Effects of Essential Oils and Monolaurin on Staphylococcus aureus: *In Vitro* and *In Vivo* Studies

Running Title: Oregano and Monolaurin as Antimicrobials

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Abstract

The antimicrobial properties of volatile aromatic oils and medium chain fatty acids derived from edible plants have been recognized since antiquity. To give examples, Origanum oil, used as a food-flavoring agent, possesses a broad spectrum of antimicrobial activity due, at least in part, to its high content of phenolic derivatives such as carvacrol and thymol. Similarly, lauric acid, present in heavy concentrations in coconuts, forms monolaurin in the body that can inhibit the growth of pathogenic microbes. Using Staphylococcus aureus in broth cultures and a micro dilution method, comparative efficacy of Origanum oil, and a constituent carvacrol, other essential oils, and monolaurin were examined. Origanum oil was the most potent of the essential oils tested and proved bactericidal in culture to two strains of Staphylococcus aureus at 0.25 mg/ml. *In vitro*, monolaurin's effects mirrored Origanum oil. The combination of both was bactericidal at the 0.125 mg/ml concentration of each. In two separate *in vivo* experiments, injected Staphylococcus aureus killed all 14 untreated mice within a one-week period. In treated mice, over one third survived for thirty days when given oral Origanum oil daily for 30 days (6/14). Fifty percent of the mice survived for 30 days when receiving daily vancomycin (7/14) and monolaurin (4/8). Over sixty per cent of mice survived when receiving a daily combination of Origanum oil and monolaurin (5/8). Origanum oil and/or monolaurin may prove to be useful antimicrobial agents for prevention and therapy of Staphylococcus aureus infections.

Key Words

Origanum oil, effect on Staphylococcus

Monolaurin, effect on Staphylococcus

Staphylococcus aureus, natural antimicrobials

Introduction

Microbial resistance to antibiotics, especially among staphylococcal strains, is a major threat to public health [1]. Since resistance by certain strains of Staphylococcus to multiple antibiotics like methicillin emerged in the late 1970's [2], many strategies to control antibiotic resistance have been proposed [3]. Considering current therapeutic regimens, vancomycin usage has proven to be the most reliable to treat resistant staphylococcal infections [4-6]. However, some staphylococcal strains have become resistant, at least to some extent, even to vancomycin -- indicating a dire need for new alternative therapeutic approaches [6-9]. One specific strategy has been to use multiple antibiotics of the same spectrum and low resistance potential when single antibiotic substitutions are not effective. However, we have examined yet another strategy – use of natural products with low potential for the development of resistance.

The purpose of the present study was to determine the efficacy of a group of essential oils and the fat monolaurin alone or combined to treat Staphylococcus aureus. Of the 12 essential oils tested, Origanum oil proved to be the best for treating Staphylococcal infections. We also found that the fat monolaurin inhibits the growth of Staphylococcus aureus *in vitro* and *in vivo* to an extent similar to Origanum oil. We conclude that the daily oral administration of these natural products alone or combined may be highly effective in preventing and treating Staphylococcus aureus infections and theoretically may even be effective against antibiotic-resistant strains.

Material and Methods

Animals and Treatment

Female BALB/c mice (15-20 g) were obtained from Taconic Farms (Germantown, NY). The rodents were maintained in a controlled environment at 24^{o}C with a 12-hour light and 12-hour dark cycle and were acclimatized in the animal facility for 3-5 days before use. The mice were housed in groups of five, fed commercial rodent pellets, and given water *ad libitum* throughout the experiments. The Animal Welfare Board at Georgetown University Medical Center approved the protocol for the entire investigation.

Plant oils and chemicals

Origanum (P73 Oreganol^{TM}), other essential oils, and olive oil were provided by North American Herb and Spices, Inc., Waukegan, IL, USA. Monolaurin was obtained from the Center for Research on Lauric Oils, Bethesda, MD (www.lauric.org). Sabouraud's glucose (S.g.) broth and agar media were obtained from Difco Laboratories (Detroit, MI, USA). Carvacrol, antibiotics, and all other chemicals used in this study were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and were of analytical grade or the highest commercial grade available.

