

Preservative properties of *Calamintha officinalis* essential oil with and without EDTA

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2002/2: received 3 January 2002, revised 23 July 2002 and accepted 25 July 2002

A. NOSTRO, M.A. CANNATELLI, I. MORELLI, P.L. CIONI, A. BADER A. MARINO AND V. ALONZO. 2002.

Aims: This study was focused on the preserving properties of *Calamintha officinalis* essential oil, a plant known for its diaphoretic, expectorant and aromatic properties.

Methods and Results: The commercial aerial parts of *C. officinalis* Moench were hydrodistilled and the essential oil analysed by Gas chromatography/Electron impact mass spectrometry (GC/EIMS). The inhibition efficacy of this essence, alone (0.5 and 1.0% v/v) and in combination with 2.0 mM EDTA, was assayed, in culture medium and in cetomacrogol cream, using preservative efficacy testing against standard microorganisms (*E. coli* ATCC 25922, *Ps. aeruginosa* ATCC 9027, *Staph. aureus* ATCC 6538P, *C. albicans* ATCC 10231 and *A. niger* ATCC 16404). *C. officinalis* essential oil in cetomacrogol cream with EDTA showed long-lasting antimicrobial activity, satisfying the European Pharmacopoeia Commission (E. P.) criteria.

Conclusion: *C. officinalis* essential oil could have a potential for a future use as a cosmetic preservative.

Impact of the Study: To find natural compounds with antimicrobial activity which could be alternatives to the synthetic chemical preservatives.

INTRODUCTION

The microbiological safety of a pharmaceutical and cosmetic product has always been of special interest for industries since a microbial contamination can be a risk both for the health of the consumer and for stability of the preparation. Microbial insults from manufacture can be controlled by careful attention to sanitary processing and adequate preservatives. Extracts of plants and essential oils known for their flavouring, fragrance, and antimicrobial activities (Hammer *et al.* 1999; Dorman and Deans 2000) have recently been proposed as alternatives to the synthetic preserving agents used for cosmetic products (Kabara 1984). Johnson *et al.* (2000) demonstrated that a natural extract containing salicylic acid has an efficient preserving activity. *Thymus vulgaris* essential oil (3% v/v) exhibited an interesting preservative activity against bacteria and *C. albicans* in topically applied formulations (Manou *et al.* 1998).

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In our studies on the antimicrobial activity of natural products, we have evaluated the inhibiting efficacy of the essential oil of *Calamintha officinalis* Moench (Lamiaceae), a plant which grows by waysides and in hedges, commonly found especially in dry places and used for its diaphoretic, expectorant and flavouring properties Grieve (1967). The aim of this work was to evaluate the preservative properties of *C. officinalis* essential oil, both in culture medium and in cetomacrogol cream, alone and in association with ethylenediaminetetraacetic acid (EDTA), using preservative efficacy testing against standard microorganisms chosen to represent potential contaminants during manufacture or use of topical preparations.

MATERIALS AND METHODS

Plant material and GC/EIMS

The aerial parts of *Calamintha officinalis* Moench obtained from a commercial source (A. Minardi & Figli, Ravenna, Italy), were subjected to hydrodistillation for 2 h using a

modified Clevenger type apparatus and the essential oil was collected and stored at 4°C.

The gas-chromatographic (GC) and gas chromatography/electron impact mass spectrometry (GC/EIMS) analyses of the essence were performed with a Varian CP-3800 gas-chromatograph (Varian, Milan, Italy) equipped with a DB-5 capillary column (30 m × 0.25 mm; coating thickness 0.25 µm) and a Varian Saturn 2000 ion trap mass detector (Varian, Milan, Italy); oven temperature programmed from 60°C to 240°C at 3°C min⁻¹; carrier gas helium at 1.0 ml min⁻¹; injection of 0.2 µl (10% hexane solution); split ratio 1 : 30. Identification of the constituents was based on comparison of the retention times and retention indexes with those of authentic samples and on computer matching against commercial (NIST 98; NIST, Gaithersburg, MD, USA, and ADAMS; Allured Publ. Co., Carol Stream, IL, USA) and home-made library mass spectra built up from pure substances and components of known oils, and mass spectrometry (MS) literature data (Massada 1976). Moreover, all the molecular weights of the identified substances were confirmed by gas chromatography/chemical ionization mass spectrometry (GC/CIMS) using MeOH as CI reagent gas, operating in the same conditions for GC/EIMS.

Organisms and media

The following organisms were used in this study: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 6538P), *Candida albicans* (ATCC 10231) and *Aspergillus niger* (ATCC 16404).

