

RESEARCH ARTICLE

Antibacterial evaluation of *Elettaria cardamomum* (L.) Maton, *Lavandula angustifolia* Mill. and *Salvia fruticosa* Mill. essential oil combinations in mouthwash preparations

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Abstract

The aim of this present study was to evaluate *Elettaria cardamomum* (L.) Maton., *Lavandula angustifolia* Mill. and *Salvia fruticosa* Mill. essential oils in mouthwashes formulated with different combinations such as 0.1/0.25/0.1; 0.2/0.25/0.1; 0.3/0.1/0.1 in 10 mL (v/v), and their *in vitro* antibacterial activity performance. The characterization of the main essential oil components was performed by GC-FID and GC/MS analyses. The antimicrobial evaluation was performed by using the disc diffusion method against human pathogenic *Staphylococcus aureus* ATCC 6538, *Escherichia coli* NRLL B-3008, *Bacillus cereus* ATCC 14579, and *Salmonella typhii* (clinical isolate), respectively. In the present study, among the tested bacteria *S. typhii* was the most sensitive, while *B. cereus* and *E. coli* were the most resistant pathogens in the applied mouthwash formulations. The essential oil combination containing mouthwash formulations can be used as a functional natural based cosmetics.

Keywords: Formulation, Lavender, Cardamon, Sage, mouthwash, antimicrobial

Introduction

The oral cavity is very sensitive to infections and other pathologies. In fact, the most common pathologies are dental diseases, periodontitis, oral mucosa infections and oral cancer. The oral mucosa can be colonized by several opportunistic pathogens. More specifically, the healthy oral cavity can be colonized by fungi, viruses, and while bacteria being the most predominant (Coll et al., 2020). Various local and systemic factors such as smoking, pregnancy, diet, nutrition, age and oral hygiene, increase the amount of indigenous bacteria causing various oral infections. Orofacial infections can induce significant discomfort to the patients and unnecessary economic burden. Thus, the early detection and management of such infections are highly significant (Bandara & Samaranayake, 2019). The local treatment of such oral cavity pathologies offers various advantages compared to systemic drug administration given that the diseased area is directly targeted with minimum systemic side effects (Sankar et al., 2011). It has been reported that semisolid or liquid dosage forms are most commonly used because of their ease of administration and patient compliance (Nguyen & Hiorth, 2015). Mouthwashes are oral solutions or liquids which are applied to rinse the mouth and eliminate bacteria, act as an astringent, deodorize the oral cavity, and for their therapeutic effect by relieving the infection. They are considered as the most sufficient and safe formulations which can deliver molecules able to reduce oral bacteria delivery systems (Sekita et al., 2016). Normally, the combination of mouthwashes (Yousefimanesh et al., 2015) and mechanical oral hygiene such as brushing and flossing is applied to prevent various oral disorders such as infection, inflammation, relieve pain and decrease halitosis. In general, mouthwashes contain antiseptics that are used in the treatment of

such infections (Alshehri, 2018; Parashar, 2015). Nonetheless, many strategies focus on the biological activities of alternative natural products due to the increased microbial resistance of common antibiotics (Jain & Jain, 2016).

It is well known that essential oils have a broad spectrum antimicrobial activity against many pathogens (Tabanca et al., 2001; Azaz et al., 2002; Başer et al., 2002; Baser et al., 2006; Tabanca et al., 2007; Polatoglu et al., 2010; Karadağ et al., 2019;). There are several reports on the antimicrobial activity of *Salvia triloba* L. (syn. *Salvia triloba* L.) essential oil (Longaray Delamare et al., 2007; Pierozan et al., 2009). According to previous studies, *S. triloba* essential oil is a potent antimicrobial agent against *S. aureus*. In addition, the antifungal activity studies with *Lavandula angustifolia* Mill. essential oil have shown that it possesses significant antifungal activity (Adam et al., 1998; D'Auria et al., 2005; de Rapperet al., 2013; Jianuet al., 2013). Lavender essential oils also showed antibacterial activity (Cavanagh & Wilkinson, 2002; Thosar et al., 2013). It was also reported that various *Elettaria cardamomum* Maton. preparations inhibited the oral mucosa pathogens (Aneja & Joshi, 2009; Kaushik et al., 2010; Kubo et al., 1991; Masoumi-Ardakani et al., 2016; Özkan et al., 2018; Singh et al., 2008).