Organisms

Standard strains of Staphylococcus (ATCC #14154 and #14775) were obtained from ATCC, Fairfax, VA, USA, and were grown and maintained on S.g. agar slants.

Susceptibility Testing

A micro-broth dilution technique was employed to determine the susceptibility of the strains of Staphylococcus aureus to oil of Origanum, carvacrol, other essential oils, and monolaurin [10-12]. Susceptibility was express as minimum inhibitory (MIC) and/or minimum bactericidal (MBC) concentration. The stock solutions of Origanum oil, other essential oils, olive oil, and carvacrol were dissolved in 50% ethanol-Tween 80 solvents. Antibiotics were dissolved in 50% ethanol and used as positive controls. Solvent controls (addition of carrier without essential oil and/or monolaurin) were also included for reference in addition to regular controls.

The Sabouraud's glucose (S.g.) broth containing varying amounts (logarithmic, serially and 2-fold diluted) of Origanum oil, other essential oils, carvacrol, monolaurin and various controls were inoculated with actively dividing Staphylococcus aureus cells. The cultures were incubated for 24 h and 48 h at 30^{O}C on a metabolic rotary shaker (220 rev/min), and the growth was monitored both visually and colorimetrically (at 540 nm). The minimum inhibitory concentration (MIC) was defined as the lowest concentration required to arrest the growth of the bacteria at the end of 24 h of incubation. Minimum bactericidal concentration (MBC) was determined by sub-culturing a 0.01 ml aliquot of the medium drawn from the culture tubes after 48 hours on S.g. agar plates and incubated further for bacterial growth. The plates were scored for growth of the bacteria colonies. The lowest concentration of the antimicrobial agent causing negative growth (fewer than three colonies) was considered as MBC. In every case, the MIC and MBC were virtually the same, so we will only report the MBC in the results.

In Vivo Protocol

In two separate experiments, groups of mice (6 and 8 respectively) infected with Staphylococcus aureus (5 x LD_{50}) were gavaged daily with Origanum oil, carvacrol, monolaurin or combined Origanum oil - monolaurin in 0.2 ml of olive oil for 30 days. The amount of bacterial agents administered was calculated based on the body weight of the mice. Control mice received daily gavages of either olive oil alone (negative control) or olive oil orally plus vancomycin (400 mcg) *i.p.* (positive control). In the first *in vivo* experiment, the daily dose of Origanum oil was either 2.0 ul (1.6 mg) or 4.0 ul (3.2 mg). The mice gavaged with carvacrol received doses comparable to the phenol content in the 2 doses of Origanum oil. In the second *in vivo* study, the daily dose of oil of Origanum was the same as the higher dose used before, i.e., 4.0 ul (3.2 mg). Monolaurin was given at the same dose; and when both agents were combined, the same individual doses were used, i.e., 3.2 mg of each. Both experiments were terminated at the end of 30 days. The body weight, the disease status, and the overall health of the mice during the experiments were recorded. The pathological status of the mice was determined by visual examination of the internal organs after their death or sacrifice at the completion of the experiment. Culturing aliquots of kidney homogenates on S.g. agar plates further tested the renal burden of Staphylococcus aureus.

Results

Table 1 shows results from a micro dilution study on Staphylococcus aureus grown in broth over 48 hours (ATCC#14154). In examining the comparative effects of various antibiotics and essential oils at the selected concentrations, penicillin had essentially no effect, and streptomycin did not

completely kill the bacteria. However, vancomycin completely destroyed the bacteria. Addition of the carrier alone to the plates did not alter growth. Of the oils examined, olive oil (the diluent), pumpkin, fenugreek, and myrtle had virtually no effect at any concentration. Allspice, sage, lavender and bay leaf were bactericidal only at the higher concentrations. Cumin had an intermediate effect, while cinnamon, cassia, and Origanum oil were quite effective at lower concentrations. Carvacrol was added at a concentration equivalent to the total phenol content in a similar volume of Origanum oil (63% v/v). While Origanum oil proved completely bactericidal at a concentration of 0.25 mg, the carvacrol was effective only at the concentration comparable to the 0.5 mg dose of Origanum oil.

