

**Antibacterial and antioxidant activities of essential oils isolated from *Origanum*
spp and *Calamintha baetica***

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ABSTRACT

Aims: *Staphylococcus aureus* and *Bacillus cereus* are two Gram positive foodborne pathogens and the use of essential oils isolated from aromatic plants can constitute a natural and equally effective to other chemicals agents in the control of these pathogens avoiding the development of antibacterial resistance. The essential oils can also constitute a good alternative to the synthetic antioxidants, generally used in food industry, but without the adverse effects described for this kind of compounds.

Methods: The oils were isolated by hydrodistillation and analysed by gas chromatography and gas chromatography coupled to mass spectra. The antibacterial activity of essential oils of *Origanum* spp and *Calamintha baetica* was tested against four strains of *St. aureus* and three strains of *Bacillus cereus* using the disc agar diffusion technique. Chloramphenicol and water were used as positive and negative control, respectively. The antioxidant activity was evaluated by two different spectrophotometric methods. **Results:** The main components of *Origanum* spp. and *C. baetica* were γ -terpinene and isopulegone, respectively. The *Calamintha* essential oil did not demonstrate to have activity against either *St. aureus* or *B. cereus* tested strains. Oregano essential oil demonstrated to have an inferior activity ($P < 0.05$) in comparison to the antibiotic activity. It was found amongst the strains of *B. cereus* strain the most resistant strain either to the essential oils tested and the antibiotic and the most susceptible strain to the same agents. The antioxidant activity of the essential oil of oregano, by both methods showed higher results in comparison to the oil of *C. baetica* for preventing lipid oxidation. The antioxidant ability of oregano oil was practically similar to that of BHT, a synthetic antioxidant. **Conclusions:** The use of *Origanum* spp essential oil in the control of the foodborne pathogens *St.aureus* and *B. cereus* as well as in the prevention of lipid oxidation constitutes an alternative method in food industry.

INTRODUCTION

Besides the extraordinary development on food technology food safety actually did not experiment less attention, by the contrary is actually an issue with increasing importance around the world. Food borne diseases is part of the concern as a significant part of the population get sick each year due to consume of contaminated food. The most affected are the young and the elderly or the immunocompromised individuals (CDC, 2004). *Staphylococcus aureus* and *Bacillus cereus* are two important foodborne pathogens. The food implicated in most outbreaks of staphylococcal food poisoning are firstly poorly cooked or extensively handled meats, namely poultry and cured meat products such as ham and secondly dairy products and pastries (Mossel et al., 1995). Two main types of food poisoning caused by the consumption of food with the *B. cereus* have been recognized. The first type the symptoms mimic those of *Clostridium perfringens* characterized by the onset of watery diarrhoea, abdominal cramps and pain but vomiting is usually absent. The second category by contrast is accompanied by vomiting and for this is designated by emetic type. The consumption of boiled or fried rice that has been allowed to stand at inappropriate temperatures after preparation has been associated to this type (Mossel et al., 1995). Moreover, it was recently reported that *B. cereus* is a common isolate in the environment but emetic strains are rare what reinforces the need of clarification of the survival and tolerance mechanisms of this food pathogen (Altayar and Sutherland, 2006).

In nowadays the development of antibiotic resistance among microorganisms is a public health concern and this drives the search for new and more safely antimicrobial agents. The essential oils can constitute a powerful tool to reduce the development and dissemination of antimicrobial resistance. Oregano is an aromatic plant extensively applied on Mediterranean type of food and is spread throughout southern Portugal. The potential of this plant as a therapeutic agent only recently became accepted. The antimicrobial activity has been subject of a vast number of studies (Faleiro et al., 2005; Lin et al., 2004; Baydar et al., 2004; Şahin et al. 2004; Burt and Reinders, 2003; Lambert et al., 2001; Skandamis et al., 2000; Sivropoulou et al. 1996). Another interesting plant that has been subjected to several studies exploring biological activities is the plant that belongs to the genus *Calamintha* Mill. (*Labiatae*). In the Iberian Peninsula can be found several species of this plant including *Calamintha baetica* that is spread along the Portuguese territory (Morales and Luque, 1997) and is well known as a spice specially used in olives preparation (vulgarly known as olive herb) but their

therapeutic properties are also recognized in folk medicine being used in the treatment of respiratory diseases. The antimicrobial activity of the essential oils of several species of the genus *Calamintha* is also explored in several studies (Couladis et al. 2003; Nostro et al. 2004; Flamini et al. 1999).

