ANTIMICROBIAL SENSITIVITY AND RESISTANCE DEVELOPMENT CAUSED

BY NUTRACEUTICALS

by

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And approved by

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For centuries, people have used herbal supplements to treat a host of medical ailments. Their use had declined with the discovery of potent pharmaceuticals, however, in recent years, the use of nutraceutical products has seen a huge increase and the industry has grown exponentially. With this increasing use of nutraceutical products, there still remains little knowledge concerning the effects of herbal products on commonly used antibiotics and antimicrobials. These studies were conducted to examine the effects of a small sample of herbal products on antibiotic resistance and sensitivity in bacteria. The herbal products studied were Bee Pollen, Black Walnut, Calendula, Copaiba, Clove, Eucalyptus and Prickly Ash in the form of tinctures, essential oils and 1:1 dilutions of essential oils. Two test strains, *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 were used as representatives of Gram-positive and Gram-negative organisms. These studies showed a thirty-fold increase in the ampicillin MIC values for the Bee Pollen and Prickly Ash exposed *Staphylococcus aureus* ATCC 29213 as well as a four-fold increase in the Bee Pollen and 1:1 diluted Eucalyptus oil exposed *Escherichia coli* ATCC 25922. Additionally, a four-fold decrease in tetracycline and norfloxacin MIC values was observed for the Bee Pollen and
Prickly Ash exposed *Staphylococcus aureus* ATCC 29213 and a four-fold decrease in the sulfamethazine MIC values was observed in the Prickly Ash exposed *Staphylococcus aureus* ATCC 29213. There was neither a substantive increase nor decrease in MIC values for the other products in this study.
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Introduction

For centuries people have used plants for healing. Plant products, as parts of foods or botanical potions and powders, have been used with varying success to cure and prevent diseases throughout history [1]. Written records regarding medicinal plants date back at least 5000 years to the Sumerians, and archeological records suggest even earlier use of medicinal plants [1]. However, the strong bond between plants and humans began to unwind and the twentieth century became a triumph for the synthetic-chemistry-dominated pharmaceutical industry, particularly in the United States [2].

The discovery of new drugs resulted in treatments for conditions that were once considered fatal [2]. The development of antimicrobials, such as sulfonamide in the mid-1930s, made it possible to treat some common and equally life-threatening infections contracted by injury, surgery, or epidemic [2, 3]. This introduction of new, and often extremely useful, synthetic drugs replaced the old botanical products. By the mid-1900s, almost all botanical remedies disappeared from the shelves of pharmacies [3]. By the 1960s, the medicines available in the United States, unlike those utilized by other countries, were primarily synthetic [3].

In the 70s and 80s, however, scientific and clinical reports began to come out of some European countries, especially Germany, indicating that the herbal remedies, which had never been discarded, had many substantial therapeutic and economic benefits for the consumer [3, 4]. With this new knowledge and the rising widespread recognition of recurring problems in treating disease with manufactured drugs, Americans began to
demand herbal products [2, 3]. Answering the call, companies began to supply these products to the public and by the late 1990s annual sales in the USA had reached almost $4 billion [3]. Currently, it is reported that annual sales have increased remarkably creating a $30 billion industry which continues to grow at 5% annum [5].

In the past, food was only thought of as something having taste, aroma or general nutrition. Today, consumers recognize additional categories of foods [6]. Within the last decade, consumers have made increasing reference to the terms “nutraceuticals” and “functional foods”, recognizing the relationship between nutrition and health and the potential of forgoing the use of pharmaceutical drugs [7]. The term “nutraceutical”, a hybrid term between nutrients and pharmaceuticals, was coined in 1989 by the Foundation for Innovation in Medicine, to provide a name for this rapidly growing area of biomedical research [8, 5]. A nutraceutical is defined as “any substance that may be considered a food or part of a food that provides medical or health benefits, including the prevention and treatment of disease” [5]. They are generally sold in medicinal forms not usually associated with food; usually in pill, capsular or ampoule form [9]. Alternatively, functional foods are foods that, by virtue of the presence of physiologically-active components, provide a health benefit beyond basic nutrition [10].

