.

2.5. NIR spectroscopy

Before quantification by GC−FID/GC−MS, NIR spectra were recorded directly at the manually cut (5 to 10 mm pieces) plant root material. NIRS analyses were carried out on a Fourier-Transform (FT)-NIR spectrometer (Multi-Purpose Analyser MPA, Bruker Optics GmbH, Germany). Spectra were recorded in the wavenumber range from 3,800-9,000 cm^{-1} with a spectral resolution of 8 cm^{-1}. Approximately 20 g of dried roots were measured in three replicates by splitting the material into individual glass Petri dish and spectra were collected during rotation of the dish using the integrating sphere for measuring in diffuse reflection. Each spectrum is composed of 32 scans which were obtained at 30 seconds. Spectra were recorded and pre-processed using OPUS 6.5 software (Bruker Optics). The raw spectra were centered, corrected for scattering effects, and baseline shifting using weighted multiplicative scatter correction (WMSC). WMSC results in linear transformation of each individual spectrum to fit it best to the mean spectrum of the whole spectra set and is widely applied for scattering samples measured in diffuse reflection. Weighting through iterative weight determination decreases the influence of minor signals for reducing impact of spectral artifacts as described in literature (Rinnan et al., 2009). Only averaged spectra of the three replicates were utilized for the later chemometric analysis.

2.6. Chemometrics

To estimate the variability of *F. assa-foetida* populations and for the development of relevant calibration models, principal component analysis (PCA) and partial least square analysis (PLS) were carried out using the Quant 2 module of OPUS 6.5 software. Calibration models were created by 10-fold cross-validation (leaving out 10 % of samples for internal validation, each). Characteristic spectral ranges were identified by PCA and lists of characteristic vibrations in comparison to reference substances. Spectra were normalized and baseline corrected by WMSC as described in section 2.5. Therefore, averaged plant-wise NIR spectra and GC data of each plant were correlated. For estimation of the individual validation results, the R^2 (coefficient of multiple determination), the RMSEP (root mean square error of prediction), LVs (number of latent variables) and the BIAS (the average difference between the NIR-predicted value and the real value) were taken into consideration.

2.7. Statistical analysis

Extractions and chemical analyses of plants from the different populations were conducted in a completely randomized experimental design with eight to ten replications. The means and standard deviations (±) were compared using least significant difference method (LSD) at $p < 0.05$ level with SPSS (SPSS, Chicago, IL, USA) software package from version 16.

Hierarchical cluster analysis (HCA) was performed using SPSS to classify *F. assa-foetida* populations according to the squared Euclidean distances. To determine the correlation between the EO content, its main constituents and environmental factors, the Pearson's correlation coefficient at $p < 0.01$ was applied using SPSS. According to the EOs constituents the PCA analysis was performed using MetaboAnalyst (Chong et al., 2019).

SEMs were applied using partial least squares (PLS) regression using Warp PLS 6.0 to evaluate the effect of environmental factors (Kock & Lynn, 2012). The PLS regression was chosen over covariance based approaches because it suited our small sample size and, compared to covariance structure analysis, can accommodate both reflective and formative scales more easily. Furthermore, PLS does not require any a priori distributional assumptions. The partial model fit scores (R^2), individual standardized path coefficients (β), and overall model *p*-values were generated using resampling estimations coupled with Bonferroni like corrections (Kock, 2010). To authenticate the models, three model-fit indices [average path coefficient (APC), average R-squared (ARS), and average variance inflation factor (AVIF)] were calculated for each region. For model fit, it is recommended that *p*-values for APC and ARS are both lower than 0.05 (i.e. significance at the 0.05 level). The AVIF index controls for multi-collinearity and should be below 5 (Kock, 2010). In the SEM analysis, we set paths from geographic factors (latitude, longitude and altitude), climatic factors (rainfall and temperature), soil texture (relative proportion of clay, silt and sand), constituents (N, P, K, Al, Ca, Fe, Mg, Mn, S, Na and Zn), C content, C/N ratio, CEC and pH-value directly to EO content and its constituents. Furthermore, we included the possible effects of the geographic factors on climatic and soil factors.

3. Results

3.1. Chemical profile and characterization of chemotypes using GC−MS

The variation of the EO content of *F. assa-foetida* roots is shown in Figure 2, indicating significant differences ($p < 0.05$) between the populations. It ranged from 0.14 to 0.83 % dry matter (V/W) with the lowest content of EOs in Neyriz and Yasuj populations and the highest EO content in the Tabas population. The volatile composition detected in the EOs of roots of *F. assa-foetida* is presented in Table 3. One hundred and four components have been identified representing 97.08 to 99.05 % of total EOs. The most abundant constituents were disulfide components such as *E*-1-propenyl *sec*-butyl disulfide (0.18-49.25 %) and *Z*-1-propenyl *sec*-butyl disulfide (0.62-15.86 %); eudesmane sesquiterpenoids such as γ-eudesmol (0.24-20.81 %), 5-*epi*-7-*epi*-α-eudesmol (0.07-16.47 %), dihydro-eudesmol (0.12-15.41 %) and 7-*epi*-α-eudesmol (0.12-13.00 %); monoterpenes including α-pinene (0.04-27.79 %),

limonene (0.14-17.79 %), camphene (tr-12.62 %) and myrcene (tr-10.93 %); other compounds were *α*-agarofuran (0.03-16.55 %) and agarospirol (0.07-6.04 %).

Monoterpenes represented the major fraction in the EOs from Azeran (≥ 70.13 %), Neyriz (≥ 17.72 %), and Yasuj (≥ 18.33 %) populations. According to our results, disulfide components were the major fraction in the EOs from Hotkan (≥ 59.46 %), Qaleasgar (≥ 54.90 %), Ashkezar (≥ 36.78 %), Taft (≥ 28.93 %) and Tabas (≥ 24.55 %), whereas eudesmane sesquiterpenoids constituted the major fraction in the sample from Khajuei (≥ 44.00 %), Taft (≥ 39.00 %), Jandaq (≥ 35.50 %), Ashkezar (≥ 31.50 %) and Tabas (≥ 28.50 %).

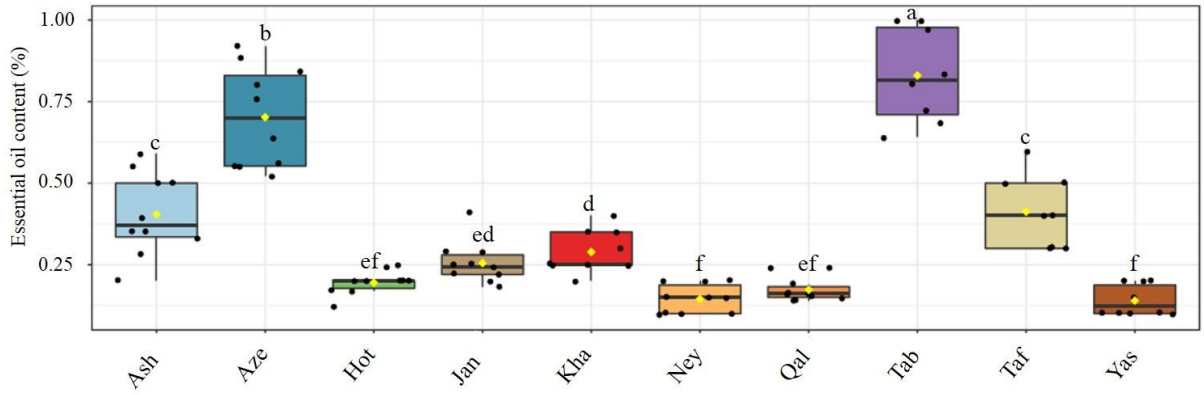

Figure 2. Variation of EO content of *F. assa-foetida* populations.

HCA and PCA were carried out to determine the phytochemical variation based on EO components in *F. assa-foetida* populations. According to the top 26 constituents (≥ 1 %), the investigated populations were distributed into three main groups by HCA representing three distinct chemotypes (Figure 3). Cluster I is composed of two sub-clusters consisting of three populations with much higher monoterpene content compared to the other two chemotypes. Sub-cluster Ia contains the Azeran population which is characterized by higher amounts of the monoterpenes *α*-pinene, limonene, camphene and myrcene and lower amounts of eudesmane sesquiterpenoids and disulfide components. Sub-cluster Ib consists of two populations (Neyriz and Yasuj) which are characterized by higher amounts of *α*-pinene and *Z*-1-propenyl *sec*-butyl disulfide.

Based on the percentage of each compound in chemotype II, it was divided into two sub-clusters containing five populations. Sub-cluster IIa consists of the Jandaq population which is characterized by high amounts of *α*-agarofuran, dihydro-eudesmol and *γ*-eudesmol. Furthermore, sub-cluster IIb including Ashkezar, Taft, Khajuei and Tabas populations

characterized by higher amounts of eudesmane sesquiterpenoids such as *γ*-eudesmol, 5-*epi*-7-*epi*-*α*-eudesmol, 7-*epi*-*α*-eudesmol, followed by *Z* and *E*-1-propenyl *sec*-butyl disulfide and by lower quantities of monoterpenes such as *α*-pinene, limonene and camphene. According to the dendrogram, chemotype III consists of two populations (Hotkan and Qaleasgar) characterized by high amounts of disulfide components, i.e. *Z* and *E*-1-propenyl *sec*-butyl disulfide followed by bis (1-methyl (thio)) ethyl disulfide and lower quantities of eudesmane sesquiterpenoids and monoterpenes.

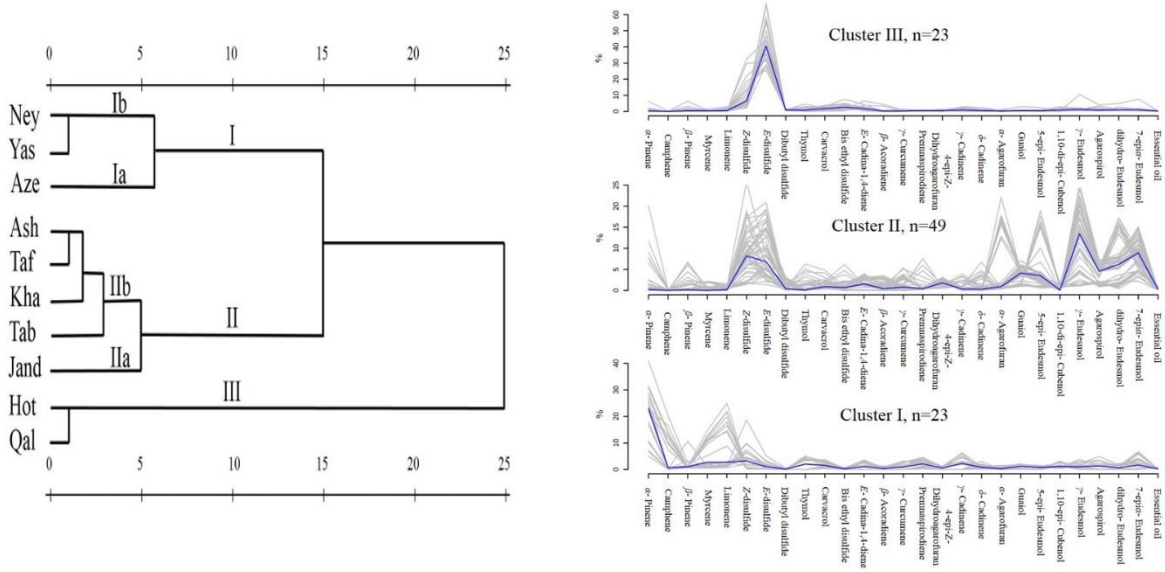

Figure 3. Hierarchical cluster analysis of *F. assa-foetida* populations, consistent with the top 26 EO constituents.

The PCA analyzed the average values of the 26 most abundant EO compounds and revealed a similar distinction between the populations of *F. assa-foetida* as the HCA approach. The first two PCs represented 78.10 % of the total variation in the data set. Monoterpenes, especially *α*-pinene (0.29) and *E*-1-propenyl *sec*-butyl disulfide (-0.93), were found as the unique compounds that influence the PC1 value, whereas eudesmane sesquiterpenoids such as *γ*-eudesmol (0.49) and 7-*epi*-*α*-eudesmol (0.30), contribute more than the other compounds to PC2. The PCA (Figure 4) revealed three chemotypes according to the HCA. Azeran, Neyriz and Yasuj populations are characterized by monoterpenes, while populations from Qaleasgar and Hotkan are characterized by high content of *E*-1-propenyl *sec*-butyl disulfide. In addition, populations from Jandaq, Ashkezar, Taft, Khajuei and Tabas are characterized by eudesmane sesquiterpenoids as well as by *α*-agarofuran (Figure 4).

Table 3. Phytochemical composition of *Ferula assa-foetida* populations.[a]

compound	RI[b]	RI[c]	population									
			Ash	Aze	Hot	Jan	Kha	Ney	Qal	Tab	Taf	Yas
2,5-dimethylthiophene	906	894	tr	0.32	0.09	tr	0.08	tr	tr	0.35	tr	0.06
α-pinene	937	939	0.05	27.79	1.65	0.38	1.08	13.99	0.13	0.26	0.04	12.57
camphene	952	954	tr	12.62	tr	0.12	tr	0.17	tr	0.2	tr	0.3
β-pinene	981	979	0.11	1	1.23	0.24	3.48	1.42	0.74	0.13	tr	1.82
myrcene	993	992	-	10.93	0.54	0.36	0.49	1.04	0.29	-	-	1.85
methyl *sec*-butyl disulphide	993		0.07	0.09	0.22	0.33	-	-	tr	1.14	tr	0.08
2,3,4-trimethylthiophene	1017		0.05	tr	1.66	0.09	0.34	0.18	1.76	0.12	0.08	0.16
p-cymene	1027	1025	tr	0.57	0.51	0.12	0.03	0.21	0.15	0.51	0.03	0.21
limonene	1032	1030	0.16	17.79	1.2	0.56	0.15	1.1	0.37	0.14	0.15	1.79
Z-*β*-ocimene	1038	1039	-	-	1.16	-	0.88	0.63	1.12	0.12	-	0.96
E-*β*-ocimene	1049	1050	tr	-	1.89	0.03	0.57	0.67	1.88	-	0.08	0.55
γ-Terpinene	1062	1062	-	0.59	-	0.1	-	0.2	-	-	-	0.18
ethyl 1-methylpropyl disulfide	1077		0.13	0.16	0.04	0.06	tr	0.05	0.12	0.16	0.04	0.11
terpinolene	1093	1089	-	0.83	-	-	-	tr	-	-	-	tr
linalool	1100	1100	0.28	0.33	0.65	0.4	0.31	0.95	0.46	0.11	0.27	0.75
n-nonanal	1104	1103	-	-	0.1	-	-	-	0.13	0.06	0.07	0.08
dipropyl disulfide	1111	1109	0.09	-	0.18	0.05	0.04	-	0.3	-	0.19	-
endo-fenchol	1118	1114	-	0.38	-	-	-	tr	-	-	-	tr
allo-ocimene	1131	1128	-	-	0.31	-	0.24	0.24	0.32	-	-	0.38
neo-allo-ocimene	1144	1140	-	-	0.32	-	0.08	-	0.24	0.05	-	-
camphor	1151	1150	-	0.15	-	-	-	tr	-	-	-	tr
n-propyl *sec*-butyl disulfide	1168	1169	0.36	0.4	0.75	0.46	0.6	tr	1.44	0.52	0.58	0.07
Z-1-propenyl *sec*-butyl disulfide	1174		15.86	0.62	5.74	2.51	8.52	11.76	9.24	8.07	11.12	6.15
E-1-propenyl *sec*-butyl disulfide	1180		19.2	0.18	49.25	4.49	4.48	3.14	39.97	12.6	15.69	2.27
α-terpineol	1195	1190	-	-	0.13	0.04	0.028	0.05	-	-	-	0.06
bis (1-methyl propyl) disulfide	1214		tr	0.04	0.16	0.31	0.1	0.08	0.23	0.28	0.06	0.08
dibutyl disulfide	1219		0.35	0.02	0.79	1.98	0.06	0.7	0.97	2.8	0.41	0.19
fenchyl acetate (*endo*)	1226	1225	-	3.17	-	0.2	0.04	0.45	0.11	0.2	-	1.29
fenchyl acetate (*exo*)	1242	1229	-	0.36	0.1	0.12	-	0.19	0.14	0.12	-	0.1
carvacrol methyl ether	1247	1241	tr	0.1	0.2	0.2	0.03	0.23	0.18	tr	0.08	0.22
thymol	1292	1295	tr	1.86	0.36	tr	1.04	2.92	2.36	0.16	0.04	4.52
decadienal (2*E*,4*Z*)	1295	1296	-	-	-	-	0.07	0.1	0.25	-	-	0.17
carvacrol	1303	1305	0.74	0.62	1.59	1.32	1.28	2.66	2.37	0.4	0.93	2.56
Z- pinocarvyl acetate	1306	1305	-	-	-	-	0.32	0.67	0.06	-	-	0.28
decadienal (2*E*,4*E*)	1319	1315	0.08	0.11	0.1	-	-	-	0.23	-	0.05	-
α-terpinyl acetate	1355	1346	-	0.29	-	-	-	tr	-	-	-	tr
bis (1-methyl (thio)) ethyl disulfide	1357		1.01	tr	2.93	2.54	tr	0.43	3.31	0.56	1.13	0.5
α-cubebene	1359	1360	0.14	0.54	0.36	0.06	tr	0.18	0.24	0.03	0.13	1.31
α-copaene	1387	1387	-	1.06	0.05	0.03	-	0.71	0.2	0.08	-	0.37
(1) tetradecene	1391	1389	0.1	-	-	-	-	0.25	0.23	0.19	0.04	0.16
β-elemene	1402	1397	0.68	1.64	0.09	tr	0.02	0.66	0.18	0.19	0.17	0.14
E-trimenal	1420	1419	-	0.14	-	-	-	tr	-	-	-	tr
Z-*α*-bergamotene	1424	1420	0.49	0.14	-	-	-	-	-	-	tr	0.12
β-cedrene	1430	1423	0.05	0.25	0.16	0.12	tr	0.24	0.31	0.19	0.09	1.27
Z-*β*-farnesene	1435	1428	tr	tr	tr	0.17	0.08	0.14	0.33	0.75	0.07	1.81
β-gurjunene	1440	1431	-	-	-	0.04	0.07	1.21	0.68	-	-	0.33
aromadendrene	1442	1439	-	0.4	0.11	0.19	-	0.33	0.62	1.07	-	0.74
γ-elemene	1443	1440	-	0.66	-	-	-	tr	-	-	-	tr
α-guaiene	1449	1440	-	0.11	0.2	0.03	0.31	0.55	0.33	0.24	-	0.73
6,9-guaiadiene	1455	1442	-	0.49	-	-	-	tr	-	-	-	tr
allo-aromadendrene	1455	1448	0.52	tr	0.14	0.09	0.03	1.91	0.11	0.17	0.4	1.01
dihydroaromadendrene	1458	1457	0.59	tr	0.41	1.27	6.18	1.5	3.24	1.2	0.3	1.96
Z-muurola-4(14),5-diene	1460	1458	0.34	-	0.15	0.21	0.15	0.47	0.3	0.11	0.32	0.38
E-*β*-farnesene	1460	1461	-	1.07	-	-	-	tr	-	-	-	tr
α-patchoulene	1463	1463	-	-	0.08	0.18	-	0.56	-	0.14	-	-

Table 3. continued,

compound	RI[b]	RI[c]	population									
			Ash	Aze	Hot	Jan	Kha	Ney	Qal	Tab	Taf	Yas
α-humulene	1469	1465	0.25	0.3	0.71	0.07	tr	0.26	0.57	0.2	0.18	0.21
dauca-5,8-diene	1474	1471	0.04	tr	0.12	0.33	tr	0.07	0.07	0.36	0.07	0.12
E-cadina-1(6),4-diene	1478	1475	2.9	0.36	1.93	1.38	0.06	1.99	1.34	2.52	2.18	2.64
β-acoradiene	1481	1482	2.2	-	0.41	0.37	-	0.66	-	2.77	0.12	0.74
γ-muurolene	1484	1483	-	0.28	-	-	-	tr	-	-	-	tr
aristolochene	1484	1484	-	-	0.59	-	0.1	-	-	0.17	-	-
α-muurolene	1488	1484	-	0.17	-	-	-	tr	-	-	-	tr
γ-curcumene	1488	1485	0.41	-	0.61	3.32	-	1.8	0.14	0.97	0.37	2.49
ar-curcumene	1490	1493	0.05	-	0.69	0.13	0.15	0.12	0.97	0.31	0.11	0.07
germacrene D	1496	1497	0.08	tr	0.32	0.1	tr	1.21	0.12	0.25	0.14	0.87
α-selinene	1498	1498	0.64	0.87	1.24	1.03	0.38	1.46	1.17	1.96	0.67	1.72
β-selinene	1502	1500	0.32	0.85	0.62	0.87	0.69	1.62	0.33	0.73	0.63	2.7
valencene	1508	1503	0.44	tr	0.44	0.41	1.05	1.9	0.38	0.42	0.58	2.03
viridiflorene	1511	1504	-	1.09	-	-	-	tr	-	-	-	-
premnaspirodiene	1514	1505	0.44	0.64	0.38	0.53	0.25	2.79	0.62	0.36	0.36	3.98
β-bisabolene	1516	1508	0.42	0.27	0.36	0.09	0.19	0.59	0.18	0.26	0.25	0.95
4-*epi*-*Z*-dihydroagarofuran	1519	1509	1.9	-	0.69	1.64	2.83	0.89	0.28	0.7	2.48	0.84
Z-dihydroagarofuran	1524	1519	0.45	-	0.55	0.39	0.54	1.23	1.17	0.28	0.53	1.51
γ-cadinene	1528	1528	0.26	tr	0.72	0.34	tr	3.7	1.91	1.1	0.31	3.03
myristicin	1530	1529	-	0.43	-	-	0.18	-	-	0.22	-	-
bicyclogermacrene	1533	1534	-	-	-	0.05	0.18	-	-	0.83	-	-
δ-cadinene	1535	1539	0.31	1.06	0.48	0.27	tr	0.87	1.03	3.98	0.29	0.92
E-*γ*-bisabolene	1541	1541	-	tr	0.29	-	-	0.6	0.09	-	-	0.69
Z-muurol-5-en-4*β*-ol	1555	1550	-	1.02	0.13	-	-	0.29	-	1.4	-	0.37
β-calacorene	1557	1554	-	tr	0.05	-	0.18	0.4	0.5	-	-	0.36
*E*lemicin	1558	1555	-	-	0.16	0.87	-	0.34	0.14	-	0.15	0.18
*E*lemol	1561	1560	tr	0.15	0.13	0.38	1.37	0.51	0.16	0.2	0.64	0.51
α-agarofuran	1565		1	0.03	0.28	16.55	0.49	0.66	0.24	0.56	1.28	0.6
E-nerolidol	1568	1564	-	-	0.05	0.1	-	0.3	0.07	0.42	-	0.25
germacrene B	1573	1567	0.08	tr	0.09	tr	0.05	0.26	0.45	0.35	0.03	0.22
E-isoelemicin	1578	1568	0.33	-	0.2	0.24	0.03	0.23	0.11	2.15	0.06	0.23
neryl isovalerate	1585	1582	-	-	0.1	0.19	0.03	0.88	0.25	-	-	0.52
spathulenol	1594	1593	-	-	0.74	0.02	-	1.58	0.52	0.15	0.05	2.53
ethyl dodecanoate	1595	1594	0.11	-	0.39	-	0.06	-	0.17	0.29	0.03	0.13
rosifoliol	1603	1600	0.31	0.19	0.39	0.51	0.54	0.84	0.79	0.27	0.5	0.93
guaiol	1611	1602	3.56	0.22	0.51	4.45	4.81	1.53	0.4	1.37	4.71	1.5
5-*epi*-7-*epi*-*α*-eudesmol	1622	1616	3.06	0.07	0.38	2.16	3.73	1.05	0.34	16.47	4.86	0.96
1,10-di-*epi*-cubenol	1631	1623	0.24	1.97	0.66	0.03	0.08	1.09	1.85	1.01	0.11	0.74
γ-eudesmol	1640	1641	14.15	0.24	1.36	11.32	20.81	2.03	0.84	5.26	17.03	1.26
valerianol	1650	1648	0.7	0.19	0.24	0.55	0.71	0.36	0.19	0.59	0.77	0.37
α-cadinol	1652		1.05	0.09	0.25	0.72	1.16	0.44	0.28	1.68	1.3	0.53
agarospirol	1654	1649	4.92	0.07	1.05	4.05	6.04	2.28	0.67	1.27	5.61	1.74
α-cadinol	1657	1652	0.36	tr	0.38	0.19	0.49	0.53	0.23	1.17	0.52	0.43
dihydro-eudesmol	1662	1661	5.31	0.12	1.06	15.41	6.72	1.38	0.96	1.77	6.6	1.65
7-*epi*-*α*-eudesmol	1671	1662	9.21	0.12	0.9	7	13	4.04	0.77	5.21	10.99	3.67
β-bisabolol	1680	1683	0.58	0.13	0.26	0.45	0.35	0.74	0.28	0.69	0.5	0.45
bulnesol	1684	1690	0.4	Tr	0.32	0.36	0.18	0.52	0.17	0.39	0.29	0.88
α-bisabolol	1696	1700	0.17	tr	0.44	0.13	0.26	0.32	0.4	2.43	0.22	0.44
2*Z*,6*Z*-farnesol	1701	1712	0.35	0.03	0.3	1.47	0.18	0.5	0.29	0.51	0.4	0.45
Essential oil content (%)			0.4	0.7	0.19	0.25	0.28	0.14	0.17	0.83	0.41	0.14

[a]tr: traces < 0.02 %; (-) not detected. [b]RI, linear retention indices on the HP-5MS column, experimentally determined using a homologue series of n-alkanes. [c]Relative retention indices taken from Adams and NIST.

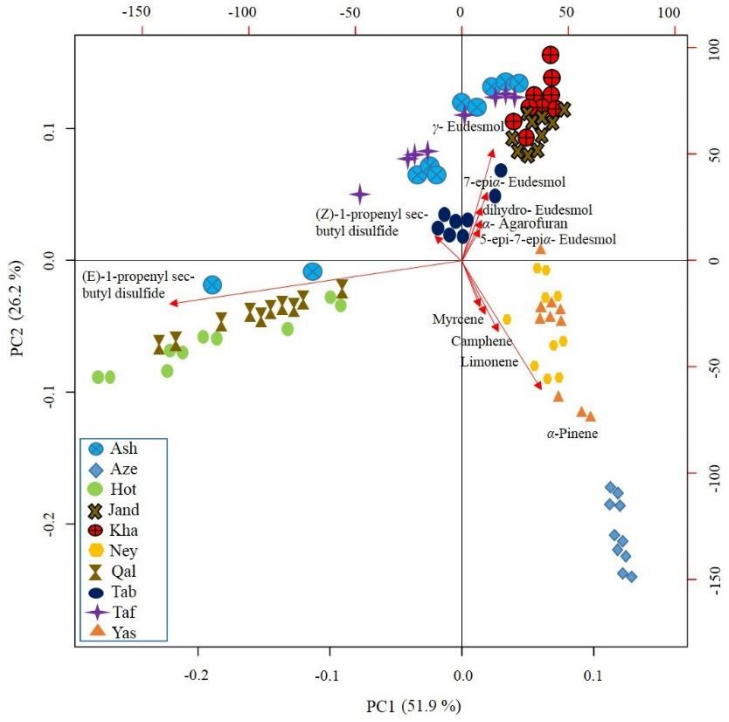

Figure 4. Score plot (PCA) obtained from the main variation of volatile constituents of *F. assa-foetida* populations.

3.2. Characterization of chemotypes of *F. assa-foetida* using NIR spectroscopy

Manually cut raw root material with particle size of up to 10 mm was investigated by NIRS for characterization of *F. assa-foetida* chemotypes directly on dried plant roots. PCA of the resulting NIR spectra showed a different clustering of the individual *F. assa-foetida* populations compared to the chemotypes identified by GC (Figure S1). Since the Tabas population was clustering further away from the other populations, we excluded this population when performing a second PCA, but this resulted in a similar classification pattern (Figure S2). The populations Azeran, Neyriz, Yasuj and Qaleasgar are grouped together in positive range of PC 1, whereas Jandaq, Ashkezar, Taft and Hotkan are located in negative PC 1 range. Khajuei has an intermediate place in the center of the PCA space. As indicated by the loading spectra for the appropriate PC 1 and 2, differentiation is mainly caused by the ratio of monoterpenes (positive PC 1 range) and eudesmane sesquiterpenoids (negative PC 1 range) content (Figure S3). PC 2 further separates the population Azeran from all others based on of the absence of disulfides whose overall concentration is about 1 % in EO.

Furthermore, partial least squares analysis of NIR spectra was applied for quantitative prediction of essential oil content and composition. The set of 95 samples (all individual

plants per population) were used, and calibration models for the overall content of EO and the most representative compounds of each compound class were validated using a tenfold cross validation ("leave 10 % out") (Krähmer et al., 2013). Only for EO content and few components such as *α*-pinene, thymol, premnaspirodiene, *δ*-cadinene, and 5-*epi*-7-*epi*-*α*-eudesmol promising models could be achieved (Table 4) with R^2 values > 0.6.

