Natural Compounds Found in Medicinal Plant of *Epilobium frigidum* Extracts by Chromatography Method

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Abstract

Epilobium genus has anti-inflammatory, antioxidant, antimicrobial, anti-tumor, anti-pain, and anti-androgenic medicinal properties in traditional medicine. In this research, the aerial parts of *E. frigidum* species were collected at the flowering stage from the heights of Kalardasht in Mazandaran province at the end of May and were phytochemically investigated for the composition of fatty acids and volatile active substances in the essential oil. The extract of plant sample was extracted with 80% methanol and evaluated by gas chromatography coupled with a mass spectrometer (GC-MS). The results showed that the main hydrocarbon compounds in the essential oil of this plant were linoleic acid with a value of 49.176 and oleic acid (32.227%) and volatile compounds 4aβ,7α,7aβ-nepetalactone with a value of 41.312 and β-caryophyllene (15.395). The chemical structure of linoleic acid, a common omega-6 fatty acid found in many nuts, seeds, and vegetable oils. Hydrocarbon terpenes had the highest concentration in the essential oil of this plant. The results of GC/MS analysis showed the presence of 24 compounds in the investigated species, which were separated into the groups of steroids and terpenes from the phytochemical point of view. The findings showed that *E. frigidum* contains important natural products such as steroids, monoterpenoids, and sesquiterpenoids. Due to the bioactive products, this plant can have more applications in the cosmetic and pharmaceutical industries.

Keywords: Phytochemical, *Epilobium*, GC/MS, Secondary metabolites, Terpenes

Introduction

Medicinal plants play an important role in human life owing to their bioactive phytochemicals with potential health and commercial benefits. In the last decades, prior to synthetic drugs development, herbs were mainly used as an alternative therapy to treat various ailments. Onagraceae is a flowering plant family known as the willowherb family or evening primrose family. Family Onagraceae is one of the most important families which have potent therapeutic effects against various diseases (Jamous et al., 2015).

The genus *Epilobium* is the largest member of the family Onagraceae and is an endemic Iranian medicinal plant that has illustrated a

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vast variety of pharmacologically important metabolites. The genus comprises 165 species and most of these species are selfcompatible and scattered worldwide (Raven, 1967), 20 species of the genus have previously been found and reported in most provinces of Iran (Azizian, 2005). Furthermore, these species grow in diverse habitats, including forests, mountainous regions, and rural areas (Dreger et al., 2016). Species belonging to *Epilobium* genus are a rich source of polyphenolic compounds, including flavonoids, phenolic acids, and tannins. They also contain lipophilic compounds, such as steroids, triterpenoids, and fatty acids (Granica et al., 2014; Gryszczyńska et al., 2018; Jürgenson et al., 2012; Kaškonienė et al., 2016; Maruška et al., 2017). The most important active ingredients are flavonoids and ellagitannins, including oenotein B. Fireweed, has been used as an agent accelerating the healing of extensive wounds and as a disinfectant. In recent years, the genus has gained increasing importance in the treatment of benign prostatic hyperplasia. Kosalec et al. (2013) compared the antimicrobial activity of ethanol extracts from flowers and leaves of fireweed. The strains of *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Candida albicans*, *C. tropicalis*, *C. dubliniensis* and *Saccharomyces cerevisiae* were susceptible to both extracts. Researchers suggest that because of high antimicrobial activity, the extracts can be used in the adjunctive therapy of benign prostatic hyperplasia. The effectiveness of fireweed extracts in the treatment of prostate disease was confirmed by Kiss et al. (2004, 2012).

Epilobium has been drawn considerable attention due to possessing important secondary metabolites such as flavonoids (kaempferol, quercetin, and myricetin), phenolic acids (ellagic acid, valoneic acid, gallic acid, protocatechuic acid), ellagitannins (oenothein A and B), fatty acids (linolenic, palmitic, linoleic, stearic), and vitamins (Lesuisse et al., 1996; Hiermann and Buca, 1997; Velasco and Goffma, 1999; Dreger et al., 2016; Kaškonienė et al., 2016). The young leaves of *Epilobium* could be eaten as salad vegetables or tea an excellent honey from their flowers (Bunney, 1992; Dreger et al., 2016; Kaškonienė et al., 2016).

