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Dallas Rohraff
Grand Valley State University

Roderick Morgan
Grand Valley State University

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Abstract

The emergence of antibiotic resistant bacteria is of pressing concern as health care associated infections kill 99,000 people a year in the U.S. alone. Researchers are currently looking for new antibiotics in alternative sources. Essential oils are traditionally known to have medical benefits, and cinnamon, tea tree, and eucalyptus oils have shown antibiotic activity. Initial testing via standard microbiological protocols found minimum inhibitory concentration (MIC) values of 0.039% for cinnamon, 1.25% for tea tree, and 0.313% for eucalyptus. All three oils proved effective against both Gram-positive and Gram-negative bacteria. Cinnamon bark oil, *Cinnamomum cassia* Blume, appeared most effective. More thorough microbiological analysis revealed it to be bactericidal and retained antibacterial activity in the presence of human serum protein. The results revealed cinnamon bark oil may contain a promising novel antibiotic.

Introduction

Infectious diseases, particularly those caused by bacterial microorganisms, are still among the top causes of mortality in the world. The rate of infections caused by bacteria that have acquired antibiotic resistance is a staggering proportion. This is especially concerning in hospital settings where 1.7 million health care-associated infections are acquired and kill 99,000 people every year. It is estimated that these infections cause between $28 and $32 billion dollars to the health care industry (5). Several resistant strains, such as multi-antibiotic resistant *Staphylococcus aureus* (MRSA), carbapenem resistant *enterobacteriaceae* (CRE), Vancomycin Resistant *Enterococci* (VRE), and antibiotic resistant hypervariant *Clostridium difficile (*Cdiff), are commonly acquired through a nosocomial infection and demonstrate the necessity of novel antibiotics to combat bacteria that have become resistant to currently used antibiotics.

Bacteria can become resistant to antibiotics in a number of ways, one of which is random mutation. Mutations can occur in DNA which would usually code for antibiotic sensitivity and in turn code for resistance (9). Once this occurs, the bacterium can spread the mutation when it produces daughter cells (9). This mutation occurs in about one per million to one per billion cells (9). Another mode by which bacteria can become resistant is by the transfer of plasmids that code for resistance from one cell to another through conjugation. For either circumstance, once one bacterium becomes resistant, the number of resistant bacteria will begin to increase (9). There has been much concern in recent years over the misuse and overuse of antibiotics. Misuse can occur with the prescription of antibiotics when they are not necessary, and some worry antibiotics are being overused in the agriculture and livestock business where recommended doses are commonly added to feed for health purposes and increased rates of livestock growth (9). When this occurs, resistant bacteria survive and are artificially selected for and allowed to replicate. This can lead to rapid growth of resistant bacteria which are resistant to current antibiotics. There are few treatments currently available that can combat these antibiotic resistant bacteria, and the necessity of novel antibiotics is becoming evident.

Unfortunately, progress in the development of new antibiotics has tapered off as several large pharmaceutical companies have decreased their infectious disease discovery programs. Much of the current research is looking toward synthesizing novel derivatives which are modeled after current antibiotics (11). There has been low success rates in finding novel antibiotics with these derivatives, and this could be because a majority of the antibiotics used

today are produced naturally from soil streptomycetes and fungi (11). While many large companies are focusing on manmade products, and finding little success, some researchers are once again looking toward natural sources for new and improved antibiotics (11). One natural product, which has been around for thousands of years, may hold the key to finding new antibiotics: essential oils derived from plants.

EOs are aromatic liquids generally made through a steam distillation process of plant material which can be traced back over 2000 years to Egyptian, Persian and Indian roots; but can also be made through extraction, enfleurage, fermentation and expression (2). EOs are typically made from aromatic plants of warm, tropical regions and can be made from nearly any plant organ, such as leaves, bark, herbs, roots, seeds, stems, and fruits. Climate, soil, plant organ, age of plant, and harvest time all affect the quality and quantity of an essential oil yield (1). EOs have been used for pharmaceutical purposes since the 13th century in some areas of Europe (7), but their use was not widespread until they were traded in London in the 16th century (2). French physician, Du Chesne, noted that by the 17th century, EOs were a common medical practice in Europe, with pharmacies stocking 15 to 20 different essential oils at a time (7). It was not until 1881 that EOs were tested for antimicrobial properties, when De La Croix examined vapors (2). By the 19th and 20th centuries, EOs were increasingly used for fragrances and flavoring different foods (7).