Table 4. Results of NIRS modeling for prediction of essential oil content and main constituents of *Ferula assa-foetida*.[a]

compound	R^2	RMSEP [%]	Range [%]	BIAS [%]	LVs
essential oil content	0.82	0.10*	0.10. – 1.13*	-0.0111*	7
α-pinene	0.65	5.84**	0.09 – 40.86**	0.369**	3
thymol	0.64	0.99**	0.07 – 6.26**	0.0453**	7
premnaspirodiene	0.61	0.88**	0.16 – 7.55**	0.0645**	8
δ-cadinene	0.7	0.62**	0.04 – 6.73**	0.0819**	4
5-*epi*-7-*epi*-*α*-eudesmol	0.88	1.52**	0.07 – 15.15**	-0.232**	9

[a]* % in dry matter; ** % in essential oil fraction.

3.3. Influence of Environment on EO and its Composition

The geographical, climatic and edaphic conditions of *F. assa-foetida* populations grown in the natural habitats in Iran are shown in Tables 2 and 5. Average annual temperature ranged from 12.17 to 23.70 °C, annual precipitation from 40.50 to 573.20 mm $year^{-1}$, and altitude from 1262 to 2861 m. Ca, Fe, K, Al and Mg were the most abundant elements in the soil of the studied regions while these habitats contain low levels of P, Mn, S, Na and Zn; moreover, the soils were extremely poor in nitrogen. Furthermore, *F. assa-foetida* populations grew in alkaline soils with pH 7.60 to 7.80, CEC varied from 10.78 to 32.22 %, C varied from 1.67 to 5.88 % and OM from 4 to 8 %.

Based on our results detailed in Table 6, several geographical, climatic and edaphic factors show correlations with the EO composition of *F. assa-foetida*. Altitude was significantly negatively correlated with 5-*epi*-7-*epi*-*α*-eudesmol ($p < 0.05$). The correlation analysis revealed that the pH-value was considerably positively correlated with 5-*epi*-7-*epi*-*α*-eudesmol ($p < 0.05$) and negatively correlated with *α*-pinene and also, *α*-pinene was positively influenced by clay content ($p < 0.05$). In addition, *γ*-eudesmol and 7-*epi*-*α*-eudesmol were positively correlated to Ca ($p < 0.01$). *Z*-1-propenyl *sec*-butyl disulfide was positively influenced by silt content and Ca ($p < 0.05$) and negatively by N ($p < 0.05$).

Table 5. Physicochemical characteristics of natural habitats of *Ferula assa-foetida* populations.

region	pH [-]	soil texture [-]	sand [%]	silt [%]	clay [%]	CEC [%]	N [%]	C [%]	C/N [%]	OM [%]
Ashkezar	7.7	silty loamy sand	40.03	49.1	10.8	12.42	0.03	5.2	171.55	5
Azeran	7.6	sandy loam	40.9	34.9	24.1	23.53	0.12	1.68	13.17	7
Hotkan	7.7	loamy sand	50.58	35.4	14.01	14.15	0.15	4.69	29.94	8
Jandaq	7.7	loamy sand	67.3	23.6	9.06	12.82	0.07	3.37	43.58	5
Khajuei	7.6	sandy loamy silt	36.09	51.4	12.4	16.67	0.1	5.88	55.59	8
Neyriz	7.6	silty loam	25.56	55.5	18.8	20.32	0.05	3.94	71.92	6
Qaleasgar	7.7	sandy loam	50.04	29.4	20.5	30.99	0.04	1.67	38.22	4
Tabas	7.8	silty sand	55.05	39.5	5.3	10.78	0.05	4.66	79.92	4
Taft	7.7	sandy silt	26.8	65.1	7.9	13.64	0.05	4.77	90.03	6
Yasuj	7.6	clayey loam	23.8	44.1	32.08	32.22	0.14	5.08	34.09	7
region	Ca (mg/kg)	Fe (mg/kg)	Al (mg/kg)	K (mg/kg)	P (mg/kg)	Mg (mg/kg)	Mn (mg/kg)	S (mg/kg)	Na (mg/kg)	Zn (mg/kg)
Ashkezar	155365.5	13728.03	18500.14	6816.99	432.87	7691.24	323.64	102.22	332.49	32.08
Azeran	15280.34	29533.38	44866.37	18319.2	365.12	7344.92	636.98	125.18	642.76	63.89
Hotkan	16463.13	26487.32	38030.59	14041.4	312.84	5882.81	648.1	199.06	748.99	52.12
Jandaq	62886.87	16169.22	16232.91	5512.82	371.16	7942.6	396.78	424.48	1360.2	36.73
Khajuei	129511.3	18777.85	23620.65	6828.39	434.86	9443.83	258.42	310.14	485.97	32.14
Neyriz	79059.4	23997.52	26553.21	8831.68	307.61	10572.4	479.33	161.63	402.84	42.58
Qaleasgar	28766.1	18336.16	17924.34	3146.02	284.78	5458.01	454.23	63.75	466.36	31.9
Tabas	93819.49	14822.43	16057.77	5585.91	387.45	10701.3	305.24	296.34	1387.3	31.07
Taft	104694.9	16122.98	18350.35	7682.87	322.08	11228.8	353.89	431.86	530.14	62.88
Yasuj	48954.05	16344.45	19063.17	5322.1	188.53	5340.61	272.24	135.89	176.59	27.35

Table 6. Pearson correlation coefficients between environmental factors and EO content or major EO components.[a]

factors	EO	α-pin[b]	camp[c]	myrc[d]	limo[e]	*Z*-disu[f]	*E*-disu[g]	5-7-eu[h]	*γ*-eu[i]	dih-eu[j]	7-*α*-eu[k]
altit[l]	-0.16	0.45	0.51	0.54	0.54	-0.15	0.39	-0.63*	-0.42	-0.63	-0.52
temp[m]	0.54	-0.03	0.06	-0.01	0.03	0.03	-0.27	0.53	0.23	0.26	0.26
pH	0.43	0.66*	0.37	-0.47	-0.41	0.18	0.47	0.69*	0.07	0.15	0.03
sand	0.23	-0.37	-0.02	-0.1	-0.05	-0.41	0.32	0.24	-0.06	0.4	-0.2
silt	-0.03	-0.03	-0.22	-0.21	-0.23	0.65*	-0.25	0.13	0.47	-0.12	0.59
clay	-0.36	0.69*	0.37	0.5	0.44	-0.3	-0.16	-0.61	-0.61	-0.51	-0.56
CEC	-0.39	0.47	0.22	0.33	0.27	-0.2	-0.03	-0.52	-0.56	-0.47	-0.52
C	-0.11	-0.44	-0.58	-0.56	-0.58	0.46	-0.1	0.33	0.58	0.19	0.58
N	-0.16	0.42	0.32	0.41	0.39	-0.65*	0.02	-0.36	-0.32	-0.24	-0.35
C/N	0.14	-0.43	-0.39	-0.45	-0.43	0.46	-0.01	0.32	0.52	0.18	0.57
OM	-0.06	0.35	0.23	0.31	0.28	-0.29	-0.08	-0.45	0.08	-0.14	0.06
Ca	0.13	-0.46	-0.43	-0.48	-0.48	0.72*	-0.29	0.41	0.81**	0.37	0.88**
Fe	-0.01	0.69*	0.66*	0.69*	0.69*	-0.49	-0.09	-0.48	-0.52	-0.47	-0.6
Al	0.14	0.68*	0.74*	0.76*	0.77**	-0.48	0.09	-0.43	-0.42	-0.46	-0.52
K	0.3	0.67*	0.77**	0.77**	0.79**	-0.46	-0.01	-0.31	-0.31	-0.35	-0.4
P	0.49	-0.26	0.1	-0.01	0.03	0.17	-0.13	0.36	0.62	0.38	0.52
Mg	0.4	-0.14	-0.13	-0.19	-0.18	0.33	-0.43	0.58	0.51	0.22	0.58
Mn	0.01	0.46	0.55	0.54	0.58	-0.44	0.39	-0.46	-0.6	-0.36	-0.27
Na	0.49	-0.27	-0.01	-0.08	-0.04	-0.43	-0.01	0.58	0.02	0.42	-0.03
S	0.15	-0.41	-0.26	-0.31	-0.29	-0.15	-0.25	0.39	0.62	0.52	0.61
Zn	0.25	0.41	0.57	0.53	0.57	-0.28	0.05	-0.24	-0.06	-0.12	-0.16

[a]significance levels: *$p < 0.05$; **$p < 0.01$. [b]*α*-pinene; [c]camphene; [d]myrcene; [e]limonene; [f]*Z*-1-propenyl *sec*-butyl disulfide; [g]*E*-1-propenyl *sec*-butyl disulfide; [h]5-*epi*-7-*epi*-*α*-eudesmol; [i]*γ*-eudesmol; [j]dihydro-eudesmol; [k]7-*epi*-*α*-eudesmol; [l]altitude; [m]temperature.

There was a highly positively correlation between monoterpenes and K ($p < 0.01$), Fe and Al ($p < 0.05$). No significant correlations between CEC, OM, C, C/N, P, Mg, Mn, Na, S, Zn, temperature and EO content and its phytochemical constituents have been observed.

To investigate the contribution of single environmental factors on EO and EO constituent content, a SEM approach was applied (Figure 5). The model fit and quality indices for EO content [APC = 0.664 ($p = 0.001$), ARS = 0.775 ($p < 0.001$), AVIF = 2.444]; monoterpenes [APC = 0.732 ($p < 0.001$), ARS = 0.738 ($p < 0.001$), AVIF = 1]; 5-*epi*-7-*epi*-*α*-eudesmol [APC = 0.761 ($p < 0.001$), ARS = 0.788 ($p < 0.001$), AVIF = 1]; dihydro-eudesmol [APC = 0.681 ($p < 0.001$), ARS = 0.908 ($p < 0.001$), AVIF = 1.104]; 7-*epi*-*α*-eudesmol and *γ*-eudesmol [APC = 0.651 ($p = 0.001$), ARS = 0.782 ($p < 0.001$), AVIF = 1.048] and *Z*-1-propenyl *sec*-butyl disulfide [APC = 0.731 ($p < 0.001$), ARS = 0.731 ($p < 0.001$), AVIF = 1.074] were calculated. Site-specific parameters, expressed as latitude, longitude and sometimes as altitude, show a high variability of soil textures in the sampling areas. The clay content and the cations (Al^{3+}, Fe^{2+}, K^{+}) in the soil, which are firmly bound or exchangeable as clay minerals, often show a direct or indirect influence on the content of EO and EO components.

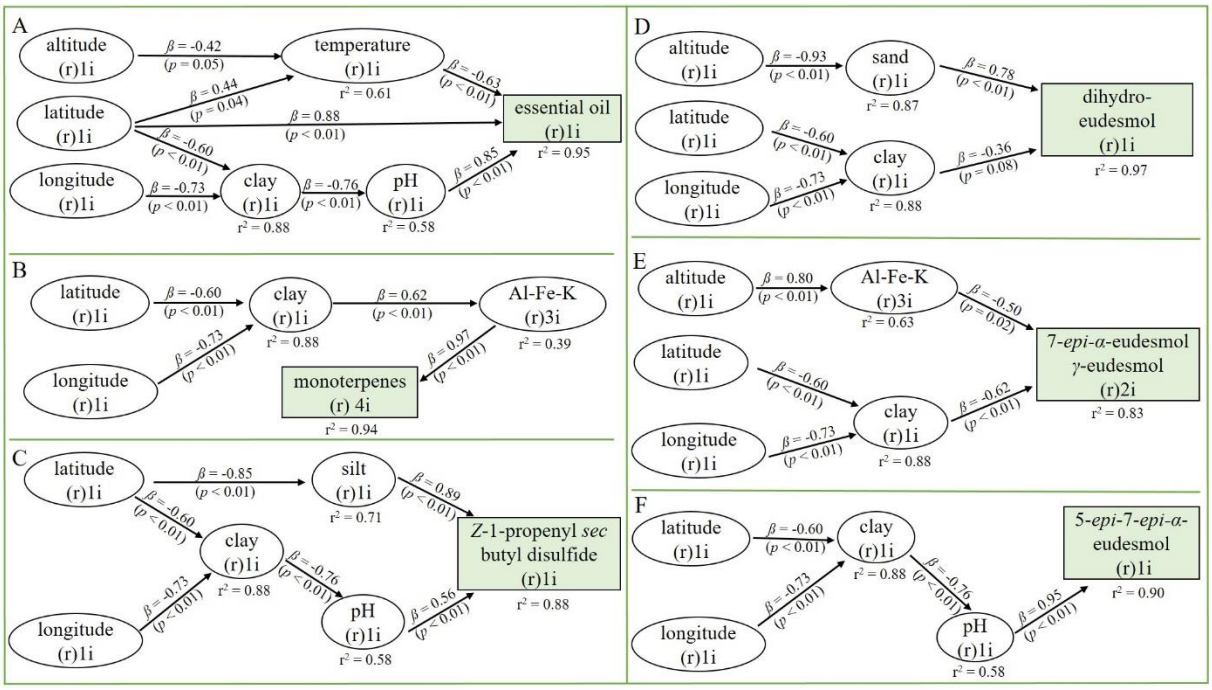

Figure 5. Hypothetical SEMs describe the relationships between environmental and edaphic factors and (A) essential oil; (B) monoterpenes; (C) *Z*-1-propenyl *sec*-butyl disulfide; (D) dihydro-eudesmol; (E) *γ*-eudesmol and 7-*epi*-*α*-eudesmol; and (F) 5-*epi*-7-*epi*-*α*-eudesmol content of *F. assafoetida*. r^2: coefficient of determination, indicating the variability explained for each variable. *β*-values indicate the path coefficients. *p*, significance level for relationship.

The pH-value (positively) and temperature (negatively) directly influenced the EO content. Altitude had a negative effect on temperature while latitude had a positive effect on temperature. Besides, the portion of clay had a negative effect on pH-value (Figure 5A). Monoterpenes were directly positively affected by clay minerals Al, Fe and K, which were positively influenced by the portion of clay (Figure 5B). The pH-value and the portion of silt had a directly positive influence on *Z*-1-propenyl *sec*-butyl disulfide while silt content was negatively affected by latitude and pH-value by the proportion of clay (directly), latitude and longitude (indirectly) (Figure 5C). On the one hand, dihydro-eudesmol was directly positively influenced by proportion of sand, which was negatively affected by altitude; on the other hand, it was negatively influenced by the proportion of clay (Figure 5D). 7-*epi*-α-eudesmol and γ-eudesmol were directly negatively influenced by the proportion of clay and the clay minerals Al, Fe and K (Figure 5E). Furthermore, latitude and longitude had a negative effect on clay content; the proportion of clay had a negative effect on pH-value, which positively affected 5-*epi*-7-*epi*-α-eudesmol (Figure 5F).

4. Discussion

EOs are produced by aromatic plants that are characterized by a distinctive scent. The diversity in EO compounds in plants occurs at different levels: among genera, among species within genera, and as polymorphic variation within species. Observed variations in EO content and constituents in aromatic plants have been ascribed to several environmental factors. The secondary compounds of plants have multiple ecological functions, which contribute to the diversity of EO composition due to different environmental effects. EO compounds are mediating the defense of plants against herbivores and parasites (Borges et al., 2018). Other functions are the mediation of plant competition via allelopathic effects on other plant species, pollinator attraction, soil microbe activity and climatic adaptation (Tölke et al., 2020; Elshamy et al., 2019; Khare et al., 2019; Thompson et al., 2007). Moreover, edaphic factors might constrain or enhance the production of specific compounds and a variation in edaphic factors contributes to the variability of EO composition and the presence of certain chemotypes. Given the variability of factors restricting and enabling variation in EO production and composition, it was questionable whether environmental metabolomics can help to predict the presence of certain chemotypes or a high content of certain compounds.

Moreover, studies on patterns of variation in secondary compound production in relation to ecological variation are relatively rare. Thus, we studied EO variation in the roots of *Ferula assa-foetida*, a plant that produces, besides EOs, many other secondary metabolites from different chemical classes in high amounts. The environmental factors promoting these compounds might superimpose a clear correlation of environmental factors and EOs. Hence,

in this study, we determined the relation of the variability of EO content and its constituents of *Ferula assa-foetida* populations to geographical, climatic and edaphic conditions.

EO content of *F. assa-foetida* roots ranged from 0.14 to 0.83 %. According to previous studies, there are big variations in EO yields of *F. assa-foetida* obtained from different organs. The EO yield of *F. assa-foetida* roots varied between 0.8 to 1.6 % whereas EO yields obtained from OGRs ranged from 2 to 9 %, respectively, depending on the collection site and the season (Hadavand & Hasanloo, 2014; Kavoosi & Rowshan, 2013; Mirzaei & Hasanloo, 2009). The content of the EO of *F. assa-foetida* in the leaves and fruits varied between 0.04 and 0.49 %, respectively (Bamoniri & Mazoochi, 2009). Thus, plant part, harvesting time and different environmental factors can influence the EO content and its constituents in *F. assa-foetida*.

Based on our results, disulfide components, eudesmane sesquiterpenoids, monoterpenes and other components such as *α*-agarofuran and agarospirol were the major compounds of *F. assa-foetida*. Moghaddam and Farhadi (2015) divided nine populations of *F. assa-foetida* into four main clusters by HCA that did not represent any chemotype whereas Hassanabadi et al. (2019) distinguished four chemotypes of *F. assa-foetida* accessions. We applied HCA and PCA to determine the chemotypes of the ten *F. assa-foetida* populations studied in correspondence with their major EO components and recognized three different chemotypes, namely type I: monoterpenes and *Z*-1-propenyl *sec*-butyl disulfide; type II: eudesmane sesquiterpenoids and *α*-agarofuran; and type III: *Z*- and *E*-1-propenyl *sec*-butyl disulfide. These classes showed the predominant constituents in the studied populations of *F. assa-foetida* growing in Iran. The variations in the phytochemical profile of *F. assa-foetida* populations can be used for a vast selection of chemotypes of this valuable species for domestication and breeding programs for agricultural, medical and industrial purposes.

The presence of specific compounds and of chemotypes found in our study are according to previous reports, where *E*-1-propenyl-*sec*-butyl disulfide, *Z*-1-propenyl-*sec*-butyl disulfide, *α*-pinene, *β*-pinene, thiophene, agarospirol, germacrene D, 7-*epi*-*α*-eudesmol and 10-*epi*-*γ*-eudesmol were described as main components of *F. assa-foetida* (Delavar et al., 2014; Sahebkar & Iranshahi, 2011). In compliance with our results, disulfide components especially *Z*- and *E*-1-propenyl-*sec*-butyl disulfide, the main compound of the Hotkan and Qaleasgar populations (Chemotype III), have been detailed as the most abundant component of EO of *Ferula* species in previous studies (Kanani et al., 2011; Khajeh et al., 2005; Takeoka, 2001). In our study, plants of Chemotype I, represented by Azeran, Neyriz and Yasuj populations, contained higher amounts of monoterpenes and lower quantities of eudesmane sesquiterpenoids. Among different *Ferula* species, monoterpenes such as *α*-pinene, *β*-pinene, limonene, camphene and myrcene were found to be the main components in the oils of, e.g. *F. gummosa* (Kouyakhi et al., 2008), *F. badrakema* (Asili et al., 2009), and *F. szowitsiana* (Dehghan et al., 2007). Contrariwise, populations from Jandaq, Ashkezar, Taft, Tabas and

Khajuei (Chemotype II) were rich in eudesmane sesquiterpenoids, while monoterpenes were minor constituents in these populations. Mirzaei and Hasanloo (2009), reported 10-*epi*-*γ*-eudesmol and 7-*epi*-*α*-eudesmol as two of the major components of the EO of *F. assa-foetida*. Bahrami et al. (2013) determined *epi*-*α*-cadinol, 5-*epi*-7-*epi*-*α*-eudesmol, germacrene B and *δ*-cadinene as main components of this species.

Our results show that the environmental metabolomics approach is a useful tool to predict the content of EO compounds of *F. assa-foetida* populations with respect to the different environmental conditions. However, for some environmental factors the predictive power is not as good for total EO content or presence of specific compounds as in plants with a high EO content (Karimi et al., 2020). The survival and propagation of plants under harsh environmental conditions, such as high solar radiation, temperature, drought, or lack of essential nutritional conditions, are depended on the capacity of the plant to employ multifaceted physiological and morphological mechanisms in order to adjust to the conditions to which they are exposed (Thompson, 2005). Plant chemical responses, e.g. adjusting the levels of secondary metabolites in the plant tissues are thus varying with environmental factors and resource accessibility (Ncube et al., 2012). For example, the sample regions are characterized by very low total nitrogen content, partly resulting from a steep slope, and might thus not display enough variation in available nitrogen to show effects on EO content (Karimi et al., 2020; Karchegani et al., 2012). It is suggested that a high C/N ratio led to an allocating of carbon resources to the synthesis of terpene defense metabolites without growth reduction (Ormeño et al., 2008). High soil nitrogen or fertilization can enhance leaf EO content in Mexican oregano (Martínez-Natarén et al., 2012), or decrease it in *Tanacetum vulgare*, while EOs in the roots were not affected (Kleine & Müller, 2013). Several studies have reported that environmental conditions affect EO content and its constituents in medicinal species like *Arnica montana* L. (Albert et al., 2009), *Zataria multiflora* Boiss. (Karimi et al., 2020), and *Ferula xylorhachis* Rech. (Mazangi et al., 2016).

In our study, the Pearson correlations indicated no effect of environmental factors on total EO content. In a similar approach on *F. assa-foetida* accessions and EO content of OGRs, no correlations were observed between temperature, altitude, precipitation rate and EO yield (Hassanabadi et al., 2019). However, Moghaddam and Farhadi (2015) reported that EO yields increase with increasing temperature in regions with lowest altitude and precipitation rate. In our study, edaphic factors (mainly the content of K, Fe and Al) show a highly significant positive correlation with monoterpenes, while Ca was significantly positively correlated with *γ*-eudesmol, 7-*epi*-*α*-eudesmol and *Z*-1-propenyl-*sec*-butyl disulfide. It is also known from other studies that macro and micro nutrients in the soil can affect EO content and modulate its composition, e.g. in *Mentha spicata* L., K level affects EO oil composition (Chrysargyris et al., 2017), while Fe level increases EO and menthol content in *Mentha arvensis* L. (Misra

& Sharma, 1991). Ca fertilization is known to change EO composition in sweet basil (Dzida, 2010), while an increase in soil Ca content has an increased EO and carvacrol and a decreased linalool content in *Zataria multiflora* (Karimi et al., 2020). An increase in the amount of Al in the soil of habitats of *Thymus pulegioides* was accompanied by decrease in the amount of EOs (Vaičiulytė et al., 2017).

SEMs are a valid tool to predict the chemical profile of a species and its different chemotypes on the basis of environmental conditions (Karimi et al., 2020). As shown in the present study, EO content and EO constituents were affected by different environmental conditions. Mainly the soil type is directly or indirectly affecting EO content and composition, while the geographic location determines the soil type. For example, the interaction between soil clay percentage and K, Fe and Al content in increasing plant α-pinene as shown by Pearson correlations can be explained by the SEM for monoterpenes. Here clay percentage is determining K, Fe and Al content directly and thus has an indirect effect on monoterpene content. It is known that clay percentage can determine the amount of exchangeable K (Addiscott & Johnston, 1975). Our SEM model revealed a negative effect of clay percentage on soil pH and an increase in EO content with increasing soil pH-value. The latter relationship has been shown for roots of the bonnet bellflower (Lee et al., 1998), and also for leaves of geranium (Ram et al., 1997). Average daily air and soil temperatures had a positive and altitude a negative effect on EO content in wild Sicilian oregano populations (Tuttolomondo et al., 2014). According to our SEM model EO content in roots of wild Iranian *F. assa-foetida* populations is decreasing with average daily air temperature, which is decreasing with increasing altitude. The altitude therefore has no direct but an indirect influence on the EO content via a reduced temperature. Latitude and longitude (together with altitude) reflect soil type and composition and temperature, but may also reflect non-considered sources of variations associated to geography, which may encompass environmental or soil variables not measured here, but that are affecting EO content and composition of *F. assa-foetida*.

Rapid and reliable identification of chemotypes concerning authenticity and quality is crucial for selecting plants or plant parts as appropriate raw material for pharmaceutical or agricultural use. Spectroscopy techniques as fast and easy handling technologies are nowadays widely applied directly on plant material for qualitative and semi-quantitative characterization. Different studies describe the application of NIRS, IRS and Raman for differentiation of chemotypes and prediction of EO composition in various medicinal and aromatic plants (Karimi et al., 2020; Bittner et al., 2017; Krähmer et al., 2013). In contrast to GC-analysis of isolated EO, sample classification by NIR spectroscopy of root material is affected by the entire root composition, fiber and water content and in some cases also root shape. For example, the different root shape of plants from the Tabas population, which had very thin and small roots compared to all other samples might have affected NIR reflection

resulting in separate clustering as shown in PCA (Figure S1). For further investigation, a better homogenization should be performed, i.e. by milling the roots for reducing impacts of sample shapes. Since in NIRS not individual compounds but absorption patterns caused by functional groups were considered, a different clustering compared to GC is obtained (Figure S2). For example, C-C double bonds were present in all compound classes (monoterpenes, eudesmane sesquiterpenoids, unsaturated disulfides), OH groups in both terpenoid classes. Therefore, NIRS classification reflects the diversity of many chemical structures, not individual compounds or classes alone. Although, impacts of the root matrix (e.g. lignin, cellulose or starch) further contribute to spectral diversity, NIRS of *F. assa-foetida* roots offers fast evaluation of compositional diversity and allows differentiation of specific populations regarding overall EO content (highest in the Tabas population). For improving the prediction models, more homogeneous material needs to be used for reducing physical reflection impacts due to varying sample shape as discussed before. The highly varying EO composition results in low prediction quality for most of the individual compounds. Nevertheless, for some of the main components promising models could be obtained as shown in Table 4. For possible application in quality control or product authentication further measurements are needed with an enlarged sample set covering the individual concentration ranges homogeneously.

5. Conclusion

In conclusion, the huge phytochemical diversity of medicinal and aromatic plant species varies with climatic and environmental conditions and with genetic factors. To fully utilize the pharmacological, industrial, therapeutic and agricultural properties of these species, the determination of individual chemotypes or populations with specific chemical features is a prerequisite. *F. assa-foetida* is an important species not only due to pharmacological properties but is also a popular spice and can be a source of compounds with agricultural relevance. Although EOs are not the main secondary compounds in *F. assa-foetida*, the variation in EO content and the composition in *F. assa-foetida* populations in Iran divides the populations into three different chemotypes. An environmental metabolomics approach using GC−MS or NIR-spectroscopy together with statistic models like SEMs can help to identify the effect of environmental factors on EO content and its components. This information can be used to predict and select populations with distinct chemotypes or bioactive constituents for use in breeding purposes, biological protection of agricultural plants or other bioeconomy needs.

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Appendix. Supplementary data

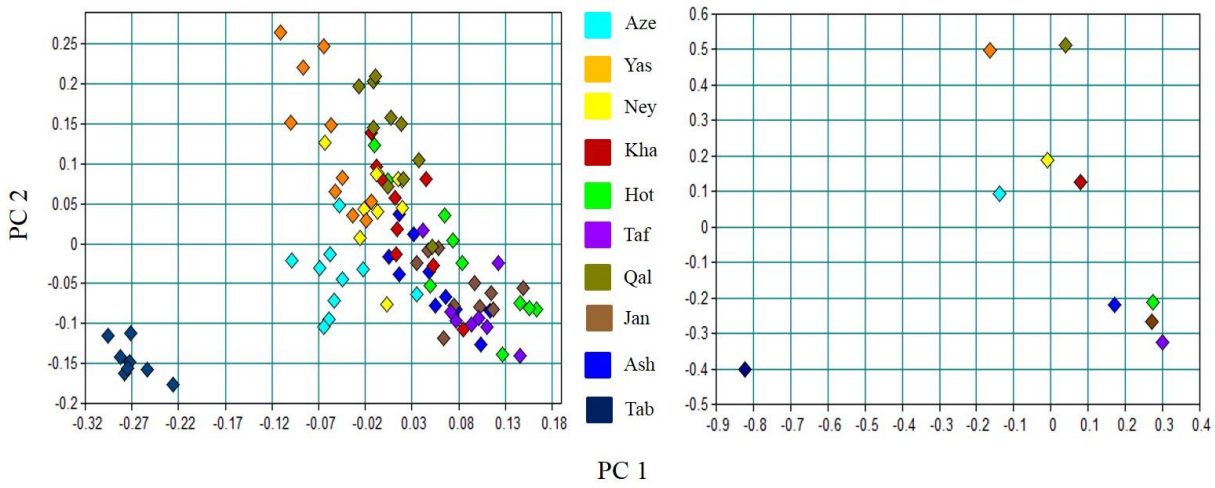

Figure S1. Score plot (PCA) of NIR spectra of the raw root material of the investigated populations of *Ferula assa-foetida*. Spectral range of 3,800 to 9,000 cm^{-1} was used and spectra were treated by 1st derivative. Mean spectra per plant (left) and per population (right) were investigated.