Table 2 shows data from a micro dilution study performed with another strain of Staphylococcus aureus (ATCC#14775). Although the results from the two strains were generally similar, there were some minor differences. Myrtle appeared relatively more effective against the second strain. Cumin seemed to work as well as cassia. Origanum oil again inhibited down to 0.25 mg, and addition of carvacrol once more inhibited at a phenol content equivalent to that in 0.5 mg Origanum oil.

Table 3 shows data from an *in vitro* experiment on Staphylococcus aureus (ATCC#14775) where the effects of monolaurin alone and combined with Origanum oil were compared to the effects of Origanum oil alone. In this experiment, a few colonies grew after 2 days of incubation in 0.125 mg of Origanum oil or 0.125 mg of monolaurin. However, a combination of 0.125 mg Origanum oil and 0.125 mg monolaurin prevented any growth.

The *in vivo* therapeutic effects of the natural edible Origanum oil and one of its major constituents, carvacrol, were examined in a murine systemic bacteremia model (Table 4). Preliminary studies showed that Staphylococcus aureus (ATCC#14154) did not cause mortality or death in mice, whereas Staphylococcus aureus (ATCC#14775) caused mortality rapidly. Groups of 6 mice infected with Staphylococcus aureus (ATCC#14775)(5 X LD_{50}) were gavaged with varying amounts of Origanum oil (2.0 ul – 1.6 mg and 4.0 ul – 3.2 mg) or injected i.p. with vancomycin (400 ug) daily for 30 days. A dose-dependent survival was observed in mice receiving Origanum oil. Fifty per cent of the mice (3/6) receiving Origanum oil (3.2 mg) survived over the thirty days, while only one of six receiving 1.6 mg Origanum oil survived for 30 days. Also, 50% of those receiving vancomycin (400 ug) survived for 30 days, while all in the Control group died within a three-day period. Although prolonged survival was noted in rats gavaged with carvacrol, none of the twelve at the two doses equivalent to the total phenol content in 1.6 and 3.2 mg of Origanum oil survived beyond 21 days. In all 30-days survivors, no internal abscesses were noted at *post mortem,* and renal cultures showed no bacterial growth. In contrast, numerous abscesses were noted and renal cultures were positive in all dying mice.

In a separate *in vivo* study, the effects of monolaurin alone and combined with Origanum oil were compared to the effects of Origanum oil alone (Table 5). Groups of 8 mice were infected with five times the LD_{50} of Staphylococcus aureus (ATTC #14775). Fifty percent of the mice (4/8) injected daily with vancomycin (400 ug i.p.) survived for the thirty days of study in contrast to the control group where all eight mice died within a week's time. In the three test groups, 3/8 survived for 30 days in the Ori-

ganum oil group (3.2 mg), 4/8 survived in the monolaurin group (3.2 mg), and 5/8 survived in the group receiving the combination of Origanum oil (3.2 mg) and monolaurin (3.2 mg). Again, no abscesses were seen on inspection, and no bacterial growth was found in the kidney tissue of the 30-day survivors.

Discussion

The carriage and subsequent dissemination of antibiotic-resistant Staphylococcus aureus by hospital staff and patients is a recognized risk for nosocomial infections [13]. Although use of antibiotics has generally been tempered to avoid antibiotic resistance, the development of resistance was inevitable [2-9]. Therefore, what alternatives now exist to treat antibiotic-resistant organisms? Essential oils, especially Origanum oil, and the fat monolaurin are natural substances reported to have the ability to kill Staphylococcus aureus and other microbes in culture [13-18]. Nevertheless, *in vivo* studies designed to test the antimicrobial effects of various essential oils and monolaurin are lacking.