Today, only a small percentage of the essential oils created are used for aromatherapy. EOs have countless uses, such as perfumes, cosmetics, dentistry, food preservatives and flavoring, and more (1). In recent years, more research has been done to determine the antimicrobial nature of numerous EOs against different bacteria (1, 7). Essential oils have also been found to have antiviral, antiparasitic, insecticidal, antitoxigenic, antiseptic, and tumor inhibiting activities as well (1, 2, 10). Because EOs are made from various plant components, it stands to reason that they would contain properties that the plant uses to protect itself from bacteria, viruses, and unwanted insects, while containing fragrances that could help attract insects that would be beneficial for pollination (1). Studies show EOs have the potential to solve a variety of different medical problems, including the crisis of antibiotic resistance of infectious bacteria.

We initially tested several essential oils using the Agar Disk Diffusion Test, or the Zone of Inhibition Test, to determine if the oil had any antibiotic activity. If an oil demonstrated a promising zone, further testing for Minimum Inhibitory Concentrations (MICs) were performed against *Staphylococcus aureus* and *Escherichia coli*. Cinnamon bark oil showed a very low MIC, meaning that it remained effective in small doses, and was tested using a Time Kill experiment to determine if it is bacteriostatic or bactericidal.

Methods
Essential Oils
The essential oils tested were from a variety of different brands. The following oils were Plant Therapy brand: pine (*Pinus Sylvestris*), cinnamon bark (*Cinnamomum cassia Blume*), spearmint (*Mentha spicata*), peppermint (*Mentha piperita*), and juniper berry (*Juniperus communis*). Lavender (*Lavandula officinalis*) and ginger (*Zingiber officianale*) were Now

Essential Oils. Orange oil was of the brand LorAnn Oils. The tea tree (*Melaleuca alternifela)*, eucalyptus oils tested were Sundown Naturals and Aura Cacia, respectively.

Anthranilic Acid Derivative- GV-2

GV-2 was prepared by the Chemistry Department at GVSU and identified using analytical (C, H, N) and spectral (IR, HNMR, CNMR, Mass) data. GV-2 shows antimicrobial activity against Gram-positive bacteria, but is not effective against those that are Gram-negative. GV-2 is used as a positive control against *Staphylococcus aureus* and a negative control against *Escherichia coli* for this reason. GV-2 has an MIC value of 16.0 µg/mL against *S. aureus*, but this value increases in the presence of Human Serum Protein to 128 µg/mL.

Zone Of Inhibition Test

Initial testing to determine antimicrobial activity of the test compounds was by the zone of inhibition test, or agar disc diffusion method (4). The zone of inhibition test, begins by swabbing a plate with overnight cultures of *Staphylococcus aureus* and *Escherichia coli* to create a "lawn." Five microliters of essential oil were pipetted onto 6 millimeter sterile paper disks which were placed atop the bacterial "lawn" and incubated for 18-24 hours at 37°C. Areas of clearing, or zones of inhibition, around the disks after incubation are measured and indicate that the compound has some antimicrobial activity. Diameter of zones of inhibition were measured in millimeters and recorded.

Determination of MIC

The MIC's of the tested essential oils were determined by the Clinical Laboratory Standards Institute's method of broth microdilution (3). The MIC's were determined by inoculation of serial dilutions of the essential oil being tested in Mueller-Hinton broth with *S. aureus* or *E. coli*. Cultures were incubated at 37°C for 18-24 hours. If appropriate, 100% Human Serum was added to a final concentration of 10%.

Time Kill

The Time Kill Assay performed on Cinnamon Oil used a solution of four or eight times the MIC of the essential oil. The solution contained final concentrations of 10% test compound, 80% Mueller-Hinton broth, and 10% inoculum. Inoculum was created by diluting a 0.5 Macfarland 1:20. Growth controls of 9.68% and 4.84% DMSO were utilized to mimic the DMSO concentrations in the Cinnamon 8X and 4X tubes, respectively. Solutions are allowed to incubate for 24 hours with one hundred microliter samples taken initially (0 hours), and at 3, 6, and 24 hours after inoculation. Samples are serially diluted and plated in duplicate to determine the number of colony-forming-units per mL (CFU/mL) at the given sample time.