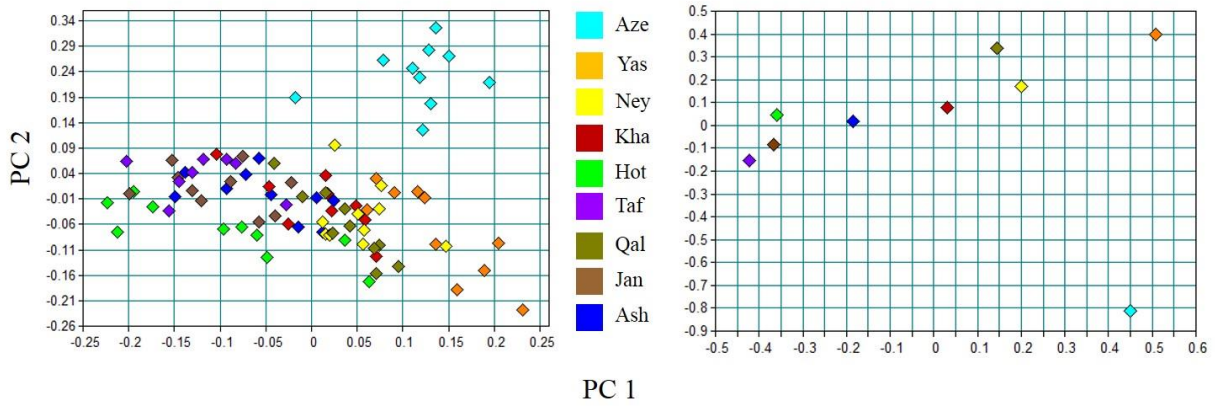

Figure S2. Score plot (PCA) of NIR spectra of the raw root material of the investigated populations of *Ferula assa-foetida* without Tabas population. Spectral range of 3,800 to 9,000 cm^{-1} was used and spectra were treated by 1st derivative. Mean spectra per plant (left) and per population (right) were investigated.

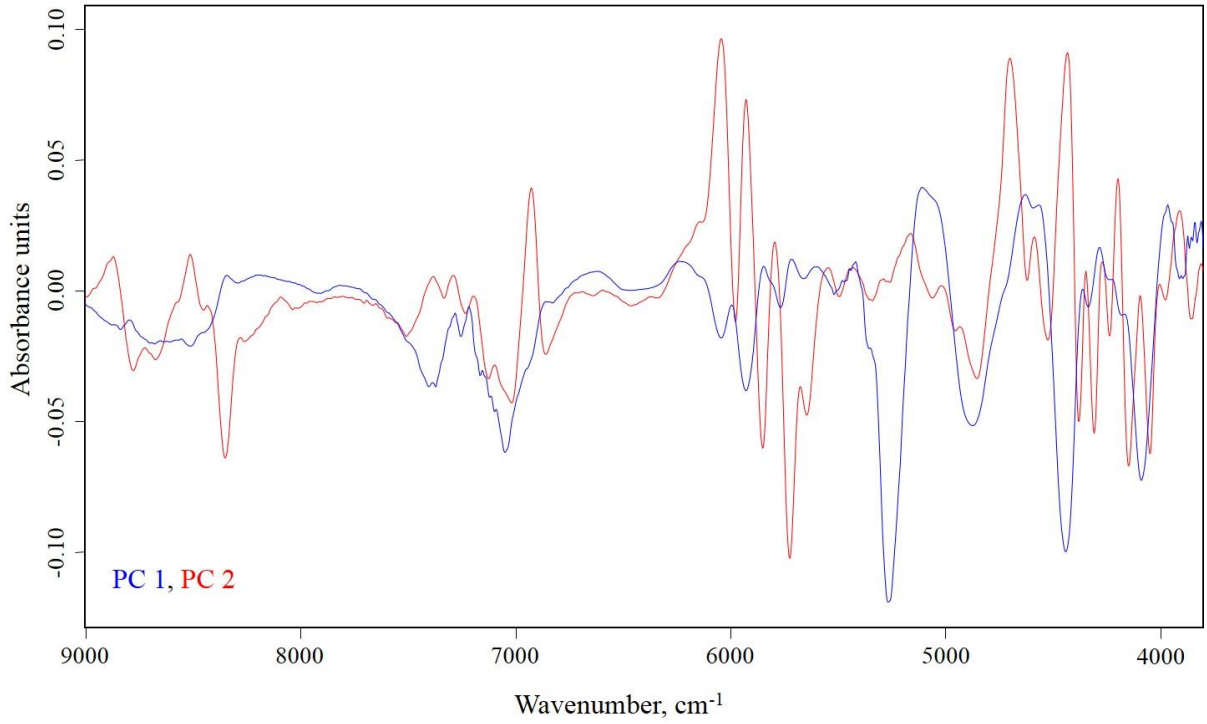

Figure S3. Loading spectra for PC 1 and PC 2 for the score plot (PCA) shown in Figure S2 (right) for *Ferula assa-foetida* population, Spectral range of 3,800 to 9,000 cm^{-1}, 1^{st} derivative spectra.

Variation of secondary metabolite profile of *Zataria multiflora* Boiss. populations linked to geographic, climatic and edaphic factors.

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Abstract

Geographic location and connected environmental and edaphic factors like temperature, rainfall, soil type and composition influence the presence and the total content of specific plant compounds as well as the presence of a certain chemotype. This study evaluated whether geographic, edaphic and climatic information can be utilized to predict the presence of specific compounds from medicinal or aromatic plants. Furthermore, we tested rapid analytical methods based on near infrared spectroscopy (NIR) coupled with gas chromatography/flame ionization (GC−FID) and gas chromatography/mass spectrometry (GC−MS) analytical methods for characterization and classification metabolite profiling of *Zataria multiflora* Boiss. populations. *Z. multiflora* is an aromatic, perennial plant with interesting pharmacological and biological properties. It is widely dispersed in Iran as well as in Pakistan and Afghanistan. Here, we studied the effect of environmental factors on essential oil (EO) content and the composition and distribution of chemotypes. Our results indicate that this species grows predominantly in areas rich in calcium, iron, potassium and aluminum, with mean rainfall of 40.46 to 302.72 mm year^{-1} and mean annual temperature of 14.90 to 28.80 °C. EO content ranged from 2.75 to 5.89 %. Carvacrol (10.56-73.31 %), thymol (3.51-48.12 %), linalool (0.90-55.38 %) and *p*-cymene (1.66-13.96 %) were the major constituents, which classified 14 populations into three chemotypes. Corresponding to the phytochemical cluster analysis the hierarchical cluster analysis (HCA) based on NIR data also recognized the carvacrol, thymol and linalool chemotypes. Hence, NIR has the potential to be applied as a useful tool to determine rapidly the chemotypes of *Z. multiflora* and similar herbs. EO and EO constituent content correlated with different geographic location, climate and edaphic factors. The structural equation models (SEMs) approach revealed direct effects of soil factors (texture, phosphor, pH) and mostly indirect effects of latitude and altitude directly affecting e.g. soil factors. Our approach of identifying environmental predictors for EO content, chemotype or presence of high amounts of specific compounds can help to select regions for sampling plant material with the desired chemical profile for direct use or for breeding.

Keywords: near infrared spectroscopy; essential oil; carvacrol; linalool; chemical diversity; environmental factors; soil chemistry; *Zataria multiflora* Boiss.

1. Introduction

All over the world, plants face different local climatic regimes as well as different edaphic factors. To predict how different environmental factors affect species dispersal, the abundance of populations and chemotypes as well as the content of specific compounds can be a valuable tool to understand plant variation in chemical features. It can also facilitate prospecting plants with high amounts of specific compounds for nutrition, pharmaceutical or agricultural use. In most cases, plant essential oils (EOs) are characterized by a strong aroma, which is mainly produced by secondary metabolites. EO compounds are coupled with environmental acclimatization and play vital biological roles. Several factors such as environmental and edaphic conditions, geographical regions, season of collection, harvesting time, genotype and ecotype influence the quantitative and qualitative composition of EO (Morshedloo et al., 2018; Zgheib et al., 2016; Milos et al., 2001). For example, in Matricaria chamomilla L. climatic conditions, altitude, soil properties and irrigation influence the phytochemical composition and antioxidant activity of EO (Formisano et al., 2015).

Zataria multiflora Boiss. (Lamiaceae) is an aromatic and perennial shrub growing wild in Iran, Pakistan and Afghanistan. This aromatic plant is known by the Persian name of *Avishan Shirazi*, which is also entitled Sattar or Zattar, meaning thyme. *Z. multiflora* can be identified by the orbicular, densely gland-dotted, grey-green ovate leaves and the thickly white hairy round buds in the leaf axils. Its inflorescence is verticillate, and the flowers are very small and white (Simbar et al., 2008). *Z. multiflora* has shown pharmacological (antimicrobial, antinociceptive, spasmolytic and anti-inflammatory) properties, is utilized in traditional folk remedies for its antiseptic, analgesic, carminative, anthelmintic and antidiarrheal properties and it is also a condiment (Mohajeri et al., 2018; Khazdair et al., 2017; Moazeni et al., 2014; Iranian Herbal Pharmacopoeia, 2002). Currently, some pharmaceutical forms of this plant, such as syrups, oral drops, soft capsules and vaginal creams are produced (Mahboubi, 2019; Sajed et al., 2013).

The EO of *Z. multiflora* is rich in phenolic oxygenated monoterpenes. The main chemical constituents are carvacrol, thymol, linalool and *p*-cymene (Mahmoudvand et al., 2017; Saedi Dezaki et al., 2016; Hadian et al., 2011a). Although there are some studies based on *Z. multiflora* EO constituents (Niczad et al., 2019; Saleem et al., 2004), there is hardly any information on the environmental factors affecting EO content and composition. *Z. multiflora* is not only harvested for local markets but is also one of the valuable species for industry, so this plant is under severe threat from overharvesting. Thus, a deep perception of its phytochemical and environmental characteristics in its natural habitats is crucial to foretell its behavior under man-made cultivation. Today, the standard method for EO analysis is gas chromatography coupled with different detection techniques like mass spectrometry. In the

last two decades, numerous vibrational spectroscopy methods including mid-infrared (IR), near-infrared (NIR), and Raman spectroscopy have been described as a useful tool to examine the plant secondary metabolites which are commonly applied in the chemical fingerprinting of plants (Gudi et al., 2014; Schulz et al., 2005, 2004). However, up to now, no studies have been performed utilizing this capable approach to differentiate and characterize various *Z. multiflora* chemotypes.

The aim of this study was to evaluate how different environmental factors affect species dispersal with respect to EO production, chemotype as well as the content of specific compounds of *Z. multiflora* populations. Besides, we aimed to evaluate whether geographic, edaphic and climatic information can predict the presence of specific compounds. Furthermore, we tested rapid analytical methods based on NIRS coupled with GC−FID/GC−MS methods for characterization and classification metabolite profiling of *Z. multiflora* populations.

2. Materials and Methods

2.1. Study area

To determine the effects of geography, climate and edaphic conditions on EO yield and composition of *Z. multiflora*, plant materials were collected in 2018 in 14 natural habitats across five provinces from the center to the south of Iran including their major growing areas Isfahan, Kerman, Yazd, Fars and Hormozgan provinces (Figure 1).

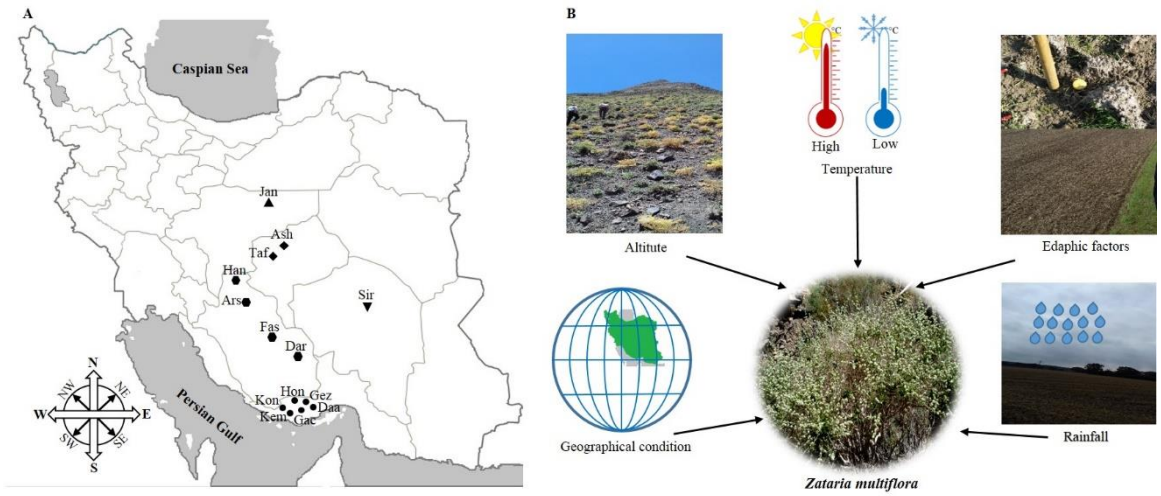

Figure 1. Collection sites (A) and overview on geographic, climatic, and edaphic factors (B) affecting *Zataria multiflora* populations from Iran.

2.2. Plant material and chemicals

Plant samples were collected in June 2018 at the flowering stage. At each region, 6-11 individual shrubs were collected depending on the population size with a minimum distance of 100 m. Voucher specimens (No. MPH-1799) were authenticated and deposited in the Herbarium of Medicinal Plants and Drugs Research Institute (MPH), Shahid Beheshti University, Tehran, Iran. Geographical data and altitude for each sampling area were recorded using GPS (Table 1). Besides, climate data for five years were taken from metrological stations closest to the habitats (Table 2). Carvacrol, linalool, *p*-cymene and γ-terpinene were purchased from Sigma-Aldrich-Fluka (Germany), and thymol and α-pinene from Roth (Germany).

Table 1. General information on natural habitats of *Zataria multiflora* populations.

Population name	Code	Province	Location	Latitude (N)	Longitude (E)	Altitude (m)
Jandaq	Jan	Esfahan	Jandaq toward mesr desert	33° 57' 44''	54° 31' 02''	1235
Ashkezar	Ash	Yazd	Zarband village	31° 48' 49''	54° 00' 26''	1946
Taft	Taf	Yazd	Darreh-ye Gahan mountains	31° 42' 26''	54° 10'	1697
Siriz	Sir	Kerman	Hamsij village	30° 55' 43''	55° 57' 01''	1763
Fasa	Fas	Fars	Kohankouye village	28° 59' 27''	53° 42' 25''	1516
Arsenjan	Ars	Fars	Tange laykhare mountains	29° 53' 49''	53° 16' 20''	1865
Haneshk	Han	Fars	Haneshk village, Safashahr	30° 49' 16''	53° 18' 19''	1898
Darab	Dar	Fars	Tange Talar Jangi mountains	28° 44' 27''	54° 34' 41''	1276
Gezeh	Gez	Hormozgan	Cheshmeh-ye seyyed	27° 06' 35''	54° 04' 46''	731
Hongooyeh	Hon	Hormozgan	Darreh-ye Baraveh	27° 06' 19''	54° 04' 07''	820
Daarbast	Daa	Hormozgan	Daarbast	26° 58' 02''	54° 01' 59''	1009
Gachooyeh	Gac	Hormozgan	Gachooyeh	26° 58' 28''	53° 58' 06''	1055
Kemeshk	Kem	Hormozgan	Kemeshk	27° 03' 13''	53° 50' 41''	937
Konar Siah	Kon	Hormozgan	Konar Siah	27° 09' 05''	53° 57' 04''	981

2.3. Soil analysis

Soil samples from the surface layer (0 to 30 cm depth) were taken from five randomly selected plots in each sampling site. The five soil samples were combined into a single 500 g sample that was dried at room temperature (20-25 °C) and sieved to 2 mm. A duplicate soil sample was sieved through a 2 mm filter once again for determination of soil chemical characteristics including the soil texture (percentage content of sand, silt, and clay), the amount of abundant nutrients (N, P, K, Ca, Al and Fe), pH-value and organic matter. The total heavy metal and nutrient contents of soil samples were determined after pressure dissolution with 69 % supra

pure nitric acid (according to A2.4.3.1, VDLUFA, 1991) by ICP-AES (iCAP™ 7600 Duo, Thermo Fischer Scientific). Contents of total carbon and total nitrogen were determined with CNS elemental analyzer (Vario EL Cube, Elementar Analysesysteme GmbH). Pedological base parameters (soil particle size, pH-value, C/N) were collected for characterization. The particle size determination of soil texture was performed according to DIN 19683-2 (1997).

Table 2. Edaphic factors and climatic characteristics in natural habitats of *Zataria multiflora*.[a]

Region	Rainfall	M-Temp	Soil texture	pH	OM	N	P	K	Ca	Fe	Al
	mm/year	[°C]			[%]	[%]			[mg/kg]		
Jandaq	55.86	21.50	silty sand	7.70	4.43	0.06	394	5581	69376	16424	17012
Ashkezar	40.46	21.10	loamy sand	7.80	5.17	0.04	409	7171	155569	14486	20489
Taft	49.76	20.30	sandy loamy silt	7.70	5.74	0.04	354	7925	112373	19637	20137
Siriz	107.80	20.20	sandy loam	7.70	4.44	0.03	416	6863	75182	20339	20521
Fasa	278.48	20.30	clayey loam	7.60	6.97	0.06	329	8441	96355	23471	27656
Arsenjan	215.22	20.30	sandy loamy silt	7.70	7.54	0.18	346	6151	117935	20298	20936
Haneshk	180.06	14.90	loamy sand	7.60	4.00	0.09	342	12427	12756	24538	34280
Darab	276.38	24.30	silty loam	7.60	10.0	0.23	312	5306	116218	16820	18424
Gezeh	302.72	28.80	sandy loam	7.80	7.02	0.05	293	3609	174266	11979	12212
Hongooyeh	302.72	28.80	silty loamy sand	7.90	7.54	0.03	355	3640	139953	14064	12378
Daarbast	302.72	28.80	sandy loam	7.80	6.59	0.03	232	1993	197820	8190	6945
Gachooyeh	302.72	28.80	loamy sand	7.80	7.02	0.03	222	2368	195214	9272	7986
Konar Siah	302.72	28.80	loamy sand	7.80	7.02	0.05	275	2272	178396	7442	6327
Kemeshk	302.72	28.80	loamy sand	7.80	7.02	0.04	249	2320	186805	8357	7157

[a]M-Temp, Mean annual temperature; OM, organic matter.

2.4. Isolation of the essential oils

The aerial plant parts were dried at room temperature (20-25 °C) in the shade, then the leaves of each plant were separated and 10 g of each plant sample were ground manually. The EO of each sampled plant (10 g of leaves) was isolated by hydro-distillation for 2 h utilizing a clevenger-type system (Pavela et al., 2018). The distilled oils were dried over anhydrous sodium sulfate and stored at 4 °C in sealed glass vials for analysis. The yield of the essential oil was calculated based on the dry weight of the plant material.

2.5. GC−FID and GC−MS analyses

EOs were analyzed by GC−FID using an Agilent gas chromatograph 6890N, equipped with a HP-5 column (30 m × 0. 25 mm i.d., with a film thickness of 0.5 μm). The oven temperature was programmed at 50 °C for 2 min, then from 50 to 320 °C at 5 °C min^{-1}, and held at 320 °C for 6 min.

Both injector and detector temperatures were 250 °C. Hydrogen was used as carrier gas with a constant flow rate of 1 mL min^{-1}, and one µl of the diluted EOs (1/500 v/v in isooctane) was injected automatically (Gerstel MPS) in a splitless mode. Nitrogen was used as make-up gas, which was set at a flow of 45 mL min^{-1}.

Mass spectrometry of the EOs was performed using an Agilent MSD 5975B/GC 6890N, equipped with a 30 m × 0.25 mm i.d., 0.5 µm, HP-5MS column. The injector temperature was 250 °C and the initial GC oven temperature was 50 °C, held for 2 min, then raised to 320 °C at 5 °C min^{-1} and held for 6 min. Helium was used as carrier gas with a flow rate of 1 mL min^{-1}. One µl of the diluted EOs (1/500 v/v in isooctane) was injected automatically (Gerstel MPS) in a splitless mode. Injector and detector temperatures were set at 250 °C. The EI^{+}-MS operating parameters were as follows: ionization energy, 70 eV and ion source temperature 230 °C. The quadrupole mass spectrometer was scanned over 35 to 350 *m/z*. The runtime and solvent delay were set at 60 min and 5 min, respectively (4.45 scans/s). Carvacrol, thymol, linalool, *p*-cymene, *γ*-terpinene and *α*-pinene were used as standard. 6-Methyl-5-hepten-2-one was used as internal standard and was added to the dilution before the analysis. The oil components were identified by comparison of mass spectra and retention indices with those recorded in the Adams (Adams, 2014), NIST mass spectral databases SRD 69 (NIST Chemistry WebBook, 2002), standard constituents and the previously published data. The retention indices of individual components were calculated using a series of n-alkanes (C8-C40) (Sigma-Aldrich-Fluka, Germany) (1/100 in n-Pentan). The relative percentage composition of individual compounds was computed from the GC peak areas obtained without using correction factors.

2.6. NIR spectroscopy and Chemometrics

Before isolation of EO, vibrational spectroscopy was performed directly on the homogenized plant material. NIRS analyses were carried out on a Fourier-Transform (FT)-NIR spectrometer (Multi-Purpose Analyser MPA, Bruker Optics GmbH, Germany). Spectra were recorded in the wavenumber range of 4,000-12,000 cm^{-1} with a spectral resolution of 8 cm^{-1}. Approximately 7 g of dried leaves were put in a glass Petri dish and spectra were collected during rotation of the dish using the integrating sphere for measuring in diffuse reflection. Spectra were acquired at 30 seconds. Each sample was analyzed with threefold repetition. The raw spectra were centered and corrected for scattering effects and baseline shifting using WMSC of the OPUS 6.5 software (Bruker Optics). Only averaged spectra of the three replicates were used for the later chemometric analysis.

2.7. Statistical analysis

Statistical analysis was performed using hierarchical cluster analysis (HCA) with SPSS version 16 to classify and cluster the populations of *Z. multiflora* based on the squared Euclidean distances. Pearson's correlation coefficients were estimated among the EO content, major components and

edaphic factors using SPSS (SPSS, Chicago, IL, USA) software package from version 16. The calculation of means, standard deviations (SD) and t-test were used to express the significance of differences ($p < 0.05$) using SAS 9.1 program (SAS Inc. USA). For chemometrics (based on NIR), HCA was performed to evaluate the diversity of the samples. Characteristic spectral ranges were identified by comparison with spectra appropriate reference standards and HCA. Calibration models were built by 10-fold cross-validation using a partial least squares (PLS) algorithm. Therefore, GC data of each plant and averaged plant wise spectra of the population were correlated.

Furthermore, we set up structural equation models (SEMs) for each region using partial least squares (PLS) regression using Warp PLS 6.0 (Kock & Lynn, 2012). The PLS regression was chosen over covariance based approaches because it suited our small sample size and, compared to covariance structure analysis, can accommodate both reflective and formative scales more easily. Moreover, PLS does not require any a priori distributional assumptions (Chin & Newsted, 1999). We present individual standardized path coefficients (β), partial model fit scores (R^2), and overall model *p*-values calculated by resampling estimations coupled with Bonferroni like corrections (Kock, 2010). To validate the models three model-fit indices [average path coefficient (APC), average R-squared (ARS), and average variance inflation factor (AVIF)] were calculated for each region. For model fit, it is recommended that *p*-values for APC and ARS are both lower than 0.05 (i.e. significance at the 0.05 level). The AVIF index controls for multicollinearity and should be below 5 (Kock, 2010). In the SEM analysis we set paths from geographic factors (latitude, longitude, altitude), climatic factors (rainfall, temperature), soil texture (relative proportion of clay, silt and sand), constituents (N, P, K, Al, Ca, Fe) and pH-value directly to EO content and compounds; furthermore, we included the possible effects of the geographic factors on climatic and soil factors.

3. Results

3.1. Phytochemical analysis of essential oil

The EOs were obtained and analyzed by hydro-distillation and GC−FID/GC−MS respectively. There was a significant difference in EO content among the studied populations. The EO content ranged from 2.75 (for population Siriz) to 5.89 % in dry matter (DM), (for population Konar Siah) (Figure 2). Fifty-six compounds were identified with significant differences between the populations (Table 3). The oils mainly consisted of carvacrol (10.56-73.31 %), thymol (3.51-48.12 %), linalool (0.90-55.38 %), *p*-cymene (1.66-13.96 %), γ-terpinene (0.99-6.28 %), α-pinene (0.93-4.01 %), carvacrol methyl ether (0.39-3.71 %), myrcene (0.94-2.77 %), *E*-caryophyllene (1.09-2.37 %) and α-terpinene (0.39-1.61 %).

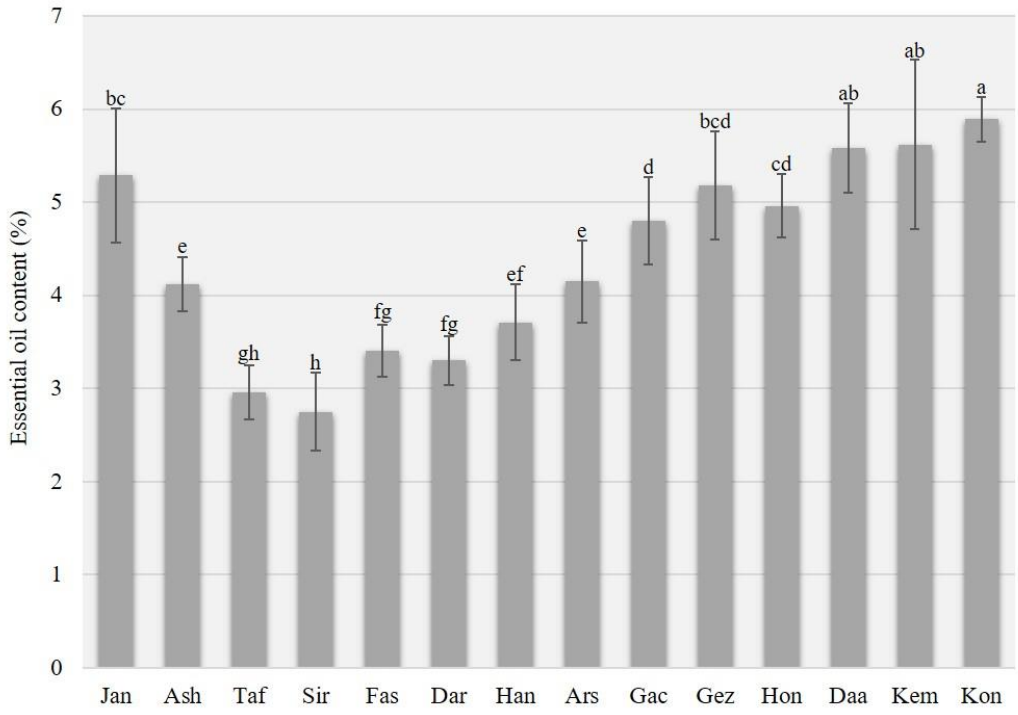

Figure 2. Essential oil content of *Zataria multiflora* populations.

The Pearson correlations indicated positive and negative significant correlations between phytochemical compounds. Carvacrol had been positively correlated with carvacrol acetate (r = 0.70), carvacrol methyl ether (r = 0.54), and negatively correlated with linalool (r = -0.69), thymol (r = -0.64) and limonene (r = -0.79) while thymol was in significant negative correlation with carvacrol (r = -0.64). Furthermore, linalool had a significant positive correlation with *E*-*β*-ocimene (r = 0.99), myrcene (r = 0.97), limonene (r = 0.72), *Z*-*β*-ocimene (r = 0.69) and a negative correlattion with *α*-terpinene (r = -0.72), *γ*-terpinene (r = -0.70), carvacrol (r = -0.69), *p*-cymene (r = -0.61), carvacrol acetate (r = -0.56) and carvacrol methyl ether (r = -0.53).

To determine the degree of phytochemical variation, HCA based on the phytochemical profiles was performed (Figure 3). According to the major components, three chemotypes can be distinguished thus populations of *Z. multiflora* were divided into three main clusters. Cluster I consist of two populations (Siriz and Haneshk) characterized by higher content of linalool. Cluster II contains two populations (Fasa and Darab) which are characterized by higher amounts of thymol, carvacrol, *p*-cymene and linalool. Cluster III contains ten populations including Jandaq, Ashkezar, Taft, Arsenjan, Gezeh, Hongooyeh, Daarbast, Gachooyeh, Konar Siah and Kemeshk characterized by lower quantities of *α*-pinene, myrcene, *α*-terpinene, linalool and carvacrol methyl ether and higher amounts of carvacrol, thymol, *p*-cymene and *γ*-terpinene.