In this present work, the *in vitro* antimicrobial activity of three different mouthwash formulations composed of a combination of *E. cardamomum*, *L. angustifolia* and *S. fruticosa* essential oils in various proportions were studied. The chemical composition of the essential oils were also analysed. To the best of our knowledge, this is the first study on mouthwashes, consisting of three different combinations of *E. cardamomum*, *L. angustifolia* and *S. fruticosa* (syn. *S. triloba* L. f.) essential oils.

Materials and Methods

Chemicals and plant material

E. cardamomum fruits and *L. angustifolia* flowers (Herbarium no: IMEF 1076 and IMEF 1077, respectively) were aquired from a local market in Istanbul, Turkey and *S. triloba* (Herbarium no: IMEF 1078) aerial parts were collected from Iskilip, Çorum, Turkey in 2018, which were identified by AEK, voucher specimens were deposited at Istanbul Medipol University Herbarium.

The dry plant samples were crushed individually prior hydrodistillation, where a Clevenger apparatus was used for the isolation of the essential oils for the combinations of the mouthwash formulations. Sodium chloride, sodium bicarbonate, sodium saccharin and ethanol were purchased from Sigma (Germany).

Analysis

An Agilent 7890B GC-FID (Santa Clara, CA, USA) coupled with an Agilent 5977E electron impact mass spectrometer (Santa Clara, CA, USA) via a two-way capillary splitter was utilized to identify and quantify essential oil components. An Agilent G4513A (Santa Clara, CA, USA) auto injector was used for sample injections. The compounds were identified by comparing their spectral data obtained from commercial libraries such the Wiley Registry of Mass Spectral Data 9th edition with NIST 11 Mass Spectral Library (NIST11/2011/EPA/NIH) and from literature. The GC- FID/MS analysis conditions were listed in Table 1, and the main components were listed in Table 2.

Table 1. GC-FID/MS Conditions

Column	Agilent DB-Wax (60 m, 0.25 mm, 0.25 µm)
Injection	Split (50:1), 1 µL
Injector Temperature	220 °C
Helium Carrier Gas Flow	1.5 mL/min

Table 1. GC-FID/MS Conditions (cont.)

Ion Source Temperature	230 °C
Quadrupole Temperature	150 °C
MSD Transfer Line Temperature	250 °C
Ion Source (eV)	70 eV
Mass Scan Range	<i>m/z</i> 35–300
FID Temperature	220 °C
FID Air Flow	400 mL/min
FID H ₂ Flow	30 mL/min
Oven Temperature Programme	70 °C isothermal for 15 min 2 °C/min ramp to 180 °C 180 °C isothermal for 5 min 5 °C/min ramp to 230 °C 230 °C isothermal for 15 min

Mouthwash formulations

The mouthwashes were prepared using combinations of the essential oils. Initially, the mouthwash solutions were formulated using 4.5-5.5 % of essential oil. Mouthwash formula 1 (MF1) contains 1% *E. cardamomum*, 2.5% *L. angustifolia*, 1% *S. triloba* essential oil; mouthwash formula 2 (MF2) contains 2% *E. cardamomum*, 2.5% *L. angustifolia*, 1% *S. triloba*; mouthwash formula 3 (MF3) 3% *E. cardamomum*, 1% *L. angustifolia*, 1% *S. triloba* essential oils. As sweetener saccharine sodium was applied. Furthermore, the essential oils were weighed, and dissolved in ethanol while sodium chloride and sodium bicarbonate were added gradually using a mechanical stirrer (500 rpm, 30 minutes), respectively. The combination blend was filtered, and the volume of the filtrate was completed to 10 mL by using distilled water. No preservative was added since the mouthwashes included high content of ethanol (> 15 %)(Kulaksiz et al., 2018), as well as essential oils.

Table 2. The mouthwash formulations containing the essential oils (EO)

Formulation codes	<i>S. triloba</i> EO (%)	<i>E. cardamomum</i> EO (%)	<i>L. angustifolia</i> EO (%)	Sodium Chloride (%)	Sodium Bicarbonate (%)	Sodium Saccharine (%)	EtOH (%)	Distilled Water
MF1	1	1	2.5	0.1	0.05	0.001	60	q.s. 10 mL
MF2	1	2	2.5	0.1	0.05	0.001	60	q.s. 10 mL
MF3	1	3	1	0.1	0.05	0.001	60	q.s. 10 mL

Colour and odour

Physical and organoleptic parameters like odour and colour were examined by plain visual examination.