While there has been a growing popularity in nutraceutical products and functional foods, it is noteworthy to mention that these markets are not well-regulated by the U.S. Food and Drug Administration (FDA) [7]. The FDA had long insisted that to obtain approval as a drug in the United States, herbs had to be supported by the same
amount of costly evidence of efficacy required for synthetic drugs [3]. However, the natural products industries faced diverse challenges.

The composition and contents of active constituents in natural plants vary depending on season, climate, temperature, humidity, soil and several other factors [7]. Therefore, the collection, identification and maintenance of uniform quality, quantification and standardization methods are of critical importance [7]. Also, the manufacturing processes, use of solvents and/or additives, purification and drying techniques, and storage conditions can play a major role on the occurrence of significant amounts of contaminants, chemical, physical and biological, in the products [7]. These issues made it very difficult for companies to create a standardized product, which deterred them from investing in the costly tests and research to prove their product’s efficacy. Consequently, since the standards were too high, only a handful of herbs have ever received drug approval in the U.S. [3].

The laws that have eventually shaped the dietary supplement industry as we know it today began in 1906, when Congress passed the Pure Food and Drugs Act and the Meat Inspection Act, both defining food as “articles used for food, drink, confectionery, or condiment by man or other animals, whether simple, mixed or compounded” [11]. They primarily focused on adulteration, misrepresentation or sale of otherwise unfit products [12]. In the following years, the laws governing food and drugs were periodically tweaked and modified.
The landmark 1958 Food Additive Amendment (FAA) to the Federal Food, Drug and Cosmetic Act (FFD&C) addressed concerns for safety and accomplished several things [11]. Firstly, it changed the meaning of the term “food additive” from a technological term to one of legal status by defining the term as a substance not generally recognized as safe and created an entirely new class of substances; those that are generally recognized as safe (GRAS) [12]. The amendment also defines who can determine what GRAS is and the process by which these experts may determine something is GRAS [12].

In 1994, the passage of the Dietary Supplement Health and Education Act (DSHEA) to the FFD&C delineated the role of the FDA in regulating nutraceutical products and dietary supplements and has essentially deregulated the industry [13]. This act does not permit the FDA to consider a new product a “drug” or “food additive” if it falls under the definition of a “dietary supplement” [6]. Dietary supplements are defined in the DSHEA as a product, other than tobacco, intended to supplement the diet that bears or contains one or more of the following ingredients: a vitamin, mineral, herb or other botanical or amino acid [13]. The DSHEA makes manufacturers responsible for the safety of products marketed without pre-marketing safety determination by the FDA and allows certain nutrition support statements on labels also without FDA approval [13]. With this new relaxed law, the industry has grown essentially exponentially [14].

As part of the passage of DSHEA, a new law requiring pre-market safety notification for new dietary ingredients (NDI) became part of the new regulatory
landscape [14]. According to the law, every dietary ingredient in the market before October 15, 1994 is considered an old dietary ingredient and presumed to be safe [14]. Any dietary ingredient marketed after this date is considered new and requires FDA pre-market review for safety [14]. Although the concept may seem easy to understand, it has not been as easy to follow and many marketers are much less aware of the requirements than they are concerned about their implications [14].

Though herbal remedies have been used for centuries, modern chemical pharmaceuticals began to overtake the market, making the use of folk medicine almost nonexistent, particularly in the United States. However, nutraceutical products have gained tremendous popularity over the past few decades and the response of Congress to a public anxious to preserve its access to dietary supplements resulted in a more flexible approach to the law governing food [12]. Where the 1958 Amendment changed the role of the FDA in GRAS matters to gatekeeper, aggressive enforcement of dietary supplements on the part of the Agency prompted Congress to enact amendments to the FD&C Act, specifically for dietary supplements, reducing the burden of proof of safety [12, 14]. Now, the FDA must prove that a dietary supplement is unsafe before appropriate action could be taken [12].

