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Short communication: Cheese supplemented with *Thymus algeriensis* oil, a potential natural food preservative

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ABSTRACT

The essential oil of *Thymus algeriensis* was analyzed as a potential preservative in soft cheese. We developed a novel method to test the preserving properties of essential oil in soft cheese. Contamination incidence of *Penicillium aurantiogriseum* was absent after 30 d of storage at 4°C with 25 μL of essential oil added. The antimicrobial activity was tested against 8 bacteria and 8 fungi. *Thymus algeriensis* oil showed inhibitory activity against tested bacteria at 0.03 to 0.09 mg/mL, and bactericidal activity was achieved at 0.05 to 0.15 mg/mL. For antifungal activity, minimum inhibitory concentrations ranged between 0.01 and 0.04 mg/mL and minimum fungicidal concentrations between 0.01 and 0.04 mg/mL. Furthermore, the oil was also screened for antiradical activity using the 1,1-diphenyl-2-picrylhydrazyl assay. The results showed that the oil was active and achieved half-maximal inhibitory activity at 0.132 mg/mL. We used gas chromatography, gas chromatography-mass spectrometry, and nuclear magnetic resonance spectrometry to investigate the volatile compounds from the oil. Carvacrol was identified as the main compound in the oil, represented by 80.9% of the total constituents, followed by *p*-cymene (7.7%).
Key words: *Thymus algeriensis*, essential oil, carvacrol, antimicrobial, cheese preservation

Short Communication

Dairy products can be easily contaminated by pathogens or food spoilage microorganisms, which can produce unwanted appearance, decrease commercial value, and, most importantly, compromise the safety of products. Molds on the surface of cheese cause undesirable flavors and many quality problems. The most frequently isolated mold genus from cheese is *Penicillium* spp., which may produce mycotoxins such as ochratoxin A and citrinin (Torkar and Vengust, 2008). Some studies have shown that natural compounds may protect cheese from food contaminant microorganisms (Smith-Palmer et al., 2001; Lucera et al., 2012); Gammariello et al. (2010) reported that chitosan and lemon extract improve shelf life of Mediterranean fresh cheese.

Plant products and their derived compounds are of growing interest due to the wide range of bioactivities that make them useful as natural additives in different types of food. For centuries, they have been used for treatment of infections and diseases worldwide (Rios et al., 1988; Skrinjar and Nemet, 2009; Gottardi et al., 2016). In recent years, utilization of plant products [e.g., essential oils (**EO**), extracts, and their secondary metabolites] has gained considerable interest for the food industry as an alternative to synthetic antimicrobials or additives (Bukvicki et al., 2014a,b, 2015; Tyagi et al., 2014).

The majority of the thyme species are widely used as medicinal herbs (Oussalah et al., 2007) and known to have biological activities, such as antispasmodic (Babaei et al., 2008), sedative (Fachini-Queiroz et al., 2012), antioxidant (Lee et al., 2005), and antimicrobial (Oussalah et al., 2007; Hazzit et al., 2009) activities. The focus of our study was potential application of *Thymus algeriensis* EO in soft cheese, and we aimed to investigate the effect of the oil as preservative in this dairy product. For such purposes, we developed a novel method to test antimicrobial food-preserving properties of substances in soft cheese. Furthermore, chemical characterization of the EO and its antiradical attributes and antimicrobial activities were investigated.

Thyme plants were collected from Gharyan city (Al Gabel Al-Garbe, Libya) in April 2012. The plant was identified by A. Giweli and then confirmed by P. D.

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Marin. One hundred grams of air-dried aerial parts of plant samples, with woody parts removed, were submitted to hydrodistillation (Bukvicki et al., 2014a).