Table 3. Variation of the phytochemical compositions (%) among the studied populations of *Zataria multiflora*.[a]

compound	RI[b]	RI[c]	population				
			Jandaq	Ashkezar	Taft	Siriz	Fasa
α-thujene	933	929	0.43 ± 0.06	0.18 ± 0.10	0.41 ± 0.03	0.14 ± 0.05	0.30 ± 0.03
α-pinene	940	939	0.93 ± 0.28	1.82 ± 0.23	2.01 ± 0.14	1.85 ± 0.33	1.64 ± 0.18
camphene	955	954	0.10 ± 0.03	0.12 ± 0.01	0.12 ± 0.01	0.10 ± 0.01	0.10 ± 0.01
2,4(10)-thujadiene	960	957	-	-	-	tr	-
1,3-octanol	969	973	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.09 ± 0.01	0.08 ± 0.01
sabinene	979	977	0.11 ± 0.01	0.07 ± 0.01	0.11 ± 0.01	0.09 ± 0.02	0.08 ± 0.01
β-pinene	983	978	0.23 ± 0.03	0.28 ± 0.03	0.37 ± 0.01	0.34 ± 0.06	0.33 ± 0.01
myrcene	995	992	1.35 ± 0.51	1.15 ± 0.16	1.39 ± 0.14	2.77 ± 0.51	1.45 ± 0.17
3-octanol	997	995	0.05 ± 0.01	0.06 ± 0.01	0.10 ± 0.05	0.21 ± 0.04	0.10 ± 0.06
α-phellandrene	1010	1005	0.15 ± 0.04	0.17 ± 0.01	0.19 ± 0.01	0.29 ± 0.03	0.21 ± 0.01
δ-3-carene	1015	1011	0.02 ± 0.01	0.04 ± 0.02	0.02 ± 0.01	0.03 ± 0.01	-
α-terpinene	1021	1019	0.92 ± 0.08	1.00 ± 0.07	1.38 ± 0.26	0.39 ± 0.14	1.61 ± 0.09
p-cymene	1030	1025	5.88 ± 1.38	5.69 ± 0.31	7.29 ± 1.17	1.66 ± 0.95	7.21 ± 0.42
limonene	1034	1030	0.37 ± 0.10	0.49 ± 0.07	0.54 ± 0.12	0.81 ± 0.07	0.61 ± 0.03
1,8-cineole	1037	1035	0.24 ± 0.10	0.05 ± 0.01	0.08 ± 0.01	0.46 ± 0.10	0.03 ± 0.01
Z-*β*-ocimene	1039	1039	0.28 ± 0.08	0.41 ± 0.04	0.44 ± 0.10	0.69 ± 0.21	0.37 ± 0.05
E-*β*-ocimene	1050	1050	0.15 ± 0.10	0.07 ± 0.01	0.12 ± 0.04	1.31 ± 0.12	0.28 ± 0.05
γ-terpinene	1063	1062	3.65 ± 0.72	3.14 ± 0.26	5.24 ± 1.12	0.99 ± 0.57	6.28 ± 0.40
Z-sabinene hydrate	1071	1070	0.30 ± 0.01	0.14 ± 0.06	0.27 ± 0.03	0.08 ± 0.02	0.18 ± 0.01
Z-linalool oxide	1076	1074	0.04 ± 0.01	0.02 ± 0.01	0.05 ± 0.01	1.67 ± 0.57	0.15 ± 0.03
terpinolene	1092	1089	0.22 ± 0.09	0.21 ± 0.03	0.27 ± 0.10	1.62 ± 0.42	0.35 ± 0.03
linalool	1103	1100	1.19 ± 0.23	1.62 ± 0.60	3.35 ± 2.20	55.38 ± 6.4	9.59 ± 1.83
E-*γ*-caryophyllene	1106	1106	0.11 ± 0.04	0.04 ± 0.01	0.06 ± 0.04	1.37 ± 0.17	0.27 ± 0.05
1-octenyl-3-acetate	1111	1113	-	-	-	0.20 ± 0.10	-
p-menth-2-en-1-ol	1124	1122	-	-	-	0.32 ± 0.18	-
allo-ocimene	1131	1132	-	-	-	0.16 ± 0.11	0.11 ± 0.02
1,3,8-*p*-menthatriene	1133		-	-	-	0.12 ± 0.01	-
borneol	1172	1171	tr	-	0.02 ± 0.01	0.63 ± 0.18	0.21 ± 0.04
Z-linalool oxide (pyranoid)	1177	1173	0.10 ± 0.01	0.02 ± 0.01	0.05 ± 0.02	0.10 ± 0.01	0.08 ± 0.01
4-terpineol	1183	1179	0.09 ± 0.01	0.14 ± 0.03	0.14 ± 0.01	0.25 ± 0.03	0.15 ± 0.01
p-cymenol-8	1188	1184	0.46 ± 0.04	0.46 ± 0.02	0.49 ± 0.04	0.23 ± 0.03	0.43 ± 0.02
α-terpineol	1195	1190	0.44 ± 0.13	0.56 ± 0.05	0.56 ± 0.11	0.68 ± 0.05	0.45 ± 0.02
Z-dihydro carvone	1202	1200	0.15 ± 0.01	0.17 ± 0.01	0.10 ± 0.03	0.15 ± 0.06	0.06 ± 0.04
nerol	1234	1228	0.07 ± 0.02	0.06 ± 0.01	-	0.10 ± 0.02	-
thymol methyl ether	1238	1237	-	0.08 ± 0.03	0.35 ± 0.19	0.20 ± 0.08	0.88 ± 0.06
carvacrol methyl ether	1248	1241	1.04 ± 0.37	1.50 ± 0.40	1.00 ± 0.21	0.39 ± 0.10	0.49 ± 0.02
geraniol	1260	1263	0.04 ± 0.01	-	0.06 ± 0.03	1.00 ± 0.43	0.11 ± 0.02
geranial	1280	1273	-	-	-	tr	-
p-thymol	1286		0.07 ± 0.03	0.08 ± 0.01	0.10 ± 0.02	0.02 ± 0.01	0.10 ± 0.01
thymol	1295	1295	3.51 ± 3.27	9.94 ± 2.03	25.32 ± 12.7	6.17 ± 3.00	48.12 ± 2.9
carvacrol	1309	1305	73.31 ± 4.3	65.14 ± 1.8	42.23 ± 15.3	10.56 ± 3.9	12.42 ± 2.8
thymol acetate	1358	1359	0.02 ± 0.01	0.10 ± 0.03	0.49 ± 0.27	0.10 ± 0.03	0.87 ± 0.07
carvacrol acetate	1377	1368	0.80 ± 0.21	0.80 ± 0.13	0.60 ± 0.19	0.20 ± 0.07	0.16 ± 0.01
β-bourbonene	1384	1378	-	-	-	0.09 ± 0.04	-
E-caryophyllene	1435	1427	1.09 ± 0.22	1.61 ± 0.21	2.16 ± 0.42	1.56 ± 0.60	1.85 ± 0.20
aromadendrene	1455	1436	0.29 ± 0.06	0.40 ± 0.07	0.37 ± 0.08	0.14 ± 0.10	0.19 ± 0.05
α-humullene	1469	1452	0.10 ± 0.01	0.11 ± 0.01	0.14 ± 0.02	0.14 ± 0.04	0.13 ± 0.01
9-*epi*-*E*-caryophyllene	1477	1474	-	-	-	-	-
E-*β*-guaiene	1504	1498	-	-	-	0.02 ± 0.01	-
viridiflorene	1510	1505	0.24 ± 0.10	0.26 ± 0.05	0.28 ± 0.08	0.19 ± 0.10	0.18 ± 0.02
spathulenol	1594	1578	0.38 ± 0.09	0.35 ± 0.03	0.36 ± 0.06	0.59 ± 0.17	0.39 ± 0.02
isoaromadendrene epoxide	1597		0.08 ± 0.01	0.11 ± 0.01	0.12 ± 0.02	0.47 ± 0.09	0.14 ± 0.02
caryophyllene oxide	1601	1599	0.18 ± 0.03	0.20 ± 0.01	0.20 ± 0.05	0.76 ± 0.11	0.21 ± 0.02
caryophylla-4(12),8(13)-dien-5-*β*-ol	1654	1663	0.07 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.32 ± 0.03	0.11 ± 0.01
14-hydroxy-9-*epi*-*E*-caryophyllene	1673	1669	0.03 ± 0.02	0.07 ± 0.02	0.08 ± 0.01	0.34 ± 0.06	0.09 ± 0.01
khusinol	1686	1674	0.05 ± 0.03	0.13 ± 0.01	0.08 ± 0.01	0.27 ± 0.03	0.09 ± 0.01
Essential oil content (%)			5.29 ± 0.72	4.12 ± 0.29	2.96 ± 0.29	2.75 ± 0.42	3.41 ± 0.28

Table 3. continued,

compound	RI[b]	RI[c]	population				
			Gachooyeh	Kemeshk	Gezeh	Hongooyeh	Arsenjan
α-thujene	933	929	0.29 ± 0.06	0.27 ± 0.11	0.47 ± 0.13	0.41 ± 0.09	0.33 ± 0.03
α-pinene	940	939	2.57 ± 0.74	2.59 ± 0.73	4.01 ± 0.97	3.23 ± 1.15	1.43 ± 0.20
camphene	955	954	0.14 ± 0.02	0.14 ± 0.02	0.11 ± 0.04	0.17 ± 0.04	0.10 ± 0.01
2,4(10)-thujadiene	960	957	-	-	-	-	-
1,3-octanol	969	973	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.07 ± 0.01
sabinene	979	977	0.09 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.10 ± 0.01
β-pinene	983	978	0.47 ± 0.05	0.48 ± 0.06	0.64 ± 0.11	0.57 ± 0.17	0.32 ± 0.02
myrcene	995	992	1.11 ± 0.13	1.04 ± 0.18	1.12 ± 0.17	1.26 ± 0.13	1.32 ± 0.15
3-octanol	997	995	0.13 ± 0.08	0.02 ± 0.01	0.10 ± 0.01	0.15 ± 0.13	0.04 ± 0.02
α-phellandrene	1010	1005	0.16 ± 0.01	0.17 ± 0.01	0.17 ± 0.01	0.17 ± 0.01	0.19 ± 0.02
δ-3-carene	1015	1011	-	0.02 ± 0.01	0.03 ± 0.02	-	0.03 ± 0.02
α-terpinene	1021	1019	1.20 ± 0.14	1.34 ± 0.44	1.17 ± 0.08	1.32 ± 0.20	0.92 ± 0.05
p-cymene	1030	1025	6.46 ± 2.47	5.50 ± 1.54	9.44 ± 1.18	10.27 ± 0.7	5.93 ± 0.48
limonene	1034	1030	0.42 ± 0.07	0.41 ± 0.16	0.58 ± 0.21	0.67 ± 0.09	0.42 ± 0.09
1,8-cineole	1037	1035	0.10 ± 0.06	0.11 ± 0.06	0.08 ± 0.05	0.02 ± 0.01	0.12 ± 0.07
Z-*β*-ocimene	1039	1039	0.40 ± 0.05	0.32 ± 0.13	0.46 ± 0.25	0.55 ± 0.13	0.41 ± 0.08
E-*β*-ocimene	1050	1050	0.04 ± 0.02	0.06 ± 0.02	0.07 ± 0.01	0.08 ± 0.01	0.20 ± 0.06
γ-terpinene	1063	1062	5.45 ± 0.77	6.16 ± 2.30	5.08 ± 0.34	5.57 ± 0.99	3.49 ± 0.23
Z-sabinene hydrate	1071	1070	0.21 ± 0.01	0.11 ± 0.04	0.21 ± 0.02	0.19 ± 0.02	0.26 ± 0.01
Z-linalool oxide	1076	1074	-	-	-	tr	0.09 ± 0.05
terpinolene	1092	1089	0.18 ± 0.01	0.18 ± 0.02	0.20 ± 0.02	0.21 ± 0.02	0.28 ± 0.04
linalool	1103	1100	0.90 ± 0.17	1.01 ± 0.55	1.13 ± 0.22	1.28 ± 0.54	8.88 ± 3.17
E-*γ*-caryophyllene	1106	1106	0.06 ± 0.02	0.05 ± 0.03	0.07 ± 0.01	0.06 ± 0.02	0.11 ± 0.04
1-octenyl-3-acetate	1111	1113	-	0.02 ± 0.01	tr	0.02 ± 0.01	-
p-menth-2-en-1-ol	1124	1122	-	-	-	-	-
allo-ocimene	1131	1132	-	-	-	-	0.09 ± 0.04
1,3,8-*p*-menthatriene	1133		-	-	-	-	-
borneol	1172	1171	-	0.02 ± 0.01	-	-	0.24 ± 0.11
Z-linalool oxide (pyranoid)	1177	1173	-	tr	tr	0.02 ± 0.01	tr
4-terpineol	1183	1179	0.20 ± 0.02	0.20 ± 0.07	0.18 ± 0.02	0.20 ± 0.01	0.17 ± 0.02
p-cymenol-8	1188	1184	0.48 ± 0.04	0.44 ± 0.02	0.50 ± 0.04	0.54 ± 0.03	0.44 ± 0.02
α-terpineol	1195	1190	0.54 ± 0.06	0.47 ± 0.18	0.63 ± 0.30	0.73 ± 0.17	0.47 ± 0.07
Z-dihydro carvone	1202	1200	0.14 ± 0.01	0.12 ± 0.06	0.16 ± 0.02	0.14 ± 0.03	0.13 ± 0.01
nerol	1234	1228	-	tr	0.03 ± 0.02	-	0.06 ± 0.01
thymol methyl ether	1238	1237	0.08 ± 0.01	0.05 ± 0.01	0.13 ± 0.07	0.15 ± 0.06	0.13 ± 0.07
carvacrol methyl ether	1248	1241	1.94 ± 0.78	1.87 ± 1.19	3.58 ± 0.88	2.90 ± 1.67	1.84 ± 0.39
geraniol	1260	1263	0.02 ± 0.02	-	-	0.03 ± 0.02	tr
geranial	1280	1273	-	-	-	-	0.02 ± 0.01
p-thymol	1286		0.10 ± 0.05	0.18 ± 0.10	0.15 ± 0.03	0.17 ± 0.01	0.07 ± 0.01
thymol	1295	1295	10.24 ± 6.6	7.34 ± 4.01	8.72 ± 1.97	14.67 ± 7.8	12.28 ± 2.2
carvacrol	1309	1305	60.26 ± 10.2	64.22 ± 7.5	54.17 ± 6.3	48.16 ± 6.7	55.10 ± 3.1
thymol acetate	1358	1359	0.09 ± 0.07	0.05 ± 0.03	0.19 ± 0.13	0.21 ± 0.06	0.08 ± 0.02
carvacrol acetate	1377	1368	0.66 ± 0.13	0.58 ± 0.39	1.39 ± 0.10	0.92 ± 0.45	0.55 ± 0.06
β-bourbonene	1384	1378	-	-	-	-	-
E-caryophyllene	1435	1427	1.95 ± 0.40	2.37 ± 0.40	1.85 ± 0.30	1.64 ± 0.29	1.38 ± 0.25
aromadendrene	1455	1436	0.43 ± 0.09	0.29 ± 0.04	0.49 ± 0.05	0.40 ± 0.08	0.20 ± 0.05
α-humullene	1469	1452	0.15 ± 0.02	0.15 ± 0.02	0.16 ± 0.01	0.15 ± 0.02	0.10 ± 0.01
9-*epi*-*E*-caryophyllene	1477	1474	0.02 ± 0.02	-	0.05 ± 0.02	0.02 ± 0.02	-
E-*β*-guaiene	1504	1498	-	-	-	-	-
viridiflorene	1510	1505	0.31 ± 0.07	0.21 ± 0.01	0.35 ± 0.03	0.28 ± 0.08	0.15 ± 0.03
spathulenol	1594	1578	0.44 ± 0.13	0.27 ± 0.05	0.63 ± 0.04	0.59 ± 0.11	0.28 ± 0.02
isoaromadendrene epoxide	1597		0.11 ± 0.02	0.11 ± 0.01	0.14 ± 0.01	0.15 ± 0.03	0.10 ± 0.02
caryophyllene oxide	1601	1599	0.21 ± 0.06	0.17 ± 0.03	0.30 ± 0.05	0.28 ± 0.06	0.19 ± 0.03
caryophylla-4(12),8(13)-dien-5-*β*-ol	1654	1663	0.09 ± 0.02	0.07 ± 0.01	0.11 ± 0.04	0.12 ± 0.02	0.08 ± 0.01
14-hydroxy-9-*epi*-*E*-caryophyllene	1673	1669	0.06 ± 0.04	0.07 ± 0.01	0.11 ± 0.01	0.11 ± 0.02	0.06 ± 0.02
khusinol	1686	1674	0.08 ± 0.02	0.07 ± 0.01	0.11 ± 0.01	0.11 ± 0.02	0.07 ± 0.01
Essential oil content (%)			4.8 ± 0.47	5.62 ± 0.91	5.18 ± 0.58	4.96 ± 0.34	4.15 ± 0.44

Table 3. continued,

compound	RI[b]	RI[c]	population				methods[d]
			Daarbast	Konar Siah	Darab	Haneshk	
α-thujene	933	929	0.40 ± 0.24	0.43 ± 0.25	0.36 ± 0.03	0.28 ± 0.17	RI, MS
α-pinene	940	939	3.89 ± 2.00	3.31 ± 2.50	2.62 ± 0.28	1.54 ± 0.95	RI, MS
camphene	955	954	0.19 ± 0.07	0.14 ± 0.07	0.15 ± 0.01	0.10 ± 0.03	RI, MS
2,4(10)-thujadiene	960	957	-	-	-	-	RI, MS
1,3-octanol	969	973	0.07 ± 0.01	0.15 ± 0.01	0.16 ± 0.24	0.08 ± 0.02	RI, MS
sabinene	979	977	0.11 ± 0.03	0.21 ± 0.10	0.10 ± 0.01	0.10 ± 0.02	RI, MS
β-pinene	983	978	0.65 ± 0.28	0.56 ± 0.30	0.51 ± 0.04	0.30 ± 0.13	RI, MS
myrcene	995	992	1.10 ± 0.27	1.15 ± 0.29	0.94 ± 0.11	2.34 ± 0.76	RI, MS
3-octanol	997	995	0.18 ± 0.10	0.13 ± 0.10	0.04 ± 0.01	0.16 ± 0.09	RI, MS
α-phellandrene	1010	1005	0.18 ± 0.01	0.18 ± 0.01	0.13 ± 0.01	0.34 ± 0.11	RI, MS
δ-3-carene	1015	1011	0.03 ± 0.01	0.02 ± 0.01	-	-	RI, MS
α-terpinene	1021	1019	1.15 ± 0.15	1.25 ± 0.25	1.04 ± 0.06	0.81 ± 0.41	RI, MS
p-cymene	1030	1025	6.34 ± 2.69	6.42 ± 2.21	13.96 ± 1.1	4.33 ± 2.76	RI, MS
limonene	1034	1030	0.58 ± 0.27	0.58 ± 0.27	0.61 ± 0.09	0.76 ± 0.13	RI, MS
1,8-cineole	1037	1035	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.05	0.19 ± 0.16	RI, MS
Z-*β*-ocimene	1039	1039	0.52 ± 0.30	0.59 ± 0.33	0.45 ± 0.04	0.68 ± 0.30	RI, MS
E-*β*-ocimene	1050	1050	0.08 ± 0.02	0.09 ± 0.03	0.04 ± 0.02	0.94 ± 0.50	RI, MS
γ-terpinene	1063	1062	4.73 ± 0.97	4.79 ± 1.03	2.93 ± 0.24	2.60 ± 1.76	RI, MS
Z-sabinene hydrate	1071	1070	0.20 ± 0.04	0.25 ± 0.05	0.21 ± 0.01	0.33 ± 0.27	RI, MS
Z-linalool oxide	1076	1074	0.10 ± 0.01	0.04 ± 0.01	0.04 ± 0.02	0.45 ± 0.30	RI, MS
terpinolene	1092	1089	0.21 ± 0.04	0.23 ± 0.06	0.21 ± 0.03	0.78 ± 0.28	RI, MS
linalool	1103	1100	1.25 ± 0.70	1.73 ± 1.10	1.69 ± 0.97	37.65 ± 20.6	RI, MS
E-*γ*-caryophyllene	1106	1106	0.06 ± 0.02	0.09 ± 0.06	0.10 ± 0.04	0.93 ± 0.50	RI, MS
1-octenyl-3-acetate	1111	1113	0.03 ± 0.01	0.07 ± 0.06	-	0.11 ± 0.07	RI, MS
p-menth-2-en-1-ol	1124	1122	0.04 ± 0.03	tr	-	0.16 ± 0.10	RI, MS
allo-ocimene	1131	1132	-	-	-	0.38 ± 0.26	RI, MS
1,3,8-*p*-menthatriene	1133		-	-	-	0.10 ± 0.08	MS
borneol	1172	1171	-	-	-	0.99 ± 0.68	RI, MS
Z-linalool oxide (pyranoid)	1177	1173	-	tr	0.12 ± 0.02	0.13 ± 0.06	RI, MS
4-terpineol	1183	1179	0.19 ± 0.04	0.16 ± 0.05	0.18 ± 0.02	0.18 ± 0.05	RI, MS
p-cymenol-8	1188	1184	0.48 ± 0.07	0.48 ± 0.05	0.59 ± 0.03	0.29 ± 0.09	RI, MS
α-terpineol	1195	1190	0.72 ± 0.37	0.81 ± 0.40	0.58 ± 0.04	0.60 ± 0.09	RI, MS
Z-dihydro carvone	1202	1200	0.19 ± 0.04	0.17 ± 0.08	0.03 ± 0.02	0.21 ± 0.11	RI, MS
nerol	1234	1228	0.02 ± 0.01	-	0.02 ± 0.01	0.06 ± 0.04	RI, MS
thymol methyl ether	1238	1237	0.08 ± 0.01	0.10 ± 0.08	1.52 ± 0.12	0.59 ± 0.50	RI, MS
carvacrol methyl ether	1248	1241	3.71 ± 3.00	3.23 ± 3.00	1.62 ± 0.38	0.92 ± 0.72	RI, MS
geraniol	1260	1263	tr	0.02 ± 0.01	tr	0.60 ± 0.28	RI, MS
geranial	1280	1273	-	-	-	-	RI, MS
p-thymol	1286		0.09 ± 0.05	0.07 ± 0.01	0.22 ± 0.03	0.05 ± 0.04	MS
thymol	1295	1295	5.26 ± 3.85	8.65 ± 8.30	41.61 ± 4.14	17.55 ± 10.9	RI, MS
carvacrol	1309	1305	61.4 ± 13.3	58.3 ± 13.3	21.81 ± 4.91	15.74 ± 12.9	RI, MS
thymol acetate	1358	1359	0.10 ± 0.10	0.17 ± 0.13	0.75 ± 0.09	0.21 ± 0.13	RI, MS
carvacrol acetate	1377	1368	1.12 ± 0.61	1.45 ± 1.23	0.31 ± 0.09	0.20 ± 0.09	RI, MS
β-bourbonene	1384	1378	-	-	-	0.04 ± 0.03	RI, MS
E-caryophyllene	1435	1427	1.93 ± 0.62	1.59 ± 0.36	1.59 ± 0.38	1.71 ± 0.62	RI, MS
aromadendrene	1455	1436	0.47 ± 0.07	0.38 ± 0.16	0.24 ± 0.09	0.19 ± 0.11	RI, MS
α-humullene	1469	1452	0.14 ± 0.02	0.12 ± 0.03	0.11 ± 0.02	0.16 ± 0.04	RI, MS
9-*epi*-*E*-caryophyllene	1477	1474	0.03 ± 0.01	0.03 ± 0.03	-	tr	RI, MS
E-*β*-guaiene	1504	1498	0.02 ± 0.01	tr	-	-	RI, MS
viridiflorene	1510	1505	0.38 ± 0.13	0.32 ± 0.18	0.14 ± 0.05	0.36 ± 0.15	RI, MS
spathulenol	1594	1578	0.48 ± 0.24	0.44 ± 0.24	0.35 ± 0.05	0.77 ± 0.31	RI, MS
isoaromadendrene epoxide	1597		0.10 ± 0.03	0.09 ± 0.04	0.21 ± 0.02	0.27 ± 0.10	MS
caryophyllene oxide	1601	1599	0.21 ± 0.04	0.20 ± 0.07	0.35 ± 0.03	0.45 ± 0.13	RI, MS
caryophylla-4(12),8(13)-dien-5-*β*-ol	1654	1663	0.09 ± 0.02	0.07 ± 0.05	0.12 ± 0.01	0.21 ± 0.06	RI, MS
14-hydroxy-9-*epi*-*E*-caryophyllene	1673	1669	0.07 ± 0.03	0.05 ± 0.04	0.13 ± 0.01	0.16 ± 0.06	RI, MS
khusinol	1686	1674	0.08 ± 0.02	0.06 ± 0.04	0.12 ± 0.01	0.17 ± 0.05	RI, MS
Essential oil content (%)			5.58 ± 0.48	5.89 ± 0.24	3.31 ± 0.26	3.71 ± 0.41	

[a]tr, trace < 0.02 %; (-), not detected. [b]RI, linear retention indices on HP-5MS column, experimentally determined using homologue series of n-alkanes. [c]Relative retention indices taken from Adams and NIST. [d]MS, by comparison of the mass spectrum with those of the computer mass libraries Adams and NIST.

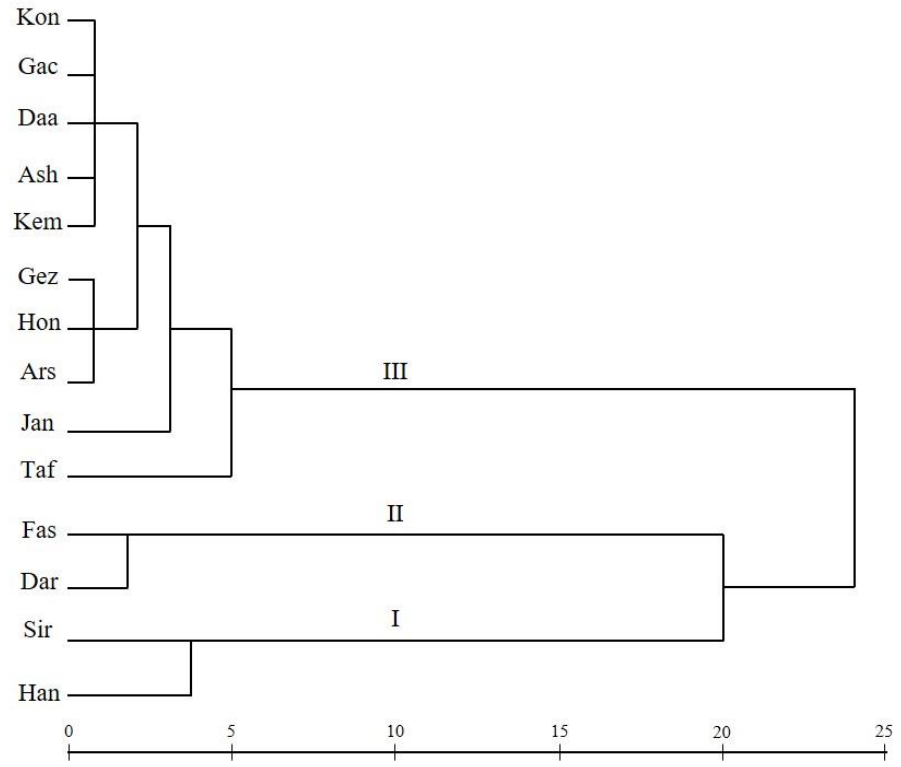

Figure 3. Hierarchical cluster analysis of *Zataria multiflora* populations based on phytochemical composition.

3.2. Environmental Characteristics

Geographical, climatic and edaphic characteristics of *Z. multiflora* natural habitats are exhibited in Tables 1 and 2. Our results indicate that this species grows in areas characterized by a mean rainfall of 40.46 to 302.72 mm year^{-1} and mean annual temperature of 14.90 to 28.80 °C. The altitude ranges from 731 to 1946 m. The percentage of organic matter (OM) ranged from 4 % to 10 % (Haneshk and Darab regions, respectively). The soil of regions were rich in calcium (Ca), iron (Fe), potassium (K) and aluminum (Al) whereas nitrogen (N) and phosphor (P) were resent in lower levels. Furthermore, *Z. multiflora* grows on soils with alkaline pH (7.60 to 7.90).

The volatile constituents were influenced by edaphic factors (Table 4). Carvacrol was significantly positively correlated with pH, Ca and temperature [0.69 ($p < 0.01$), 0.62 and 0.54 ($p < 0.05$) respectively] and there was a highly negative correlation between carvacrol and Al, Fe and K. The correlation analysis indicated that linalool was considerably positively correlated with Al, Fe and K ($p < 0.01$). No statistically significant correlations were detected among N and EO content and phytochemical constituents.

Table 4. Pearson correlation coefficients between EO content, major components and edaphic factors.[a]

edaphic factors	essential oil	linalool	thymol	carvacrol	*p*-cymene
altitude	-0.73**	0.53*	0.20	-0.41	-0.45
temperature	0.73**	-0.59*	-0.30	0.54*	0.37
OM	0.13	-0.55*	0.45	0.02	0.80**
pH	0.67**	-0.42	-0.64*	0.69**	0.01
Sand	0.22	0.41	-0.63*	0.15	-0.64*
Silt	-0.12	-0.56*	0.50	0.06	0.64*
Clay	-0.30	0.14	0.53*	-0.52	0.27
N	-0.31	-0.04	0.47	-0.27	0.49
P	-0.56*	0.44	0.04	-0.28	-0.23
K	-0.73**	0.57*	0.41	-0.63*	-0.25
Ca	0.62*	-0.65*	-0.29	0.62*	0.23
Fe	-0.78**	0.60*	0.50	-0.72**	-0.15
Al	-0.75**	0.55*	0.49	-0.68**	-0.17

[a]Significance levels: *$p < 0.05$; **$p < 0.01$.

The SEM approach was used to dissect the contribution of environmental factors on EO and EO constituent content. Significant SEMs for EO [APC = 0.641 ($p < 0.001$), ARS = 0.571 ($p = 0.002$), AVIF = 1.001], thymol [APC = 0.874 ($p < 0.001$), ARS = 0.770 ($p < 0.001$), AVIF = 1.435], carvacrol [APC = 0.602 ($p = 0.001$), ARS = 0.560 ($p = 0.002$), AVIF = 1.536] and linalool [APC = 0.489 ($p = 0.005$), ARS = 0.655 ($p = 0.008$), AVIF = 1.019] were obtained. The portion of clay and phosphor had a direct negative influence on EO content. The altitude had a positive effect on phosphor content while latitude had a negative effect on clay content in the soil (Figure 4A). Thymol content was positively affected by clay amount in the soil and indirect negatively via the negative effect of latitude on clay (Figure 4B). Carvacrol was directly positively influenced by silt content and pH-value in the soil, which was positively depended on the amount of sand in the soil (Figure 4C). Latitude had a negative effect on soil silt and a positive one on the soil sand portion. The linalool content was affected on the one hand, directly by longitude (positively) and on the other hand by silt (negatively) while silt content itself was negatively affected by latitude (Figure 4D).