Further curtailing the FDA’s authority are the DSHEA amendments [12]. Though it did establish standards for good manufacturing practices for dietary supplements, DSHEA did not establish standards of quality for individual products and this lack of quality assurance is the biggest single problem in the entire field today [3]. Repeated
studies have shown that the quality of products, even those purported to be standardized on the basis of active or marker compounds varies enormously [3]. There is absolutely no way that consumers can be assured that what is on the label is actually in the package, other than the reputation of the producer [3]. This leaves even knowledgeable consumers bewildered. Until new, stricter laws are created and enforced however, the decision making power will still lie in the hands of the consumers and it is the consumer who will ultimately have to decide what is right for them.
Survey of Literature of Herbal Products and Antimicrobial Properties

A- General Characteristics of Herbals

Centuries of human experience with plants demonstrated that some plants have strong physiological effects that can relieve or cure disease [15]. Many herbalists claimed that all plants have medicinal value, but scientifically, that has not been shown. Plants used for food, for example, are not strictly medicinal though it is clear that they may contain factors such as vitamins and minerals that prevent disease [15].

Plants are continuously in contact with microorganisms, including viruses, bacteria and fungi. Some of these interactions are beneficial to plants, for example the symbiotic relationship between the nitrogen-fixing bacteria rhizobia with leguminous plants [16]. However, many plant-associated microbes are pathogens that affect plant development, reproduction and ultimately yield production [16].

It has been observed that plants produce materials such as starches, fibers, latexes, vitamins and minerals that are necessary for life as well as several distinctive secondary products, or metabolites [15]. And in many cases, it is these secondary metabolites that can serve as plant defense mechanisms against predation by microorganisms and also insects, herbivores and environmental conditions [17, 16]. They may contribute to a plant’s medicinal value [15, 17].

There are numerous secondary metabolites; about 12,000 have been isolated, a number estimated to be less than 10% of the total [17]. A select few however, often
serve as therapeutic chemicals [18]. These include alkaloids, bioflavonoids, essential oils, glycosides, resins, saponins, sterols, tannins, terpenes and other phytochemicals (Table 1). These secondary metabolites are found in specific groups of plants [18]. Of the 310 or so families of seed plants, medicinal plants occur in perhaps 200 to 250 [15]. The daisy family (Compositae), the mint family (Labiatae), the bean family (Leguminosae), the lily family (Liliaceae), the buttercup family (Ranunculaceae), the rose family (Rosaceae) and the carrot family (Umbelliferae) are especially rich in medicinally useful species [15].
Table 1 - Categories of Herbal Chemicals [18]

<table>
<thead>
<tr>
<th>Class</th>
<th>Definition</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>Basic amines (names end in “-ine”).</td>
<td>Includes potent drugs and narcotics. Over 12,000 known; over 13 classes.</td>
</tr>
<tr>
<td>Bioflavonoids</td>
<td>Plant pigments; vitamin-like.</td>
<td>Over 4000 known; over 14 classes.</td>
</tr>
<tr>
<td>Essential Oils</td>
<td>Isoprene derivatives; oxidized terpenes and phenylpropanoids.</td>
<td>Used in perfumes and in aromatherapy. Over 9 classes. Also known as volatile oils, ethereal oils, essences.</td>
</tr>
<tr>
<td>Glycosides</td>
<td>Sugar derivatives attached to aglycones.</td>
<td>Over 10 classes. Over 3000 known.</td>
</tr>
<tr>
<td>Resins</td>
<td>Oxidation products of terpenes; resins are insoluble in water.</td>
<td>Includes oleoresins, gum resins and balsams.</td>
</tr>
<tr>
<td>Saponins</td>
<td>Soap-like glycosides; cause hemolysis if directly introduced into the blood serum.</td>
<td>Various groups of chemicals. Some are involved in steroid metabolism.</td>
</tr>
<tr>
<td>Sterols</td>
<td>Steroid and vitamin D precursors.</td>
<td>Found in soy and other plants; also produced by microorganisms (e.g., sitosterol, stigmasterol).</td>
</tr>
<tr>
<td>Tannins</td>
<td>Polyphenolics, mostly based on gallic acid.</td>
<td>Astringent compounds, bind to protein (tanning); reduce diarrhea, act as hemostatics.</td>
</tr>
<tr>
<td>Terpenes (Terpenoids)</td>
<td>Derived from 5-carbon isoprene units (10, 15, 20, 30, 40, &gt;40)</td>
<td>Over 20,000 known; 6 classes. Most structurally varied phytochemicals.</td>
</tr>
</tbody>
</table>
Alkaloids

