Research Article
Volume 7:4, 2021

Journal of Antimicrobial Agents

ISSN: 2472-1212

Open Access

Antibacterial and Antifungal Properties of Essential Oil Blends

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Abstract

Pathogenic microorganisms represent an ever-increasing human health concern. Simultaneously, there is an increased desire for naturally derived, antimicrobial agents for use in consumer products. Essential oils, which are derived from natural plant materials and typically have a long history of use, are sources of alternative, non-antibiotic antimicrobial agents. In particular, oregano (*Origanum vulgare*) oil and its respective constituents have been shown to have antimicrobial properties. In this study, the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of two essential oil formulations containing oregano oil as the main constituent were determined. Results showed that the essential oil formulations effectively inhibited growth of *Escherichia coli* with MBCs of around 2.0-5.7 mg/mL *in vitro*. Additionally, the formulations exhibited MFCs of 2.8-4.0 mg/mL and 1.4-2.0 mg/mL against the fungal species *Candida albicans* and *Candida auris*, respectively. The demonstrated antibacterial and antifungal properties of these naturally derived antimicrobial formulations provide a 'greener' alternative than traditional synthetic chemicals that may be useful in consumer products, such as cleaning agents.

Keywords: Oregano oil • Essential oil • Antibacterial • Antifungal • Antimicrobial

Introduction

Pathogenic microorganisms including *Escherichia coli* (*E. coli*), *Candida albicans* (*C. albicans*), and *Candida auris* (*C. auris*) represent an ever-increasing human health concern. These concerns stem from the demonstrated increase in antibiotic resistance, rising cost of treatments for infections, and propensity to form biofilms [1-3]. This has facilitated the search for alternate and natural sources of antimicrobials that can be used as non-antibiotic alternatives in food and consumer products. Essential oils are one alternative source that have increased in recognition recently for protection of food and other sources of microbial contamination that may pose a risk to humans [4,5]. Their demonstrated antimicrobial effects, along with their low-cost, biocompatibility, low toxicity to humans, and environmentally-friendly properties, make them an ideal choice as a natural alternative to traditional synthetic chemical agents (e.g., bleach) [6]. In addition, naturally derived products are generally considered 'greener' alternatives to traditional chemical agents based on their greater environmental benefits and the reduction of the use of potentially toxic agents [7].

Essential oils are generally defined as mixtures of chemical compounds from the same chemical family, such as terpenoids, that are derived from plant material [8]. Because the chemical composition of essential oils can be affected by various factors, such as species and subspecies, geographical location, harvest time, and extraction method, their antimicrobial activity is generally thought to arise from the synergistic effect of multiple compounds [9]. Oregano (*Origanum vulgare*) oil is an essential oil that has been used in traditional and natural medicine and culinary applications for hundreds of years [10]. Additionally, oregano oil is currently used as food flavoring agent and has been identified as generally recognized as safe (GRAS) for direct human consumption by the United States Food and Drug Administration (US FDA) [11].

The main constituents of oregano oil are the monoterpenoid phenols carvacrol and its closely related constituent thymol [12]. Carvacrol and thymol have been shown to disrupt cell membrane integrity in both Gram-negative and Gram-positive microorganisms, thereby causing structural and functional damage resulting in cytotoxicity and cell death [13,14]. Additionally, oregano oil and its constituents can disrupt membrane-embedded proteins and lipids, along with efflux pump functionality [6]. These antimicrobial and antifungal properties of essential oil components contribute to the natural defense mechanism of plants and provide useful properties for products with applications to humans.

In this study, the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of two essential oil formulations containing oregano oil were determined. The ability of these essential oil formulations to inhibit the growth of both bacterial cultures (*E. coli*) and fungal cultures (*C. albicans* and *C. auris*) *in vitro* was demonstrated.

