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Essential oil extraction, chemical analysis and anti-*Candida* activity of *Foeniculum vulgare* Miller – new approaches

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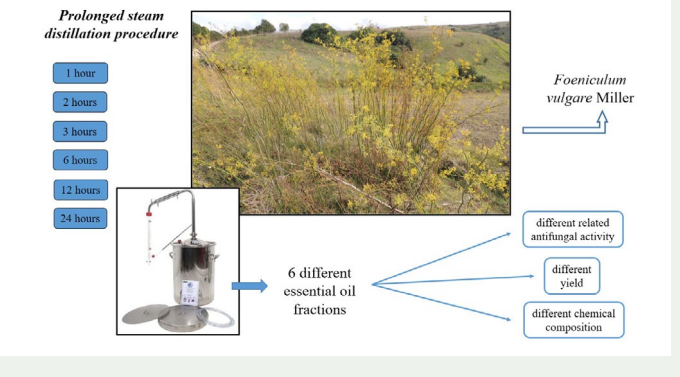
ABSTRACT

A comprehensive study on essential oil samples of *Foeniculum vulgare* Miller from Tarquinia (Italy) is reported. A 24-h systematic steam distillation was performed on different harvested samples applying different extraction times. The GC-MS analysis of the residue outcome showed *o*-cymene, *α*-phellandrene, *α*-pinene and estragole as the major constituents. The predominance and continued presence of *o*-cymene makes this fennel oil a rather unique chemotype. An evident correlation between the antifungal activity and phenological stage is demonstrated. The most active fractions were particularly rich in estragole, as well as a significant amount of fenchone that possibly exerts some additive effect in the expression of overall antifungal potency. Pre-fruiting material produced oil particularly rich in *o*-cymene. With reference to the duration of the extraction, the maximum amount of oil was released within the first 3 h, whereas the reproductive phase material needed at least 6 h for the extraction.



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1. Introduction

Essential oils (EOs) are well known since ancient times due to their different biological activities, i.e. antibacterial and antifungal, or antioxidant (Baratta et al. 1998). Generally, these activities depend on the chemical composition of EO related to the plant genotype, as well as other factors such are geographical, environmental and agronomic which may also greatly influence components and ratio (Celikel and Kavas 2007). Many EOs and their ingredients have an unexpectedly large range of applications. Their cosmetic use is probably the most ancient one, both for their functional, perfume and preservative properties (Carvalho et al. 2016).

Furthermore they have been increasingly used in foods (Burt 2004), because of their antioxidant and antimicrobial activities that confer additional properties (Tongnuanchan and Benjakul 2014). Other common uses are as disinfectants (i.e. bactericidal, virucidal and fungicidal), as medications and also flavourings and fragrance (Bakkali et al. 2008). A new approach, based on 24-h steam distillation extraction procedure, applied on six wild-growing plants, was performed taking into consideration different harvest periods and extraction time, has been recently reported in detail (Garzoli et al. 2015; Božovic, Garzoli, et al., 2017, Božovic, Navarra, et al., 2017). During these studies, it was observed that different EO chemical composition was obtained depending on the extraction time, with variations in their related experimental biological activity. Finally analysing, a similar systematic EOs extraction, chemical composition analysis and antifungal activity evaluation are reported in relation to *Foeniculum vulgare* Miller (FV) collected in Tarquinia (Figures S1–S3, Sections S1–S2 in Supplementary Material).

2. Results and discussion

2.1. EO Extraction

Dried aerial parts of FV were submitted to steam distillation and the oil was collected at various intervals (1, 2, 3, 6, 12 and 24 h). FV was monitored from August to October, including vegetative and reproductive stages, and did not show any significant differences in the first two harvesting periods. On the contrary, a great increase in EO amount, up to five times, was noticed in October during plant fruiting (Figure S4 and Table S1 in Supplementary Material).