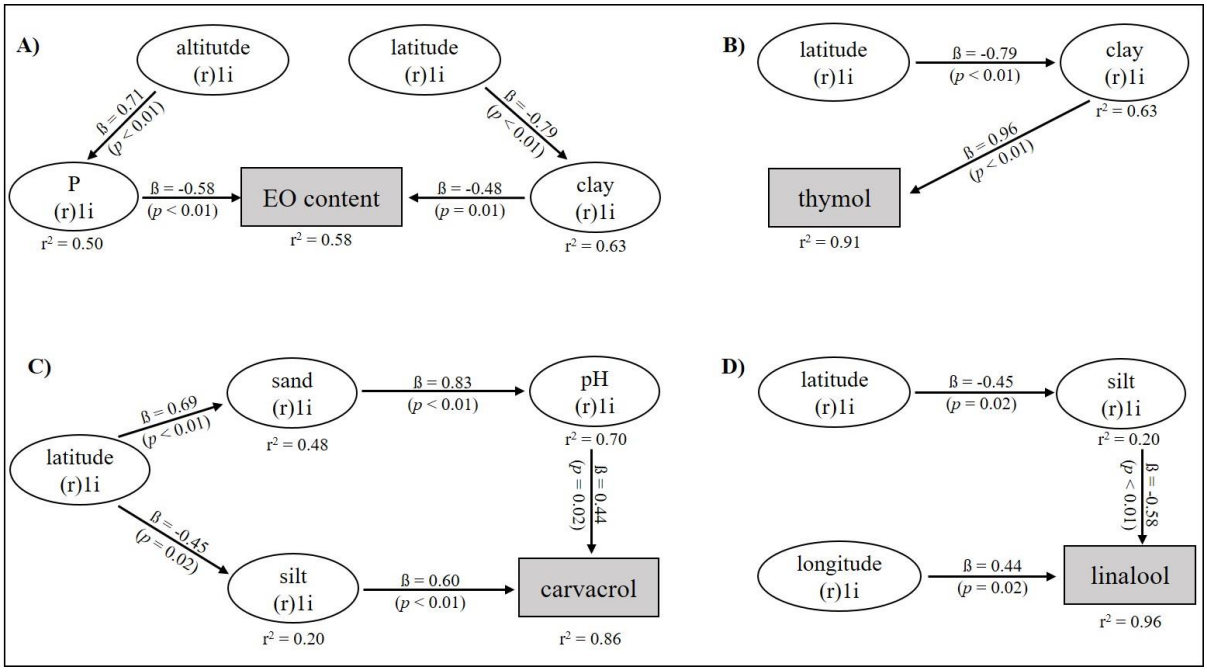

Figure 4. Hypothetical structural equation models (SEMs) to describe the relationships between geographical and edaphic factors and A) EO content, B) thymol, C) carvacrol, and D) linalool content of *Zataria multiflora*. The climatic factors, temperature and rainfall were included in the full model but did not explain EO or EO constituent content. R^2: coefficient of determination indicating the variability explained for each variable. *β*-values indicate the path coefficients, *p*: significance level for relationship.

3.3. Quantitative Analysis of EO composition by NIRS

The dried leaves of specimens of *Z. multiflora* from different regions were analyzed by near infrared spectroscopy and hierarchical cluster analysis (HCA, Wards Algorithm). The NIR spectra of *Z. multiflora* were characterized by combination, first and second overtone vibrations in the range of 4,000-12,000 cm^{-1}. HCA was used to group samples according to their spectral appearance determined through their chemical profile. Figure 5 presents the appropriate HCA plot showing the separation of *Z. multiflora* populations into different clusters. In contrast to GC analysis, NIRS combines spectral features of chemically similar structures. Hence, carvacrol, thymol and *p*-cymene, all characterized by an isopropyl- and methyl-substituted aromatic ring system, show all nearly identical NIRS absorption patterns. Therefore, for NIRS not only the quantity of individual EO components are relevant, but the amount of structurally related substances. As shown in Figure 5, HCA resulted on highest level of heterogeneity in the clustering of samples according to the ratio of aromatic EO

compounds (thymol + carvacrol + *p*-cymene) to aliphatic, isolated C=C structures (linalool). On the next level, types with a high content of aromatic structures are divided into sub-clusters with high amounts of carvacrol (cluster IIIB), high thymol and high linalool or high *p*-cymene (cluster IIIA) or high carvacrol and high p-cymene (cluster II).

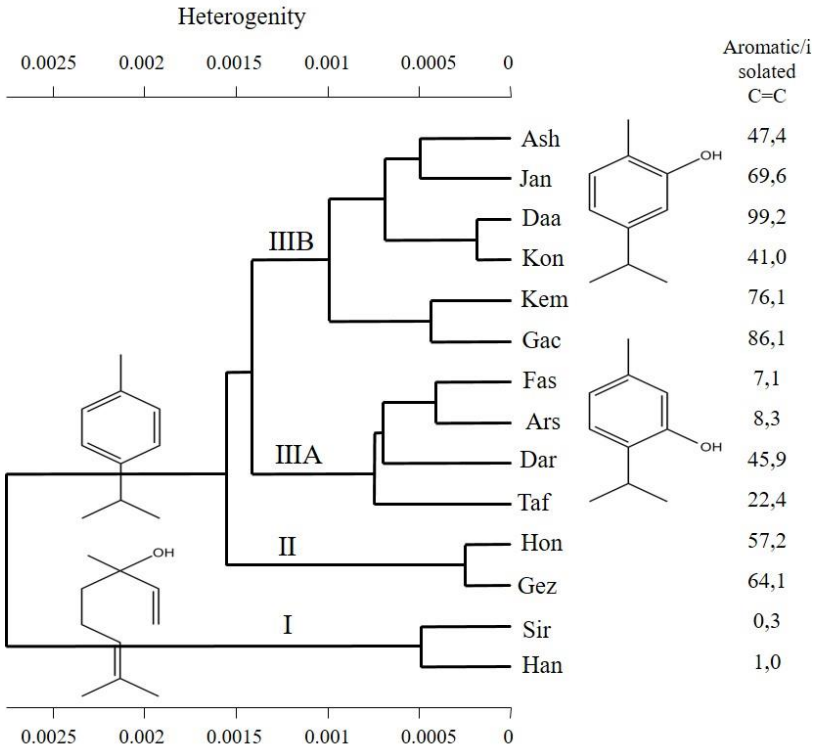

Figure 5. Hierarchical cluster analysis of the studied populations of *Zataria multiflora* based on the NIR spectra.

Chemometrics of superintended pattern identification based on PLS-DA of GC combined with NIR spectroscopy was endeavored to categorize fourteen populations of *Z. multiflora*. Quantification models for the EO content and for major compounds were developed by 10-fold cross-validation procedure according to literature (Krähmer et al., 2013). Therefore, averaged spectra for each plant were correlated with GC reference data for carvacrol, thymol and linalool as well as EO content. For all constituents, appropriate prediction models were achieved. Figure 6 shows the results of cross-validation according to plant wise averaged NIR spectra from all populations. Generally, coefficients of determination (R^2) were higher than 0.82 for individual components and EO content. As shown in Figure 6A, NIRS offers a fast tool for estimation of EO content with a coefficient of determination $R^2 = 0.85$ and a root mean square error of prediction (RMSEP) below 10 % of mean EO content (the mean of EO

content over all samples used in the model, according to Figure 6 something about 4 to 5 ml / 100 g) (RMSEP = 0.431 %). Furthermore, for major EO components, prediction quality was best for linalool (R^2 = 0.97) followed by R^2 = 0.87 and R^2 = 0.82 for carvacrol and thymol (Figure 6D, B and C), respectively.

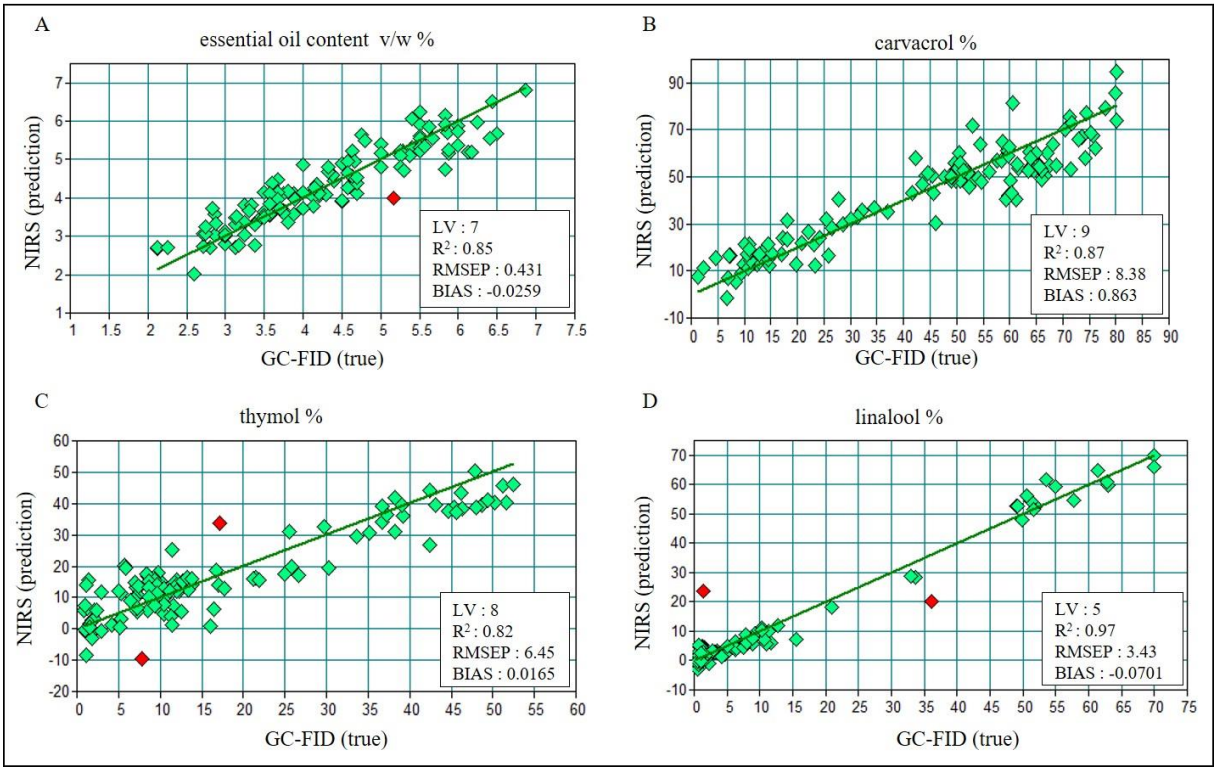

Figure 6. Results of 10-fold cross-validation of NIR and GC data for the A) EO content, B) carvacrol, C) thymol, D) linalool by correlation of averaged spectra for each population.

4. Discussion

This study investigated the effect of different environmental factors on EO production, the content of specific EO compounds as well as on chemotype of different *Z. multiflora* populations. The EO values (up to 5.89 % dry weight) detected in 14 populations in Iran were higher than those reported previously in the literature including 1.2 % to 3.4 % (Hadian et al., 2011a), 2.91 % to 4 % (Sadeghi et al., 2015) and 1.93 % to 2.22 % (Golkar et al., 2020). The EO content can be affected by geological, climatic and edaphic characteristics as well as harvesting time. Saei-Dehkordi et al. (2010) described that the largest quantity of the EO content of *Z. multiflora* was collected in mid-May with 1.57 % (v/w). Thus, knowledge on the

season, phenological stage and harvesting time during the day is necessary to obtain high quantities of EO content. Of the chemical constituents detected, carvacrol, thymol, linalool, *p*-cymene, *γ*-terpinene and *α*-pinene were found as the main compounds of *Z. multiflora*. In other studies, the highest diversity was shown for the monoterpenes, including carvacrol, thymol, linalool and *p*-cymene (Ziaee et al., 2018; Mahboubi & Bidgoli, 2010; Abkenar et al., 2008; Shafiee & Javidnia, 1997).

Carvacrol, the major compound of the Jandagh population, has been previously reported as one of the most important components of EO among various members of the Lamiaceae family (Santos et al., 2019; Stefanaki et al., 2018; Hadian et al., 2011b; Ebrahimi et al., 2008). The main component of Darab and Fasa populations was thymol (41.61 % and 48.12 % respectively), which is an isomer of carvacrol. Saei Dehkordi et al. (2010) and Sharififar et al. (2007) had depicted thymol as the most abundant component in the essential oil profile of *Z. multiflora* from different areas in Iran. Contrariwise, two other studies showed carvacrol as the main constituent of *Z. multiflora* (Khosravi et al., 2009; Basti et al., 2007). Moreover, EO of *Z. multiflora* contains other important monoterpene constituents like linalool, *p*-cymene, *γ*-terpinene and *α*-pinene. Siriz and Haneshk populations were rich in linalool (55.38 and 37.65 % respectively) and *p*-cymene was one of the main components of Darab population (13.96 %).

The positive and negative correlations between EO components indicate the presence of three different chemotypes: thymol, carvacrol and linalool. Furthermore, they indicate which compounds are interlinked in a chain of monoterpene synthesis with certain branches in the predicted enzymatic pathway: while geranyl-diphosphate is the precursor of non-phenolic linalool and phenolic thymol and carvacrol, the latter are connected via *p*-cymene (Thompson, 2005). In agreement to our results, similar correlations between individual EO components were found in *Artemisia dracunculus*, where methyl chavicol as the main constituent of *A. dracunculus* was positively correlated with terpinolene and methyl eugenol, and negatively correlated with *α*-pinene, limonene, *Z-β*-ocimene and *E-β*-ocimene (Karimi et al., 2015). Hierarchical cluster analysis based on phytochemical components was proven to be a helpful tool to classify medicinal and aromatic plants accessions. For instance, cluster analysis on *Verbascum songaricum* resulted into nine groups (Selseleh et al., 2019) and for lemon balm populations three different chemotypes could be identified (Pouyanfar et al., 2018). Also grouping based on EO constituents of four Vitex specimens revealed different clusters (de Sena Filho et al., 2017). In the present study, the components of the EO measured at full flowering stage underpin the presence of the three chemotypes (carvacrol, thymol, linalool).

Rapid and reliable identification of medicinal plant species and chemotypes concerning authenticity and quality is crucial for pharmaceutical and food processing. Spectroscopy

techniques as fast and easy handling technologies are nowadays widely applied directly on plant material for qualitative and semi-quantitative characterization. Different studies describe the application of NIRS, IRS and Raman for differentiation of chemotypes and prediction of EO composition in various medicinal and aromatic plants (Farag et al., 2018; Gudi et al., 2014; Seidler-Lozykowska et al., 2010). For *Z. multiflora* the presented quantification models are not accurate for exact determination at current state, since e.g. for linalool, samples are very inhomogeneous distributed over the investigated range of concentration. Nevertheless, in combination with HCA, near infrared spectroscopy offers a fast method for chemotyping and EO estimation already on plant material. An improved prediction of EO content and main components with regard to cross-validation concerning averaged ATR−FTIR spectra can also be achieved for constituents with lower concentrations (Gudi et al., 2015). The high correlation between NIRS and GC data allows application of NIRS for authenticity and quality control directly on the plant material for the flavor and fragrance as well as pharmaceutical industries. NIR spectroscopy can be used to classify plants according to their chemotype as well as predict the content of valuable components such as carvacrol, thymol and linalool as well as other terpenes, rapidly and accurately.

The effect of soil parameters and climatic condition on plant performance and EO content has been shown for many plant species. For example, *Kelussia odoratissima* Mozaff grows in dark soil, rich in mineral content (Raiesi et al., 2013) and growth habitats of *Thymus pulegioides* were characterized by high amount of Al, Ca, Fe, K and Si, however, by low amount of P and Mn (Vaičiulytė et al., 2017). Mexican oregano populations grown in soil with high nitrogen and iron content, lower soil water availability and higher pH values showed a higher EO yield (Martínez-Natarén et al., 2012). It is widely accepted that environmental conditions affect plant EO content and its components (Mansour et al., 2010; Ormeño et al., 2008). Several studies have revealed that the predominance of carvacrol or thymol in different Lamiaceae species is related to environmental factors (Economou et al., 2011; Boira and Blanquer, 1998). In *Thymus vulgaris* such phenolic chemotypes cope better with summer drought, while non-phenolic (e.g. linalool) chemotypes cope better with early-winter freezing temperatures (Thompson et al., 2007). In our study, the Pearson correlations revealed that altitude, K, Fe and Al were significantly ($p < 0.01$) negatively correlated with EO content (Table 4). In agreement to our results, the lowest altitudes showed higher EO yield in *Lavandula angustifolia* (Demasi et al., 2018) and *Satureja rechingeri* (Hadian et al., 2014). Also, a correlation between higher EO yields at decreasing altitudes was found in *Origanum vulgare* (Giuliani et al., 2013). Notwithstanding the effect of geographical condition, EO content and EO constituents can be affected by edaphic factors and climatic conditions, for example, the soil type affects *Origanum syriacum* chemotype (El-Alam et al., 2019). In our study, EO yield showed a highly significant positive correlation with temperature, pH-value

and Ca. Former studies highlighted the same behavior in other aromatic plants and suggest that the wide variation in the chemical composition of the EO can be ascribed to habitat influences in *Origanum compactum* (Aboukhalid et al., 2017) and *Origanum vulgare* L. (De Falco et al., 2013). The influence of environmental conditions on EO of *Origanum vulgare* ssp. showed a negative correlation with altitude and a positive correlation with soil temperature and air temperature (Tuttolomondo et al., 2014). SEMs were applied to impute relationships between the different factors and revealed indirect geographic and direct edaphic effects on EO content and compounds, while climate factors do not have an influence. Chemotype and high amount of specific compounds can thus be predicted when looking for populations with specific features. Biotic factors like co-occuring vegetation (Wäschke et al., 2015) or herbivore activity (Dicke et al., 2009) can additionally influence the metabolome profile of plants and shall be considered in future studies.

5. Conclusion

In conclusion, medicinal and aromatic plants play important roles all over the world because of their wide application due to pharmacological, therapeutic, industrial and agricultural properties. The varying climate and environmental growth conditions lead to a huge phytochemical diversity of these resources. *Zataria multiflora* is a valuable medicinal plant with various pharmaceutical properties and has potential as source of compounds with agricultural relevance as plant protection agents. Ingredients such as carvacrol, thymol and linalool are responsible for the respective effects and show a high variability among the investigated populations. Environmental conditions are affecting the EO content and its components. Hence, existing variability in the chemical profile of studied populations allow selection of populations with distinct scent or bioactive components for use in pertinent industries and breeding purposes. Our approach of identifying environmental predictors for EO content, chemotype or presence of high amounts of specific compounds can help to identify regions for sampling plant material with the desired chemical profile. Based on mobile NIRS devices, fast classification of yet undescribed populations and individual plants together with an EO profiling can be performed directly in the field.

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Comprehensive metabolite profiling of *Zataria multiflora* Boiss. by HPLC-ESI-QTOF-MS and bioassays reveal candidate compounds for biobased protection against five important fungal pathogenes

Abstract

Due to increasing demand for the use of natural compounds for food preservatives and control plant pathogens, plant extracts with bioactive secondary metabolites can be used as an effective and ecofriendly approach. In this study, hydroalcoholic leaf extracts of 14 *Zataria multiflora* Boiss. populations were investigated as a source of phenolic and flavonoid components. The aims were to (i) determine metabolite fingerprints of *Z. multiflora* populations, (ii) screen the antifungal activity of *Z. multiflora* extracts and fractions, and (iii) designate bioactive compounds correlating with antifungal activity. Three major chemical classes were found among *Z. multiflora* populations. A total of 32 metabolites were annotated including flavonoid conjugates, hydroxycinnamic acid derivatives and phenolic terpenes. The extracts showed weak (≤ 37 %, for populations in class I), moderate and high inhibition rates (40 % to 65 %, for populations in class II and III) against *Fusarium culmorum*, *Fusarium sambucinum*, *Botrytis cinerea*, *Alternaria dauci* and *Colletotrichum lindemuthianum*, which exhibited significant differences among populations. Furthermore, among the five fractions of hydroalcoholic extracts, one fraction was the most active fraction against all fungi (≥ 48 % inhibition). When screening *Z. multiflora* extracts for bioactive metabolites, flavonoid compounds such as dihydroquercetin ($r^2 \geq 0.74$), dihydrokaempferol ($r^2 \geq 0.76$), eriodictyol ($r^2 \geq 0.78$) and naringenin ($r^2 \geq 0.71$) showed strong correlation with antifungal activity. These results revealed antifungal activity in *Z. multiflora* extracts are potent and correlated with the presence of flavonoids, which could help to develop new natural fungicide to use in food production, agricultural and pharmaceutical industries.

Keywords: Hydroalcoholic extract, Flavonoids, Naringenin, Dihydrokaempferol, Bioactive compounds, Pathogenic fungi

1. Introduction

Medicinal and aromatic plants have been a valuable source of biologically active compounds used not only as food preservation and diseases control, but also as bio-based fungicides and/or insecticides on a smaller scale (Walia et al., 2017; Burketova et al., 2015), for agricultural and pharmaceutical purposes. Due to increasing resistance of pathogens to synthetized fungicides, herbicides and insecticides, the demand for alternative plant protection have been increased. An alternative to chemical pesticides is using bio-based compounds to promote plant disease resistance (Raveau et al., 2020). In last decades, fungal pathogens with a rapid growth have become a big challenge caused severe pre- and postharvest losses (Bebber and Gurr, 2015), so developing successful strategies such as bio-based fungicides are essential to combat increasing crop yield losses rates. Among fungal pathogens, the genera *Fusarium*, *Botrytis*, *Alternaria* and *Colletotrichum* have high potential to infect and damage various plant species in the pre- and postharvest stage. For example, *B. cinerea* has high potential losses in small fruit crops (i.e. grape, strawberry) and vegetables that can produce phytotoxic metabolites cause potential risk to human and animal health (Williamson et al., 2007). Among plant species, medicinal and aromatic plants that contain secondary metabolites particularly phenolic and flavonoid compounds could be used as an alternative environmentally friendly plant protection and/or food preservation.

The Lamiaceae family comprises several important herbs with valuable secondary metabolites, which have been used in pharmacological, agricultural and food industries for decades. *Zataria multiflora* Boiss. is a flavoring herb of this family used not only as spice and/or medicine in traditional folk remedies based on its anti-inflammatory, antiseptic, analgesic, carminative, anthelmintic, antimicrobial and antidiarrheal properties, but also as pharmaceutical forms (i.e. syrups and oral drops) (Khazdair et al., 2020; Sajed et al., 2013). *Z. multiflora* is a thyme-like plant native to southwestern Asia (grows wildly in Iran, Afghanistan, and Pakistan), and contains a wide range of biologically active components. Many phytochemical studies so far examined the chemical composition of its essential oils (EOs). The main EO compounds of *Z. multiflora* are carvacrol, thymol, linalool, γ-terpinene and *p*-cymene (Karimi et al., 2020a). Although, many studies have been investigated the EOs compounds of *Z. multiflora* and antibacterial and antifungal activity of its EOs (Karimi and Meiners, 2021; Ziaee et al., 2018; Mohajeri et al., 2018), there are very few hints about chemical compounds of hydroalcoholic extract of *Z. multiflora*. However, few chemical compounds of hydroalcoholic extract of *Z. multiflora* have been investigated, including rosmarinic acid, luteolin, luteolin O-glucuronide, naringenin, Vicenin-2 and α-tocopherolquinone (Pourhosseini et al., 2020; Izadiyan et al., 2019; Mohagheghzadeh et al., 2004; Ali et al., 2000). There are also no hints about bioactivities of chemical compounds of

Z. multiflora hydroalcoholic extract. Among examined compounds, rosmarinic acid, naringenin and luteolin were characterized in thyme and sweet marjoram (Fecka and Turek, 2008), oregano, sage, basil and thyme (Hossain et al., 2010). It has been reported that phenolic and flavonoid compounds have many pharmacological properties, for instance, naringenin shown anti-diabetic and antifibrotic properties (Tsai et al., 2012). Therefore, chemical characterization and screening of antifungal activity of *Z. multiflora* hydroalcoholic extracts could provide valuable details about this species and its biological properties.

In previous studies, we found three main groups among 14 *Z. multiflora* populations according to the EOs compounds, each representing a distinct chemotype with linalool, thymol and carvacrol as the major components (Karimi et al., 2020a). Furthermore, we screened antifungal activity of the EOs of chemotypes against *Fusarium culmorum*, *Fusarium sambucinum*, *Botrytis cinerea*, *Alternaria dauci* and *Colletotrichum lindemuthianum*; results indicated that low concentrations of carvacrol and thymol, but not of linalool chemotype EOs inhibited the mycelial growth of these pathogens (Karimi and Meiners, 2021). Based on these findings, we expected that populations of *Z. multiflora* referring to their chemical compounds of hydroalcoholic extract, have a wide chemical diversity and antifungal activities like the patterns of EOs studies. Hence, we applied liquid chromatography-electrospray ionization-quadrupole-time-of-flight mass spectrometry (LC-ESI-QTOFMS) to analyze the metabolome profile of *Z. multiflora* hydroalcoholic extracts and identify metabolites with promising antifungal activities. Nevertheless, to our knowledge only one study using HPLC technique investigated the metabolite fingerprinting of *Z. multiflora* hydroalcoholic extract considering as potential source of valuable metabolites including flavonoids and hydroxycinnamic acid derivatives (Izadiyan et al., 2019).

In the present study, we have examined hydroalcoholic extracts of *Z. multiflora* populations in order to determine its metabolite fingerprinting and identify compounds with promising antifungal activities. The aims were i. assessment of metabolite fingerprints based on semi-polar metabolites of *Z. multiflora*; ii. evaluation of *Z. multiflora* extracts against various pre- and postharvest fungal pathogens; iii. determination of active fraction and identification of compounds in the metabolome; and iv. designation of bioactive compounds correlating with antifungal activity.

2. Materials and Methods

2.1. Plant material and chemicals

Leaf samples of 123 *Z. multiflora* plants representing a vast geographic and environmental variation were randomly collected at flowering stage in 14 natural habitats in Iran (see Table 1). Six to eleven individual shrubs were sampled at each region and a voucher specimen (No. MPH-1799) were deposited in the Herbarium of Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Iran. LC/MS-grade ethanol (≥ 99.9 %) and methanol (≥ 99.95 %) were supplied by Th.Geyer (Berlin, Germany), and formic acid (≥ 98 %) was purchased from Sigma-Aldrich (Germany). Ultrapure water (resistivity ≥ 18.2 MΩ cm) was obtained from an Arium 611 water purification system (Sartorius, Göttingen, Germany). Sources of internal standards (umbelliferone and biochanin A) and reference compounds including rosmarinic acid, luteolin and apigenin (Sigma-Aldrich and Carl Roth, Germany) were used for metabolite identification.

Table 1. General climatic and geographical information of natural habitats of *Z. multiflora* populations.

Sample name	Origin	Province	Latitude (N)	Longitude (E)	Altitude (m)	Temperature (°C) (av. annual)	Precipitation (mm/year)
Ash	Ashkezar	Yazd	31° 48' 49''	54° 00' 26''	1946	21.1	40.5
Taf	Taft	Yazd	31° 42' 26''	54° 10'	1697	20.3	49.8
Ars	Arsenjan	Fars	29° 53' 49''	53° 16' 20''	1865	20.3	215.2
Dar	Darab	Fars	28° 44' 27''	54° 34' 41''	1276	24.3	276.4
Fas	Fasa	Fars	28° 59' 27''	53° 42' 25''	1516	20.3	278.5
Han	Haneshk	Fars	30° 49' 16''	53° 18' 19''	1898	14.9	180.1
Jan	Jandaq	Esfahan	33° 57' 44''	54° 31' 02''	1235	21.5	55.9
Sir	Siriz	Kerman	30° 55' 43''	55° 57' 01''	1763	20.2	107.8
Daa	Daarbast	Hormozgan	26° 58' 02''	54° 01' 59''	1009	28.8	302.7
Gac	Gachooyeh	Hormozgan	26° 58' 28''	53° 58' 06''	1055	28.8	302.7
Gez	Gezeh	Hormozgan	27° 06' 35''	54° 04' 46''	731	28.8	302.7
Hon	Hongooyeh	Hormozgan	27° 06' 19''	54° 04' 07''	820	28.8	302.7
Kem	Kemeshk	Hormozgan	27° 03' 13''	53° 50' 41''	937	28.8	302.7
Kon	Konar Siah	Hormozgan	27° 09' 05''	53° 57' 04''	981	28.8	302.7

2.2. Non-targeted metabolomics

Air-dried leaf material of each sample (approx. 3-4 gr) was ground to fine powder (5 min at 30 s^{-1}) using a mixer mill (Retsch MM2) and a steel ball of 8 mm diameter. Homogenized leaf material (30 ± 1 mg) was weighed into a 2 mL polypropylene centrifuge tube. Afterwards 200 µL internal standard solution [100 µM Umbelliferone and 100 µM Biochanin A in

methanol/water, 1/1 (v/v)] and 1.3 mL 80 % (v/v) aqueous methanol were added. The mixture was thoroughly vortex-mixed for 20 s and sonicated for 10 min at 20-25 °C (checked and eventually adjusted temperature of the ultrasonic bath by adding crushed ice). After centrifugation (10 min, 12.000 g, 20 °C), the supernatant was transferred to a 10 mL volumetric flask. The remaining residue was extracted twice again with 1.5 mL 80 % (v/v) aqueous methanol as described above. The resulting extracts were combined and their volume adjusted to 10 mL using 80 % (v/v) aqueous methanol. One mL aliquot of the resulting solution was transferred to a 1.5 mL polypropylene tube and centrifuged (10 min, 12.000 g, 20 °C). Afterwards, 200 µL of the supernatant were transferred into a vial and stored in a fridge at 6 °C until LC/ESI-QTOFMS analysis. In addition, aliquots of the 123 extracts were combined to give a quality control (QC) sample within and between analytical batches (see Tais et al., 2021). One µl extract was analyzed by LC/ESI-QTOFMS in positive and negative ion mode.