Materials and Methods Materials and Methods

Liquid products

Traditional Germ-a-CLENZ™ and mycelized Germ-a-CLENZ™, which was designed to be a more water soluble form of Germ-a-CLENZ™, were provided by North American Herb and Spice (Buffalo Grove, IL, USA). According to the manufacturer, the main constituent of both the traditional and mycelized Germ-a-CLENZ™ formulations is a proprietary blend of wild oregano (P73 Oreganol™) oil. The primary constituents of oregano oil are shown in Table 1. The density of both Germ-a-CLENZ™ and mycelized Germ-a-CLENZ™ was calculated to be 1.09 g/mL using a standard internal gravimetric method.

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Received date: 07 March 2021; **Accepted date:** 21 March, 2021; **Published date:** 28 March, 2021

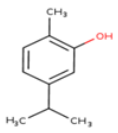
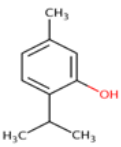
Active Ingredient	CAS Number	Systematic Name	Chemical Formula	Structural Formula
Carvacrol	499-75-2	2-methyl-5-propan-2-ylphenol	$C_{10}H_{14}O$	
Thymol	89-83-8	5-methyl-2-propan-2-ylphenol	$C_{10}H_{14}O$	

Table1. Summary of the main active components in oregano oil.

Organisms

Microorganism studies were conducted by Analytical Food Laboratories (Grand Prairie, TX, USA). *Escherichia coli* (ATCC#8739), *Candida albicans* (ATCC#10231), and *Candida auris* (CDC B11903) were selected as challenge organisms for this study. Organism cultures were prepared from lyophilized stocks according to the manufacturer's instructions or from stock plates. The *E. coli* culture was transferred into Tryptic Soy Broth (TSB, Hardy Diagnostics, Santa Maria, CA) and incubated at 35 ± 2°C for 24 hours, while the *C. albicans* and *C. auris* cultures were transferred into Sabouraud Dextrose Broth (SDB, Hardy Diagnostics) and incubated at 25 ± 2°C for 5 days. Following incubation, the concentration of the *E. coli* inoculum was 4,500,000 CFU/mL (log 6.65), the concentration of the *C. albicans* inoculum was 3,300,000 CFU/mL (log 6.52), and the concentration of the *C. auris* inoculum was 3,600,000 CFU/mL (log 6.56). The bacterial culture and fungal cultures were verified by streaking onto Tryptic Soy Agar (TSA, Hardy Diagnostics) and Sabouraud Dextrose Agar (SDA, Hardy Diagnostics).

Susceptibility testing

Serial dilutions of the Germ-a-CLENZ™ and mycelized Germ-a-CLENZ™ products were employed to determine the susceptibility of the bacterial (*E. coli*) and fungal (*C. albicans* and *C. auris*) strains to the essential oil formulations. Stock formulations of Germ-a-CLENZ™ (5.83% active ingredients; 63.6 mg/mL) and mycelized Germ-a-CLENZ™ (4.17% active ingredients; 45.4 mg/mL) were serially diluted 1:2 with sterile TSB or SDB media in preparation for inoculation with bacterial or fungal cultures, respectively. Two replicates for each dilution were prepared for inoculation with each challenge organism. For each challenge organism experiment, two controls were included: an uninoculated matrix control that contained full strength (1.09 g/mL) Germ-a-CLENZ™ product and an inoculated positive control that contained no product (0 g/mL).

Post-inoculation, samples were incubated at 30 ± 2°C for up to 48 hours. Samples were evaluated visually, however, turbidity was observed even at comparatively low product concentrations. Therefore, bacterial or fungal inhibition was confirmed by plating each tube on either TSA (for tubes inoculated with the *E. coli* culture) or SDA (for tubes inoculated with either *Candida* culture). Control tubes for each set were also plated. TSA plates were incubated at 35 ± 2°C for 48 ± 2 hours, while SDA plates were incubated at 25 ± 2°C for 5 days.

After incubation, plates were visually examined for significant (i.e. ≥3 colonies) growth. Susceptibility was expressed as the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC). The MBC was defined as the lowest product concentration that prevented significant growth (fewer than 3 colonies) of the *E. coli* challenge organism after culture on TSA plates. The MFC was defined as the lowest product concentration that prevented significant growth (fewer than 3 colonies) of the *C. albicans* or *C. auris* challenge organism after culture on SDA plates.