2.1.1. GC/MS analysis

The GC-MS analysis of the 33 FVEO distilled samples revealed the presence of 26 different chemical constituents chemical constituents (Tables S2–S7, Supplementary material). In general, a typical chemical profile was identified, and there is a substantial difference in oil composition obtained from the fruiting material can be observed. While pre-flowering and flowering materials gave oils rich in *o*-cymene (OCY) and *α*-phellandrene (APH), the oil from October harvested material was characterised by a very high content of estragole (EST) and *α*-pinene (PIN). Detailed overview of the literature data and comparison with our results are available in Supplementary Material Section S3. OCY can be considered the most characterising compound since it was always present in any harvesting month and any distilled fraction. However, significantly lower amounts were found in October. Its percentages reached the maximum during the flowering period in September (up to 52.5%). In every

month, a decrease in its amount with the extraction progress is observed, being most abundant in the first 3 h. Although OCY presence in FV was already reported (Bowes and Zheljazkov 2005; Esquivel-Ferriño et al. 2012), the *p*-isomer occurs much more frequently (Özcan et al. 2006; Afify et al. 2011; Mokhtar et al. 2014). APH was the characterising compound mainly in the first 6 h of extraction. EO particularly rich in APH was obtained from plant harvested in August, reaching the maximum percentage in the A6 h fraction (20.5%). APH is a monoterpene commonly found in Apiaceae EOs, usually co-present with its *β*-isomer. The presence of APH in FVEO from Tarquinia is in agreement with numerous literature data (Özcan et al. 2006; Cosge et al. 2009; Rehab et al. 2015). EST was recognised at its highest-level and the main constituent for FVEO obtained from October gathering. It was particularly abundant in the first four fractions (30.1–57.6%) and drastically decreased after 12 and 24 h. It was also identified in the plant material from August, but in much lower amount (up to 18.5%) and interestingly, it was not revealed in the flowering material. EST is a common constituent of FV oil, usually present in fruits or flowers, as reported in numerous papers (Özcan et al. 2006; Cosge et al. 2009; Afify et al. 2011; Rehab et al. 2015). A significant increase in the PIN concentration during the FV fruiting period was also observed. In the case of PIN a reverse evolution with the extraction duration was noticed, since the percentage became higher with the process progression, becoming particularly abundant after the first three extraction hours. It reached the maximum in O6 h fraction (29.3%). This compound was also present in the EOs obtained by the materials coming from the other months, although in lower amounts. The amount of PIN found in this study is in good agreement with previous reported studies (Cosge et al. 2009; Mokhtar et al. 2014).

The presence of other constituents is related to the extraction time or harvesting month, but some of them are always present in substantial amounts. For instance, limonene was present in every month, with its maximum in September. It was particularly abundant in the first 6 h of the extraction process, with percentages ranging from 10.9 (S3 h) to 15.1 (S6 h). Monoterpenoids *β*-myrcene, *β*-phellandrene, *γ*-terpinene and *p*-cymen-8-ol are also present in every month and in almost every fraction. Although present in every month, thymol, myristicin and piperitenone oxide were found as the characterising compounds of some fractions (usually after 6 h). Some compounds were found in significant amount only in specific fractions, such as *p*-menth-en-2-one in A12 h (9.7%), S12 h (9.6%) and S24 h (13.9%), while others are characteristic of particular months: e.g. fenchone for August and October, pulegone and *cis*-sabinol for August and September, and *β*-pinene, terpinolene and cinerolone only for October. These observations may indicate that some constituents are markers of the reproductive period.

Regarding the FVEO chemical composition complexity, certain fractions from August material (20 and 21 compounds in A12 h and A6 h, respectively) were more complex than others (16–18 different chemical contituents were observed in September and October harvests). The prepared mixtures, taking into account relative yields of the main characterising compounds, reflect quite well the compositions of the original fractions (Tables S5-S7, Supplementary Material). Thus, OCY was still the main characterising compound in August and September, while EST and PIN are the most representative in October mixtures.

2.2. *Anti*-Candida *activity evaluation*

The anti-*Candida* activitity of 33 FVEO samples are reported in Table S8 (Supplementary Material). Similarly to the chemical analytical differences above reported, the samples from

October greatly differ in antifungal activity expression. Whereas the majority of August samples was found not active at the used concentration range, active EOs were observed from September and October, being the latters the most potent. With few exceptions, the MIC values ranged from 3.12 to 12.48 mg/mL for the oils extracted in August and September, and from 1.56 to 12.48 mg/mL for the samples obtained from October harvest. The most potent fractions were isolated from the fruiting material during the first 6 h of the extraction process (1–6 h). Regarding the mixtures, their potencies reflect quite well the final result of the original fractions, being October samples the most effective.