PH measurement

Mouthwashes are evaluated for their pH values, since the oral tissues can be affected by the low pH (Shaik et al., 2017). Thus, the pH of the mouthwashes was measured via a calibrated pH meter (Mettler Toledo, Switzerland) and reported as the average value out of triplicates.

Antimicrobial evaluation

The *in vitro* antimicrobial potential was evaluated *via* the disc diffusion method following the methodology described by the Clinical and Laboratory Standards Institute as previously reported in detail (Siafaka et al.,

2016 and 2019). *Staphylococcus aureus* ATCC 6538, *Escherichia coli* NRLL B-3008, *Bacillus cereus* 14579, *Salmonella typhi* (clinical isolate) human pathogenic strains were used. The inoculation of the pathogens was performed using Mueller Hinton Broth (MHB, Merck, Germany) at 37°C under aerobic conditions for 24 h, and standardized to 1×10^8 CFU/mL using McFarland No: 0.5 in sterile saline (0.85%). The mouthwash samples stock solution were prepared in dimethylsulfoxide (DMSO) at 10 mg/mL concentration, and the antibacterial evaluation was performed in triplicates, where the results were reported as average, as reported in Table 3. Tetracycline was used as standard antibiotic for comparison.

Results and Discussion

In the present work, the GC-FID and GC-MS analyses revealed the main components of *E. cardamomum* essential oil as 1,8-cineole (43.6%), terpinyl acetate (28%), linalool (8.8%), 4-terpineol (4.2%), and linalyl acetate (2.3%); the major components of *L. angustifolia* essential oil were identified as linalool (23.6%), linalyl acetate (12.1%), camphor (11.8%), linalool oxide B (10.7%), and borneol (7.1%), respectively. Furthermore, the main volatile components of *S. triloba* essential oil was characterized as 1,8-cineole (40%), camphor (11.3%), α -pinene (7.3%), myrcene (4.5%), and camphene (3.9%), respectively.

In the present work we report on the antibacterial activity of different combinations of *E. cardamomum*, *L. angustifolia* and *S. triloba* essential oils as shown in Tables 2 and 3. Table 2 shows the amounts of the essential oils and other ingredients, which constituted the mouthwash formulations comparatively. The three formulations presented the same amount of the excipients, however in different concentrations of the essential oils.

There are several reports on the chemistry and antimicrobial activity of *S. triloba* essential oils (Shimoni et al., 1993; Longaray Delamare et al., 2007; Pierozan et al., 2009). Also many publications on the bioactivity and chemistry of *L. angustifolia* essential oil exist (Adam et al., 1998; Cavanagh & Wilkinson, 2002; D'Auria et al., 2005; de Rapperet al., 2013; Jianuet al., 2013 Thosar et al., 2013). *E. cardamomum* essential oils were also previously investigated for the chemistry and biological activities (Aneja & Joshi, 2009; Kaushik et al., 2010; Kubo et al., 1991; Masoumi-Ardakani et al., 2016; Özkan et al., 2018; Singh et al., 2008). The antimicrobial activities of essential oils rich in linalool are also remarkable (Özek et al., 2010). In the composition of essential oils used in this study, linalool is also a major constituent. As it was identified by the GC analysis, the compounds 1,8-cineole, linalool and camphor are major volatile components in the composition of essential oils in mouthwashes. Previous studies exhibit that these three volatiles are known for their strong antimicrobial activity (Jirovets et al., 2005; Park et al., 2012; Vuuren et al., 2007). As a result, it can be said that combinations of all these antimicrobial active ingredients in certain concentrations may have a synergistic effect.

In this present study, the pH values of the prepared mouthwashes were measured between 7.19 and 7.84. The pH values of the formulations were compatible and suitable for use as a mouthwash. Due to the essential oil combinations a pleasant odour was present, resulting from the examination of the prepared mouthwashes. Noteworthy was also that the formulations were clear and homogenous, which is also important since a blurred mouthwash can confuse the patient and prevent its use (Fig.1).