Results

Essential oil formulations exhibit antibacterial and antifungal activity *in vitro*

The antimicrobial and antifungal properties of two formulations of an essential oil blend, traditional Germ-a-CLENZ™ and mycelized Germ-a-CLENZ™, were examined *in vitro*. Susceptibility of bacterial and fungal challenge cultures were determined by inoculating the cultures with serially diluted essential oil blend formulations. The MBC of Germ-a-CLENZ™ was determined to be 2.0 mg/mL against *E. coli* in culture, while the MBC for mycelized Germ-a-CLENZ™ was determined to be 5.7 mg/mL (Table 2).

Regarding fungicidal effects, MFCs of 4.0 mg/mL and 2.8 mg/mL were observed for the Germ-a-CLENZ™ and mycelized Germ-a-CLENZ™ products, respectively, against *C. albicans* (Table 2). MFCs of 2.0 mg/mL and 1.4 mg/mL were observed for the Germ-a-CLENZ™ and mycelized Germ-a-CLENZ™ products, respectively, against *C. auris* (Table 2).

Germ-A-CLENZ™							Mycelized Germ-A-CLENZ™						
Organism	***E. coli***		***C. albicans***		***C. auris***		***Organism***	***E. coli***		***C. albicans***		***C. auris***	
Concentration (mg/mL)	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Concentration (mg/mL)	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
Uninoculated (control)	-	-	-	-	-	-	Uninoculated (control)	-	-	-	-	-	-
63.6	-	-	-	-	-	-	45.4	-	-	-	-	-	-
31.8	-	-	-	-	-	-	22.7	-	-	-	-	-	-
15.9	-	-	-	-	-	-	11.4	-	-	-	-	-	-
7.9	-	-	-	-	-	-	5.7	-	-	-	-	-	-
4	-	-	-	-	-	-	2.8	+	+	-	-	-	-
2	-	-	+	+	-	-	1.4	+	+	+	+	-	-
1	+	+	+	+	+	+	0.7	+	+	+	+	+	+
0 (control)	+	+	+	+	+	+	0 (control)	+	+	+	+	+	+
Rep: replicate													
+ : observed growth (≥ 3 colonies)													
- : no observed growth (<3 colonies)													

Table 2. Effect of essential oil blends on growth of bacterial and fungal species.

Test Substance	MIC (challenge organism)	MBC (challenge organism)	MFC (challenge organism)	Reference
Traditional Germ-A-CLENZ™	NR	2.0 mg/mL (*E. coli*)	4.0 mg/mL (*C. albicans*); 2.0 mg/mL (*C. auris*)	This study
Mycelized Germ-A-CLENZ™	NR	5.7 mg/mL (*E. coli*)	2.8 mg/mL (*C. albicans*); 1.4 mg/mL (*C. auris*)	
Oregano oil	0.250-1.0 mg/mL (*E. coli*)	NR	NR	[16]
Oregano oil	0.125 mg/mL (*C. albicans*)	NR	0.25 mg/mL (*C. albicans*)	[17]
NR: Not reported				

Table3. Comparison of MBC and MFC values for traditional and mycelized Germ-a-CLENZ™ formulations to values reported in the literature for oregano essential oils.

Discussion

Based on the observed MFCs, the traditional Germ-a-CLENZ™ and mycelized Germ-a-CLENZ™ products similarly inhibit the growth of fungal challenge organisms (*C. albicans* and *C. auris*), whereas a 2.8-fold difference in MBC was observed for the bacterial challenge organism (*E. coli*). The mycelized formulation was slightly more effective than traditional Germ-a-CLENZ™ against both *C. albicans* and *C. auris*. Conversely, traditional Germ-a-CLENZ™ was more efficacious than the mycelized formulation against *E. coli*. Thus, the more soluble, mycelized version of the formulation appears to inhibit fungal growth more effectively, but bacterial growth less effectively, than traditional Germ-a-CLENZ™. However, given the small sample size, the statistical significance of the differences in efficacy between the traditional and mycelized formulations against the challenge organisms was not determined. It is worth noting that Gram-negative bacteria (e.g., *E. coli*) are generally less susceptible to the antimicrobial activity of essential oils and their components due to the presence of an outer lipid membrane containing lipopolysaccharides, which is lacking in Gram-positive bacteria [8]. It is possible that the more soluble, mycelized Germ-a-CLENZ™ formulation is less effective at penetrating this outer membrane than the traditional Germ-a-CLENZ™ formulation. Indeed, formation of micelles in the aqueous phase, which may suppress their attachment to the microorganism, has been proposed as a mechanism to explain reduced efficacy of some essential oil treatments [15].