FVEO and extracts were thoroughly investigated and literature survey revealed plenty of data (Section S3, Supplementary Material). However, the majority of studies included only particular plant parts, and with refernce to data exposed in our previous document, comparable activities are only the ones from October samples, the fruiting phase. The samples having moderate potency (some of them even very notable, e.g. 1 and 3 h) are in agreement with most of the reported data (Section S3, Supplementary Material). However, the majority of all those studies reported *trans*-anethole as the main EO constituent. This compound was not detected in the samples from Tarquinia, and according to some authors, that it is the main active component responsible for the antimicrobial activity of fennel oil (Çetin et al. 2010; Kazemi et al. 2012). In contrast, our samples abound in EST that could be a less potent compound then *trans*-anethole. For instance, investigating the EOs of three fennel cultivars (with various *trans*-anethole/EST ratio), the highest antifungal efficacy was observed in the ones containing *trans*-anethole in prevalence; the EST-rich cultivar was the most effective against Gram(+) bacteria (Shahat et al. 2011). The activities of the fruit-oils of six fennel accessions collected in Portugal were tested against various food spoilage fungi and a selection of bacterial strains (Mota et al. 2015). The EST-rich oil showed the lowest antifungal activity, whereas all were found to exert a broad spectrum of antibacterial activity (with the MIC value varying from 62.5 to 700 µg/mL). However, the authors highlighted the highest potency of the accession having an equilibration amount of the three main EO compounds: *trans*-anethole, fenchone and EST. Moreover, they concluded that its antibacterial activity might be attributed mainly to synergetic effects between them and the proportions in which they are present within a complex mixture. Further, antimicrobial activity of EO samples obtained from different fennel organs (root, stem, leaves and fruit) was evaluated (Kawther 2007). The author reported the fruit-oil as the only one that shows activity against commonly encountered *Candida* species (18–20 mm inhibition zone), whereas no inhibition zone was recorded in the case of fennel root, stem and leaves. This report fits in with the results here reported, since August material (only vegetative parts of the plant) failed to show any significant effect.

Last but not least, a relevant connection between the anti-*Candida* activity and phenological stage is shown. Potency was found to increase in the reproductive period, being the most potent EOs obtained from fruit-containing material. The most active fractions were particularly rich in EST (39.1–57.6%), along with the significant amount of fenchone (8.6–14.1%) that possibly exerts some additive effect in the expression of overall activity, since its good antimicrobial potential has already been reported (Kazemi et al. 2012). In the case of September oils, moderate activity of some fractions can be observed. Taking into account their chemical compositions, and complete lack of EST, the possible enhancing effect can be attributed to the higher presence of limonene and thymol with already proved antimicrobial potencies (Kazemi et al. 2012; Vimal et al. 2013).

3. Conclusion

Steam distillation is the most common technique to isolate EO from plant material. Classical procedure is usually reported to be completed in 2–4 h. In line with our previous research (Garzoli et al. 2015; Božovic, Garzoli, et al., 2017), therefore we report a systematic 24-h EO extraction procedure applied to FV. The study has included chemical analyses of obtained EOs, as well as the related antifungal activity. The extraction method applied gave fractions that differ greatly in their chemical compositions. In spite of the fact that the main characterising compounds are usually present in every fraction, amount variations are particularly evident between the first three fractions and the last ones (after 12 or 24 h). Furthermore, some compounds appear only with the development of the extraction process, and gradually increase in amount, being significantly present only in the last few fractions. The chemical profile has been found to be heavily influenced by harvesting period. Whilst the pre-fruiting materials from August to September gave OCY and APH rich EOs, on the other side the oil from October was characterised by high amounts of EST and PIN.

FV samples differ greatly in their antifungal activity expression. Whereas the majority of August samples were not active in the tested concentration range, the potency increased in the reproductive period, reaching the maximum from fruit-containing material derived EOs. The most active EO fractions were particularly rich in EST, along with significant amounts of fenchone. Moderate potency of some fractions from September could be observed, and taking into account their chemical compositions and complete lack of EST, the possible enhancing effect can be related to higher percentage of both limonene and thymol.

Due to continuous interest in food ingredients that may serves as 'active ingredients' in salutistic products (i.e. functional foods, supplements and cosmeceuticals) (Sacchetti et al. 2005; Ziosi et al. 2010) at first anti-*Candida* activity was explored to disclose mild solutions to systemic as well topical problems related to this fungi. Further investigations are currently ongoing in order to highlight other biological activities and potential application in dermatological products.

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