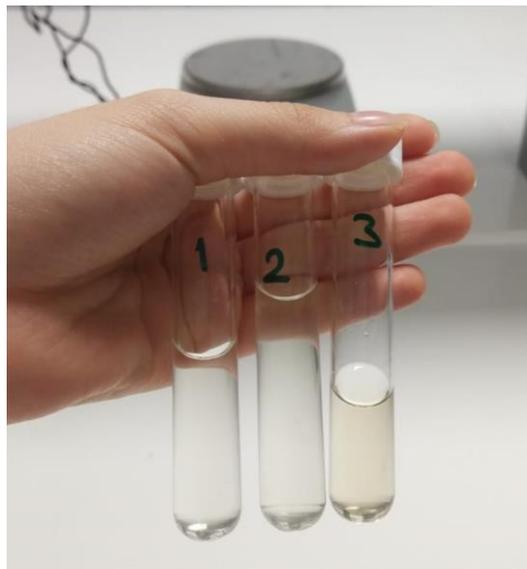


Figure 1. Visual observation of the prepared mouthwashes (MF1, MF2 and MF3)

To prevent oral and dental infections, mouthwash solutions or formulations with antimicrobial activity may prevent but also eliminate pathogenic bacteria (Jones et al., 2018; Müller et al., 2017). However, mouthwashes can affect the oral tissue, the tooth enamel and also deteriorate symptoms if the pH is too acidic, due to ethanol or other compounds. Thus, the pH values are important (Pelino et al., 2018).

Mouthwashes containing plant based preparations may mask malodour and provide a pleasant flavor (Ahmad et al., 2018). The oral cavity contains many habitats (teeth, gingival sulcus, tongue, palates, and tonsils) providing space for the colonization by various microorganisms such as bacteria, fungi and viruses. Bacteria are the predominant components of this microflora (Allaker & Ian Douglas, 2015). Many antimicrobials and other strategies have been approved for oral and dental infections (Allaker & Ian Douglas, 2015). Nonetheless, the majority of the oral bacterial infections are polymicrobial in nature (El-Awady et al., 2019). Thus, the combination of antimicrobial molecules is essential to eliminate the polymicrobial infection. From the antibacterial drugs, chlorhexidine is the gold standard. Nonetheless, this active molecule, as well as the other antibacterial molecules, present various side effects, such as pigmentation, taste alteration, etc (Marchetti et al., 2011). The essential oils are applied for cleaning (Adelakunet al., 2016), for wound disinfection (Mori et al., 2016), and to treat infections precisely due to their effectiveness in killing microbes (Valeriano et al., 2012). Since the antibiotic resistance has risen, many researchers believe that essential oils are the next generation of antibiotics (Yapet al., 2014). Hence, the study of the antimicrobial effectiveness of essential oils against pathogens is in high demand. Several studies show that the combination of essential oils can lead to the optimization of the medical activities (De Rapper et al., 2013). It has been shown that formulations containing lavender essential oil act to a greater extent against *S. aureus* (Hossain et al., 2017; Thosar et al., 2013). Another study showed that *E. cardamomum* seems to have significant antibacterial activity and could be a very useful in the discovery of novel antibiotics (Abdullah et al., 2017; Kaushik et al., 2010). Furthermore, *S. triloba* essential oils have been identified as strong antimicrobial agents (Fu et al., 2013; Gali-Muhtasib et al., 2000).

Table 3 summarizes the antimicrobial activity results of the essential oils and their combinations. *S. triloba* essential oil showed activity against *S. aureus* and *B. cereus* whereas *L. angustifolia* essential oil against *S. aureus* and *E. coli*. *E. cardamomum* exhibited activity against *S. aureus*, *S. typhii* and *E. coli*. The studied microorganisms have been identified as common pathogens of oral infections.

In this study, significant antimicrobial activity was observed against *S. aureus*, which is the cause of throat infections (McCormack et al., 2015). As the results showed, MF3 is the mouthwash with the highest antibacterial activity. MF1 presents antibacterial activity only against *S. typhii* whereas MF2 against *S. aureus* and *S. typhii*. MF3 is a formulation containing mainly *E. cardamomum* essential oil (3%). It has been also observed from previous studies that the *E. cardamomum* essential oil demonstrates high antimicrobial activity against *S. aureus* (Singh et al., 2008). Besides, *E. cardamomum* essential oil showed antimicrobial activity against *S. aureus* and *S. typhii* strains. In the combination of *E. cardamomum*, *L. angustifolia* and *S. triloba* oils (MF3), the activity increased. Based on this, it can be claimed that the formulations demonstrated a synergistic effect due to the different combinations. In addition, it can be concluded that MF3 can be used as an antibacterial mouthwash due to the enhanced synergistic antimicrobial activity.