Alkaloids have some of the most potent effects on animals and humans, and they demonstrate both therapeutic and toxic properties [18]. They are basic amines, heterocyclic nitrogen-containing substances, which are clear, crystalline and non-volatile [18]. They lack odor, but are bitter in taste and insoluble in water, although their salts are soluble [18]. Included in this class of over 12,000 known agents are purines, pyrrolidines, piperidines, pyridines and quinolines [18]. Some of the best known herbal drugs are alkaloids, including atropine, capsaiacin, morphine, quinine, methylxanthines (such as caffeine and theophylline) and nicotine [17, 18].

Many alkaloids have been shown to possess some antimicrobial effects. Diterpinoid alkaloids, commonly isolated from the plants of the Ranunculaceae, or buttercup family are commonly found to have antimicrobial properties [17]. One chemical compound, berberine, is an especially important representative of the alkaloid group [17]. It is potentially effective against trypanosomes and plasmodia [17]. The mechanism of action of highly aromatic planar quaternary alkaloids such as berberine is attributed to their ability to intercalate with DNA [17].

Phenols and Polyphenols

There are about 8000 known plant phenolics [18]. The simplest phenol is phenol (hydroxybenzene or carbolic acid), which was the first surgical antiseptic used in modern medicine [20]. Other phenols include eugenol (used as a dental analgesic and
disinfectant), carvacrol and thymol, all antiseptics [20]. They are found in essential oils of clove, cinnamon, thyme, oregano and savory and are bactericidal and antifungal [20].

Other classes of phenols include coumarins and bioflavonoids. Coumarins are phenolic substances made of fused benzene and α-pyrone rings and are responsible for the characteristic odor of hay [17]. Their fame has come mainly from their antithrombotic, anti-inflammatory and vasodilatory activities, as demonstrated by the well-known coumarin and warfarin; however there are several other coumarins that have antimicrobial properties [17]. General antimicrobial activity was documented in woodruff (*Galium odoratum*) extracts and coumarin was also found in vitro to inhibit *Candida albicans* [17].

Bioflavonoids or simply flavonoids are therapeutically the most important group of polyphenols that can be broken down into 12 classes: flavans, flavones, flavanones, flavonols, flavanolols, isoflavones, leukoanthocyanins, chalcones, dihydrochalcones, aurones, anthocyanidins and catechins [20]. Several thousand bioflavonoid compounds are known, occurring freely or as glycosides [18]. In general, they are phenolic structures containing one carbonyl group, lack nitrogen, and usually contain two 6-carbon rings joined by three carbon atoms, but 5-membered rings (aurone) and open-chain (chalcone) compounds are included in this group [17, 18]. The flavonoids are often pigmented, appearing yellow or other colors of petals, fruits, and berries in higher plants [18]. They are found in high concentrations in many flowers, and in foods such as citrus fruits, tomatoes, red wine, onions and tea [18].
Since they are known to be synthesized by plants in response to microbial infection, it should not be surprising that they have been found in vitro to be effective antimicrobial substances against an array of microorganisms [17]. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls [17]. More lipophilic flavonoids may also disrupt microbial membranes [17].