The volatile composition of the *T. algeriensis* EO was determined according to a previously published procedure (Bukvicki et al., 2016). The components were identified by comparison of mass spectra with reference from commercial libraries (Wiley 7, NIST 11, and Adams 4; McLafferty, 2005; Adams, 2007; NIST, 2011). Structural determination of each component was confirmed by comparison of retention indices (**RI**) obtained by the standard method (Vandendool and Kratz, 1963) with the Adams 4 RI library. Quantitative ^{1}H nuclear magnetic resonance (**NMR**) analysis of the *T. algeriensis* EO was performed by using a Bruker Avance III 500 NMR spectrometer (500 MHz, Bruker, Rheinstetten, Germany) in deuterated chloroform ($CDCl_3$), with tetramethylsilane as a reference and 2,6-bis(1,1-dimethylethyl)-4-methylphenol (butylated hydroxytoluene, **BHT**) as an internal standard. Essential oil (5.260 mg) and BHT (5.050 mg) were dissolved in 500 μL of $CDCl_3$ in a 5-mm NMR tube. Proton NMR spectra were run with a standard pulse sequence with 16 scans, and with 3 different relaxation delays (d1) times of 1, 4, and 10 s. The BHT was selected because its ^{1}H NMR signals (aromatic proton) are clearly distinguishable from those of EO components (Figure 1). Peak area of characteristic protons used for quantitative calculation was manually integrated. Signal of 1 proton of carvacrol [chemical shift (**δ**) 7.03 doublet, coupling constant (J) = 7.7 Hz], 2 signals of 4 protons of *p*-cymene (δ 7.07–7.15 multiplets), and signal of 2 protons of BHT (δ 6.98 singlet) were used for relative percentage calculation, based on the equation

$$Px(\%) = \frac{Mx}{Ms} \times \frac{m_s}{m_{E0}} \times \frac{Ix}{Is} \times \frac{n_s}{n_x} \times 100,$$

where Mx and Ms are the molar masses of the analyte and the internal standard (BHT); m_{E0} and m_S are the weighed mass of the essential oil of *T. algeriensis* and BHT; Ix and Is are the peak areas of the analyte and BHT; and n_x and n_s are the numbers of the protons generating the corresponding peak of the analyte and BHT, respectively. The 1,1-diphenyl-2-picrylhydrazyl (**DPPH**) assay was done according to a previously published procedure (Dzamić et al., 2015), as were the assays for antibacterial and antifungal activity (Bukvicki et al., 2016).

Soft cheese used in our experiment was obtained from a market located in Belgrade (Serbia). Soft cheese (domaći mladi sir, without preservatives; Président/Group Lactalis, Laval, France), with nutritional values of 5.5% milk fat, 4% carbohydrates, and 12.5% protein, was stored at 4°C for until further use. Cheese was sliced into equal cuboid shapes, with an approximate weight per slice of 20 g, and placed in sterile petri dishes. For the in situ antifungal assay, we developed a convenient assay for all investigations. Slices of soft cheese were pierced (5 mm deep and 5 mm wide), creating 3 wells per slice. Each treatment had 2 replicates with 5 slices of cheese per replicate. Essential oil of *T. algeriensis* was dissolved in different concentrations of sterile 0.2% Tween 80 (vol/vol). Essential oils were applied at 1, 5, 10, 15, 20, and 25 μL, and then 25 μL of EO emulsion was placed into each well made in cheese. After 1 h, 25 μL of a conidial suspension (2×10^4 conidia/mL) of *Penicillium aurantiogriseum* was added to each well in sliced cheese. As a positive control of fungal growth, *P. aurantiogriseum* was inoculated on cheese without EO. The treated slices of cheese were stored at 4°C for 30 d, respectively. After storage, the number of wells that showed contamination symptoms (fungal growth) was recorded, and contamination incidence (%) was calculated as

$$\text{Contamination incidence} = (\text{number of infected wells}/\text{total wells per replicate}) \times 100.$$

To evaluate possible changes in color, texture, and flavor, cheese was sprayed with EO at levels of 1, 5, and 15 μL per surface of sliced cheese [diluted EO at 1, 5, and 15 μL in 100 μL of 0.2% Tween 80 (vol/vol) in water], with 1 control sample not sprayed with EO. Test was made in initial shelf-life, third day after production. Results were expressed as average grades given by 20 panelists (Bukvicki et al., 2014a).

Based on GC and GC-MS analysis, 21 compounds were identified, representing 99.7% of the total oil. Carvacrol was the main compound in the oil (80.9% of total oil), followed by *p*-cymene (7.7%). In addition, 2 components of analyzed EO were identified and quantified by ^{1}H NMR (Figure 1). Relative mass percentage of carvacrol was 72.8 and *p*-cymene 7.6; moreover, substitutional isomers of cymene (*o*-, *m*-, *p*-) have similar mass spectra, and RI, GC, and GC-MS analysis could be time consuming. Otherwise, differences among proton NMR spectra of isomers of cymene provide clear identification. The NMR analysis could be the method for quality control of chemical composition of EO with few dominant compounds.

Thymus algeriensis EO, its main component carvacrol, and butylated hydroxyl anisole had good antiradical potential as investigated by the DPPH assay (results in Figure 2). Our investigations showed that *T. algeriensis* EO exhibited notable free radical scaveng-