Bacteria were cultured on Tryptone Soya Agar (TSA; Oxoid, Milan, Italy) for 24 h at 37°C. *Candida albicans* and *A. niger* were grown on Sabouraud Dextrose Agar (SDA; Oxoid) at 25°C for, respectively, 48 h and 5 d. Cells were harvested into 0.1% peptone water by gentle agitation. The peptone water used for harvesting *A. niger* contained 0.05% (v/v) of Tween 80 (Sigma-Aldrich, Milan, Italy).

Antimicrobial tests

Disc diffusion test. The cell suspensions, adjusted to yield approximately 1.0×10^8 cfu ml⁻¹ for bacteria and 1.0×10^8 spores ml⁻¹ for fungi, were streaked with a calibrated loop on plates containing an appropriate solid medium. Filter paper discs (6.0 mm diameter; Oxoid Ltd) were placed on the inoculated agar surfaces and impregnated with 10 µl of essential oil and carvone pure substance (Sigma-Aldrich). The plates were observed for up to 48 h at 37°C for bacteria and up to 5 d at 25°C for fungi. All tests were performed in duplicate and the antimicrobial activity was expressed as mean inhibition diameters (mm) produced by the essential oil.

Antimicrobial activity on culture medium. Preliminary studies on the inhibiting efficacy of *C. officinalis* essential oil alone (0.5 or 1.0% v/v) and in combination with 2.0 mM EDTA (Sigma-Aldrich) were conducted on 20 ml of Tryptic Soya Broth (TSB; Oxoid) and Sabouraud Liquid Medium (SLM; Oxoid). The standardized microbial suspensions were inoculated in media in order to obtain a final concentration of 10^6 cfu ml⁻¹. The incubation was performed at 37°C for bacteria and at 25°C for fungi. After a contact time of 0, 2.0, 7.0, 14, and 28 d, the samples (1.0 ml) were removed and placed into 9.0 ml of neutralizing medium Leethen broth (Difco, distributed by Becton Dickinson, Milan, Italy). Cell viability was determined by the plate count method in TSA or SDA plates and cfu were counted after a 5-d incubation at 37°C and 30°C for bacteria and fungi, respectively. All determinations were performed in duplicate and two growth controls with the medium alone and with EDTA were included.

Microbial challenge test

Preparation of cetomacrogol cream. Cetomacrogol cream BP 1988 was prepared by slowly adding 30 g of warm emulsifying ointment to 70 ml of warm water. The mixture was stirred continually until it set. The EDTA (2 mM) and the *C. officinalis* essential oil (0.5% or 1.0% v/w) were added prior to setting, then the cream was left at room temperature for 24 h.

Challenge test. The microbial challenge test was performed following the standards proposed in the latest draft of the European Pharmacopoeia Commission (E.P.) (1996) concerning topical preparations. Since in the preliminary studies only the combination essential oil-EDTA showed preserving activity on all five strains, the challenge test was performed on two formulations: cetomacrogol + 0.5% essential oil + EDTA cream and cetomacrogol + 1.0% essential oil + EDTA cream. These formulations (samples of 20 g) were placed in sterile containers and separately inoculated with bacterial and fungal suspensions to give a final level of approximately 10^6 cfu g⁻¹. The preparations were well mixed to ensure a homogeneous microorganism distribution and incubated at 20°C to 25°C. The viability of the inoculated cells and their ability to grow were evaluated by two growth controls consisting of cetomacrogol cream and cetomacrogol-EDTA cream. After a contact time of 0, 2, 7, 14, and 28 d, the samples (1.0 g) were removed and counted according to the method described above. The results were confirmed in three different experiments.

According to guidelines of E.P. (1996) a topical preparation is defined to be effectively preserved if the number of the bacteria recovered per gram is reduced by a factor of 10^3 (criteria A) and 10^2 (criteria B) within 2 d of challenge with

no subsequent evident cell proliferation at the 7th and up to the 28th day. As for fungi, the number recovered per gram must be reduced by a factor of 10^2 (criteria A) and 10^1 (criteria B) within 14 days of challenge.

RESULTS

The main components of *C. officinalis* essential oil identified by GC/EIMS analysis are listed in Table 1; this essence has a high content of carvone and pulegone.

The results of the disc diffusion test are reported in Table 2. The essential oil and carvone are active against *E. coli*, *Staph. aureus*, *C. albicans*, and *A. niger* but are inactive against *Ps. aeruginosa*.