Catechins, the most reduced form of the C₃ unit in flavonoid compounds, deserve special mention [17]. These flavonoids have been extensively researched due to their occurrence in oolong green teas [17]. It was noted that teas contain a mixture of catechin compounds, which have shown in in vitro studies to inhibit Vibrio cholera O1, Streptococcus mutans, Shigella spp., and other bacteria and microorganisms [17]. The catechins inactivated cholera toxin in Vibrio cholera and inhibited isolated bacterial glucosyltransferases in S. mutans, possibly due to complexing activities [17].

There are two possible explanations for the mechanism of action of flavonoids. Some research has found that flavonoids lacking hydroxyl groups on their β-rings are more active against microorganisms than those with the hydroxyl groups, which supports the proposed idea that their microbial target is the membrane [17, 18]. Lipophilic compounds would be more disruptive of this structure [17, 18]. However, there has been research that has found the opposite effect, which proves that there is no clear predictability for the degree of hydroxylation and toxicity to microorganisms [17, 18].
Essential Oils and Terpenoids

The antimicrobial effects of essential oils have been reported and used in herbal medicine in many countries [21]. Essential oils, also known as volatile oils or essences, constitute the attractant odors of flowers and the attractant or defensive components of the other parts of the plant [18, 21]. These oils are secondary metabolites that are highly enriched in compounds based on an isoprene structure (C$_5$H$_8$) [17, 18]. Some are terpenes (C$_{10}$H$_{16}$) and can occur as diterpenes, triterpenes, tetraterpenes, hemiterpenes and sesquiterpenes [17]. When the compounds contain additional elements, usually oxygen, they are termed terpenoids [17].

Terpenoids have been shown to be active against many bacteria, fungi, viruses and protozoa [17, 18]. In 1977, it was reported that 60% of essential oil derivatives examined at the time were inhibitory to fungi while 30% inhibited bacteria [17, 18]. The mechanism of action of terpenes remains not fully understood; however, it was speculated that terpenes disrupt cell membranes [17, 18].

Food scientists found the terpenoids present in essential oils of plants to be useful in the control of *Listeria monocytogenes* [17]. Also, the ethanol-soluble fraction of purple prairie clover yielded a terpenoid called petalostemumol, which showed excellent activity against *Bacillus subtilis* and *Staphylococcus aureus* and lesser activity against gram-negative bacteria as well as *Candida albicans* [17].
**Glycosides and Saponins**

Numerous plant chemicals contain a carbohydrate residue, or glycone, attached to a noncarbohydrate residue, or aglycone, to form a glycoside [18]. If hydrolysis yields glucose, the originating glycoside is termed a glucoside, in contrast to non-glucosides that yield other sugars [18]. Glycosides usually have a characteristic odor of bitter almonds and are usually bitter tasting; however, some are sweet [18, 20].

Saponins are glycosides with terpenoid aglycone components [18]. They possess two major characteristics: they have soap-like surfactant effects, and they cause hemolysis when directly introduced into the blood stream [18]. There are three major classes of saponins, steroidal, terpenoid and glycoalkaloid saponins, and a number of them have potential medicinal effects, including anti-inflammatory, immune-boosting and expectorant properties [18, 20].

**Resins and Gums**

When a plant is injured, it may exude a hard-setting material to cover the wound [18]. This material, known as a resin, is a semi-solid, amorphous substance that, in general, is soluble in alcohol and ether [20]. Resins are composed of resin alcohols, resenes, esters and other compounds; the main components are oxidation products of terpenes [18]. There are three main types of resins: oleoresins, gum resins and balsamic resins [20].
Oleoresins are a mixture of resin components and volatile oils [18]. Turpentine is a primary example of an oleoresin. Gum resins are complex oleoresins that are soluble in alcohol and form emulsions with water [18, 20]. Myrrh and frankincense are both gum resins. Balsam of Peru and benzoin are examples of balsamic resins. Balsamic resins are resins combined with cinnamic or benzoic acids or their esters [18]. They dissolve in alcohol, but not water [20].