Table 3. Growth of inhibition zones of essential oils (EO) and mouthwash formulations (in mm)

Sample	<i>E. coli</i>	<i>S. aureus</i>	<i>S. typhii</i>	<i>B. cereus</i>
<i>S. triloba</i> EO	-	10	-	8
<i>L. angustifolia</i> EO	8	9	-	-
<i>E. cardamomum</i> EO	7	7	8	-
MF1	-	-	7	-
MF2	-	7	8	-
MF3	6	10	13	7
Tetracycline	23	20	19	22

To conclude, this is the first report on the antimicrobial activity of mouthwashes prepared from different combinations of *L. angustifolia*, *S. triloba* and *E. cardamomum* essential oils. The *in vitro* antimicrobial activities on different combinations of these essential oils efficiently alter the antimicrobial activity in a synergistic manner. Consequently, the functional formulations may help as a good solution for the prevention and management of oral infections.

Supplementary files

The GC-FID chromatogrammes of *L. angustifolia*, *S. triloba* and *E. cardamomum* essential oils.

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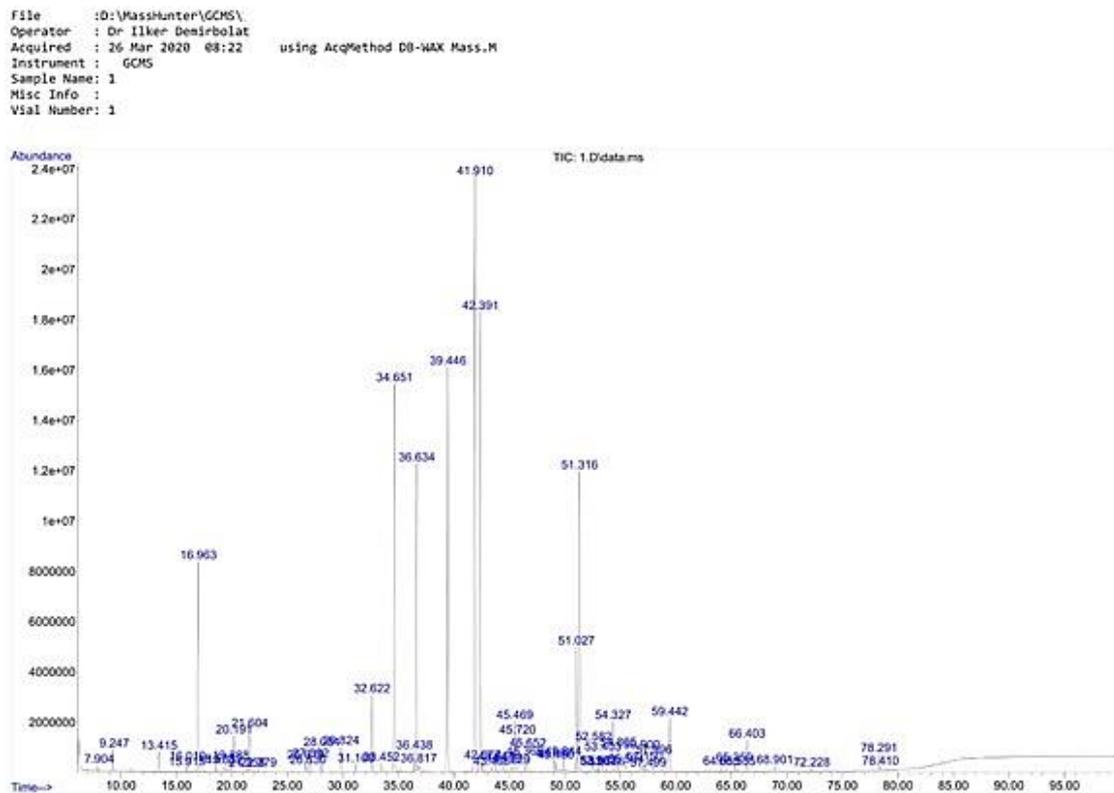
Supplementary file

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Ayşe Esra Karadağ, Esra İpekçi, Ayşe Pinar Yağcılar, İlker Demirbolat, Murat Kartal, Panoraia I. Sifaka, Neslihan Üstündağ Okur