Preliminary studies (Table 3) have pointed out that when the culture medium was treated with 1.0% (v/v) and 0.5% (v/v) essential oil with and without EDTA, the number of viable cells of *E. coli* and *Staph. aureus* dropped by 3 log₁₀ unit within 2 d of exposure, after which cell death continued at a much slower rate reaching values ≤ 2 log cfu ml⁻¹ at the 28th day (Table 3). As for fungi, *C. albicans* showed a strong sensitivity and its growth was reduced by almost 5 log₁₀ units after 2 d of incubation with essential oil 1.0% (v/v) and after 7 d with essential oil 0.5% (v/v); *A. niger* showed a similar effect only after 7 d with a dose of 1.0% (v/v) and after 28 d with a dose of 0.5% (v/v). On the contrary, the essence at 1.0% and 0.5% (v/v) did not seem to prevent the growth of *Ps. aeruginosa*, as confirmed by the

increased growth observed during the incubation time. However, the essential oil in combination with 2 mM EDTA reduced the viability of *Ps. aeruginosa* to the same extent as that reported for *E. coli* and *Staph. aureus*.

The results of the challenge test on cetomacrogol + essential oil + EDTA cream are reported in Table 3. Reduction of the inoculum in the 1.0% essential oil-EDTA formulation satisfied criteria A of the E.P. (1996). On the contrary, the 0.5% essential oil-EDTA formulation fully satisfies only criteria B. As regards criteria A, a 0.5% concentration seems to be insufficient for a rapid antimicrobial activity of *Staph. aureus* and *A. niger*, since the reduction of the prefixed logarithmic unit was obtained after 28 d.

DISCUSSION

In the present work, *Calamintha officinalis* essential oil, in culture medium, showed antimicrobial activity against *E. coli*, *Staph. aureus*, *C. albicans*, and *A. niger*; on the contrary, its activity against *Ps. aeruginosa*, an organism intrinsically resistant to a wide variety of antimicrobial agents, has been unsatisfactory, probably due to the impermeability of the outer membrane (OM) to hydrophobic and high molecular weight hydrophilic drugs (Vaara 1992; Russell and Chopra 1996). The good activity against *Ps. aeruginosa* of the association essential oil-EDTA confirms that while the essence itself hardly crosses the OM of this bacterium, its combination with EDTA allows it to reach the inner part of the cells. In fact EDTA chelates the Ca⁺⁺ and Mg⁺⁺ ions that play an important role in the stability of the outer membrane complex (Nikaido and Vaara 1985). EDTA has been also reported to reverse the resistance of Gram negative organisms against some antimicrobial agents, and to enhance the effect of several cosmetic preservatives, such as the parabens, imidazolidinyl urea, and tert-butyl hydroxyanisole (Hart 1984). Besides EDTA is a safe, inexpensive, and effective product and its addition to cosmetic and toiletry formulations maintains clarity, protects the fragrance of the components, and stabilizes the colouring agents (Hart 1984).

The preserving activity of the *C. officinalis* essential oil in cetomacrogol cream was proved to be satisfying, although less than that observed in culture medium. Emulsions and creams, generally, require higher concentrations of preservatives than other preparations. When added to a formulation the preservative distributes itself into the oil and aqueous phases and its antimicrobial activity is largely attributed to its free concentration in the last phase. Although essential oils are often thought highly lipophilic compounds insoluble in the aqueous phases, in effect, in many cases they have a relative hydrophilicity given by the presence of constituents with polar functional groups. Carvone, the major component of *C. officinalis* essential oil has a water solubility of 916 ± 20 p.p.m. and belongs to Group I of compounds

Table 1 Major components of *C. officinalis* essential oil as determined by GC/EIMS

Retention (min)	Compound	% Peak area
6.74	β -pinene	1.0
8.41	limonene	3.3
8.54	1,8-cineol	4.3
14.47	menthol	1.2
16.30	<i>trans</i> -carveol	1.1
16.91	<i>cis</i> -carveol	1.4
17.16	pulegone	10.9
17.49	carvone	64.3

Table 2 Disc diffusion test

Microorganisms	<i>C. officinalis</i> carvone (inhibition diameters, mm)	
	<i>C. officinalis</i>	carvone
<i>E. coli</i> ATCC 25922	14	15
<i>Staph. aureus</i> ATCC 8538	16	18
<i>Ps. aeruginosa</i> ATCC 9027	—*	—*
<i>C. albicans</i> ATCC 10231	33	34
<i>A. niger</i> ATCC 16404	30	30

* Inhibition zone was less than 6 mm.