As shown in Table 3, the essential oil formulations evaluated herein exhibited MBC and MFC values that are on the order of those reported in the literature for oregano essential oils against *E. coli* and *C. albicans* [4,16,17]. The MBC and MFC values provided herein against the *C. auris* challenge organism appear to be the first for an oregano essential oil, as no values were identified in the published literature. In addition, some values provided in the literature, such as an MFC of 0.02%-0.05% oregano oil against various Candida species [13], could not be accurately compared to the values provided herein (in mg/mL) as density information was not provided in the publications. It is important to note that minimum inhibitory concentrations (MICs) are generally defined as the concentration of test compound at which no macroscopic sign of cellular growth is detected after incubation in media (e.g., by turbidity), whereas MFC and MBC are typically used to refer to measurements of growth after plating [4,17]. Thus, MFC, MBC, and MIC values may not be directly comparable and slight variations are expected. Additionally, caution should be used when comparing the MBC values reported for *E. coli* herein to MIC or MBC values reported in the literature, as these literature values may have been obtained following incubation of the bacterial cultures at 35°C-37°C. In this study, an incubation temperature of 30°C was used for both bacterial and fungal cultures based on the optimized protocol for fungal species provided by Manohar and colleagues [17]. Additionally, Manohar et al. (2001) specified the use of less than three (<3) CFU to represent significant inhibition of bacterial or fungal growth (i.e., the MBC or MFC) [17]. As such, differences in the determination of 'significant' inhibition of growth among publications reporting MIC, MBC, or MFC values may arise.

Studies have demonstrated that individual components of oregano oil, such as carvacrol and thymol, give an additive effect to the overall efficacy of oregano oil against microorganisms [12,14,17]. The Germ-a-CLENZ™ essential oil formulations evaluated herein have the benefit of combinatorial effects of the individual components of oregano oil, such as carvacrol and thymol, along with active components of other essential oils (e.g., cumin oil) in the formulation. The combinatorial effects of multiple active components from multiple essential oils in the Germ-a-CLENZ™ formulations also explain the slight differences in observed MFC, MBC, and MICs compared to those presented in the literature. The resulting Germ-a-CLENZ™ formulations provide antibacterial and antifungal activity against the challenge organisms tested herein. Additional studies will be conducted to further evaluate the antimicrobial properties of these essential oil formulations against a wider range of challenge microorganisms of medical importance, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* and viruses including the human coronavirus.

Conclusion

The essential oil formulations evaluated herein showed efficacy against a pathogenic Gram-negative bacteria and two fungal species that represent public health concerns. The demonstrated antibacterial and antifungal properties of these naturally derived Germ-a-CLENZ™ antimicrobial formulations provide a 'greener' alternative to synthetic chemicals that may be useful in consumer products including, but not limited to, cleaning agents. Additional studies will determine the efficacy of these essential oil blends against a larger set of challenge organisms and include a time kill analysis. In addition, further exploration in both clinical and nonclinical settings should be conducted to confirm the safety and efficacy of these essential oil products.

Conflict of Interest Statement

None declared.

Funding Sources

This work was adapted in part from a report developed by the authors with funding from North American Herb and Spice. However, the funding source had no influence on study design, data analysis, or the decision to submit the article for publication. The results reflect the opinions of the authors and not necessarily the opinions of their employer or funding organization.

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How to cite this article: Jillian A Parker. "Antibacterial and Antifungal Properties of Essential Oil Blends". J Antimicrob Agents 7 (2021)