Antioxidant capacity of essential oils is other possibility to use them as natural agents for food preservation, replacing the synthetic antioxidants. This possibility will be interesting mainly in those specific sectors of industry of food preservation when antioxidant and aroma properties are simultaneously desired.

Our study was conducted in order to explore the antibacterial and antioxidant properties of essential oils of *Origanum* spp and *Calamintha baetica*. The antioxidant activity of these oils was evaluated by two different methods one of them measured the formation of secondary (malonaldehyde) components of the oxidative process of a lipid matrix and the other one measured the free radical scavenging activity (DPPH method). The antibacterial activity was determined against several strains of *St. aureus* and *B. cereus* using agar diffusion technique.

MATERIAL AND METHODS

Plant material

Plants grew up in the experimental field of “Direcção Regional de Agricultura do Algarve” (DRAALG), all of them in three different blocks with 25 plants each, in order to get a representative sample. All these plants are endogenous of Algarve. After flowering, plants were cut and dried in a solar drier in the same conditions.

Isolation of essential oils

The oils were isolated from dried material by hydrodistillation, for 4 hours, using a Clevenger-type apparatus.

Chemical analysis of essential oils

Solid phase micro extraction procedure. 20 µl of oils obtained by hydrodistillation were mixed with 1000 µl of pentane into a 20 ml vial. A 65 µm PDMS-DVB (polydimethylsiloxane – divinylbenzene) coated fibre was exposed 20 min in the headspace at laboratory temperature (20 ± 2 °C), after the fibre was withdrawn into the needle and transferred to the injector of the GC-MS, where the analytes were thermally desorbed from the fibre during 5 min. Similar proceedings was done with GC. Also 1 µl of the mixture was direct injection in GC.

GC-MS analysis. A Shimadzu 17-A chromatograph equipped with Shimadzu QP-5000 mass spectrometer was used. The separation was achieved using a J&W Scientific DB-1701P column of 30 m x 0,25 mm i. d. and 0,25 µm of film thickness. GC oven temperature was programmed from 40 °C (5 min), to 230 °C at a rate of 5 °C/min and then 5 min at 230 °C. The carrier gas was helium with a column-head pressure of $1,4 \times 10^5$ Pa. Mass spectra were recorded in the electron impact (EI) mode at 70 eV, scanning the m/z 30 to 300. Interface temperature was 250 °C. Data acquisition and data processing were carried using Class5K programme. Peaks in TIC (total ion current) or MIC (Multi Ion Chromatogram) profiles for both analyses were characterized or tentatively identified from their mass spectral data using National Institute of Standards and Technology (NIST12 or NIST62) and Wiley 229 mass spectrometry libraries. Identification was confirmed using standard compounds when available.

Bacterial strains and antibacterial activity

Four strains of *Staphylococcus aureus* (CFSA1; CFSA2; CFSA3; CFSA4) and three strains of *Bacillus cereus* (C1010; C1060; C1062) were used. Strains were kept at

-80°C and maintained in BHI agar (Brain Heart Infusion) at 4°C during the study. The cultures were recovered from freezing by growth in liquid BHI and followed by growth on solid media for 24-48h at 37°C prior to the assay. From this plate a loop was used to inoculate 10 ml of liquid BHI for about 2 hours until middle exponential phase was reached. The antibacterial activity was determined by agar diffusion method, and 0.1 ml of the previously achieved exponential culture was used to inoculate BHI agar plates. Sterile filter paper disks of 6 mm (Oxoid, UK) were distributed on the agar surface. The essential oil was eluted in 2-propanol in a proportion of 1:5. In each disk were distributed 4 µl of the essential oil solution. Sterile water and the antibiotic chloramphenicol (30µg/disk) were used as control. Inhibition zones were determined after an incubation period of 24-48h at 37°C.

Antioxidant activity measurement

From each sample, different concentrations of essential oils were prepared in methanol: 100, 250, 500, 750 and 1000mg/L. The antioxidant activity of essential oils was carried out using two different methods: free radical scavenging activity using DPPH and by the TBARS assay.

Free radical scavenging activity. The free radical scavenging activity of oils were measured by 2,2-diphenyl-2-picryl-hydrazil (DPPH). A solution of DPPH in methanol (24µg/ml) was prepared and 2ml of this solution was added to 50µl of extracts solution in methanol at different concentrations (100, 250, 500, 750 and 1000mg/L). The absorbance was measured at 517nm in a spectrophotometer Shimadzu 160-UV. The DPPH radical concentration was calculated using the following equation: Scavenging effect % = $[(A_0 - A_1) / A_0] * 100$ where A_0 was the absorbance of the control sample (without essential oil) and A_1 was the absorbance in the presence of the sample.

