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HPLC Analysis of Terpenoid Content of Flowers of Lavender

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Running title: **Terpenoids in Lavender Flowers**

Abstract
In the recent years there is an increasing interest in plant extracts, because of their valuable biological and pharmaceutical activities. So the purpose of this study was to investigate oleanolic acid, ursolic acid, betulinic acid, betulin and corosolic acid in the flowers of *Lavendula angustifolia* Mill., naturally grown in Bulgaria. Terpenoid profiles of investigated species were determined by a simple, precise and accurate HPLC method. Chromatographic separation was performed on reversed phase C 18 column and compounds were detected at 210 nm with a flow rate of 0.4 ml/min. The mobile phase used was methanol (A) and 0.1 % formic acid (B) with a ratio of 92:8 (A : B, v/v). HPLC analysis showed that all samples were with high concentration of triterpenic acids - oleanolic and ursolic acids (20.26 - 95.27 mg/g DW and 24.07 – 82.36 mg/g DW, respectively).

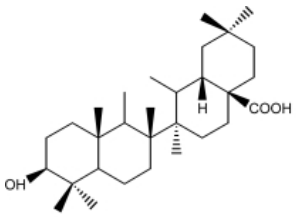
Practical applications
The presence of high quantity of triterpens (oleanolic and ursolic acids) in lavender flowers permit their possible application in pharmacy and cosmetics with improved health benefits for human health.

Key words: HPLC, lavender flowers, terpenoids

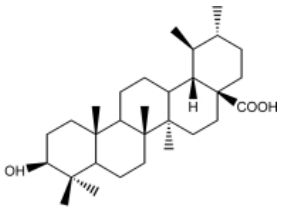
Introduction

Genus *Lavandula* belongs to Lamiaceae family and includes about 39 species. However, the most important species are lavender (*Lavandula angustifolia* Mill.), lavandin (*Lavandula intermedia* Emeric.) and spike lavender (*Lavandula spica* L.) (Kara et al., 2013). Lavender (*Lavandula angustifolia* Mill.) is perennial plant and is cultivated in many European countries. The main countries where lavender grows are Bulgaria and France and smaller areas in Morocco, Yugoslavia, Hungary, Italia, Russia, Spain, Romania, Ukraine, Turkey, and others (Zheljazkov et al., 2012). Lavender flowers contain the essential oil with linalool, geraniol, cymene, camphene, pinene, cumarin, as main oil components, as well as phenolic acids (12%), flavonoids, tannins and triterpenoids (Robu et al., 20110). Lavender oil is known for its antibacterial, antifungal, carminative, antiflatulence, antiholic, sedative and antidepressive activities (Sabara et al., 2009). Lavender is reported to be an effective medicinal plant for treating inflammation, depression, stress, headache, migraine, gastrointestinal and rheumatic disorders (Hajhashemi et al., 2003).

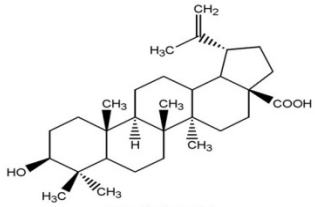
Oleanolic acid (OA) and ursolic acid (UA) (Figure 1) are the two major terpenoids in *Lavandula angustifolia* (Jäger et al., 2009). These compounds, ubiquitous in plant kingdom, are known to possess different biological activities that could be involved in the therapeutic effects of *L. angustifolia* flowers and of other herbal preparations containing these secondary metabolites. In particular, OA shows antifungal (Tang et al., 2000), anti-inflammatory, anti-HIV (Kashiwada et al., 2000), diuretic (Alvarez et al., 2002) and anticancer (Li et al., 1999) activities. UA also possesses anti-angiogenic (Shon et al., 1995), anticancer (Es-saady et al., 1996), and anti-inflammatory activities (Baricevic et al., 2003).



