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Enzyme-assisted extraction of essential oils from thyme (*Thymus capitatus* L.) and rosemary (*Rosmarinus officinalis* L.): Impact on yield, chemical composition and antimicrobial activity

Karim Hosni[a,*], Imed Hassen[b], Hedia Chaâbane[c], Maroua Jemli[c], Sana Dallali[d], Houcine Sebei[d], Hervé Casabianca[e]

[a] Laboratoire des Substances Naturelles, Institut National de Recherche et d'Analyse Physico-chimique (INRAP), Biotechpole de Sidi Thabet, 2020, Tunisia
[b] Laboratoire des Méthode et Techniques Analytiques, Institut National de Recherche et d'Analyse Physico-chimique (INRAP), Biotechpole de Sidi Thabet, 2020, Tunisia
[c] Laboratoire de microbiologie, Institut National de Recherche et d'Analyse Physico-chimique (INRAP), Biotechpole de Sidi Thabet, 2020, Tunisia
[d] Département de production végétale, Ecole Supérieure d'Agriculture de Mograne, 1121 Zaghouan, Tunisia
[e] Service Central d'Analyse, Centre National de recherche Scientifique (CNRS), 69360 Solaize, Lyon, France

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ABSTRACT

The main goal of the present study is to evaluate the effects of enzymatic pre-treatment on the yields, chemical composition and antimicrobial activities of the essential oils of *Thymus capitatus* and *Rosmarinus officinalis* leaves. In *T. capitatus*, application of cellulase, hemicellulase and combination of both enzymes induced 63.55, 23.72 and 109% increase in the essential oil yields. It also induced increment by 2.7, 31 and 38% in the amount of its main component carvacrol. In *R. officinalis*, enzymatic treatment resulted in enhanced oil yields by 5, 50 and 20% for cellulase, hemicellulase and the combination of both enzymes, respectively. In contrast to *T. capitatus*, the amount of the main component 1,8-cineole dropped by 17.73, 36.92 and 15.46% in oils extracted from cellulase, hemicellulase and cellulose/hemicellulase treated samples of *R. officinalis*. At the same time, the essential oils (at 1/32 and 1/4 dilution for *T. capitatus* and *R. officinalis*, respectively) were evaluated for their antimicrobial activities against 6 food-borne pathogens (*Escherichia coli, Salmonella typhimurium, Streptococcus agalactiae, Staphylococcus aureus, Enterococcus feacium* and *Candida albicans*). All investigated oils exhibited antimicrobial activity with those issued from hemicellulase treated samples being the most effective. Enzymatic pre-treatment could be useful for enhancing yield and antimicrobial activity of the essential oils, and hold a good potential for use in food and pharmaceutical industries.

1. Introduction

The Lamiaceae family is one of the most employed as a worldwide source of spices and a consolidated source of functional ingredients (Sacchetti et al., 2004). Within this family, the genus *Thymus* and *Rosmarinus* have received particular attention due to their aromatic composition and their food-related biological properties. In fact, many species of these genera namely *Thymus capitatus* and *Rosmarinus officinalis* are commonly used as a condiment to season and to improve the organoleptic of food, and as a source of antioxidant compounds for food conservation (Celiktas et al., 2007). In traditional medicine, they are used as antiseptic, astringent, spasmolytic, expectorant and antitussive (De Lisi et al., 2011). They are also recognized for their antioxidant, antibacterial, anti-inflammatory, antifungal, anticancer, and antiviral properties (Gachkar et al., 2007; Chizzola et al., 2008; Okoh et al., 2010). Health-promoting properties of these species have been ascribed to their inherent secondary metabolites namely volatile and phenolic compounds (Jordán et al., 2013). At this point, extracts from rosemary (rich in phenolic acids such as rosmarinic and carnosic acid) and thyme (rich in phenolic acids and flavonoids such as cafeic acid, syringic acid, genistic acid and luteolin) with strong antioxidant activities have been proposed to be used as preservation for certain foods and pharmaceutical products (Chizzola et al., 2008). The antioxidant and antibacterial activities of the basic components of the essential oils of thyme (carvacrol and thymol) and rosemary (1,8-cineole, α-pinene and camphor) have been demonstrated (Jordán et al., 2013). Due to their intriguing biological activities, essential oils of thyme and rosemary are widely applied in the cosmetic industry producing various Cologne waters, bathing essences, hair lotions and shampoos and as a component of