The pharmacological effect of *Epilobium* could be explained by the presence of steroids (in particular sitosterol and its esters), triterpenes, fatty acids, macrocyclic tannins, and flavonoids (in particular myricitrin, isomyricitrin, quercitrin, and quercetin 3-O -d-glucuronide) in the aerial parts (Barakat et al., 1997; Hiermann and Radl, 1998).

The study conducted by Abbasi-Karin et al (2023) showed that *E. frigidum* species is a rich source of antioxidants, whose antioxidant activity was meaningfully related to climatic data.

Due to the lack of information about the compounds found in *E. frigidum* species, this study was conducted to investigate the natural compounds found in the extract of this species by GC and GC/MS methods.

Material and methods

Plant Material

With the beginning of the growing season, *Epilobium* aerial parts were sampled from Kalardasht region, Mazandaran province (geographical coordinates: E: 51° 09'14", N: 36° 30'13" and altitude 1250 meters) at the end of May and using Authentic sources of botany such as Flora Iranica, Iranian plants, Flora of Iran and Flora Orientalis were identified. The collected plant sample was transferred to the botanical laboratory.

Extraction method

To extract light greenish extract from the samples, 2 g of leaf tissue were powdered and 4 ml of water with 16 ml of methanol (methanol: water, 20:80) was added and mixed well, then the samples were placed on a shaker and incubated at room temperature for 24 hours. The contents of each sample were filtered through filter paper and centrifuged at 3100 rpm for 4 minutes. The obtained light greenish extract was divided into 4 to 7 test tubes of 2 ml and kept in a freezer until phytochemical tests were performed (Rohloff et al., 2015).

Analysis of free fatty acids by Gas Chromatography (GC)

A gas chromatograph (Varian CP3800) connected to a FID detector and equipped with a polar silica column (TR-CN100 poly (bicyanopropyl) siloxane capillary) (column length: 60 meters, inner diameter: 0.25 mm), film thickness: 0.2 µm (Teknokroma Co, Barcelona, Spain) was used for separation and identification of fatty acids, Helium gas with a flow of 1 ml/min in the column was used as the carrier gas and the gap ratio in the injection chamber was 25:1. The temperature program of the column started with a temperature of 175 °C for 2 minutes and then continued with an increase in temperature of 3 °C per minute until it reached 230 °C and then remained at the same temperature for 3 minutes. The temperature of the injection chamber and the detector was 290 °C and the volume of the extract for injection was 1 microliter. The components of each sample were analyzed using Workstation software (V 6.4). The oil content of the leaf sample was analyzed based on its dry weight percentage. At the final stage, the amount of fatty acids based on the total oil was calculated and reported by comparing the area under the peak with standard samples (C:14-C:22, Sigma Company).

GC/MS-Based Metabolite Profiling

Crude extracts were first placed in a Speed vac device without heat for 24 hours to dry to perform metabolite profiling by GC/MS method. The dried sample was prepared as a solution in 80 microliters of 20 mg/ml methoxyamine hydrochloride in pyridine and treated at 30 °C for 90 minutes. Then, the samples were dehydrated with 80 microliters of (N-Methyl-N-MSTFA (trimethylsilyl) trifluoroacetamide) at 37 °C in an incubator for 30 minutes. Finally, the samples were transferred to automatic 1.5 ml glass sampling tubes and stored at -20 °C until gas chromatography/mass spectrometry (GC/MS) (Rohloff et al., 2015). In order to profile metabolites, the samples were used by the gas chromatography device GC 6890 /5975 of the company system. Agilent Technologies Inc., Palo Alto, (Agilent/MS CA) were analyzed. The device is equipped with a capillary column HP-5MS 30 meters