We found that Origanum oil, a constituent carvacrol, certain other essential oils, and monolaurin killed Staphylococcus aureus effectively *in vitro* (Tables 1-3). More importantly, however, we also found that Origanum oil and monolaurin are protective *in vivo* (Tables 4,5). Figure 1 combines the data from tables 4 and 5. In the 14 rats used as control, Staphylococcus aureus (ATCC #14775) killed all the mice within 7 days. In contrast, 37.5%-50% of the mice survived for thirty day after receiving daily gavages of vancomycin (7/14), Origanum oil (6/14), and monolaurin (4/8). The survival with combined Origanum oil and monolaurin was slightly better –

62.5% (5/8). Obviously, more studies are needed to establish whether a combination of natural products is superior to individual use, especially if higher doses of individual agents are used. As a final point, no abscesses were found at post mortem; and Staphylococcus aureus could not be grown out of the kidneys of the 30-day survivors suggesting a cure.

Interestingly, carvacrol gavaged in a dose calculated to be equivalent to the phenol content in the gavaged Origanum oil delayed death but could not cure the mice. In the first *in vivo* study where carvacrol was examined, all untreated mice died within three days, whereas all mice receiving carvacrol lived beyond 3 days, but died by 21 days. Staphylococcus aureus was grown out of their kidneys after death. Thus, carvacrol alone could not duplicate, to the same extent, the beneficial effects of Origanum oil. Either the addition of thymol and/or other phenols to carvacrol is necessary or there are other significant factors present in the Origanum oil.

What do we really know about essential oils and their antimicrobial properties? The benefits of essential oils to preserve various foods have been known since the days of the explorers [17]. The previous results of studies using broth micro dilution techniques support the notion that plant essential oils and extracts may have a role as nutraceuticals and preservatives (19). An advantage of essential oils over antibiotics may be that bacteria do not develop resistance to essential oils (14). In addition, some oils actually stimulate immune function. The major components of oregano with antibiotic properties are two phenols (carvacrol and thymol) [20,21]. Phenols are antiseptic substances found in Lysol, Pinesol, Chloroseptic, and throat lozenges.

Many essential oils have been shown to be effective against a number of organisms. Origanum oil, cinnamon, and clove were judged "very active" by examining their inhibitory effects on Clostridium botulinum 33A [15]. In addition to effects against Klebsiella pneumonia and Staphylococcus aureus, Origanum oil is fungicidal [12,14]. Also, antiviral actions of Origanum and clove oils against RNA and DNA viruses have been reported [22]. As a potential mechanism of action, the outer protective membrane of the viruses disintegrated after exposure to the Origanum oil when viewed by electron microscopy (22). Importantly, most essential oils of spices are classified as GRAS (generally recognized as safe) indicating that consumers can eat them reasonably without fear [15]. Accordingly, the benefits/risks ratio of essential oils in treating microbes would seem to be very high.

Considering other natural products with antimicrobial properties, Kabara championed the use of certain lipids [23]. He measured the antimicrobial activity of fatty acids and their corresponding monoglycerides and reported that the optimum chain length was C12 [24]. Of the saturated fatty acids, lauric acid (C12) has greater antiviral activity than caprylic acid (C8), capric acid (C10) or myristic acid (C14). In contrast to monolaurin, the dilaurin derivative was inactive. It is now generally accepted that monoglycerides are active: diglycerides and triglycerides are inactive

A broth dilution method was used to determine the minimal inhibitory concentration of a series of fatty acid ester of polyhydric alcohols against gram-negative and gram-positive organisms [25]. Gram-negative organisms were not affected. Gram-positive organisms were affected to the greatest

extent by monolaurin [25]. Monolaurin is effective in blocking or delaying the production of exotoxins by pathogenic gram-positive bacteria [26] and inhibits the synthesis of most staphylococcal infections and other exoproteins -- it does so at the level of transcription [27]. Monolaurin also inhibits the expression of virulence factors in Staphylococcus aureus and the induction of vancomycin resistance in Enterococcus faecalis [28,29]. Monolaurin also inhibits spore growth in milk [27].

When the anti chlamydial effects of several fatty acids and monoglycerides were studied by incubating Chlamydia trachomatis bacteria, the results indicate that the lipid kills the bacteria, possibly by disrupting the membranes(s) of the elementary body [30]. Corroborating evidence is available from viral studies suggesting that the bactericidal effects are via disintegration of membranes by fatty acids [31-33] similar to a report of the action of Origanum oil on viruses [22].