* Corresponding author. Tel.: +216 71537666; fax: +216 71537888.
E-mail addresses: Karim_hosni1972@yahoo.fr, karim.hosni@inrap.rnrt.tn (K. Hosni).

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disinfectant and insecticides (Bousbia et al., 2009). As a result, the market demand for these valuable products has increased remarkably. This has driven researcher and manufacturers to search for new methods of extraction in order to improve the yield without alteration of the qualitative traits of the product extracted. Essential oils were usually extracted by steam distillation, traditional hydrodistillation and organic solvent extraction (Okoh et al., 2010). Recently, a lot of alternative techniques such solvent-free microwave extraction technique, supercritical fluid extraction and microwave-assisted solvent extraction have been successfully applied for the extraction of the essential oil from rosemary leaves (Bousbia et al., 2009; Okoh et al., 2010). Another alternative approach to favour essential oil release is the partial or complete hydrolysis of the cell walls by means of appropriate enzymes (Bhat, 2000). The use of enzymes for flavour extraction from a few spices such as garlic, celery, cumin, fenugreek, pepper, mustard, chilli and citrus peel has been reported (Sowbhagya et al., 2009, 2010, 2011). These authors have showed that the pre-treatment of garlic, celery seeds and cumin results in significant increase in the yields of the essential oil and their main components (Sowbhagya et al., 2011). Eight years earlier, Shamala et al. (2003) have demonstrated that the application of enzymes to ginger and garlic improved the essential oil yields to an extent of 50%. In a recent report, Jiao et al. (2012) have reported that the application of enzymes for the extraction of essential oils from *Fructus forsythia* resulted in significant increase in the oil yield. Despite that enzyme-assisted extraction procedure has been shown to achieve high extraction yields for essential oil of the aforementioned species, there are no reports on the application of this procedure for thyme and rosemary. Bearing this in mind, and taking into account the increased market demand of the essential oils from both species, the present contribution aimed at evaluating the effects of the enzymatic pre-treatment of thyme (*T. capitatus* L.) and rosemary (*R. officinalis* L.) leaves on yield, quality and the antimicrobial activities of their essential oils.

2. Material and methods

2.1. Plant material and reagents

Fresh leaves were collected from wild plants of thyme (*T. capitatus* L.) and rosemary (*R. officinalis* L.) in the locality of Mograne (Northeaster Tunisia; latitude 36°25′38″ (N), longitude 10°05′41″ (E), altitude 149 m). The plant material was botanically identified by Dr. Nadia Ben Brahim (Department of botany, National Institute of Agronomic Research, Tunis, Tunisia) and according to the morphological description presented in Tunisian Flora (Pottier-Alapetite, 1981). Leaves were air dried at room temperature (20 ± 2 °C) for one week, and subsequently essayed for their essential oil composition.