2.3. Preparation of ethanolic extracts for bioassay

To evaluate the antifungal activity of *Z. multiflora* extract, three pooled samples per population were prepared by mixing homogenized dried leaf material from 6-11 individual plants. Homogenized leaf material (2.5 g) was weighed into a 15 mL polypropylene centrifuge tube. Afterwards 10 mL 50 % (v/v) aqueous ethanol were added and the mixture was thoroughly vortex-mixed for 60 s and sonicated for 15 min at 20-25 °C. The mixture was shaken again (2000 min^{-1}, ambient temperature, 30 min) and centrifuged (10 min, 12.000 g, 20 °C). The supernatant was transferred to a 25 mL volumetric flask. The remaining residue was extracted once again with 10 mL 50 % (v/v) aqueous ethanol as described above. The resulting extracts were combined and their volume adjusted to 25 mL using 50 % (v/v) aqueous ethanol. The extract were stored at 6 °C until antifungal assays and LC/ESI-QTOFMS analysis.

2.4. Fractionation of semi-polar metabolites

For fractionation *Z. multiflora* extract, 10 g of pooled sample from Konar Siah population were extracted with 100 ml 50 % (v/v) aqueous ethanol using an ultrasonic bath as described in section 2.3. The remaining residue was extracted once again with 100 mL 50 % ethanol. Both supernatants were combined (as original extract) and 10 mL were kept at 6 °C for bioassay and LC−MS analysis. The rest of extract was evaporated to dryness using a rotary evaporator under vacuum. The weight of the dried extract (2.95 g) was determined and dissolved in 20 % (v/v) aqueous methanol to obtain concentration of 10 mg/mL. The dissolved extract was fractionated on Strata C18-E solid phase cartridges (Strata Giga-tube C18-E,

silica-based, Phenomenex, Germany). For conditioning and equilibration, the cartridge was eluted with 60 mL methanol and finally washed with 60 mL 20 % methanol, respectively.

After conditioning and equilibration, a total of 25 mL of the dissolved extract in 20 % methanol transferred to the cartridge and the aqueous methanol phase was collected into 100 mL flask (fraction I). Furthermore, 25 mL of 20 % methanol were applied and the aqueous methanol phase was collected in fraction I. Subsequently, fraction II was eluted with 50 mL of 40 % aqueous methanol. Fraction III was eluted with 50 mL of 60 % methanol. Fraction IV was washed with 50 mL of 80 % aqueous methanol and eluate was collected. Finally, fraction V was eluted with 100 mL of pure methanol and elution was collected into a 200 mL flask. Elution from the column was proceeded into flasks by gravity flow. After fractionation, 500 µl of each fraction was transferred in sealed glass vial and stored at 6 °C until LC−MS analysis. The fraction I gave the highest yield (208 mg) followed by fraction II (71 mg), fraction III (19 mg), fraction V (10 mg) and fraction IV (8 mg) when the eluates were evaporated to dryness by rotary evaporation. Afterwards, the dried extracts redissolved in aqueous ethanol (50 %) to obtain concentration of 10 mg/mL for antifungal assays.

2.5. Screening for antifungal activity

Antifungal assays were accomplished according to the agar well diffusion assay used by (Jan et al., 2009) with slight modifications in order to identify antifungal activity of total extracts and fractions against *F. culmorum*, *F. sambucinum*, *B. cinerea*, *A. dauci* and *C. lindemuthianum* (from the mycological culture collection of the BBA/JKI, Berlin, Germany). Approximately twenty mL of potato dextrose agar (PDA) were poured into Petri dishes (9 cm). After solidification, a 6 mm plug was removed from the agar and holes were filled with 100 µL of each extract obtained in section 2.3 and 2.4. Then, 6 mm diameter mycelial discs of fungus (9-14 days old) were placed on the opposite side of the hole and the Petri dishes were incubated for 5-7 days at 20 °C in darkness. Antifungal activity was evaluated by measuring the radial growth of fungus in the direction of the punched hole using ImageJ software (an open platform for scientific image analysis). The inhibition rate (IR), was calculated using the formula, IR (%) = [(C-T)/C] × 100, where T represents the mycelial growth diameter in the treatment and C represents mycelial growth diameter in the control. The assay was repeated three times. Ethanol 50 % and untreated inoculum served as controls.

2.6. LC/ESI-QTOFMS

LC/MS analyses were performed according to the method described previously (Tais et al., 2021). In brief, an Infinity 1290 series UHPLC system (Agilent Technologies) interfaced to an iFunnel Q-TOF mass spectrometer (G6550A, Agilent Technologies) via a dual Agilent jet stream electrospray ion source was used. Controlling the instrument and data acquisition were

processed through the MassHunter LC/MS Data Acquisition software (version B.06.01) and for data evaluation, MassHunter Qualitative and Quantitative Analysis software (version B.07.00) was utilized. One µL of extracts were injected and separated on a Zorbax RRHD Eclipse Plus C18 column (100 × 2.1 mm, 1.8 µm particle size, Agilent Technologies) using 0.1 % (v/v) formic acid in water and 0.1 % (v/v) formic acid in methanol as eluent A and B, respectively. The following binary gradient program at a flow rate of 400 µL min^{-1} was used: 0-10 min, linear from 5 % to 95 % B; 10-13 min, isocratic, 95 % B; 13-15 min, isocratic, 5 % B. The column temperature was set at 40 °C. Mass spectra were acquired from *m/z* 100-1200 in centroid mode using an acquisition rate of 3 spectra per second.

Collision-induced dissociation (CID) mass spectra were acquired in auto-MS/MS mode using the following parameters, isolation width, narrow (1.3 m/z); collision energy, 10-60 V; collision gas, nitrogen.

2.6.1. Non-targeted data analyses

Using MassHunter Qualitative Analysis software, raw data files were converted into *mzData* format and arranged separately for each batch in fifteen sample classes (14 population and QC sample) and processed using the R package "XCMS" (Smith et al., 2006). Feature detection was carried out using the centWave algorithm [parameters: prefilter = (3, 1000); sntresh = 3; ppm = 25; peak width = (5, 12)]. Alignment was accomplished by consecutive application of the functions group.density (parameters: minfrac = 1; bw = 2; mzwid = 0.02), rector.loess (parameters: span = 1; missing = 0; extra = 0) and group.density (parameters: minfrac = 0.5; bw = 1.5, mzwid = 0.02). Missing feature intensities were set as NA values. Before statistical analyses NA values were replaced in the range of 500-600 by evenly distributed random numbers as an intensity threshold. Normalized peak area values were $\log_2$ transformed to correct for variance inhomogeneity.

2.6.2. Targeted analysis

To quantify target metabolites, the extracted ion chromatograms were integrated and generated for respective quantifier ions using MassHunter Quantitative Analysis software. Feature intensities were normalized by sample weight and $\log_2$ transformed (for detail see Tais et al., 2021).

2.7. Statistical analysis

All statistical analyses were performed using the R software (version 4.0.2). For non-targeted metabolomics, Random Forest (RF) classification models were carried out to compare metabolome patterns of *Z. multiflora* populations. The similarity of each individuals of

populations was visualized by the scatterplot of the unsupervised RF models for positive and negative ion modes. Furthermore, a Venn diagram was created using an absent-present matrix for all populations to depict how many features are common among all populations in different classes, and also how many features are unique for each class. For all identified compounds, peak areas were log_2-transformed and mean centered to visualize abundance of compounds in all populations by heatmap. For the comparison of antifungal activity of populations and fractions, analysis of variance (ANOVA) was performed using least significant difference (LSD) model. Moreover, correlation analyses (Spearman's correlation analysis and Canonical correlation analysis) were performed to analyze linear relationships between normalized abundance of identified compounds and antifungal activity of extracts. A geom-boxplot was created to visualize the inhibitory rate of plant extracts and fractions. The Bonferroni correction was applied to adjust for multiple testing.

3. Results

3.1. Metabolite fingerprinting

To investigate metabolite profiling, whole leaf samples of 14 populations (123 individuals) of *Z. multiflora* were analyzed using LC/ESI-QTOFMS in positive and negative ion mode. For non-targeted analysis, 9518 and 1839 features were obtained in positive and negative ion mode, respectively. Feature intensities were log_2-transformed and subjected to an unsupervised RF scatterplot to determine the phytochemical variation among *Z. multiflora* populations. The RF model classification of the metabolic data separated the investigated samples into three major classes. As shown in Figure 1 in positive ion mode (for negative ion mode, see Figure S1), the individuals from Siriz and Haneshk populations totally discriminated from the other populations, clustering as class I. According to LC/ESI-QTOFMS analyses, class II includes Fasa, Taft, Arsenjan, and Kemeshk populations and class III consist of Darab, Ashkezar, Hongooyeh and Gezeh populations. As revealed by the RF clustering, the individuals from Jandaq, Konar Siah, Daarbast and Gachooyeh populations were scattered in both class II and III. Presence–absence analysis shown over five thousand features were detected in all populations (Figure 2). Over thousand features were specific only for populations in class I, hundred eighty features were specific for populations in class II, ninety six features were specific for populations in class III and over two hundred fifty features were specific only for those populations that scattered among class II and III (see Figure S2 for negative ion mode).

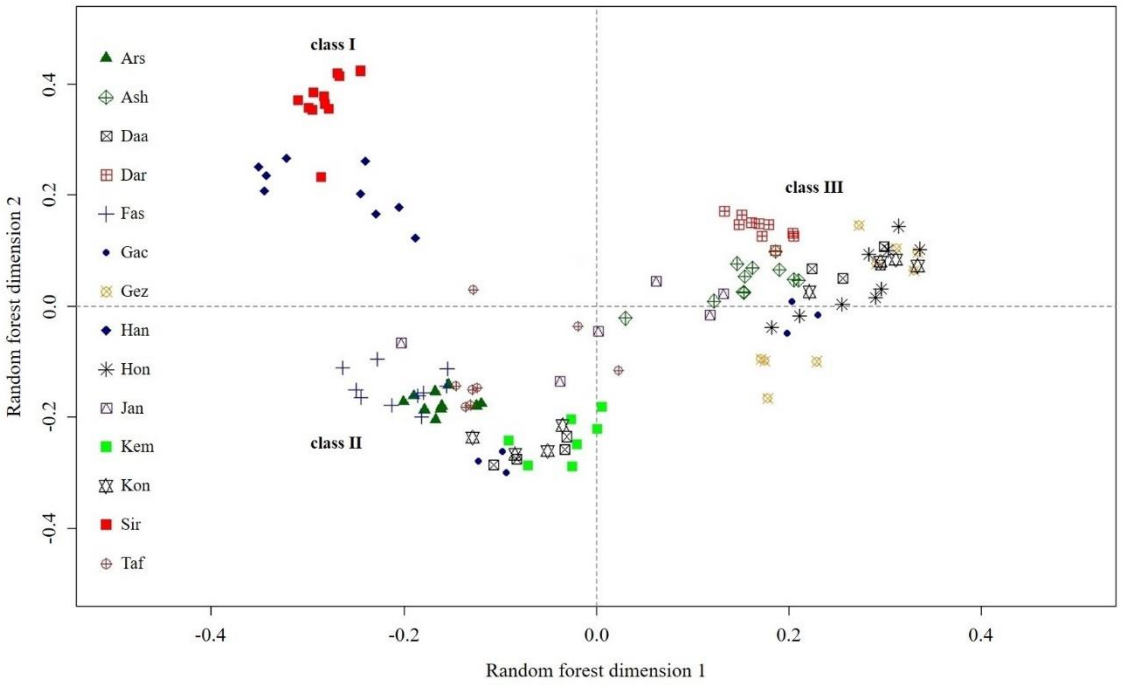

Figure 1. Comparison of metabolic-feature patterns of *Z. multiflora* populations by an unsupervised RandomForest model measured in positive ion mode.

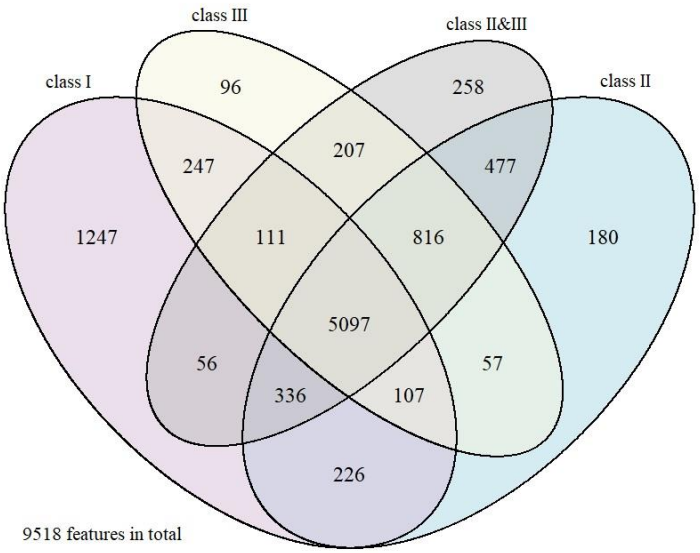

Figure 2. Common features between all four classes of *Z. multiflora* shown by a Venn diagram measured in positive ion mode.

3.2. Screening antifungal activity of extracts and fractions

The antifungal activity recorded for the ethanolic extracts from 14 *Z. multiflora* populations showed weak (populations of class I), moderate and high potential (class II and III) against the tested fungi which exhibited significant differences among populations (Table 2). The extracts from populations of Siriz and Haneshk (class I) showed only low antifungal activity against the all fungal pathogens (≤ 33 % and 37 %, respectively). We observed high antifungal activity against *F. culmorum* and *F. sambucinum* of the extracts from Konar Siah and Hongooyeh populations (≥ 59 % and 57 %, respectively). The most active extract against *B. cinerea* and *A. dauci* was the extract of the Jandaq population (≥ 54 % and 64 %, respectively) and the best activity against *C. lindemuthianum* was found with the extract of Daarbast population (≥ 65 %).

To determine the active fraction of ethanolic extracts, the extract of Konar Siah population was fractionated using Strata C18-E solid phase cartridges. Fraction IV was the most active fraction against all fungi (≥ 48 %), whilst fractions I and fraction V showed the lowest antifungal activity (≤ 25 %) compared to the other fractions and original extract (Figure 3).

Table 2. Antifungal activity of ethanolic extracts from plants of 14 *Z. multiflora* populations against five economically important plant pathogenic fungi.

Extract samples	Inhibition of mycelial growth (%)[a]				
	F. culmorum	*F. sambucinum*	*B. cinerea*	*A. dauci*	*C. lindemuthianum*
Arsenjan	51.5 ± 2.5 bcd	50.3 ± 3.8 ab	50.5 ± 1.5 abc	58.7 ± 3.1 bc	48.2 ± 5.5 de
Ashkezar	49.3 ± 3.1 cd	40.5 ± 3.3 c	46.1 ± 1.4 bcd	55.1 ± 1.5 bcd	44.8 ± 2.8 e
Daarbast	57.5 ± 2.5 ab	51.4 ± 3.6 ab	53.9 ± 3.7 ab	58.1 ± 3.9 bc	65.7 ± 4.2 a
Darab	52.2 ± 2.9 abcd	46.4 ± 2.3 bc	44.2 ± 2.3 cde	51.6 ± 1.1 de	47.6 ± 2.7 e
Fasa	52.9 ± 2.8 abcd	43.2 ± 1.5 bc	40.1 ± 7.7 de	48.4 ± 1.7 e	36.1 ± 3.7 f
Gachooyeh	54.6 ± 0.7 abcd	47.6 ± 7.4 bc	46.9 ± 1.6 abcd	53.5 ± 1.3 cd	46.2 ± 2.2 e
Gezeh	56.2 ± 3.4 abc	48.4 ± 2.7 bc	50.9 ± 1.5 abc	58.3 ± 4.7 bc	55.4 ± 2.7 c
Haneshk	36.8 ± 2.9 e	24.4 ± 10.5 d	21.7 ± 5.4 f	36.7 ± 2.1 f	22.5 ± 2.3 g
Hongooyeh	54.5 ± 3.3 abcd	57.6 ± 2.6 a	51.2 ± 2.3 abc	56.4 ± 3.5 bcd	48.7 ± 3.7 de
Jandaq	57.3 ± 3.4 ab	50.9 ± 2.3 ab	54.4 ± 0.8 a	64.9 ± 3.5 a	62.6 ± 3.3 ab
Kemeshk	56.6 ± 8.9 abc	43.3 ± 6.4 bc	50.9 ± 4.2 abc	53.6 ± 3.4 cd	53.8 ± 4.1 cd
Konar Siah	59.1 ± 1.9 a	51.7 ± 3.7 ab	51.9 ± 1.7 abc	59.6 ± 3.3 b	59.1 ± 0.6 bc
Siriz	31.4 ± 3.6 e	29.6 ± 3.8 d	18.9 ± 8.3 f	32.7 ± 4.8 f	21.1 ± 3.6 g
Taft	47.9 ± 3.8 d	41.3 ± 4.5 c	37.9 ± 0.5 e	54.1 ± 3.4 cd	36.1 ± 4.2 f

[a] Different lowercase letters indicate significant differences in each column among the means [+ SD] of antifungal activity by LSD tests ($p < 0.05$) using 9 replicates.

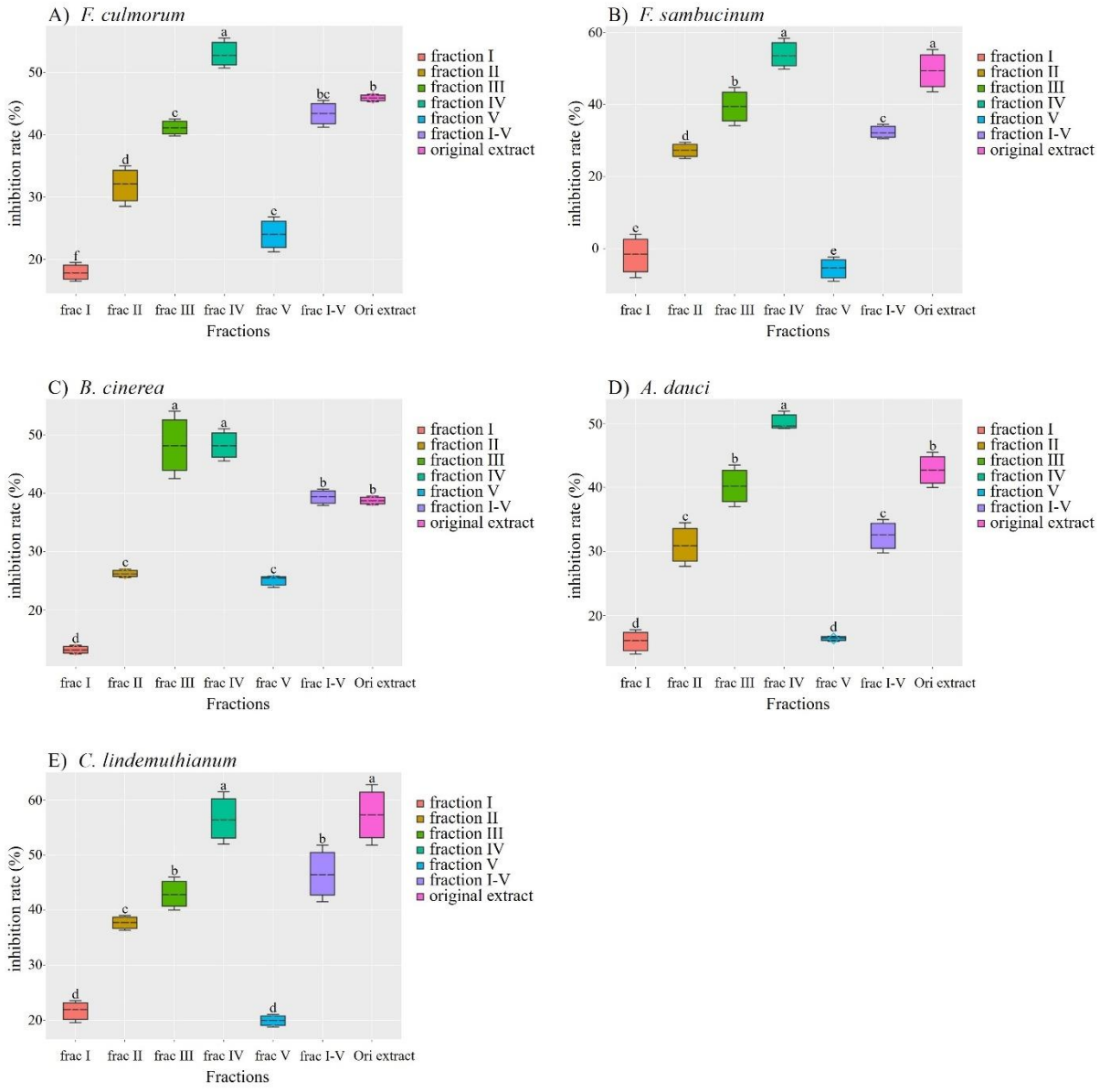

Figure 3. Box plot graphics representing the median, 25-75 % quartiles, min and max (%) of 3 replicates of the antifungal activity (%) of *Z. multiflora* fractions against A) *F. culmorum*; B) *F. sambucinum*; C) *B. cinerea*; D) *A. dauci*; E) *C. lindemuthianum*.

3.3. Recognition of candidate compounds

A total of 32 metabolites from the *Z. multiflora* extract were annotated and belonged to the three classes including 19 flavonoid conjugates and also 3 other partly unknown flavonoid compounds, 4 hydroxycinnamic acid (HCA) derivatives, 2 phenolic terpenes and 4 unknown compounds (Table 3). Flavonoids were the main compounds presented in the extracts of *Z. multiflora*, considering that two third of the annotated compounds represent flavonoid conjugates. Flavonoids detected in *Z. multiflora* populations belonged to flavone, flavonol and flavanone classes (see Figure 4).

Table 3. Characterization of phenolic compounds in hydroalcoholic extracts of *Z. multiflora*.

Compound	Peak no.	Elemental composition	Quantifier ion m/z [M+H]+	Quantifier ion m/z [M-H]-	RT [min][a]	Annotation level[b]	Fraction[c]
Apigenin 6-C-hexoside-8-C-hexoside	peak 1	C27H30O15	595.159	593.151	4.23	3	II
C12H18O4 unknown	peak 2	C12H18O4	227.128	225.113	4.45	4	II
Dihydroquercetin (Taxifolin)	peak 3	C15H12O7	305.066	303.051	4.65	2	II
Luteolin C-hexoside	peak 4	C21H20O11	449.108	447.093	4.71	3	II&III
Quercetin O-hexuronide	peak 5	C21H18O13	479.082	477.068	4.8	3	II&III
Quercetin O-hexoside	peak 6	C21H20O12	465.096	463.088	4.85	3	II&III
Apigenin C-hexoside	peak 7	C21H20O10	433.113	431.098	5.01	3	II&III
Kaempferol O-hexoside-O-deoxyhexoside	peak 8	C27H30O15	595.159	593.151	5.26	3	II&III
Kaempferol O-hexuronide	peak 9	C21H18O12	463.087	461.073	5.27	3	II&III
Kaempferol O-hexoside	peak 10	C21H20O11	449.108	447.093	5.3	3	II&III
Dihydrokaempferol	peak 11	C15H12O6	289.071	287.056	5.34	2	II&III
Rosmarinic acid -minor isomer	peak 12	C18H16O8		359.077	5.48	1	II&III
Lithospermic acid B	peak 13	C36H30O16		717.146	5.61	3	II&III
Methyl caffeate	peak 14	C10H10O4	195.065		5.65	3	II&III
Rosmarinic acid -major isomer	peak 15	C18H16O8		359.077	5.67	1	II&III
Apigenin O-hexuronide	peak 16	C21H18O11	447.092	445.078	5.71	3	II&III
Chrysoeriol O-hexuronide	peak 17	C22H20O12	477.103	475.088	5.81	3	II&III
Chrysoeriol O-hexoside	peak 18	C22H22O11	463.124	461.109	5.95	3	II&III
Eriodictyol	peak 19	C15H12O6	289.071	287.056	6.03	2	III
Naringenin	peak 20	C15H12O5	273.076	271.061	6.66	2	III&IV
unknown Flavonoid, "Apigenin + Methoxy"	peak 21	C16H12O6	301.071	299.056	6.81	4	III&IV
Luteolin	peak 22	C15H10O6	287.055	285.041	6.83	1	III&IV
unknown Flavonoid, "Apigenin + 2 Methoxy"	peak 23	C17H14O7	331.081	329.067	6.9	4	III&IV
Apigenin	peak 24	C15H10O5	271.06	269.046	7.34	1	IV
unknown Flavonoid, "Apigenin + Methoxy"	peak 25	C16H12O6	301.071	299.056	7.42	4	IV
Dihydrothymoquinone	peak 26	C10H14O2		165.092	7.72	2	IV
5-/7-O-Methyl-Naringenin	peak 27	C16H14O5	287.091	285.077	7.95	3	IV
C10H14O3 unknown	peak 28	C10H14O3	183.102		8.31	4	IV
Genkwanin	peak 29	C16H12O5	285.076	283.061	8.55	2	IV
C20H24O4 unknown	peak 30	C20H24O4	329.175	327.16	9.17	4	IV
C20H24O3 unknown	peak 31	C20H24O3	313.18	311.165	9.27	4	IV
C25H24O5 unknown	peak 32	C25H24O5	405.17	403.155	9.35	4	IV

[a] RT: retention time. [b] Annotation level: 1, verfied by measured standard; 2, verified by spectra and RT; 3, verified by spectra; 4, unknown. [c] *Z. multiflora* fractions.

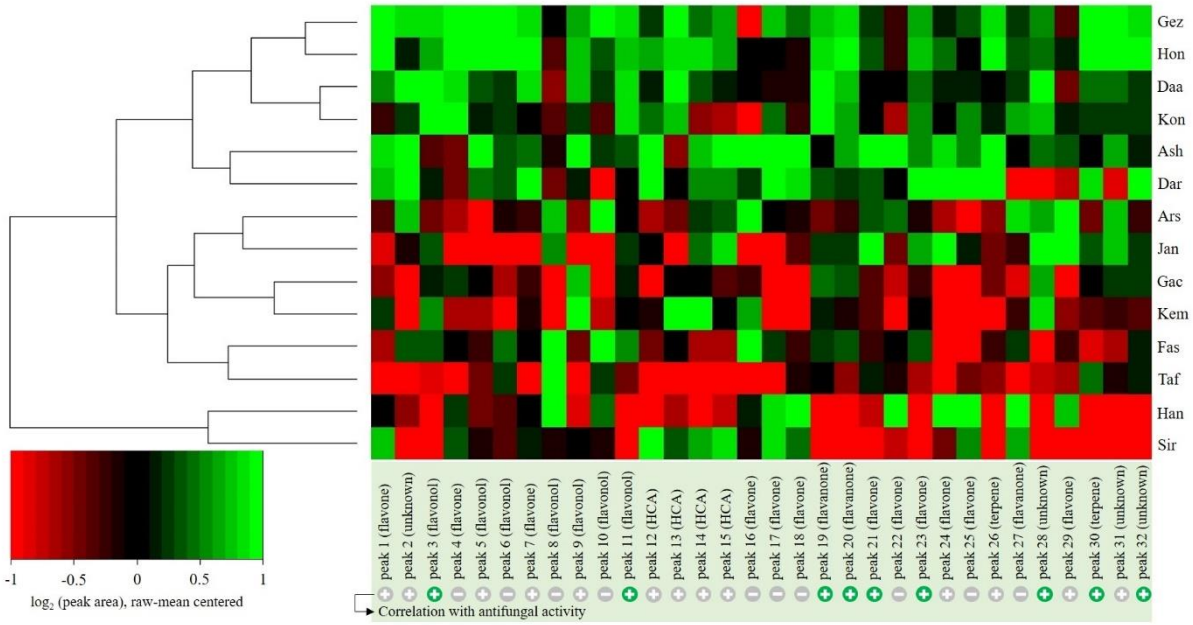

Figure 4. Abundance of annotated compounds and correlation of compound abundance with antifungal activity of Z. multiflora extracts against fungal pathogens. Significant positive correlations by linear model with $p < 0.05$ labelled in green cross, and $p > 0.05$ in grey.