TBARS assay. A modified thiobarbituric acid-reactive substances (TBARS) assay (Wong *et al.* 1995) was also used to measure the potential antioxidant capacity of essential oils. Egg yolk homogenate was used as lipid-rich media, an aliquot of yolk material was made up to a concentration of 10% (w/v) in KCl (1.15%, w/v). The yolk was then homogenized for 30s, followed by ultrasonication for a further 5 min. 500µl of 10% (w/v) homogenate and 100µl of sample, solubilized in methanol, were added to a test tube and made up to 1 ml with distilled water, followed by addition of 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% (w/v) 2-thiobarbituric acid (TBA) in 1.1% (w/v) sodium dodecyl sulphate (SDS). Each essential oil and tested substance was

assayed at the concentrations of 100, 250, 500, 750 and 1000 mg/L. This mixture was stirred in a vortex, and heated at 95°C for 1 h. After cooling, at room temperature, 5 ml butan-1-ol was added to each tube, stirred and centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer Shimadzu 160-UV. All the values were expressed as antioxidant index (AI%), calculated by the formula: Antioxidant index % = $[(A_0 - A_1)/A_0] * 100$ where A_0 being the absorbance value of the fully oxidized control and A_1 , the absorbance of the test sample.

RESULTS AND DISCUSSION

Chemical analysis

Figures 1 and 2 represent the chromatograms of essential oils of *Origanum* spp. and *C. baetica*, respectively. The main components of oregano oil were γ -terpinene and carvacrol, while the oil isolated from *C. baetica* was dominated by 1,8-cineole and isopulegone.

Antibacterial activity

The inhibitory activity of the essential oils of *Origanum* spp and *C. baetica* are summarized in table 1. The most efficient essential oil against the tested bacteria was the oil of *Origanum* spp. However is noteworthy that the most resistant strain of *B. cereus* C1060 to the essential of *C. baetica* was also the most resistant to *Origanum* essential oil. The most susceptible bacteria was *B. cereus* C1062 followed by the strain of *St. aureus* CFSA4 (Table 1). Besides the higher antibacterial activity of *Origanum* spp essential oil in comparison to *C. baetica* oil the obtained inhibition zones were inferior to the antibiotic (Table 1). Our previous data indicate that essential oil of *Origanum vulgare* L. had an activity against the food borne pathogen *Listeria monocytogenes* similar to the antibiotic (Faleiro et al. 2005). The discrepancy of results certainly is related with the different bacteria tested. Nevertheless the inhibitory activity of the *Origanum* spp essential oil against these two important gram-positive food borne pathogens is very promising. To our knowledge there is no data on the inhibitory activity of *C. baetica*. However there are studies either on composition or antimicrobial activity of *C. nepeta* (Baldovini et al. 2000; Flamini et al. 1999). It was reported by Flamini et al. (1999) an inhibitory activity against *B. cereus* slightly superior (11.00 ± 0.58 mm) to the recorded in our study. This divergence may be associated either to differences in the tested strains and in the oil composition. The main components of

C. nepeta are pulegone and menthol (Baldovini et al. 2000; Flamini et al. 1999) whereas the essential oils of *C. baetica* analysed in our study is rich on isopulegone and 1,8-cineole.

Antioxidant activity

Figures 3 and 4 picture the antioxidant activity of the oregano and *C. baetica* oils as well as the synthetic antioxidant, generally used in food industry (BHT and BHA), evaluated by two different methods. From Figure 3 it is possible to conclude that the essential oil isolated from *C. baetica* is not able to give a proton to the stable radical DPPH[•] that is the principle of this simple method for the determination of antioxidant activity. For oregano oil the antioxidant capacity was dependent on the concentration tested. For high concentrations (1000 mg/L) of oregano oil the antioxidant activity reached 73 %, while for 100 mg/L the percentage was only 16 %. The same were not observed for *C. baetica*, whose percentages of antioxidant activity ranged from 5 % to 7 % from the lowest to the highest concentrations, respectively. The best percentages of antioxidant activities were observed for synthetic antioxidants BHA and BHT, generally used in food industry, more evident for low concentrations. In both cases at 1000 mg/L, the percentages of antioxidant activities reached 100 %. If *C. baetica* oil was deprived of antioxidant ability when evaluated by the DPPH method, using the TBARS method (Figure 4), the same oil possessed some antioxidant activity reaching 58 % at 1000 mg/L. In TBARS method the capacity of preventing lipid oxidation of oregano was also superior when compared to the DPPH method. Such results may be explained for the multistep of lipid oxidation process. In this way, both essential oils were more effective against the lipid degradation (secondary oxidative process) than as free radical scavengers (primary oxidative process). Such results can be explained by the different chemical composition of the oils. The highest percentage of a phenolic compound present in the oregano oil (carvacrol) can be responsible for the highest ability to scavenger free radicals such as H[•], measured by DPPH method. The presence of available hydrogen atoms from phenol represents a good barrier against the primary oxidative process.