Results and Discussion

Initial testing of the EOs using the agar disk diffusion method resulted in zones of clearance, or no growth, listed in table 1. Cinnamon bark and eucalyptus had the largest zones on *S. aureus* with zones of 27.5 and 19.5 mm, respectively, and were comparable to our positive control, GV-2, which measured 26.2 mm in diameter. Peppermint and spearmint also had large

zones against *S. aureus*, 19.5 and 28.0 mm respectively. They were not subjected to further testing because of the growth of mutant colonies within the zone of clearance, peppermint on *E. coli* and spearmint on *S. aureus* (data not shown). The presence of mutant colony growth suggests that it is fairly easy for *S. aureus* or *E. coli* to resist either peppermint or spearmint, and thus there was no further interest in the two EOs as antibiotics.

MIC values against *S. aureus* were determined for cinnamon bark, eucalyptus, and tea tree oils; 0.04%, 0.31%, and 1.25% respectively (table 1). GV-2 has an MIC against *S. aureus* of 16.0 μg/mL, but this value increases to 128.0 μg/mL in the presence of 10% Human Serum Protein (HSP). Since cinnamon bark oil had the lowest MIC, we tested its activity in the presence of HSP, and it retained the MIC of 0.04%. GV-2, as well as numerous other compounds, bind to HSP, and thus more of the compound must be used in order to inhibit bacterial growth. Binding to HSP can cause concern when considering these compounds as medication because the desired effective concentration is very low, as it will be less toxic to the body. Because cinnamon bark oil retains its MIC in the presence of HSP, lower concentrations could be used for medicinal purposes when it could be subjected to HSP in the bloodstream.

Chemical Name	Zone of Inhibition on *S. aureus* (mm)	Zone of Inhibition on *E. coli* (mm)	MIC against *S. aureus*	MIC against *S. aureus* with 10% Human Serum Protein	MIC against *E. coli*	MIC against *E. coli* with 10% Human Serum Protein
Cinnamon Bark	27.5	12.5	0.04%	0.04%	0.04%	0.04%
Eucalyptus	19.5	15.5	0.31%	---	0.31%	---
Tea Tree	13.7	15.7	1.25%	---	---	---
GV-2	26.2	0.0	16.0 μg/mL	128.0 μg/mL	---	---
Peppermint	19.5	8.7	---	---	---	---
Pine	6.7	7.2	---	---	---	---
Lavender	13.0	8.0	---	---	---	---
Spearmint	28.0	9.0	---	---	---	---
Ginger	9.5	0.0	---	---	---	---
Juniper Berry	14.5	0.0	---	---	---	---

Table 1. Zone of Inhibition (diameter), and MIC values (percentage of oil and μg/mL for GV-2) of several essential oils and synthesized compounds. Dashes indicate no testing of the compounds.

A time kill assay was performed to determine if cinnamon bark oil kills bacteria or inhibits its growth. The values of table 2, reported in $\log_{10}$ Colony Forming Units (CFU) per milliliter, depict the increase of bacterial growth that would be expected at each of the sampling times. The concentrations of the growth control, 9.68% and 4.84% DMSO, match those of the cinnamon ark Oil at 8X and 4X the MIC. This allows for better comparison of growth between the control and the cinnamon bark oil. The growth control also depicts that DMSO is not responsible for the decrease in bacteria after incubation as the CFU/mL increases over time. A 3 $\log_{10}$ decrease or greater in bacteria when subtracting the count at 0 hours from 24 hours

constitutes the test compound as bactericidal (14). If the difference is less than a 3 $\log_{10}$, this suggests bacteriostatic (14). According to the summarized results of the time kill (table 3), GV-2, a known bacteriostatic compound, registered a 0.4 $\log_{10}$ and 2.7 $\log_{10}$ decrease in CFU/mL for 4X and 8X times the MIC, respectively, which falls in the given parameter to be determined bacteriostatic.