by 0.25 mm inner diameter and the phase thickness was 0.25 micrometers. For this purpose, first, the samples were diluted in automatic sampler tubes. In this way, 1 µL of the sample was injected at a dilution ratio of 15:1. The injection and detection temperatures were set at 230 and 250 °C, respectively. The initial temperature of the column was set at 40 °C, and to reach 250 °C, it was programmed in increments of 3.5 °C per minute, and finally was kept at 250 °C for 3 minutes (analysis time 63 minutes). The MS source or detection was set at 230°C. The carrier gas used was helium with a constant flow rate of 1 ml/ min. The temperature program of the GC column was isothermally initially set at 70 °C for 5 min, and programmed to reach 310 °C in increments of 5 °C per minute, finally at 310 °C for 10 min. It was kept for 7 minutes (analysis time: 60 minutes). The MS source or detection was set at 230°C, and a mass spectrum of 70-700 m/z was recorded. All mass spectra were obtained in electron ionization (EI) mode (70 eV). Chromatogram detection and peak area integration was performed using Agilent Chem Station software (Agilent Technologies Waldbronn, Germany) and MetAlign data alignment software (Wachningen UR, The Netherlands). The identified metabolites were quantitatively determined based on the internal standard Ribitol and the final concentration was determined in terms of (gram dry weight/ µg). AMDIS software (National Institute of Standards and Technology, Slate, Inc., USA) in combination with the Golm metabolome database, GMD (Max Planck Institute, Molecular Plant Physiology Division, Golm, Germany) database MassBank High Resolution Power Spectrometry Data (Norman Society Verneuil-en-Halatte, France), NIST05 spectrometer library (National Institute of Standards and Technology, Gaithersburg, MD) and in situ spectrometer and index library and plant metabolite derivatives were used To evaluate the mass spectrum and identify the metabolites (Rohloff et al., 2015).

Results

Epilobium species have been traditionally used as medicinal plants for centuries. The present work studies the chemical composition of the essential oil of *E. frigidum* from Iran. The essential oil of the aerial parts of the plant was extracted by water distillation and analyzed by GC and GC/MS. 10 fatty acid compounds and 24 volatile compounds were identified in the essential oil.

The results of the analysis of the essential oil obtained from the *E. frigidum* plant by GC are given in the following chromatogram (Figure 1) and Table 1. The most abundant fatty acid components identified were C18:2, c9C18:1 + c6C18:1, C16:0, C18:0 and t9C18:1 + t11C18:1 which constituted 49.17654%, 32.22771%, 8.000311%, 3.249144% and 1.93709% of the oil, respectively.

The results of metabolite profiling with GC/MS analysis in *E. frigidum* revealed 24 chemical compounds (Table 2). The most abundant volatile compounds identified were 4aβ,7α,7aβ-nepetalactone, β- Caryophyllene, 1,8-cineole, trans-

Caryophyllene and β-pinene which constituted 41.31265% ,15.39532%, 9.096448%, 4.8018% and 4.596524% of the oil, respectively (Figures 2 and 3).

The highest amount of fatty acids is related to linoleic acid and oleic acid. As in the studies conducted on some *Epilobium* species, this applies. The abundance of volatile compounds identified in this species is shown in Figure 1.