Cellulase (8.9 U/mg) and hemicellulase (13.8 U/mg) were purchased from Sigma–Aldrich (Steinheim, Germany). Reference standards including 1,8-cineole, citronellol, bornyl acetate, thymol, carvacrol, β-caryophyllene, 1-nonanol, ethyl butyrate, ethyl-2-phenol, methyl-2-pentane, 2-nonanol, 1-tridecane, β-ionone, α-terpinene, δ-3-carene and 2-methyl-1-butanol were purchased from Sigma–Aldrich (Steinheim, Germany). Limonene, α-pinene, 1-decanol, undecane, 2-ethanone, ethyl laurate, octanal, 2-pentanol, *n*-dodecane, 2-hexanol, 2-decanone and hexanal were obtained from Merck (Schuchardt, Germany). Linalool, β-myrcene, spathulenol, 2-methyl-2-butanone, decanal, nonanal, linalyl acetate, α-bisabolol and ethyl valerate were obtained from Fluka Chemicals (Buchs, Switzerland). Anhydrous sodium sulphate (Na_2SO_4) and n-alkanes (C_7–C_{20}) were obtained from Fluka Chemicals and *n*-pentane of analytical grade was purchased from LabScan (Dublin, Ireland).

2.2. Enzyme pre-treatment

Air dried leaves (100 g) were mixed with 500 mL distilled water containing 10 mg of enzymes (cellulase and hemicellulase) and the mixture cellulase/hemicellulase. The mixtures were stirred for 1 h at 40 °C then subjected to hydrodistillation for essential oil isolation. Control samples were directly subjected to hydrodistillation without any treatment.

2.3. Isolation of essential oils

Essential oil from enzyme treated samples and the control were extracted by hydrodistillation for 2 h using a Clevenger-type apparatus. The oils obtained were recovered, weighed, dried over anhydrous sodium sulphate (Na_2SO_4) and stored in amber and air-tight sealed vials at 0 °C until required.

2.4. Essential oil analyses

Gas chromatography analyses were carried out on a Shimadzu HRGC-2010 gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with flame ionization detector (FID), Auto-injector AOC-20i and auto-sampler AOC-20s. Separation of volatile components was performed using an apolar column Rtx-1 (30 m × 0.25 mm, 0.32 μm film thickness). The oven temperature was held at 50 °C for 10 min and then programmed at 2 °C/min to 190 °C and remained at this temperature for 10 min. The injector and detector temperature were programmed at 230 °C. The flow of the carrier gas (N_2) was 1.2 mL/min and the split ratio was 1:20. Injection volume for all samples was 0.5 μL of diluted oil in *n*-pentane.

Gas chromatography-mass spectrometry (GC–MS) analyses were performed on a gas chromatograph HP 6890 (II) interfaced with an HP 5973 mass spectrometer (Agilent Technologies, Palo Alto, Ca, USA) with electron impact ionization (70 eV). An HP-5MS capillary column (60 m × 0.25 mm, 0.25 μm film thickness) was used. The column temperature was programmed to rise from 40 to 280 °C at a rate of 5 °C/min. The carrier gas was helium with a flow rate of 1.2 mL/min. Scan time and mass range were 1 s and 50–550 *m*/*z*, respectively.

The volatile compounds were identified by comparison of their retention indices relative to (C_7–C_{20}) *n*-alkanes with those of literature (Paolini et al., 2005; Barboni et al., 2009; Hosni et al., 2010) and/or with those of authentic compounds available in our laboratory, and by matching their mass spectral fragmentation patterns with corresponding data (Wiley 275.L library) and other published mass spectra (Adams, 2001) as well as by comparison of their retention indices with data from the Mass Spectral Library "Terpenoids and Related Constituents of Essential oils" (Dr. Detlev Hochmuth, Scientific consulting, Hamburg, Germany) using the MassFinder 3 software (http://www.massfinder.com).

The content of the individual constituents was expressed as a peak area percent obtained from the electronic integration of the FID peak areas without the use of the correction factor.

2.5. Antimicrobial activity

2.5.1. Microbial strains

The antimicrobial activity of thyme and rosemary essential oils at 1:32 and 1:4 dilution in dimethyl sulfoxide (DMSO), was evaluated using 3 Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Enterococcus feacium* (ATCC 19434) and *Staphylococcus agalactiae*. Two Gram-negative bacteria (*Escherichia coli* ATCC 8739 and *Salmonella typhimurium* ATCC 14028) and the yeast *Candida albicans* ATCC 10231 were also used in this bioassay.