Resins are used as astringents and antiseptics, and seem to have immune-stimulating properties along with antiseptic activity [20]. They are traditionally used for wounds, both to seal and to disinfect [20]. Balsam of Peru has been shown to directly kill bacteria, fungi and parasites.

Tannins

Tannins are polymeric phenolic substances, which have an astringent effect and can precipitate proteins in the tanning process that produces leather [17, 18]. They can be divided into two main groups, hydrolyzable and condensed tannins [17, 18]. Hydrolyzable tannins are based on gallic acid, usually as multiple esters with D-glucose, while the more numerous condensed tannins called proanthocyanidins are derived from flavonoid monomers [17].

Tannins have been shown to have antimicrobial action. They form complexes with proteins through so-called nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation [17]. Thus, their mode of
antimicrobial action may be related to their ability to inactivate microbial adhesions, enzymes and cell envelope transport protein [17]. They also form complexes with polysaccharides [17].

According to studies, tannins can be toxic to filamentous fungi, yeasts and bacteria [17]. Condensed tannins have been determined to bind to cell walls of ruminal bacteria, preventing growth and protease activity [17]. Also, though still speculative, tannins are considered at least partially responsible for the antibiotic activity of methanolic extracts of the bark of *Terminalia alata* found in Nepal [17].

**Sterols**

These compounds are precursors of steroids, including ergosterol, dihydrotachysterol and ergocalciferol, which are part of the Vitamin D complex [18]. The important phytosterols, β-sitosterol, stigmasterol and campesterol, are found in popular sources such as olive oil and soy and have been shown to possibly reduce LDL cholesterol and lower serum cholesterol [18]. Similar sterol combinations in ginseng are credited with adaptogenic properties [18]. Other phytosterol benefits that are claimed include anti-inflammatory properties, immune enhancement, anti-cancer activity and antioxidant effects [17].
B- Experimental Herbs in these Studies

In this study, seven nutraceuticals were chosen based on some indication of antimicrobial activity. Many of the families which contain a high number of medicinally active species were represented, such as the daisy, mint and bean families. The herbs also represent a wide range of activity against gram-negative and gram-positive organisms as well as activity against certain types of parasites and fungi. These nutraceuticals studied are individually derived from almost every part of the plant and are used in a variety of forms (Table 2).

Calendula (Marigold)

Calendula (*Calendula officinalis*) is commonly known as pot marigold or golden marigold and is a member of the Asteraceae family. The name refers to its tendency to bear flowers by the calendar, once a month, in warm climates during the new moon [2]. “Marigold” also refers to the Virgin Mary and is traditionally used in Catholic celebrations concerning the Virgin [2].