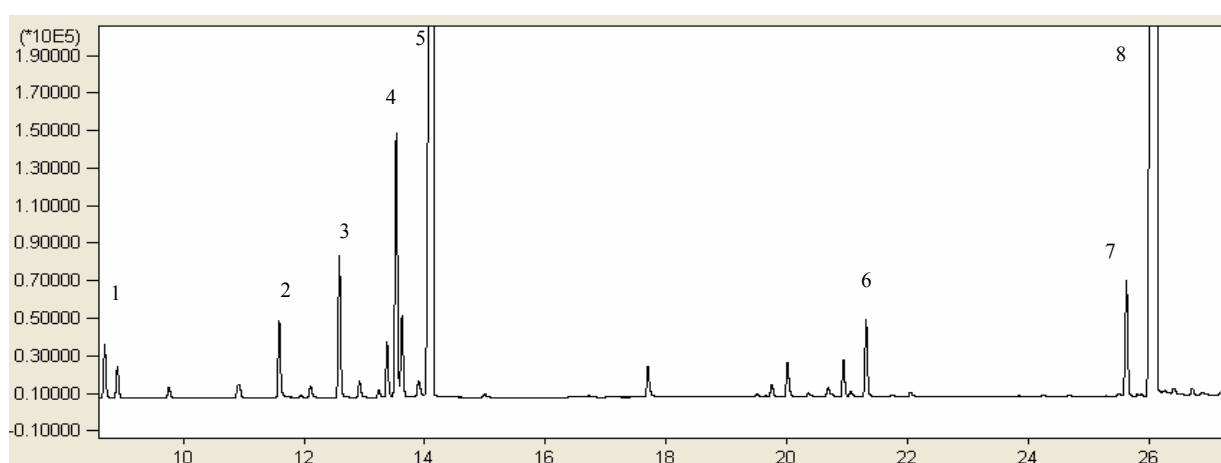
Acknowledgements

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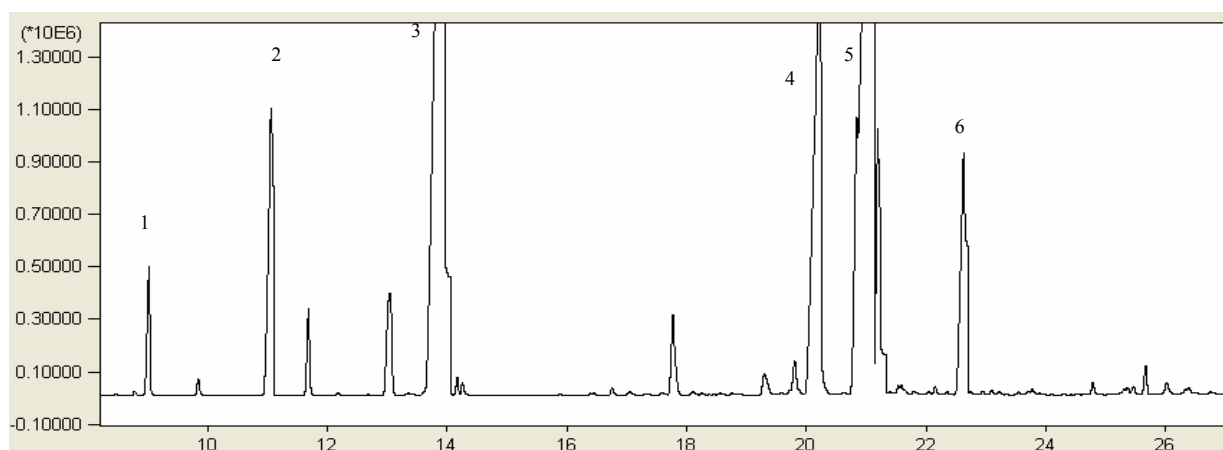
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1 – α -Thujene; 2 – β – myrcene; 3 – α - terpinene; 4 – ocimene; 5 – γ -terpinene; 6 – thymyl methyl ether; 7 – *trans* caryophyllene; 8 – carvacrol

Figure 1. Main components of the essential oils of *Origanum* spp.