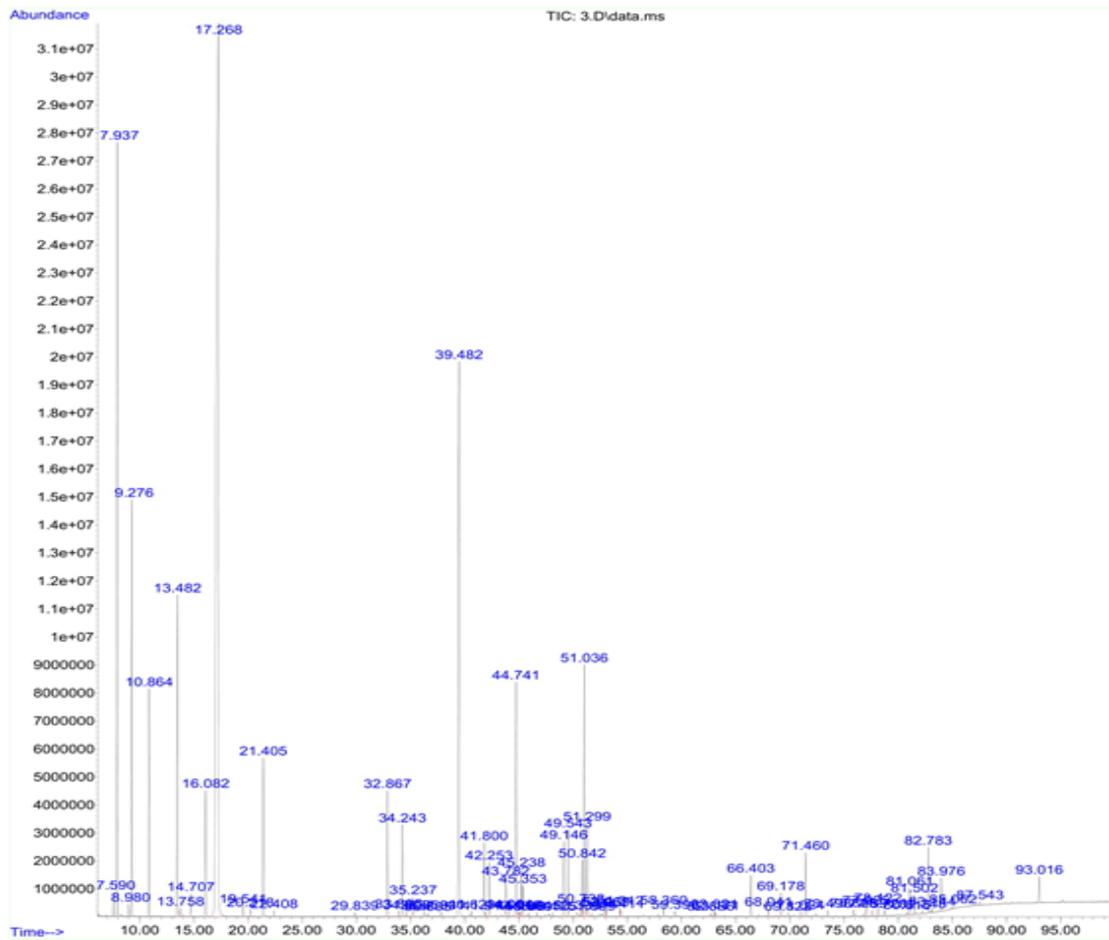
Antibacterial evaluation of *Elettaria cardamomum* (L.) Maton, *Lavandula angustifolia* Mill. and *Salvia fruticosa* Mill. essential oil combinations in mouthwash preparations

The GC-FID chromatogramme of *L. angustifolia* essential oil



The GC-FID chromatogramme of *S. triloba* essential oil

File :D:\MassHunter\GCMS\1\data\
Operator : Dr Ilker Demirbolat
Acquired : 26 Mar 2020 11:58 using AcqMethod DB-WAX Mass.M
Instrument : GCMS
Sample Name: 3
Misc Info :
Vial Number: 3



The GC-FID chromatogramme of *E. cardamomum* essential oil

File : D:\MassHunter\GCMS\
Operator : Dr. İlker Demirbolat
Acquired : 26 Mar 2020 10:06 using AcqMethod DB-WAX Mass.M
Instrument : GCMS
Sample Name: 2
Misc Info :
Vial Number: 2