Twelve flavone conjugates with apigenin, luteolin and chrysoeriol as aglycones were found in the extracts. Peak 1 was tentatively assigned to Apigenin 6-C-hexoside-8-C-hexoside ($[M+H]^+$ at *m/z* 595.158, $[M-H]^-$ at *m/z* 593.151), peak 7 to apigenin C-hexoside ($[M+H]^+$ at *m/z* 433.112, $[M-H]^-$ at *m/z* 431.098) and peak 16 to apigenin O-hexuronide ($[M+H]^+$ at *m/z* 447.092, $[M-H]^-$ at *m/z* 445.077). Peak 24 ($[M+H]^+$ at *m/z* 271.060, $[M-H]^-$ at *m/z* 269.045) was identified as apigenin. Furthermore, three other partly unknown flavone conjugates [peak 21 ($C_{16}H_{12}O_6$), peak 23 ($C_{17}H_{14}O_7$), and peak 25($C_{16}H_{12}O_6$)] were also observed, which are consistent with an apigenin plus methoxy groups. Peak 4 at 4.71 min ($[M+H]^+$ at *m/z* 449.107, $[M-H]^-$ at *m/z* 447.093) were assigned as being luteolin C-hexoside. Luteolin (peak 22) eluting at 6.83 min showed $[M+H]^+$ ions of *m/z* 287.055, and $[M-H]^-$ ions of *m/z* 285.041. Peak 17 and 18 were tentatively assigned to Chrysoeriol O-hexuronide ($[M+H]^+$ at *m/z* 477.102, $[M-H]^-$ at *m/z* 475.088) and Chrysoeriol O-hexoside ($[M+H]^+$ at *m/z* 463.123, $[M-H]^-$ at *m/z* 461.108), respectively. Peak 29 ($[M+H]^+$ at *m/z* 285.075, $[M-H]^-$ at *m/z* 283.061) was identified as genkwanin. Except for two unknown apigenin conjugates (peak 21 and peak 23) which showed significantly positive correlation with antifungal activity, no significant correlation for other flavone conjugates were observed (Figure 4, for detail see Figure S3 and Table S1).

Seven flavonol conjugates with quercetin and kaempferol as aglycones were detected. Peak 3 ($[M+H]^+$ at *m/z* 305.065, $[M-H]^-$ at *m/z* 303.051) was identified as being

dihydroquercetin. Peak 5 and 6 were tentatively assigned to quercetin O-hexuronide ($[M+H]^+$ at *m/z* 479.082, $[M-H]^-$ at *m/z* 477.067) and quercetin O-hexoside ($[M+H]^+$ at *m/z* 465.095, $[M-H]^-$ at *m/z* 463.088), respectively. Peak 8, 9 and 10 were tentatively assigned to kaempferol O-hexoside-O-deoxyhexoside ($[M+H]^+$ at *m/z* 595.158, $[M-H]^-$ at *m/z* 593.151), Kaempferol O-hexuronide ($[M+H]^+$ at *m/z* 463.087, $[M-H]^-$ at *m/z* 461.072) and Kaempferol O-hexoside ($[M+H]^+$ at *m/z* 449.107, $[M-H]^-$ at *m/z* 447.093), respectively. Peak 11 ($[M+H]^+$ at *m/z* 289.071, $[M-H]^-$ at *m/z* 287.056) was identified as dihydrokaempferol eluting at 5.34 min. Among flavonol conjugates, dihydroquercetin (peak 3) and dihydrokaempferol (peak 11) showed significantly positive correlation with antifungal activity (Figure 4, for detail see Figure S2 and Table S1). Three flavanone compounds named eriodictyol, naringenin and 5-/7-O-methyl naringenin were detected. Peak 19 eluting at 6.03 min ($[M+H]^+$ at *m/z* 289.071, $[M-H]^-$ at *m/z* 287.056) was identified as eriodictyol. Peak 20 were assigned to naringenin ($[M+H]^+$ at *m/z* 273.075, $[M-H]^-$ at *m/z* 271.061) and peak 27 eluting at 7.95 min ($[M+H]^+$ at *m/z* 287.091, $[M-H]^-$ at *m/z* 285.076) could be assigned as 5-/7-O-methyl naringenin. Correlation analysis revealed strongly positive correlation between antifungal activity and eriodictyol and naringenin (Figure 4, for detail see Figure S3 and Table S1).

Four HCA derivatives were identified in *Z. multiflora* extracts. Two rosmarinic acid isomers ($[M-H]^-$ at *m/z* 359.077, $[M+Na]^+$ at *m/z* 383.073) eluting at 5.48 min and 5.67 min (peak 12 and 15, respectively) were detected. Peak 13 ($[M-H]^-$ at *m/z* 717.146, $[M+NH4]^+$ at *m/z* 736.187) were identified as lithospermic acid B and peak 14 as methyl caffeate ($[M+H]^+$ at *m/z* 195.065). Although there were positive correlation between HCA derivatives and antifungal activity, no significant correlation were observed (Figure 4, for detail see Figure S3 and Table S1). Two phenolic terpenes were also detected. Peak 26 ($[M-H]^-$ at *m/z* 165.092) were identified as dihydrothymoquinone and peak 28 which were identified by elemental composition (C10H14O3, $[M+H]^+$ at *m/z* 183.101, eluting at 8.31 min) showed positive correlation with antifungal activity. In addition, four unknown compound were only characterized by elemental composition, including peak 2 (C12H18O4, $[M+H]^+$ at *m/z* 227.127, $[M-H]^-$ at *m/z* 225.113), peak 30 (C20H24O4, $[M+H]^+$ at *m/z* 329.174, $[M-H]^-$ at *m/z* 327.160), peak 31 (C20H24O3, $[M+H]^+$ at *m/z* 313.179, $[M-H]^-$ at *m/z* 311.165) and peak 32 (C25H24O5, $[M+H]^+$ at *m/z* 405.169, $[M-H]^-$ at *m/z* 403.155), which had positive correlation with antifungal activity (Figure 4, for detail see Figure S3 and Table S1).

As shown in Figure S4, among those nine compounds, which had significantly positive correlation with antifungal activity, six of them were detected in fraction IV including naringenin, unknown apigenin conjugates (peak 21 and 23), unknown phenolic terpenes (peak 28), and two unknown compounds (peak 30 and 32). Furthermore, eriodictyol and dihydrokaempferol along with naringenin and unknown apigenin conjugates (peak 21 and 23)

were presented in fraction III. In addition, fraction II contained dihydroquercetin and dihydrokaempferol as active compounds.

4. Discussion

Over the last few decades, crop diseases caused by fungal pathogens have become a big problem in food and agricultural industries due to losses of significant portions of production. Control of these diseases mainly relied on synthetic chemical fungicides, which are associated to negative environmental impacts, residual problems on food and toxicity to human and animals (Sales et al., 2016). Therefore, to overcome these problems, plant extracts with bioactive secondary metabolites were used as an effective and ecofriendly alternative approach (Gahukar, 2012). In the present study, *Z. multiflora* hydroalcoholic extract were considered as potential source of valuable metabolites including phenolic and flavonoid compounds which could be used as natural antifungal agents against a broad spectrum of agriculturally important fungal pathogens. Several studies have reported the bioactivity of plant metabolites such as phenolic terpenes, flavonoids and saponins against not only fungal and bacterial pathogens (Karimi and Meiners, 2021; Martins et al., 2015; Koolen et al., 2013), but also against insects, e.g. the pollen beetle which can cause high yield losses in oilseed rape (Austel et al., 2021).

According to the results shown in Figure 1, *Z. multiflora* populations differed in their metabolite profile due to their secondary metabolites (non-volatile compounds) present in the extracts. In previous studies, we found three major chemotypes among 14 *Z. multiflora* populations, named linalool, thymol and carvacrol chemotypes referring to the essential oil compounds (Karimi et al., 2020a). In comparison to essential oil classification of *Z. multiflora* populations (see Karimi et al., 2020a), populations from Siriz and Haneshk were classified as class I referring to the non-volatile compounds in their hydroalcoholic extract (see Figure 1 and Figure 4) as similar as essential oil clustering. Nevertheless, populations from Fasa and Darab, which clustered as thymol chemotype based on essential oil compounds, were classified in different classes based on their non-volatile compounds. The other 10 populations that clustered as carvacrol chemotype based on essential oil compounds, were scattered into two groups referring to non-volatile compounds (see Figure 1 and Figure 4).This phytochemical diversity of medicinal plant species can have its origin in different environmental conditions and/or genetic factors (Karimi et al., 2020b; Karimi et al., 2020a; Selseleh et al., 2019). Phytochemical diversity of one medicinal plant population based on non-volatile and/or volatile compounds might show different clustering among its populations, which can have different antimicrobial activity based on the metabolites present.

For example, *Verbascum songaricum* populations were clustered differently with respect to volatile compounds compared to non-volatile compounds (Selseleh et al., 2019).

From thirty-two compounds identified in this study, two third of the annotated compounds were flavonoids along with four HCA derivatives and two phenolic terpenes. In agreement with our results, there is a study on *Z. multiflora* reported that hydroalcoholic extract contains flavonoid and HCA compounds such as vicenin-2, luteolin-O-glcA, naringenin, lithospermic acid and rosmarinic acid (Izadiyan et al., 2019). There are several reports on other Lamiaceae species revealed that rosemary, oregano, sage, basil and thyme contained a considerable amount of flavonoid compounds such as quercetin, apigenin and luteolin (Hossain et al., 2010). In addition, the presence of phenolic and flavonoid compounds including eriodictyol, quercetin, apigenin, genkwanin, luteolin and kaempferol conjugates as well as rosmarinic acid were reported in *Thymus vulgaris* (Martins et al. 2015) and *Rosmarinus officinalis* (Jordán et al., 2012).

The majority of the studies on *Z. multiflora* have reported antifungal and antibacterial activities of essential oil compounds (Mohammadi et al., 2015; Kavoosi and Rabiei, 2015; Gandomi et al., 2009, Sharififar et al., 2007) which are related to its phenolic monoterpenes particularly thymol and carvacrol (Karimi and Meiners, 2021). Nevertheless, when screening *Z. multiflora* extracts for bioactive secondary metabolites, flavonoid compounds such as dihydroquercetin, dihydrokaempferol, eriodictyol and naringenin showed strong correlation with antifungal activity. Moreover, there is also high correlation between two unknown apigenin conjugates (peak 21 and 23), unknown phenolic terpenes (peak 28), and two unknown compounds (peak 30 and 32) and antifungal activity (see Figure 4). As shown in Figure 4, the abundance of these compounds were in the highest levels in those of populations (class II and III) who exhibited the highest antifungal activity whilst populations from Siriz and Haneshk (class I) were characterized by lower amounts of these compounds. On the other hand, it is shown that those fractions containing these compounds (fraction IV > fraction III > fraction II) are more active against studied fungal pathogens (see Figure 3 and Figure S4).

Among flavonoids exhibiting positive correlation with antifungal activity, naringenin was associated with inhibition of growth of *Phytophthora* spp (Boiteux et al., 2014). Furthermore, eriodictyol and naringenin showed the greatest activity against bacterial pathogens and yeast comparing to the other compounds of bergamot extract (Mandalari et al., 2007). Tajuddeen et al. (2014) reported that in contrast to kaempferol, dihydrokaempferol was the more active compound against bacterial pathogens. As Sunil and Xu. (2019) reviewed, dihydroquercetin had promising pharmacological activities e.g. anti-cancer, anti-oxidants and antimicrobial. Furthermore, dihydroquercetin showed antimicrobial activity against a broad spectrum of important bacterial pathogens (Artem'Eva et al., 2015). Hence, antifungal activity of active fractions and populations (class II and III) of *Z. multiflora* could be

associated with the presence of eriodictyol and naringenin along with other flavonoids. When comparing antifungal activity of *Z. multiflora* hydroalcoholic extracts with essential oils (see Karimi and Meiners, 2021), populations who contain phenolic and flavonoid compounds revealed promising antifungal activity. Nevertheless, further studies require to be performed in order to understand the effects of single compounds as well as the synergic effects of mixed compounds.

5. Conclusion

In conclusion, *Z. multiflora* extracts are a valuable source of flavonoids, phenolic terpenes and HCA derivatives. There was a huge phytochemical diversity among populations of this species, referring to the amounts of phenolic and flavonoid compounds in hydroalcoholic extracts. However, ethanolic extracts of *Z. multiflora* populations (class II and III) were rich in flavonoids and phenolic compounds particularly dihydrokaempferol, dihydroquercetin, eriodictyol and naringenin, which exhibited promising antifungal activity against studied fungal pathogens. On the other hand, extracts from Siriz and Haneshk populations (class I) which were characterized by lower amounts of these compounds showed lowest levels of antifungal activity. Overall, antifungal activity of *Z. multiflora* extracts are highly associated with the presence of flavonoids and phenolic terpenes.

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Appendix. Supplementary data

Table S1. Spearman's Correlation coefficients from correlations between antifungal activity and abundance of annotated compounds from plants of 14 *Z. multiflora* populations.

compound	Fungal pathogens				
	F. culmorum	*F. sambucinum*	*B. cinerea*	*A. dauci*	*C. lindemuthianum*
peak1	0.01	0.11	0.08	-0.07	0.15
peak2	0.38	0.42	0.40	0.38	0.39
peak3	0.85***	0.82***	0.85***	0.74***	0.81***
peak4	-0.06	0.16	-0.04	-0.12	0.01
peak5	0.02	0.15	0.03	-0.04	-0.01
peak6	-0.06	0.12	-0.05	-0.03	-0.08
peak7	-0.01	0.17	0.06	-0.08	0.11
peak8	-0.34	-0.15	-0.31	-0.12	-0.36
peak9	0.21	0.20	0.27	0.03	0.26
peak10	-0.18	-0.04	-0.16	-0.15	-0.23
peak11	0.83***	0.79***	0.84***	0.78***	0.76***
peak12	0.22	0.47	0.31	0.19	0.27
peak13	0.24	0.26	0.21	-0.05	0.24
peak14	0.12	0.27	0.27	0.11	0.31
peak15	-0.09	0.15	0.10	0.05	0.15
peak16	-0.09	-0.09	-0.05	-0.15	-0.15
peak17	-0.35	-0.16	-0.30	-0.28	-0.25
peak18	-0.45	-0.31	-0.40	-0.31	-0.35
peak19	0.86***	0.85***	0.85***	0.78***	0.78***
peak20	0.79***	0.80***	0.79***	0.72***	0.71***
peak21	0.61**	0.58*	0.67***	0.72***	0.57*
peak22	-0.33	-0.25	-0.29	-0.23	-0.32
peak23	0.78***	0.72***	0.79***	0.74***	0.70***
peak24	-0.08	0.06	0.00	0.07	0.07
peak25	-0.40	-0.23	-0.37	-0.29	-0.26
peak26	0.14	0.35	0.17	0.18	0.10
peak27	-0.33	-0.21	-0.24	-0.17	-0.21
peak28	0.72***	0.75***	0.80***	0.69***	0.71***
peak29	0.05	0.12	0.19	0.35	0.12
peak30	0.61**	0.71***	0.63**	0.65**	0.57*
peak31	0.49	0.56	0.51	0.55	0.60*
peak32	0.69***	0.76***	0.70***	0.67***	0.59*

Significance levels after Bonferroni correction: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

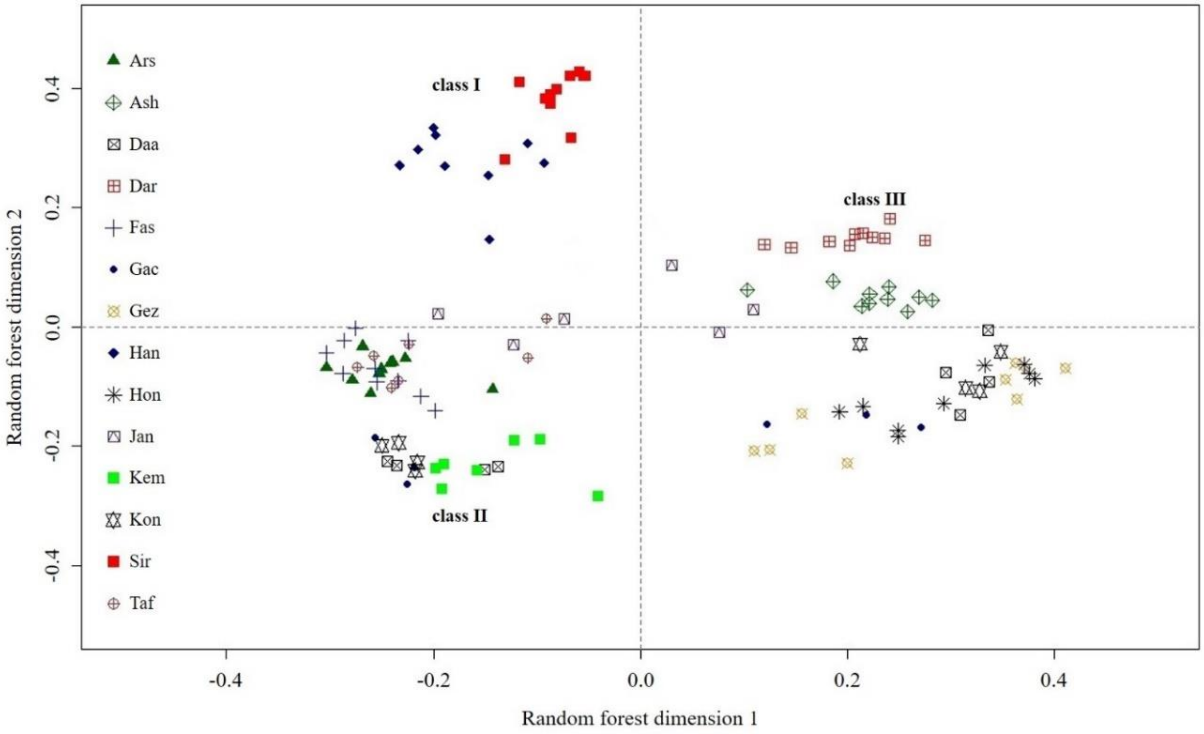

Figure S1. Comparison of metabolic-feature patterns of *Z. multiflora* populations by an unsupervised RandomForest model measured in negative ion mode.

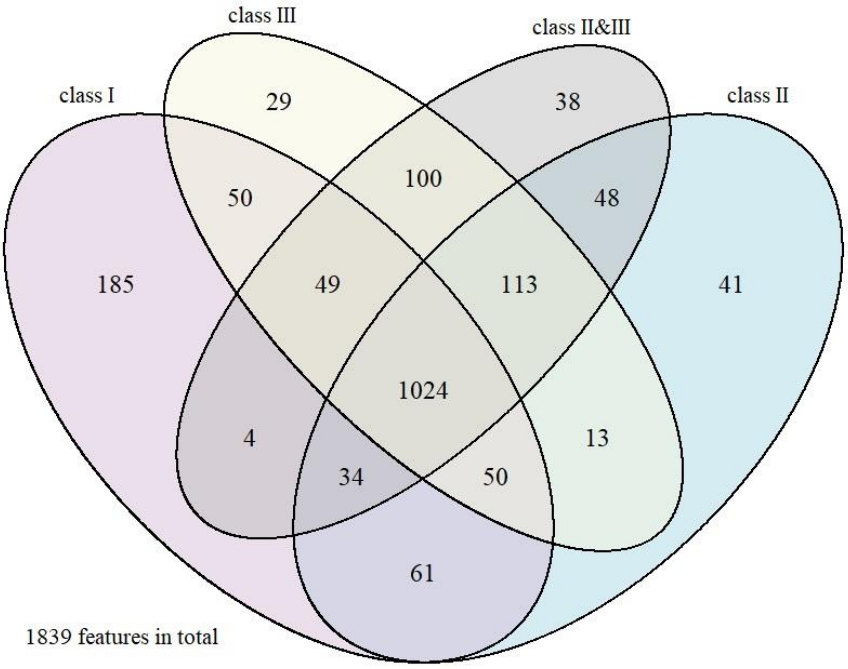

Figure S2. Common features between all four classes of Z. multiflora shown by a Venn diagram measured in negative ion mode.

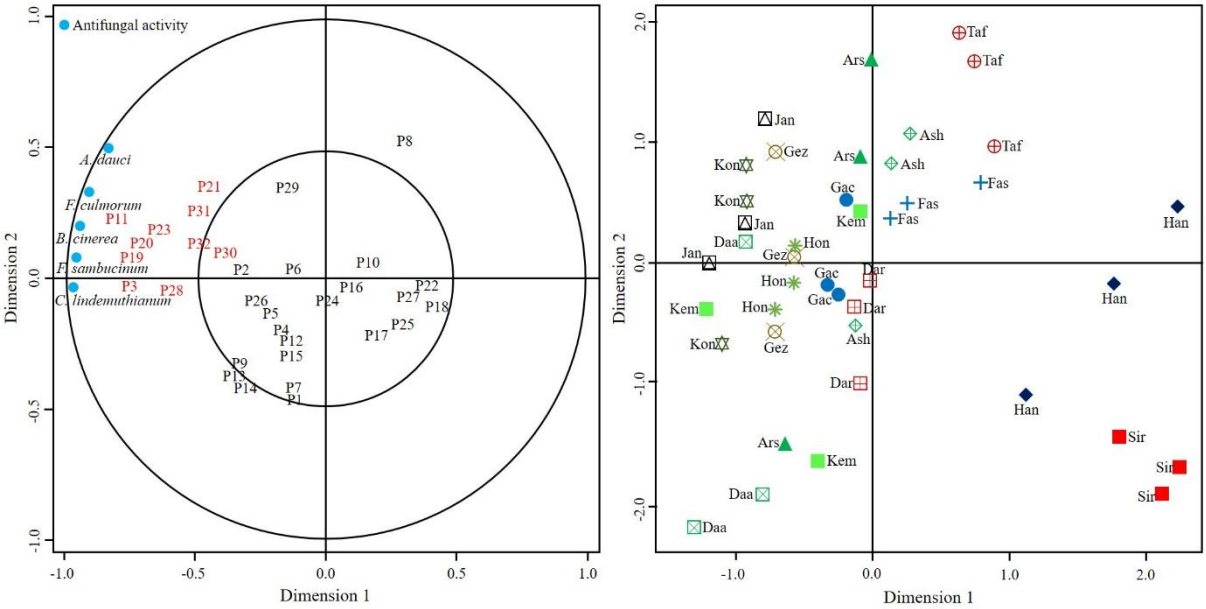

Figure S3. Canonical correlation analysis (CCA) of annotated compounds and correlation of compound abundance with antifungal activity of *Z. multiflora* extracts against fungal pathogens (left) and Score plot (PCA) obtained from the main variation of annotated compounds and antifungal activities among populations (right). P, peak area of annotated compounds; peak in red color had significant positive correlation with antifungal activity.

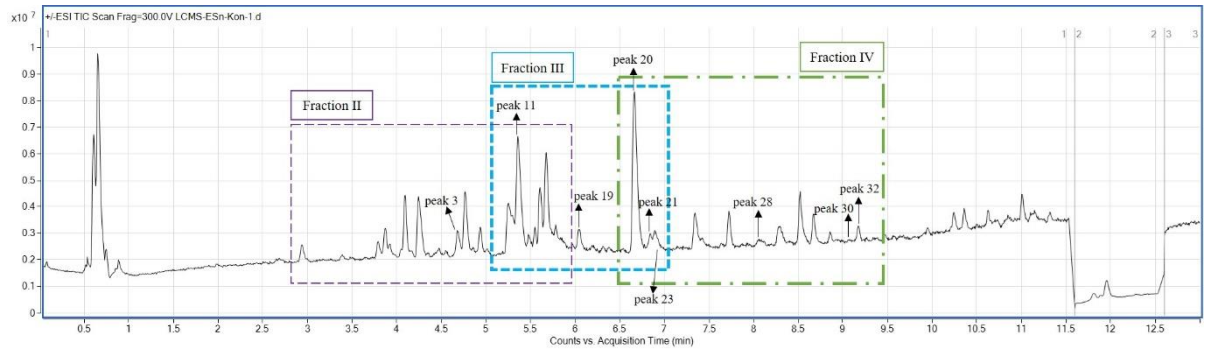

Figure S4. Fractionation of phenolic profile of *Z. multiflora* hydroalcoholic extract recorded in negative ion mode.

Antifungal activity of *Zataria multiflora* Boiss. essential oils and changes in volatile compound composition under abiotic stress conditions. https://doi.org/10.1016/j.indcrop.2021.113888

Abstract

There is an increasing need for natural compounds for pest control and food preservation in agriculture, food and dairy industries. To satisfy this need, essential oils (EOs) from aromatic plants can serve as flavors, food preservatives and ecofriendly pesticides. This study investigated the potential of different EOs from field-collected leaves of fourteen *Zataria multiflora* Boiss. populations representing three different chemotypes (carvacrol, thymol and linalool) to inhibit a broad spectrum of fungal pathogens important in food industry and agriculture and the relationship between total leaf elements concentration and EOs compounds. Furthermore, a greenhouse experiment was performed to elucidate the effects of heat stress (33 °C vs. 20 °C), drought stress (50 % reduced irrigation), and ultraviolet light intensity (3, 6 and 9 W m^{-2} UV-A radiation) on the relative content of specific volatile compounds. The results indicated that low concentrations of carvacrol and thymol, but not of linalool chemotype EOs inhibit significantly the growth of pre- and postharvest pathogens *Colletotrichum lindemuthianum*, *Fusarium sambucinum*, *Fusarium culmorum*, *Alternaria dauci* and *Botrytis cinerea* (thymol/carvacrol EOs: 0.8-1 µL, linalool EOs: 4 µL). The analyses revealed further significant correlations between the concentrations of mineral elements in *Z. multiflora* leaves and relative amounts of EO compounds and antifungal activity. Abiotic stresses, particularly heat and the interaction of drought and heat, induced changes in plants of the linalool chemotype resulting in higher relative amounts of carvacrol (22.7 % and 32.9 % vs. 1.5 %), while drought stress alone did not influence the relative amount of the main volatile compounds of *Z. multiflora* (carvacrol 1.7 %). Furthermore, the relative amount of linalool was slightly reduced in the linalool chemotype, when plants were subjected to high intensities of UV-A radiation (33.9 % vs. 44.6 %), whilst the relative amount of carvacrol was slightly increased (20.1 % vs. 9 %). Moreover, the main volatile compounds of plants from the carvacrol chemotype did not change in response to abiotic stresses. Understanding the effect of environmental conditions on aromatic plant populations and chemotype development helps agriculture and food industry fully exploiting the potential of aromatic plants as a source of natural sustainable fungicides or insecticides.

Keywords: Phenolic compounds; Pathogenic fungi; Heat stress; Drought stress; UV-A irradiation; Leaf mineral elements.

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General discussion

The aims of this thesis were:

1) to evaluate the environmental metabolomics approach for identifying regions for sampling plant material (*Ferula assa-foetida* L. and *Zataria multiflora* Boiss.) with the desired chemical profile by considering different environmental factors that might influence the secondary metabolites.
2) to investigate different plant populations and/or chemotypes of *Z. multiflora* to identify secondary plant metabolites as active compounds against agricultural plant infestations with respect to its essential oils (EOs) and hydroalcoholic extracts.
3) to enhance the production of beneficial metabolites of *Z. multiflora* by mimicking extreme environmental conditions during cultivation.

Here, I first discuss the environmental metabolomics approach and the abiotic environmental factors that might influence plant secondary metabolites and cause differentiation in plant chemotypes. Then I refer to the use of plant extracts with respect to their active compounds against fungal pathogens and application of abiotic stresses to increase beneficial metabolites production.

Can environmental metabolomics be used as a tool to indicate specific plant metabolites?

Environmental metabolomics is the application of metabolomics to analyze the interactions of organisms with their environment at the molecular level; the interactions can be investigated from individuals to populations of a species (Bundy et al., 2009). In the plant sciences environmental metabolomics is an approach that applies metabolome analyses techniques (GC−MS, LC−MS, NMR or NIR spectroscopy) to investigate the effects of environmental factors on plant metabolism (Stierlin et al., 2020); in food science it is used to detect the origin food products (Ghisoni et al., 2019). This approach has many advantages such as to identify and quantify of a wide range of metabolites in plants that are involved with diverse traits, pathways and stress responses, so it may discover unexpected or even novel responses to environmental stressors (Bundy et al., 2009). Plants produce a wide variety of chemical compounds with diverse biological functions in response to their environment.

In natural habitats, environmental factors influence secondary metabolites of plant species, and cause variation in chemical compounds determining the distribution of plant species and the abundance of specific populations in natural habitats. To date, environmental

metabolomics has been successfully applied in plant sciences, e.g. in *Thymus vulgaris* showing that seedlings of non-phenolic chemotypes are more adapted to low temperatures in winter than seedlings from phenolic chemotypes (Amiot et al., 2005).

In *chapter 2* and *3*, environmental metabolomics using GC−MS and NIR spectroscopy together with Structural equation models revealed how EO content and its compounds of *F. assa-foetida* and *Z. multiflora* plants were directly and/or indirectly influenced by environmental conditions. This approach recognized three different chemotypes among *F. assa-foetida* populations with respect to the different environmental conditions (Karimi et al., 2020a). In addition, it revealed that the non-phenolic chemotype of *Z. multiflora* is more adapted to low temperatures while phenolic chemotypes are better adapted to summer drought (Karimi et al., 2020b). The results demonstrated that the variation of specific environmental factors cause the variability of secondary metabolites and might constrain or enhance the production of specific compounds and the presence of certain chemotypes.