1- α -Pinene; 2 – β pineno + sabineno; 3 – 1-8 cineole; 4 – isopulegol; 5 – isopulegone; 6 – pulegone

Figure 2. Main components of the essential oils of *C. baetica*.

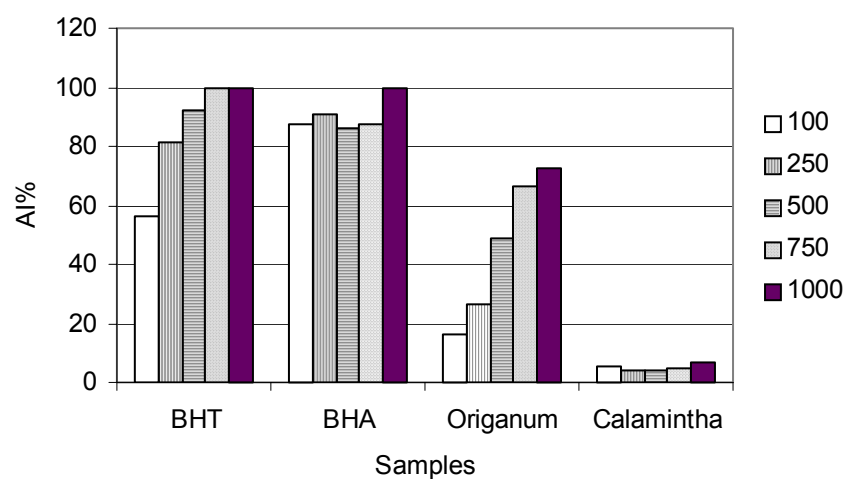


Figure 3. DPPH scavenging activity of different concentrations of the essential oils of *Origanum spp.* and *C. baetica* and the synthetic antioxidants BHT and BHA.

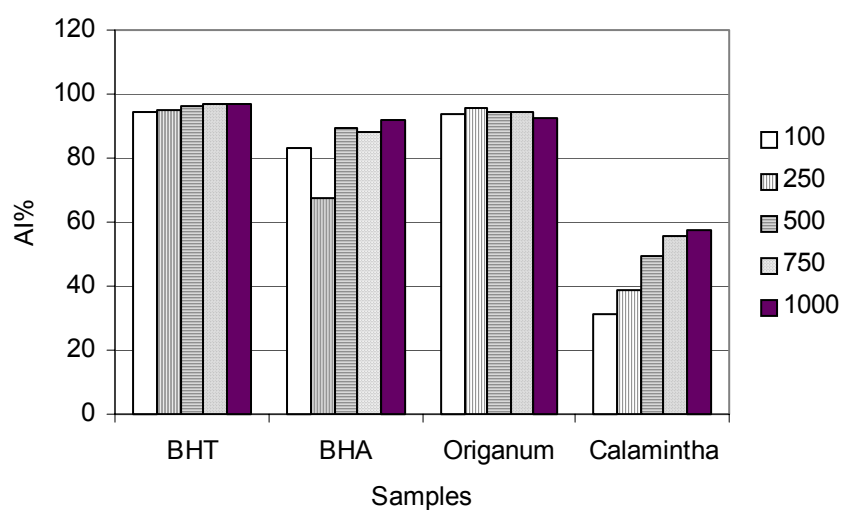


Figure 4. Antioxidant activity of different concentrations of the essential oils of *Origanum spp.* and *C. baetica* and the synthetic antioxidants BHT and BHA, measured by the TBARS method.

Table 1. Inhibitory activity of *Origanum* spp and *Calamintha baetica* essential oils. The zone of inhibition is expressed in mm±SD (n=3).

Bacteria	Essential oil	
	<i>Calamintha baetica</i>	<i>Origanum spp</i>
CFSA1	5.50±1.06	11.25±3.25
CFSA2	8.75±0.35	16.50±4.8
CFSA3	7.67±0.58	13.33±2.08
CFSA4	9.33±0.58	18.33±2.88
<i>B. cereus</i> C1010	8.67±0.56	12.33±1.52
<i>B. cereus</i> C1060	*	8.67±0.57
<i>B. cereus</i> C1062	8.67±0.58	22.00±2.64

*-no inhibition zone