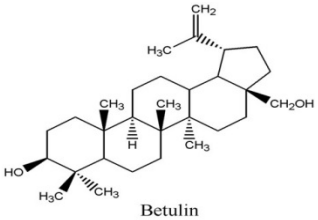
Oleanolic acid



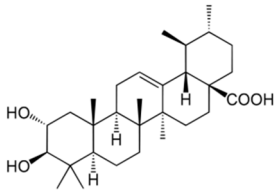
Ursolic acid



Betulinic acid



Betulin



Corosolic acid

Figure 1. Chemical structures of terpenoids

Although the health-promoting effects of *L. angustifolia* have been well described, the content of OA and UA in the extracts of *L. angustifolia* flowers should be accurately determined in order to investigate the extent to which these active components are absorbed, as well as their pharmacological activities and bioavailabilities. Thus, the development of a rapid and reproducible analytical method for the quantitative determination of the structural isomers UA and OA in *L. angustifolia* crude extracts is required.

Traditionally, target compounds from herbs could be procured using organic solvent extraction along with maceration, heat-flux, and Soxhlet extraction techniques. However, a low selectivity or extraction yield, in addition to the long extraction times, toxic solvent residues, and degradation of temperature-sensitive compounds may occur when these techniques are used (Wei et al., 2014).

The main analytical methods for analysis of triterpene acids are thin layer chromatography (TLC) (Zhang et al., 2000), gas chromatography (GC) (Janicsak et al., 2003), high-performance liquid chromatography (HPLC) (Xie et al., 2001) and capillary zone electrophoresis (Yang et al., 2007). HPLC is the most common method for quantitative analysis of the triterpene acids with high separation efficiency and short analytical time (Du et al., 2009).

Our objective was to study the contents of terpenoids composition of flowers of *L. angustifolia* Mill grown in areas of Plovdiv city. This study was concerned with high performance liquid chromatography (HPLC) qualitative and quantitative analysis of terpenoids in lavender flowers extracts obtained by Soxhlet extraction.

Materials and Methods

Reagents and standards

All reagents and solvents used were of HPLC grade. Methanol, formic acid were from Merck (Germany) and analytical standards of betulin (>98%), betulinic (>98%), ursolic (>90%), oleanolic (>97%) and corosolic (>97%) acids were purchased from Sigma® (St. Louis, MO, USA). Deionized water was purified through Ultrapure Water Systems Crystal B ® (Adrona Crystal B).

Preparation of standard solutions

Stock solutions with 1 mg/cm^3 of the oleanolic acid, ursolic acid, betulin, betulinic acid and corosolic acid were prepared by dissolving in methanol. Serial dilutions were made to prepare standard solutions at concentrations of 5, 10, 25, 50, 100, 200, 300, 400 and 500 µg/ cm^3 for each compound.

Statistical analysis

Three independent extracts from analyzed sample of lavender were prepared and each extract was analyzed for terpenoid content in triple replication. The presented values are means (n = 3) with standard deviations (± SD).

HPLC analysis

HPLC analysis was performed with Elite LaChrome Hitachi equipped with pump Hitachi L-2100/213, photodiode array detector model L-2455 of Hitachi and software ELITE La Chrome Hitachi. The samples were separated through reversed phase C_{18} column Supelco Discovery HS C_{18} (5µm, 25 cm x 4,6 mm) using an isocratic mobile phase consist of methanol : 0.1 % formic acid (92:8 v/v). The flow rate was maintained at 0.4 ml/min and temperature of the column was kept at 26^{o}C. The volume of injected samples was 20 µl and detection was carried out at 210 nm. The retention times are present in Table 1. The standard curves of compounds were linear in range of 10-400 µg/cm^3.

Table 1. Retention time of terpenoids.

№	Terpenoids	Retention time, min
1	Corosolic acid	18.04±0.21
2	Betulin	22.24±0.18
3	Betulinic acid	24.13±0.24
4	Oleanolic acid	27.22±0.23
5	Ursolic acid	28.81±0.22

Collection of plant samples

The investigated plant material from lavender was collected from different areas of Plovdiv city. (Angelova et al., 2015)

Preparation of Lavender Extracts

The plant material was air dried and stored in the darkness at room temperature prior to sample preparation step and analysis.

Powdered samples from lavender flowers were extracted with chloroform by Soxhlet extraction. The obtained extracts were filtered through filter pepper and evaporated to dryness under vacuum. Before analysis dried extracts were dissolved in methanol and filtered through a membrane filter with pore size of 0.45 µm.