Cinnamon bark oil had a 3.6 $\log_{10}$ decrease in bacteria at a concentration of 4X the MIC after 24 hours of incubation. The results for 8X the MIC are recorded as ND in tables 2 and 3 because there was no growth detected from undiluted samples. This indicates that the cinnamon bark oil killed such a great number of bacteria that they were virtually undetectable at the 3, 6 and 24 hour tubes, when the results for other concentrations and compounds needed to be diluted in order to be counted. Because there was no growth, no proper $\log_{10}$ value could be associated with the sample, yet these results conclude that the cinnamon bark oil is bactericidal. The experiment was repeated with only cinnamon at 8X the MIC, and the results were consistent with the original experiment.

Time Point (hours)	Growth Control 9.68% DMSO	Growth Control 4.84% DMSO	Cinnamon Bark Oil 8X MIC 0.32%	Cinnamon Bark Oil 4X MIC 0.16%	GV-2 8X MIC 128 µg/mL	GV-2 4X MIC 64 µg/mL
0	5.4	5.7	5.4	5.6	5.7	5.6
3	6.2	6.6	ND	4.7	4.9	5.2
6	7.0	7.8	ND	4.1	4.9	5.3
24	8.8	8.9	ND	2	3.0	5.2

Table 2. Time Kill Experiment summary of the antibacterial activity of cinnamon bark oil and a comparator agent (GV-2) versus *Staphylococcus aureus*. Results shown are reported as $\log_{10}$ colony forming units (CFU) per milliliter. Samples were taken at 0, 3, 6, and 24 hours to determine the CFU in solution at each time point. A result of "ND" indicates that there was no detectable growth from the sample.

Antibacterial Agent	Concentration (Fold-MIC)	Maximal kill at any time point	Bacterial kill at 24 hours
Cinnamon Bark Oil	0.32% (8X)	ND	ND
Cinnamon Bark Oil	0.16% (4X)	-3.6	-3.6
GV-2	128.0 µg/mL (8X)	-2.7	-2.7
GV-2	64.0 µg/mL (4X)	-0.4	-0.4

Table 3. Summarized Time-kill results for cinnamon bark oil and GV-2. Maximal Kill and Kill after 24 hours determined by Bacterial count after incubation minus the initial inoculum. A negative value indicates a net kill and a positive value indicates a net growth. Results of the time kill are reported as $\log_{10}$ CFU/mL. A result of "ND" indicates that there was no detectable growth from the sample.

Having a bactericidal quality can be useful when understanding how a compound interacts with the bacteria it is targeting. Because it is killing bacteria, there may be some concern that cinnamon bark oil could have some toxic effect for human cells. Cinnamon bark oil has been used for years to add flavor, and as a health preventative measure for different types of animals, such as lactating sows and small piglets, indicating that it does not appear to be toxic in minimal doses (2). Cytotoxicity tests have been run on various cinnamon oils, including *Cinnamomum zeylancium* (6, 13), and *Cinnamomum cassia* Blume. Fabio *et al.* tested the

cytotoxicity of several essential oils, and could not determine if cinnamon oil's (*Cinnamomum zeylancium*) antimicrobial activity was due to cytotoxicity, as the MIC was higher than the highest minimum nontoxic concentration (6). On the other hand, Ooi *et al.* concluded that *Cinnamomum zeylancium* inhibited cell growth of rat fibroblast cells in a concentration dependent matter, but did not show cytotoxicity in a time dependent-matter (13).

   While it may not be logical to distribute cinnamon bark oil as an antibiotic, one of its constituents may have antibacterial properties from which an antibiotic could be derived. Researchers have determined that cinnamon bark oil (*Cinnamomum cassia* Blume) is largely composed of trans-cinnamaldehyde, approximately 85%, and nearly 9% o-methoxy-cinnamaldehyde (12). When comparing the microbial activity of cinnamon bark oil to its major component, cinnamaldehyde, results showed nearly the same activity for both (12). As cinnamon bark oil showed promising results in the Zone and MIC tests, one next step in research may be to investigate the individual components of the oil for antimicrobial activity. Further testing on *trans-cinnamaldehyde* and *o-methoxy-cinnamaldehyde* of *Cinnamomum cassia* Blume (12) and *Cinnamomum zelanicum*, (7); and *cinnmyl cinnamate*, and *benzyl cinnamate* of *Cinnamomum zelanicum* (7) to determine MICs and cytotoxicity are worthwhile in the pursuit of novel antibiotics.

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