Discussion

Several phytochemical surveys have been performed on *Epilobium* extracts due to their importance in folk medicine and their rich biologically active compounds (Ducrey et al., 1995; Hiermann, 1995; Hevesi Tóth et al., 2009; Kiss et al., 2011; Granica et al., 2014; Monschein et al., 2015; Mohammadi Bazargani, 2019). Based on GC/MS analysis the major compounds in *E. frigidum* are

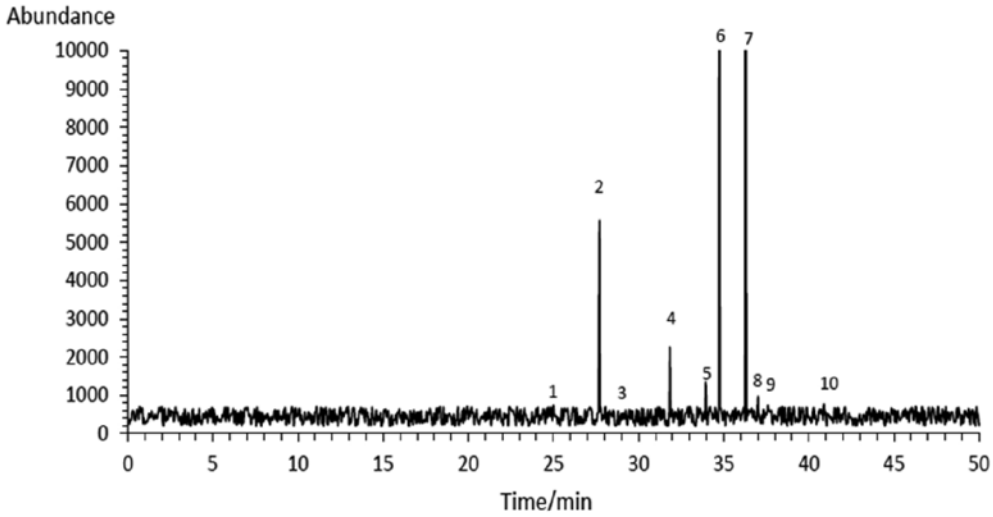

Fig. 1. General GC chromatogram of plant essential oil *E. frigidum*

Table 1. Fatty acid components identified from leaves essential oil of *E. frigidum* via Gas Chromatography (G/C)

Peak IDs	RT (min)	Concentration (%w/w)
C14:0	25.02	076588
C16:0	27.71	8.000311
C16:1	29.08	0.756627
C18:0	31.85	3.249144
t9C18:1 + t11C18:1	33.95	1.93709
c9C18:1 + c6C18:1	34.75	32.22771
C18:2	36.31	49.17654
C20:0	37.02	1.395353
c6,c9,c12C18:3	37.62	1.067286
C22:0	40.91	1.113352

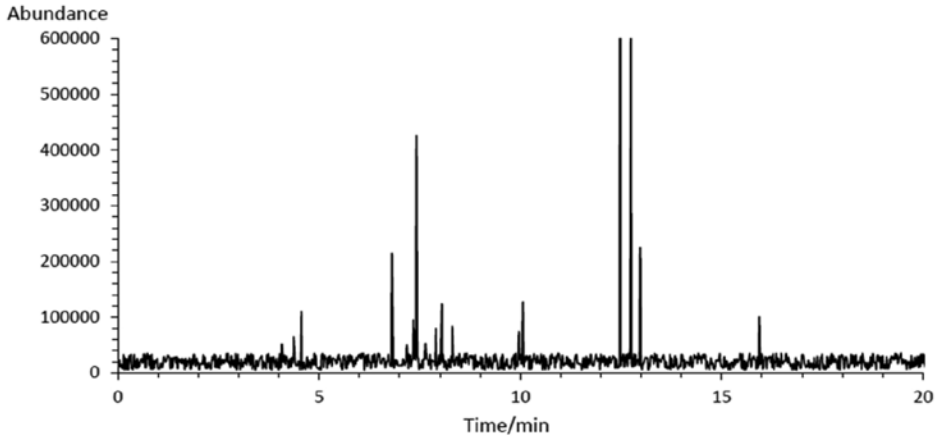

Fig. 2. General GC/MS chromatogram of plant essential oil *E. frigidum*

Table 2. The main volatile compounds identified from leaves essential oil of *E. frigidum* using Gas chromatography/mass spectrometry (GC/MS)