It is an annual aromatic plant native to the Mediterranean countries, but is found throughout central and southern Europe, western Asia, and the United States [2, 22, 23]. The plant grows between 30 and 50 cm high and on the tip of each stem, there is a many-petaled orange or yellow flowering head 4 to 7 cm in diameter [2, 23]. Though widely grown as an ornamental garden plant, it is also cultivated and the flowers harvested for use in herbal medicine throughout Eastern Europe and Latin America [2].
<table>
<thead>
<tr>
<th>Name</th>
<th>Latin Name</th>
<th>Common Name</th>
<th>Part Used</th>
<th>Antimicrobial Activity</th>
<th>Available Form</th>
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<tbody>
<tr>
<td>Calendula</td>
<td>Calendula officinalis</td>
<td>Pot calendula, pot marigold</td>
<td>Flowers</td>
<td>This herb has strong bactericidal effect and may counteract infection with <em>H. pylori</em> and <em>S. aureus.</em></td>
<td>Tinctures, cream and aerosol sprays</td>
</tr>
<tr>
<td>Clove</td>
<td>Syzygium aromaticum</td>
<td>None</td>
<td>Oil extracted from leaves and flowers and fresh dried flower buds</td>
<td>Kills some types of bacteria associated with food poisoning, including <em>P. aeruginosa,</em> <em>Shigella</em> (many species), <em>S. aureus,</em> and <em>S. pneumoniae</em></td>
<td>Tincture and essential oil</td>
</tr>
<tr>
<td>Copaiba</td>
<td>Capifera species</td>
<td>Balsam copaiba, copal, Jesuit’s balsam, mal-dos-sete-dias</td>
<td>Resin is collected, and the sap is distilled to concentrate its oils</td>
<td>Volatile oil acts as an antimicrobial and prevents secondary infections in eczema, herpes, and psoriasis</td>
<td>Oil, capsules, ointments, powders, and tinctures</td>
</tr>
<tr>
<td>Eucalyptus</td>
<td>Eucalyptus globules</td>
<td>Blue gum, red gum</td>
<td>Leaves are distilled for the oil</td>
<td>Contains the chemical eucalyptol, which kills several types of bacteria, including <em>B. subtilis</em> and several strains of <em>Streptococcus.</em></td>
<td>Tincture and essential oil</td>
</tr>
<tr>
<td>Bee Pollen</td>
<td>None</td>
<td>None</td>
<td>Microspores (male reproductive elements)</td>
<td>Acts against bacteria, including <em>Colibacilli</em> and certain strains of <em>Salmonella</em></td>
<td>Raw and micronized form</td>
</tr>
<tr>
<td>Prickly Ash</td>
<td>Xanthoxylum americanum and Xanthoxylum bungeanum (Rutaceae [citrus] family)</td>
<td>Angelica tree, xanthoxylum</td>
<td>Fruits and bark</td>
<td>The chemical constituents that give prickly ash its heat kills food borne bacteria</td>
<td>Tincture, tea, capsules</td>
</tr>
<tr>
<td>Walnut leaf</td>
<td>Juglans nigra, Juglans regia (Juglandaceae [walnut] family)</td>
<td>Black walnut and white walnut</td>
<td>Leaves</td>
<td>Contain two antibacterial agents, walnut essential oil and juglone, which act directly on infectious microorganisms</td>
<td>Teas</td>
</tr>
</tbody>
</table>
Many studies of calendula flowers have been carried out, especially in Europe, without revealing anything unique in their physiological properties [22]. However, a volatile oil, bitter principles, carotenoids, mucilage, resin, polysaccharides, plant acids, various triterpene alcohols, saponins and other glycosides and sterols were found to be present [22, 23, 25]. Many of the individual constituents in these general groups have been identified.

Calendula has been used as an antibacterial, anti-inflammatory and pain killer [22]. Numerous studies demonstrated that the flowers had antimicrobial activity due to the terpene alkaloids, lactone and flavones contained in the essential oil [23, 24]. Flavonoids isolated from calendula flowers showed positive antimicrobial activity against *Staphylococcus aureus* at a concentration of 1mg/mL [2, 23]. Other studies have demonstrated the flavones to be effective against *Klebsiella pneumoniae, Micrococcus lutea* and *Candida monosa* [23].

Calendula extract is available as an aerosol spray which is applied to cuts and scrapes to prevent infection and to help stop bleeding [2]. It is also used to treat conjunctivitis infections in the form of eye drops and taken internally as a tea [2]. It was shown to have a strong bactericidal effect on *Helicobacter pylori*, the bacterium associated with gastritis and peptic ulcers, and aids in healing duodenal and peptic ulcers [2].
Clove

One of the most popular spices throughout history, clove (Syzygium aromaticum) is a product of an evergreen tree native to the Spice Islands [2, 19]. The clove tree thrives only in a tropical climate near the sea where it grows to a height of about thirty feet [19]. The leaves resemble bay leaves and the flower buds are pinkish-red in color [19, 23]. The oil of cloves is extracted from the leaves and flower buds and is the principal form of clove used medicinally [2, 23].