Table 3 Preservative properties of *C. officinalis* essential oil in culture medium and in cetomacrogol cream

	%	Culture medium (Log cfu ml ⁻¹) Days					Cetomacrogol cream (Log cfu g ⁻¹) Days				
		0	2	7	14	28	0	2	7	14	28
<i>E. coli</i> ATCC 25922											
Essential oil	1	6.0	2.8	2.5	2.4	2.2	ND	ND	ND	ND	ND
	0.5	6.0	3.0	2.8	2.7	2.5	ND	ND	ND	ND	ND
Essential oil + EDTA (2 mM)	1	6.0	2.6	2.4	2.2	1.9	6.3	2.8	2.6	2.5	1.0
	0.5	6.0	2.8	2.6	2.5	2.3	6.3	3.4	3.0	2.8	2.7
Control EDTA		6.0	9.9	9.0	8.9	8.7	6.3	6.5	7.7	8.4	8.4
Control		6.0	10	9.5	9.2	9.0	6.3	9.7	9.2	8.9	8.8
<i>Staph. aureus</i> ATCC 8538											
Essential oil	1	6.0	2.6	2.2	2.0	1.8	ND	ND	ND	ND	ND
	0.5	6.0	3.0	2.4	2.2	2.1	ND	ND	ND	ND	ND
Essential oil + EDTA (2 mM)	1	6.0	2.4	2.4	2.4	2.0	6.4	3.4	3.1	2.6	1.6
	0.5	6.0	2.9	2.5	2.5	2.1	6.4	4.4	4.0	3.7	3.0
Control EDTA		6.0	9.7	9.0	8.9	8.7	6.4	8.1	8.1	8.4	8.0
Control		6.0	9.9	9.4	9.2	8.9	6.4	9.7	8.6	8.8	8.2
<i>Ps. aeruginosa</i> ATCC 9027											
Essential oil	1	6.0	7.8	9.1	8.7	8.6	ND	ND	ND	ND	ND
	0.5	6.0	8.4	9.3	8.9	8.7	ND	ND	ND	ND	ND
Essential oil + EDTA (2 mM)	1	6.0	2.3	2.0	1.9	1.7	6.7	2.5	2.0	1.5	1.0
	0.5	6.0	2.8	2.4	2.1	1.9	6.7	3.0	2.8	2.1	2.0
Control EDTA		6.0	9.3	9.2	9.1	8.8	6.3	6.1	7.6	8.7	8.8
Control		6.0	9.9	9.4	9.2	9.0	6.3	9.7	8.9	8.9	8.8
<i>C. albicans</i> ATCC 10231											
Essential oil	1	6.0	1.0	1.0	1.0	1.0	ND	ND	ND	ND	ND
	0.5	6.0	2.0	1.0	1.0	1.0	ND	ND	ND	ND	ND
Essential oil + EDTA (2 mM)	1	6.0	1.0	1.0	1.0	1.0	6.0	2.5	1.0	1.0	1.0
	0.5	6.0	1.5	1.0	1.0	1.0	6.0	2.7	1.0	1.0	1.0
Control EDTA		6.0	8.0	8.4	8.4	8.1	6.0	6.8	7.1	7.1	7.2
Control		6.0	8.5	8.6	8.6	8.6	6.0	7.5	7.8	7.6	7.6
<i>A. niger</i> ATCC 16404											
Essential oil	1	6.8	1.9	1.0	1.0	1.0	ND	ND	ND	ND	ND
	0.5	6.8	6.0	3.3	2.0	1.0	ND	ND	ND	ND	ND
Essential oil + EDTA (2 mM)	1	6.8	2.0	1.0	1.0	1.0	6.4	4.0	3.5	3.2	2.4
	0.5	6.8	4.6	2.0	2.0	1.0	6.3	5.2	5.1	4.6	3.1
Control EDTA		6.8	6.5	6.2	6.3	6.0	6.3	5.6	5.7	6.0	6.1
Control		6.8	6.7	7.8	7.7	7.3	6.3	5.5	6.2	6.2	6.3

Culture medium: Tryptic Soya Broth for bacteria and Sabouraud Liquid Medium for fungi.
ND, not determined.

able to form a hydrogen bond (Griffin *et al.* 1999). This compound is a constituent of many essential oils; it is known for its antimicrobial activity (Oosterhaven *et al.* 1995; Karatzas *et al.* 2000; Griffin *et al.* 2001), and it is widely used as a flavoring agent, for example for toothpaste and other cosmetics. Carvone seems to be the cause of cheilitis when used at 5% in toothpastes (Francalanci *et al.* 2000) but it is not included in the International Fragrance Research Association (IFRA) list of compounds of established sensitizing potency. It has also excited considerable interest in the pharmaceutical industry as an enhancer for drugs (Fuchs

et al. 1997; Gao and Singh 1997) and has been patented for its use as a skin penetration enhancer (Leonard *et al.* 1989).

In conclusion, *C. officinalis* essential oil in combination with EDTA has evidenced a good preservative activity both in culture medium and in cetomacrogol cream, satisfying also the European Pharmacopoeia Commission (E. P.) criteria.

ACKNOWLEDGEMENTS

We thank Angela Daniela Musolino and Francesca Procopio for their collaboration.

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