Peak IDs	RT (min)	Concentration (%)
α-thujene	4.07	1.103133
α-pinene	4.37	1.377762
Sabinene	4.56	2.346961
β-Myrcene	4.68	0.357528
β-pinene	6.81	4.596524
α-Terpinene	7.18	1.065904
p-Cymene	7.34	2.030241
Limonene	7.39	0.681558
1,8-cineole	7.42	9.096448
β-Ocimene	7.64	1.133914
γ-Terpinene	7.9	1.70352
Terinolene	8.05	2.643043
Camphor	8.14	0.458779
Nonanal	8.26	0.215135
Linalool	8.31	1.799108
Myrtenal	9.97	1.584477
α-terpineol	10.06	2.726873

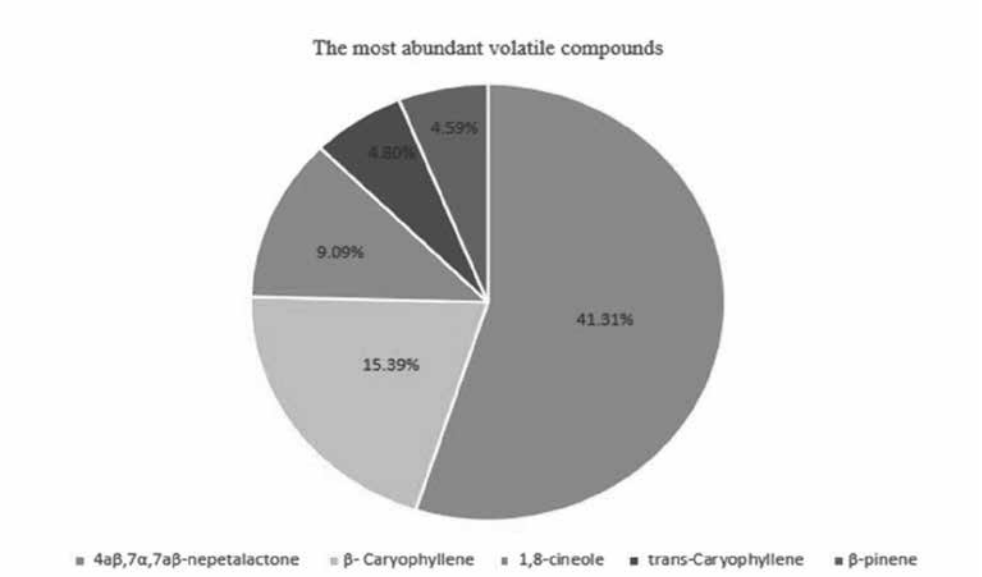

Fig. 3. The most abundant volatile compounds in *E. frigidum*

steroids, and terpenes. Several studies have also shown that species in *Epilobium* genus are a rich source of secondary metabolites especially polyphenols including flavonoids, phenolic acids, and tannins (e.g., Granica et al., 2014). Some other lipophilic metabolites such as steroids, triterpenoids, and fatty acids have also been detected in various *Epilobium* species (Granica et al., 2014). A study showed that the extract of the plant *E. hirsutum* is rich in compounds such as flavonoids, alkaloids, glycosides and tannins (Tafrishi and Taherkhani, 2022). The literature provides a comprehensive description of the habitat conditions, but the causal relationship remains to be clarified and how agroclimatic factors affect the dynamics of *E. angustifolium* development. Available publications show that factors that are important when studying the spatial distribution of this species in the field include primarily mineral nutrients and light requirements (Myerscough and Whitehead 1966, 1967). In study Bazargani et al (2021), myricetin was dominant and constituted the major component of flavonoid group in *E. hirsutum* and *E. parviflorum* populations. Similar studies have reported the occurrence of myricetin as a dominant flavonoid in several *Epilobium* species including *E. hirsutum*, *E. dodonaei*, *E. fleischeri*, *E. roseum*, *E. parviflorum*, *E. montanum*, and *E. tetragonum* whereas quercetin glycosides were a dominant flavonoid in *E. angustifolium* (Slacanin et al., 1991; Ducrey et al., 1995; Hiermann, 1995; Hevesi Tóth et al., 2009; Granica et al., 2014). The results of the study show that a positive correlation was observed among the 30 compounds identified by GC/MS (mainly flavonoids and phenolics) with the altitude above sea level, and environmental factors at higher altitudes caused an increase in the level of flavonols and phenols in *E. minutiflorum* (Bazargani, 2019).