Results

The results of HPLC analysis of terpenoids content and chromatogram are presented in Table 2 and Figure 2, respectively.

All of the analyzed samples of lavender flowers contain oleanolic, ursolic, betulin, betulinic and corosolic acids, but in different concentrations. Ursolic and oleanolic acids are the main triterpenoids in all of analyzed extracts. Ursolic acid was in highest concentration in sample 1, as oleanolic acid in sample 3 (Table 2).

Discussion

Triterpenic acids are common constituents of many medicinal herbs and plants and can strongly influence their therapeutic properties. Amongst plants matrices with a high content of ursolic acid are apple peel (*Malus domestica*), marjoram (*Origanum majorana*), rosemary leaves (*Rosmarinus officinalis*), sage (Salvia officinalis), lavender leaves and flowers (*Lavandula angustifolia*), (Szakiel et al., 2012).

Plants are a rich source of biologically active compounds with health benefits for humans.

In the present paper, triterpenic acids in commonly used plant, such as *L. angustifolia* was determined.

Five terpenoids - corosolic, betulin, betulinic, oleanolic and ursolic acids were found as oleanolic and ursolic acids were in highest concentration in all of the analyzed samples. That is of great importance, because of many valuable biological activities of these terpenoids (Tang et al., 2000; Kashiwada et al., 2000; Alvarez et al., 2002; Li et al., 1999; Shon et al., 1995; Es-saady et al., 1996).

Our results are in agreement with most of the previous work reported that the oleanolic and

ursolic acid are the major terpenoids in *L. angustifolia.* Jäger et al. (2009) reported that lavender flowers contain ursolic aicd-1.05 g/ 100g DW, oleanolic acid -0.40 g/ 100g DW and betulinic acid 0.12 g/ 100g DW. Similar results for triterpenoids content in lavender flowers were also found by Sanicsák et al., (2006). They reported for 0.479 % oleanolic acid and 1.397 % ursolic acid.

Betulin, betulinic and corosolic acids were in lower concentration in investigated samples than ursolic and oleanolic acids. However they also possess valuable biological activities. Betulinic acid is a minor bio-compound but exhibits a variety of biological and medicinal properties such as inhibition of human im- munodeficiency virus (HIV), anti-bacterial, anti-malarial, anti-inflammatory, anthelmintic, antinociceptive, anti-HSV-1, and anti- cancer activities (Moghaddam et al., 2012). Recently, interest in betulin has increased as certain derivatives from this compound were considered to be a potential agent against tumor cancer tested on brain, skin or other tumor cells (Alakurtti et al., 2006; Oh et al., 2006; Sami et al., 2006; Fulda et al., 1999; Rajendran et al., 2008), as well in the chronic hepatitis therapy (Shikov et al., 2011).

Conclusion

Our investigation demonstrated that the extract of lavender flowers contain high quantities of biologically active triterpens (oleanolic and ursolic acids). That is a base for their possible application in pharmaceutical and cosmetic products with improved health benefits for human health.

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Table 2. HPLC analysis of terpenoids content of lavender flowers.

Terpenoids, mg/g DW	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Corosolic acid	2.25 ± 0.18	8.15 ± 0.28	2.47 ± 0.18	2.33 ± 0.17	2.26 ± 0.14
Betulin	4.41 ± 0.26	4.19 ± 0.29	4.86 ± 0.22	4.46 ± 0.23	1.35 ± 0.09
Betulinic acid	15.08 ± 0.31	19.03 ± 0.34	13.34 ± 0.35	12.33 ± 0.31	4.08 ± 0.25
Oleanolic acid	55.46 ± 0.52	49.60 ± 0.45	95.27 ± 0.58	48.01 ± 0.38	20.26 ± 0.28
Ursolic acid	82.36 ± 0.64	65.01 ± 0.53	43.58 ± 0.47	68.83 ± 0.57	24.07 ± 0.31

Figure 2. HPLC chromatogram of analysis of terpenoids content of Sample 1: 1-Corosolic acid; 2-Betulin; 3-Betulinic acid; 4-Oleanolic acid; 5-Ursolic acid