This approach is a well-established method that offers higher sensitivity, selectivity, ease of use and reproducibility to study secondary plant metabolites and their interactions with the environment (Beale et al., 2018). Besides, NIR spectroscopy due to distinct advantages of being nondestructive and portability is nowadays widely applied directly on plant materials to examine the chemical profile of plants in the field. However, it should be emphasized that sample classification by NIR spectroscopy may affected by entire sample composition, fiber, water content and in some cases also the sample shape of plant materials. For example, the different root shapes of plants from the Tabas population of *F. assa-foetida*, which had very thin and small roots compared to all other populations, affected NIR reflection and caused different classification comparing to GC-analysis (Karimi et al., 2020a).

How do environmental factors influence secondary metabolites?

Plants live in a complex environment and it is known that environmental conditions, edaphic and genetic factors (Karimi et al., 2020a; Ormeño et al., 2008) as well as biotic stresses (Kessler and Baldwin, 2002) alter plant metabolites or chemotypes. Plants produce diverse secondary metabolites including volatile and non-volatile compounds in response to their environmental conditions. The variation in the composition of chemical compounds occurs not only between plant families, but even in the same species. Plants interact with the environment leading to changes in their metabolism in order to survive; under the biotic and abiotic stresses they counteract the irreparable damages (Borges et al., 2017). Changes in plant metabolites can improve plant growth and survival under abiotic and biotic stresses, for example, several classes of secondary metabolites integrate into plant metabolism and can have regulatory roles and provide precursors for primary metabolites (Erb & Kliebenstein,

2020). Therefore, secondary metabolites may have multifunctional features that are required for plant interactions with the environment (Kliebenstein, 2013).

Medicinal plants can accumulate secondary metabolites such as phenolic compounds or terpenes in their different tissues in response to certain stresses. These compounds, because of their multifunctional features, can help plants to adapt to harsh conditions (Verma and Shukla 2015). On the basis of volatile and non-volatile compounds, phytochemical differentiation of medicinal plants can be observed among their populations with respect to different geographical conditions, e.g. in *Verbascum songaricum* (Selseleh et al., 2019), and *Tanacetum vulgare* (Kleine and Müller, 2013).

In *chapter 2* and *chapter 3*, the effect of environmental factors (including geographical, edaphic and climatic parameters) on EO content and its compounds of *F. assa-foetida* and *Z. multiflora* was investigated. The results revealed that EO content and EO composition were directly influenced by edaphic factors (portion of clay, silt and sand as well as iron, potassium, and calcium content) and temperature and predominantly indirectly by geographical factors (latitude, longitude, and altitude) which cause differentiation among populations and chemotypes. Many studies reported that secondary metabolites were influenced by the environment (Thakur and Kumar, 2020), for example, Ca fertilization changes essential oil compounds in sweet basil (Dzida, 2010). In addition, the chemical composition of *Salix myrsinites* leaves varied between two elevations and was affected by warming and gall infestation (Swanson et al., 2021). In *Z. multiflora*, altitude had a significant positive effect on linalool content whilst it had a negative effect on carvacrol content. Furthermore, temperature had a significant positive correlation with the carvacrol content and a significant negative correlation with the linalool content (*chapter 3*).

The study on EO compositions of *F. assa-foetida* roots revealed that edaphic, geographical and climatic factors strongly affected EO content and its compositions causing distribution of populations into different chemotypes (see figure 4 and 5, *chapter 2*). It suggests that soil parameters and climatic condition can modulate essential oil content and its compounds in below- or above ground tissues of medicinal plants and cause accumulation of specific secondary metabolites in their tissues depending on environmental conditions of their growing regions. The findings therefore were consistent with the hypothesis that existing variability in the chemical profile, production of specific compounds and dispersal of chemotypes are affected by changes in environmental factors.

Do plant species express different chemotypes?

Individuals within a species can differ in their secondary metabolites from each other, and this variation is result of interactions with environmental conditions, genetic factors and/or

other species (Verma and Shukla, 2015), that affect the production of metabolites in specific chemotypes. Hence, there may be widely diverse chemotypes within a species according to their volatile and non-volatile compounds. The wide range of secondary metabolites in a species can lead to various medicinal and biological properties attributed to the presence of secondary metabolites belonging to different classes. For example, the great chemotypic variability of *Hypericum species* revealed significant variability in the cytotoxic activity of the crude extracts (Sarrou et al., 2018). Under such a high variations in biological properties, it is necessary to have information about the chemotypes and origin of a herb, so that desired impact can be achieved.

In *chapter 2*, the metabolomics approach revealed three distinct chemotypes among *F. assa-foetida* populations characterized by (I) monoterpenes and *Z*-1-propenyl *sec*-butyl disulfide; (II) eudesmane sesquiterpenoids and *α*-agarofuran; and (III) *Z*- and *E*-1-propenyl *sec*-butyl disulfide. Muturi et al. (2018) reported that garlic essential oil containing allyl disulfide and diallyl trisulfide as main compounds was more toxic than the disulfide chemotype of *F. assa-foetida* containing *Z*- and *E*-1-propenyl *sec*-butyl disulfide and methyl 1-(methylthio) propyl disulfide as the most abundant compounds in its EOs against larvae of *Culex pipiens* Linnaeus and *Culex restuans* Theobald. Therefore, it could be emphasized that for studying the biological properties of one species, different populations and/or chemotypes should be considered to obtain the desired impact. For example, the other two chemotypes of *F. assa-foetida* identified in this study (monoterpenes and eudesmane sesquiterpenoids chemotypes) may have more efficient effects against insects or pathogens.

In *chapter 3*, essential oils from field-collected leaves of fourteen *Z. multiflora* populations represented three different chemotypes (carvacrol, thymol and linalool). In *chapter 4*, it is reported that on the basis of non-volatile compounds of hydroalcoholic extract analyzed by HPLC-ESI-QTOF-MS, *Z. multiflora* populations containing flavonoid conjugates, hydroxycinnamic acid derivatives and phenolic terpenes as secondary metabolites were clustered into three main classes (see Figure 4 in *chapter 4*). In contrast to essential oil classification, individuals from thymol and carvacrol chemotypes of *Z. multiflora* were scattered into two groups referring to non-volatile compounds, although the linalool chemotype was classified as unique class based on volatile and non-volatile compounds (see Figure 3 in *chapter 3* and Figure 4 in *chapter 4*).

Despite similarity in classifications with respect to volatile and non-volatile compounds of *Z. multiflora*, different secondary metabolites or the same secondary metabolites in different quantities can be observed among medicinal plant populations concerning various environmental conditions that cause different chemotypes with ideal and/or undesirable biological and pharmacological activities. On the other hand, not only individuals within a medicinal plant species show phytochemical diversity in response to environmental

conditions, but also may not necessarily show responses to environmental conditions depending on where the individuals are grown. Hence, investigating more individuals or populations of a species can help to identify chemotypes with a desirable biological activity.

Do the plant extracts inhibit fungal pathogens?

In crop plants, pathogens and especially fungi cause enormous losses in yield and quality of crops and economic damage. On the other hand, mycotoxins of phytopathogenic fungi can directly affect human and animals health (Rai et al., 2020). Fungal pathogens negatively affect the growth, development, and health status of a wide variety of plants and cause considerable postharvest losses of fruits and vegetables, which can reach up to 100 % of the production. Synthesized chemical fungicides have often been used to control pathogens and diseases, but the residual problem and toxicity of chemical pesticides that is associated to negative environmental impacts, human health and beneficial microorganism, demand an environmentally friendly alternative approach for plant and food protection measures (Ogunnupebi et al., 2020). The use of plant extracts is an ecofriendly alternative approach against pathogens and pests (Domingues and Santos, 2019).

Plant extracts such as essential oils, hydroalcoholic extracts, resins and tannins have shown a potential for control of phytopathogenic microorganism, due to their content of antimicrobial compounds. The diversity in the secondary metabolites of plant extracts, such as essential oil compounds, even those obtained from the same species, may result in different responses regarding to the potential for microorganism inhibition (Karimi and Meiners, 2021). Many studies revealed the potential of natural compounds such as phenolic and flavonoid compounds as bio-based herbicides, fungicides, and insecticides (Muñoz et al., 2020; Walia et al., 2017; Martins et al., 2015). Thus, secondary metabolites can be considered as a source of phytochemicals that can control the disease. Plant extracts have numerous advantages, e.g. essential oil compounds are highly volatile, thus they do not typically cause residue problems in food products (Govindarajan et al., 2016). Moreover, the antimicrobial properties of plant extracts may exist because of the synergistic activity of many compounds, hence they are poorly subject to resistance (Burketova et al., 2015).

In *chapter 4*, when screening *Z. multiflora* extracts for bioactive secondary metabolites, twelve populations (class II and III, see Figure 1) containing high amount of flavonoid compounds such as dihydroquercetin, dihydrokaempferol, eriodictyol and naringenin showed moderate and strong antifungal activity, whereas the extracts of two populations (class I) containing lower amount of these compounds showed weak antifungal activity (≤ 37 %). These compounds had significantly positive correlation with antifungal activity. Furthermore, the presence of these compounds in active fractions of *Z. multiflora* extracts (see Figure 3 and

Figure S4, *chapter 4*) can explain that these compounds are responsible for antifungal activity, although synergistic activity of all compounds should be considered. In compliance with our results, eriodictyol and naringenin showed the greatest activity against bacterial pathogens and yeast comparing to the other compounds of bergamot extract (Mandalari et al., 2007). In addition, among extracts and fractions of *Ocimum sanctum* leaves, alcoholic extract containing phenolic compounds, flavonoids and alkaloids, effectively inhibited the growth of *Trichophyton mentagrophytes* at lower concentration (Balakumar et al., 2011).

In *chapter 5*, antifungal activity of volatile compounds using fumigation bioassays with EOs of *Z. multiflora* populations were investigated against five food and agricultural important pathogens. The carvacrol and thymol chemotypes exhibited strong growth inhibitory effects at low concentrations, whilst EO of the linalool chemotype inhibited the fungal growth only weakly. The findings were consistent with the hypothesis that phenolic compounds of EOs particularly thymol and carvacrol are the key mediator of antifungal activity, but not of linalool chemotype EOs. The concentration of these bioactive compounds depends on the environmental conditions where plants are grown (Karimi et al., 2020b). For instance, those populations of *Z. multiflora*, which grew in adverse environments, contained high amount of carvacrol and thymol and inhibited fungal growth at low concentrations, whilst essential oils of the linalool chemotype grown in cooler regions, inhibited fungal growth only weakly (Karimi and Meiners, 2021).

It is evident that the antimicrobial activity of plant extracts may be due to secondary metabolites present in their extracts, which have low environmental impact and low levels of residuals in food products. Many EOs exhibit in vitro antifungal activities (Karimi and Meiners, 2021; Zhang et al., 2019; Kordali et al., 2008), but are not always effective when used as a commercial product for postharvest fungal control in in vivo. For example, the EOs of thyme, oregano, clove and cinnamon completely were able to inhibit *Penicillium* species in vitro, whereas in in vivo experiments none of these oils were effective against the pathogens (Plaza et al., 2004). However, the possibilities for volatility of EO compounds in an indoor and outdoor environment, or use of several plant extract together would have to be carefully assessed to find an effective alternative to synthetic fungicides.

Plant elicitation to increase secondary metabolites production

Environmental stresses could affect the distribution patterns of particular plant populations. Interactions with the environment result in the adaptation of plants to the specific biotic and abiotic stress conditions and determine the variability of secondary metabolites and the presence of certain compounds (Verma and Shukla, 2015), because specific compounds help these plants to cope with an adverse environment (Xiao et al., 2020; Mahajan et al., 2020).

Thus, the accumulation of these compounds can be stimulated by elicitors. For instance, glucosinolates of the Brassicaceae family are known from the plant defense response mechanisms, which is induced after wounding, insect herbivory, exposure to diverse environmental factors and/or pathogen attack (Jahangir et al., 2009). Elicitors can switch on the enzymatic activity and the signaling pathways against stresses that potentially lead to an enhanced concentration of valuable phytochemicals (Trivellini et al., 2016), resulting in specific up- or down-regulation of their biosynthesis. It is known that environmental factors largely influence secondary metabolite production such as phenolic and flavonoid compounds, which can play critical roles in plant responses to ameliorating biotic and abiotic stresses (Manukyan, 2013). Phenolic compounds are precursors of lignin, a cell-wall component of plants involved in plant defense mechanisms (Chalker-Scott and Fuchigami, 2018). The biosynthesis and accumulation of phenols in plants are strongly affected by environmental stresses, which can be stimulated by artificial elicitors to modify metabolites and provide a beneficial outcome for industrial purpose (Trivellini et al., 2016). For example, methyl jasmonate as chemical elicitor of phenols enhanced the level of rosmarinic acid that is used in cosmetic, food and pharmaceutical industries (Bauer et al., 2009).

The study in *chapter 5* provides strong evidence that abiotic stresses, particularly heat and the interaction of drought and heat, induced changes in plants of the linalool chemotype of *Z. multiflora* resulting in higher relative amounts of carvacrol (22.7% and 32.9% vs. 1.5%), which revealed strong bioactivity against pre- and postharvest fungal pathogens. On the other hand, the relative amount of linalool was significantly decreased in response to stress conditions (Figure 3 in *chapter 5*). Furthermore, the relative amount of linalool was slightly reduced in the linalool chemotype, when plants were subjected to high intensities of UV-A radiation (33.9% vs. 44.6%), whilst the relative amount of carvacrol was slightly increased (20.1% vs. 9%). Abiotic stresses did not affect the relative content of the main volatile compounds of the carvacrol chemotype (Figure 3 in *chapter 5*).

In natural habitats, the carvacrol chemotype of *Z. multiflora* often grows in adverse environments, e.g. with high temperature (23 to 29 °C) whilst the linalool chemotype grows in lower temperature (14 to 21 °C) (Karimi et al., 2020b). Therefore, it shows that the carvacrol chemotype was adapted to harsh environments comparing to the linalool chemotype. This could explain why the linalool chemotype did respond to abiotic stresses by converting linalool to phenolic compounds, particularly carvacrol and/or thymol, but the carvacrol chemotype did not.

The study thereby provides insights into the accumulation of specific compounds in response to elicitors. The study also provides that the combined environmental stresses (e.g. drought and heat together) can be applied to modify the content of beneficial compounds, which not only are involved in plant responses to harsh conditions, but also have the potential

as source of natural fungicides in agriculture and food industry. In fact, application of a stress condition and the induction of a stress response can stimulate the plants to produce the desired chemicals, such as terpenoids, flavonoids, and alkaloids for industrial purpose (Li et al., 2020). Over all, the responses to stress conditions are specific for the specific compounds but also for the plant species and can depend on the degree of stresses.

General conclusion

Environmental factors can affect the chemical compounds and cause differentiation in individuals of a species. Environmental metabolomics investigates the differentiation of chemodiversity in plants and evaluates the natural variation in metabolite content of plant populations or individuals to characterize the interactions of plants with their environment (Stierlin et al., 2020). In this thesis, the environmental metabolomics approach provides novel insights into the distribution of compounds within *F. assa-foetida* and *Z. multiflora* populations and the interactions of both species with their environment where the presence of high amounts of specific compounds were observed in response to the specific environment. The approach of identifying environmental predictors for EO compounds, chemotype or presence of high amounts of specific compounds helped to identify regions for sampling plant material with the desired chemical profile for agriculture and food industry.

This study also increases our understanding of the potential of volatile and non-volatile compounds of *Z. multiflora* to inhibit the mycelium grows of five severe fungal pathogens. Among volatiles of *Z. multiflora* essential oils, phenolic compounds particularly carvacrol and thymol showed strong antifungal activity, whereas non-phenolic compound linalool showed only weak antifungal activity against a broad spectrum of fungal pathogens important in food industry and agriculture. Furthermore, among non-volatiles of *Z. multiflora* hydroalcoholic extracts, flavonoid compounds including naringenin, eriodictyol, dihydroquercetin and dihydrokaempferol exhibited strong correlation with antifungal activity. It shows that phenols and flavonoids of *Z. multiflora* have the potential to use as natural antifungal agent and could help to develop new natural fungicide to use in industries as alternatives to synthetic chemicals.

Furthermore, the thesis demonstrated that the concentration of these bioactive compounds can be stimulated by abiotic and biotic stress conditions to help plants to cope with an adverse environment. In the linalool chemotype of *Z. multiflora*, heat and the interaction of drought and heat were shown to elicit an increased phenolic compounds production, whilst decreased linalool content which exhibited weak antifungal activity. Hence, adjusting specific abiotic conditions can be applied to modify the content of

biologically active compounds such as the antifungal compound carvacrol in the linalool chemotype of *Z. multiflora*.

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Summary

The use of plant extracts such as essential oils (EOs) as botanical pesticides has numerous advantages, including the high effectiveness against a wide range of diseases and pest insects of agricultural and medicinal importance due to multiple mechanisms of action. EOs belong to secondary plant metabolites, which mediate direct and indirect plant defenses against biotic and abiotic stress conditions. The main objective of this thesis was to obtain plant extracts from aromatic plants, which can be used as bio-based plant protection products. The general hypothesis investigated whether a) the content of biologically active substances of two Iranian medicinal plants species (*Ferula assa-foetida* and *Zataria multiflora*) originated from a wide range of extreme environments is correlated with certain environmental factors; whether b) certain secondary plant metabolites from *F. assa-foetida* and *Z. multiflora* can be used as biologically active compounds against fungal infestations in crops; and whether c) the production of these metabolites can be artificially increased by mimicking extreme environmental conditions during cultivation.

a) First of all this study evaluated whether environmental factors can be utilized to predict the presence of specific compounds from medicinal plants by two different methods, near-infrared spectroscopy (NIRS) and gas chromatography coupled mass spectrometry (GC−MS). Using an environmental metabolomics approach (GC−MS), on the basis of EOs from roots, three distinct chemotypes were found among 10 *Ferula assa-foetida* L. populations. These were characterized by (I) monoterpenes and *Z*-1-propenyl *sec*-butyl disulfide; (II) eudesmane sesquiterpenoids and *α*-agarofuran; (III) *Z*- and *E*-1-propenyl *sec*-butyl disulfide. NIRS measurements indicated a similar, but less distinct pattern. Structural equation models (SEMs) showed that EO compounds and content were directly influenced by edaphic factors and temperature (*chapter 2*).

Essential oil compounds from leaves of 14 populations of *Z. multiflora* were classified into three main groups, each representing a distinct chemotype with linalool, thymol and carvacrol as the major components. Corresponding to the phytochemical cluster analysis, the hierarchical cluster analysis based on NIR data also recognized the carvacrol, thymol and linalool chemotypes. The SEMs approach revealed direct effects of soil factors and mostly indirect effects of latitude and altitude on EO compounds and content of *Z. multiflora* populations. Therefore, predicting EO compounds or chemotypes by environmental metabolomics can be used in medicinal plants to select populations with the desired chemical profile (*chapter 3*).

b) Due to increasing demand of natural compounds for food preservatives and plant pathogen control, plant extracts with bioactive secondary metabolites can be used as an effective and ecofriendly plant protection approach. Hydroalcoholic extracts and EOs of *Z.*

multiflora were assessed to identify biologically active compounds and/or chemotypes against the plant pathogens *Fusarium culmorum*, *Fusarium sambucinum*, *Botrytis cinerea*, *Alternaria dauci* and *Colletotrichum lindemuthianum* (*chapter 4* and *5*).

On the basis of non-volatiles compounds of *Z. multiflora* hydroalcoholic extracts analyzed by LC−MS, three major chemical classes were found among the 14 populations. A total of 32 metabolites were annotated including flavonoid conjugates, hydroxycinnamic acid derivatives and phenolic terpenes. Flavonoids were the main compounds in the extracts, considering that two third of the annotated compounds represent flavonoid conjugates. The antifungal activity of extracts from *Z. multiflora* populations showed high variability from weak (≤ 37 %) to high inhibition rates (up to 65 %). Nine compounds such as dihydroquercetin, dihydrokaempferol, naringenin and eriodictyol strongly positively correlated with antifungal activity (*chapter 4*).

Corresponding to the single volatile compounds, even low concentrations of the carvacrol and thymol, but not of the linalool chemotype EOs inhibited significantly the growth of all fungal pathogens. Bioassays revealed positive correlation between relative amounts of *p*-cymene, *γ*-terpinene, thymol and carvacrol and the inhibition of the fungal mycelium growth, whereas myrcene and linalool relative amounts had a strong negative correlation with antifungal activity (*chapter 5*).

c) To enhance the production of biologically active metabolites, carvacrol and linalool chemotypes of *Z. multiflora* were cultivated under extreme environmental conditions including UV-A radiation, heat and drought stresses. Although no significant differences were observed in extracted volatile compounds in UV-A irradiated plants, the relative content of linalool was slightly reduced in the linalool chemotype, whilst the relative amount of carvacrol was slightly increased. Drought stress alone did not alter the relative contents of volatile compounds in both chemotypes, whilst high temperatures lead to a decrease of the linalool content and an increase of the relative amount of carvacrol in the linalool chemotype. Furthermore, the interaction of drought and heat induced changes in plants of the linalool chemotype resulting in higher relative amounts of carvacrol and lower relative amounts of linalool. Moreover, the main volatile compounds of plants from the carvacrol chemotype did not change in response to abiotic stresses (*chapter 5*).

Understanding the effect of environmental conditions on populations and chemotypes of medicinal plants supports the development of natural and sustainable fungicides or insecticides. Although several hypotheses and questions have been developed and tested or answered in this study, further studies are needed to gain deeper insight into the bioactive metabolite biosynthesis of *Z. multiflora*. We have to study further, how severe stress conditions affect different chemotypes of *F. assa-foetida* and other medicinal and aromatic plant species.

Zusammenfassung

Die Verwendung von Pflanzenextrakten wie ätherischen Ölen (ÄÖ) als pflanzliche Schädlingsbekämpfungsmittel hat zahlreiche Vorteile, darunter die hohe Wirksamkeit aufgrund mehrerer Wirkmechanismen gegen ein breites Spektrum von Krankheiten und Schadinsekten von landwirtschaftlicher und medizinischer Bedeutung. ÄÖ gehören zu den sekundären Pflanzenmetaboliten, die direkte und indirekte pflanzliche Abwehrmechanismen gegen biotische und abiotische Stressbedingungen vermitteln. Das Hauptziel dieser Arbeit war die Gewinnung von Pflanzenextrakten aus Aromapflanzen, die als biobasierte Pflanzenschutzmittel verwendet werden können. Als allgemeine Hypothese wurde untersucht, ob a) der Gehalt an biologisch aktiven Substanzen zweier iranischer Arzneipflanzenarten (*Ferula assa-foetida* und *Zataria multiflora*), die aus Gebieten mit einem breiten Spektrum extremer Umweltbedingungen stammen, mit bestimmten Umweltfaktoren korreliert; ob b) bestimmte sekundäre Pflanzenmetaboliten aus *F. assa-foetida* und *Z. multiflora* als biologisch aktive Verbindungen gegen Pilzbefall in Nutzpflanzen verwendet werden können; und ob c) die Produktion dieser Metaboliten künstlich erhöht werden kann indem extreme Umweltbedingungen während des Anbaus nachgeahmt werden.

a) In dieser Studie wurde zunächst untersucht, ob Umweltfaktoren genutzt werden können, um die Abundanz spezifischer Verbindungen aus Arzneipflanzen mit zwei verschiedenen Methoden vorherzusagen: die Nahinfrarotspektroskopie (NIRS) und die Gaschromatographie gekoppelte Massenspektrometrie (GC-MS). Mit Hilfe eines Umwelt-Metabolomischen-Ansatzes wurden auf der Grundlage von per GC-MS identifizierter ÄÖ aus Wurzeln drei verschiedene Chemotypen unter 10 *Ferula assa-foetida* L.-Populationen gefunden, die durch (I) Monoterpene und *Z*-1-Propenyl-*sek*-butyldisulfid; (II) Eudesmane-Sesquiterpenoide und *α*-Agarofuran; (III) *Z*- und *E*-1-Propenyl-*sek*-butyldisulfid gekennzeichnet sind. NIRS-Messungen ergaben ein ähnliches, aber weniger ausgeprägtes Verbindungsmuster. Strukturgleichungsmodelle (SGM) zeigten, dass die ätherischen Ölverbindungen und deren Gehalt direkt von den edaphischen Faktoren und der Temperatur beeinflusst wurden (Kapitel 2).

Die ätherischen Öle aus Blättern von 14 *Z. multiflora* Populationen konnten in drei Hauptgruppen eingeteilt werden, von denen jede einen bestimmten Chemotyp repräsentiert, wobei Linalool, Thymol und Carvacrol die Hauptkomponenten sind. In Übereinstimmung mit der phytochemischen Clusteranalyse konnten bei der hierarchischen Clusteranalyse auf der Grundlage von NIR-Daten ebenfalls die Carvacrol, Thymol und Linalool - Chemotypen unterschieden werden. Der SGM-Ansatz zeigte direkte Auswirkungen der Bodenfaktoren und hauptsächlich indirekte Auswirkungen des Breitengrads und der Geländehöhe auf die Verbindungen der ÄÖ und deren Gehalt in den *Z. multiflora*-Populationen. Daher kann die

Vorhersage von ÄÖ-Verbindungen oder Chemotypen durch einen Umwelt-Metabolomischen Ansatz bei Arzneipflanzen zur Auswahl von Populationen mit dem gewünschten chemischen Profil verwendet werden (Kapitel 3).

b) Aufgrund der zunehmenden Nachfrage nach natürlichen Verbindungen für die Konservierung von Lebensmitteln und die Bekämpfung von Pflanzenpathogenen können Pflanzenextrakte mit bioaktiven sekundären Metaboliten als wirksame und umweltfreundliche Alternative verwendet werden. Hydroalkoholische Extrakte und ÄÖ von *Z. multiflora* wurden auf biologisch aktive Verbindungen und/oder Chemotypen gegen die Pflanzenpathogene *Fusarium culmorum*, *Fusarium sambucinum*, *Botrytis cinerea*, *Alternaria dauci* und *Colletotrichum lindemuthianum* untersucht (Kapitel 4 und 5).

Basierend auf den mittels LC-MS analysierten nicht-flüchtigen Verbindungen der hydroalkoholischen Extrakte von *Z. multiflora* wurden in den 14 Populationen drei Hauptklassen festgestellt. Insgesamt wurden 32 Metaboliten beschrieben, darunter Flavonoidkonjugate, Hydroxyzimtsäurederivate und phenolische Terpene. Flavonoide waren die wichtigsten Verbindungen in den Extrakten, da zwei Drittel der annotierten Verbindungen Flavonoidkonjugate waren. Die antifungale Aktivität von Extrakten aus *Z. multiflora*-Populationen zeigte eine große Variabilität von schwachen (≤ 37 %) bis zu hohen Hemmungsraten (bis zu 65 %). Neun Verbindungen wie Dihydroquercetin, Dihydrokaempferol, Naringenin und Eriodictyol korrelierten stark positiv mit der antifungalen Aktivität (Kapitel 4).

c) In Übereinstimmung mit den einzelnen flüchtigen Verbindungen hemmten selbst niedrige Konzentrationen vom ÄÖ des Carvacrol und Thymol, nicht aber des Linalool - Chemotyps das Wachstum aller Pilzerreger erheblich. Bioassays zeigten eine positive Korrelation zwischen den relativen Mengen von *p*-Cymol, γ-Terpinen, Thymol und Carvacrol und der Hemmung des Pilzmyzelwachstums, während die relativen Mengen von Myrcen und Linalool eine starke negative Korrelation mit der antifungalen Aktivität aufwiesen (Kapitel 5).

Um die Produktion biologisch aktiver Metaboliten (c) zu steigern, wurden die Carvacrol- und Linalool-Chemotypen von *Z. multiflora* unter extremen Umweltbedingungen wie UV-A-, Hitze- und Trockenstress angebaut. Obwohl bei UV-A-bestrahlten Pflanzen keine signifikanten Unterschiede bei den extrahierten flüchtigen Verbindungen festgestellt wurden, war der relative Gehalt an Linalool beim Linalool-Chemotyp leicht reduziert, während die relative Menge an Carvacrol leicht erhöht war. Trockenstress allein veränderte die relativen Gehalte an flüchtigen Verbindungen in beiden Chemotypen nicht, während hohe Temperaturen zu einem Rückgang des Linalool-Gehalts und einem Anstieg der relativen Mengen an Carvacrol im Linalool-Chemotyp führten. Darüber hinaus führte das Zusammenwirken von Trocken- und Hitzestress zu Veränderungen bei Pflanzen des Linalool-

Chemotyps, die sich in höheren relativen Mengen an Carvacrol und niedrigeren relativen Mengen an Linalool auszeichneten. Darüber hinaus veränderten sich die wichtigsten flüchtigen Verbindungen der Pflanzen des Carvacrol-Chemotyps nicht als Reaktion auf abiotische Stressfaktoren (Kapitel 5).

Das Verständnis der Auswirkungen von Umweltbedingungen auf die Populationen und Chemotypen von Arzneipflanzen trägt somit zur Entwicklung natürlicher und nachhaltiger Fungizide oder Insektizide bei. Obwohl in dieser Studie mehrere Hypothesen und Fragen entwickelt und beantwortet wurden, sind weitere Studien erforderlich, um einen tieferen Einblick in die Rolle der Biosynthese bioaktiver Metaboliten von *Z. multiflora* zu gewinnen. Auch muss untersucht werden, wie sich extreme Stressbedingungen auf verschiedene Chemotypen von *F. assa-foetida* und anderen medizinischen und aromatischen Pflanzenarten auswirken.

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Affidavit

I hereby declare that I have written this dissertation independently and authored by myself. This dissertation has not been submitted to open a doctoral procedure elsewhere. I marked parts of the text in my thesis that were taken from other sources verbatim or analogously as such by quoting the exact source.