Terpenoids (or isoprenoids) are a large and diverse class of naturally occurring organic chemical compounds found in all classes

of living things that can be assembled and modified in thousands of ways; They are the largest class of plant secondary metabolites, representing about 60% of known natural products (Ashour, 2010). Many terpenoids have substantial pharmacological bioactivity and are therefore of interest to medicinal chemists.

The steroids and sterols in animals are biologically produced from terpenoid precursors. Sometimes terpenoids are added to proteins, e.g., to enhance their attachment to the cell membrane; this is known as isoprenylation. Terpenoids play a role in plant defense as prophylaxis against pathogens and attractants for the predators of herbivores.

The studies showed that polyphenols were the main compounds occurring in *Epilobium* herb, among which flavonoids, phenolic acids, and tannins were dominating constituents (Granica et al., 2014; Jürgenson et al., 2012; Tóth et al., 2009; Barakat et al., 1997).

Several studies have also shown that species in *Epilobium* genus are a rich source of secondary metabolites especially polyphenols including flavonoids, phenolic acids, and tannins Some other lipophilic metabolites such as steroids, triterpenoids, and fatty acids have also been detected in various *Epilobium* species (Granica et al., 2014).

The extracts and isolated compounds from *Epilobium* species were shown to possess antimicrobial (Granica et al., 2014; Bartfay et al., 2012; Borchardt et al., 2008; Steenkamp et al., 2006; Battinelli et al., 2001), anti-proliferative (Vitalone et al., 2001), anti-inflammatory (Kiss et al., 2011; Hevesi et al., 2009), antinociceptive (Pourmorad et al., 2007), anti-diarrhoeal, anti-motility, anti-secretory (Vitali et al., 2006), analgesic (Tita et al., 2001) and antioxidant (Kiss et al., 2011; Tóth et al., 2009; Hevesi et al., 2009) activities.

The compounds found in *E. frigidum* plant extract in Tables 1 and 2 are reported. The highest amount of fatty acids is related to linoleic acid and oleic acid.

The present research showed that the extract of *E. frigidum* plant is rich in terpenoid compounds such as monoterpenes, diterpenes, triterpenes, and sesquiterpenes.

The α-humulene compound identified in the essential oil of *E. frigidum*, also known as α-caryophyllene, is a natural monocyclic sesquiterpene (C15H24) containing an 11-membered ring consisting of 3 isoprene units containing three double bonds. C=C is non-conjugated, two of which are substituted three times, and it is replaced once or twice (Tinseth, 1993). Humulene is an isomer of β-caryophyllene, and the two are often found as a mixture in many aromatic plants.

The results of this research showed that the extract of *E. frigidum* is rich in essential fatty acids, oleic acid (omega 9 fatty acids) and polyunsaturated fatty acid linoleic acid (omega 6 fatty acids), in terms of fatty acid profile. Also, the extract of this plant contains There are significant amounts of plant terpenes, especially 4aβ, 7α, 7aβ-nepetalactone and β-Caryophyllene. For this reason, if the nutritional value of *E. frigidum* species extract is confirmed, it can be used in pharmaceutical and food industries.

In the future, the antioxidant and

antimicrobial activities of different extracts of the mentioned plant can be researched. It is also expected that the antioxidant and biological effects of different fractions of the extract of this plant will be investigated with different extraction methods and solvents with different polarities in the next studies.

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