This pilot study raises a number of important considerations. The bactericidal effects of Origanum oil, other essential oils, and monolaurin *in vivo* indicate a need for further studies to establish the importance of these natural products in combating pathogenic microbes, especially those resistant to antibiotics. Because of the potential for fewer side-effects with natural products compared to pharmaceuticals, the health care provider might eventually recommend taking them prophylactically to prevent future infections. While combining Origanum oil and monolaurin produces additive effects, more studies are necessary to determine if synergy exists. It is also possible that these agents might also work well in the presence of antibiotics and make them more effective.

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Figure-Legend

Fig. 1 Combined data from two *in vivo* studies. See text for details.

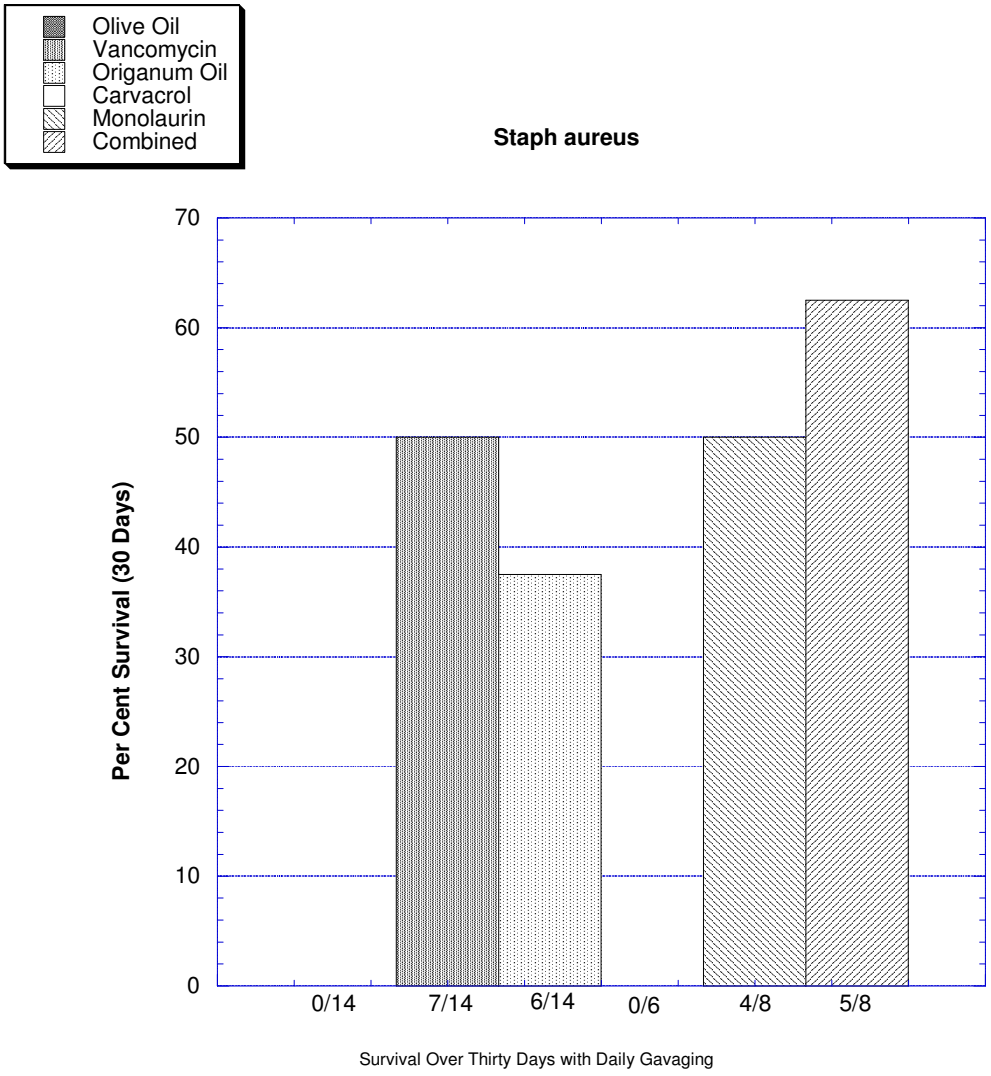

Table 1