The use of clove in medicine dates back to ancient China and was widely used throughout the ages [2]. In China, the members of the upper classes would suck on the spice to cure halitosis [2]. Early physicians prescribed the spice as an aid to digestion, believing that it strengthened the stomach, liver and heart [19].

Today, clove has been shown to be antiseptic, antibacterial, antifungal, antiviral, spasmolytic and a local anesthetic [2, 23]. There are a number of compounds discovered in the oil including flavonoids, tannins, triterpenes, steroids and volatile oil that contribute to its medicinal properties [23]. In one study, conducted by Nascimento, et al. [25], the phytochemical eugenol, which was extracted from clove and is a constituent of the essential oil, was found to have the highest antimicrobial activity against a number of microorganisms including Klebsiella pneumoniae and Proteus spp. The clove oil kills other types of bacteria, including Pseudomonas aeruginosa, Shigella (many species), Staphylococcus aureus, and Streptococcus pneumonia, all of which can be involved in food poisoning [2]. Also, most importantly, another study reported clove oil to have a
high antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Bacillus subtilis* [21].

Clove is also taken internally for relieving the sensation of gas that frequently troubles patients with peptic ulcers [2, 23]. It is thought that the chief component of the volatile oil, eugenol, depresses nerve impulses that convey the feeling of bloating [2]. It has also been used around the world for generations to relieve pain from toothache and dental treatment [2, 19, 23].

**Copaiba**

Copaiba is a member of the Fabaceae (legume) family. Other common names for this medicinal plant are balsam copaiba, copal and Jesuit’s balsam [2]. It is a giant tropical legume found in the rainforests of Brazil, Colombia and Venezuela and can grow in height from sixty to one hundred feet [2]. The part used medicinally is the resin oil that accumulates in cavities within the tree trunk [2, 23, 26]. The tree is tapped, much like a rubber tree, to harvest the oil [2]. Once collected, it is distilled to concentrate the essential oils [2]. The resin oil consists of diterpinoid oleoresins and essential oil made up of alpha-and beta-caryophyllene, beta-bisabolene and L-cadinene [23]. The resin oil (oleoresin) ranges in viscosity from very liquid to a resin-like substance, and in color from a pale yellow to a red or fluorescent tint [23].

The medicinal properties of copaiba oils were known among Native American, who observed that animals rubbed themselves on copaiba tree trunks to heal their wounds
The effects attributed to copaiba oils in folk medicine include anti-inflammatory, anti-tetanus, anti-tumor, anti-blenorrhagea, and urinary antiseptic activities [23, 27].

Laboratory research showed that the resin acted by reducing the permeability of capillary walls to histamine, the chemical responsible for painful swelling [2]. The oleoresin also showed a bacteriostatic effect on the urinary tract and has antimicrobial properties that are used to prevent secondary infections in eczema, herpes and psoriasis [2, 23]. Studies indicated that the main constituents of the copaiba oil, sesquiterpenes and diterpenes, have bactericidal activity against a wide spectrum of gram-positive organisms, including methicillin-resistant *Staphylococcus aureus* (MRSA), for which there are very few therapeutic alternatives [26, 27].

**Eucalyptus**

The plant family Myrtaceae is comprised of 3800 species distributed in 140 genera occurring along tropical and subtropical regions of the world [28]. This family represents an important source of essential oils with biological activities such as being bacteriostatic, fungistatic and having anti-inflammatory properties [28]. Within this family, the *Eucalyptus* genus has been cultivated and exploited on a large scale for many years [28].

Eucalyptus (*Eucalyptus globulus*) is one of the fastest growing trees in existence [19]. Also known as red gum or blue gum, the Eucalyptus tree is a native of Australia and Tasmania, but is now currently cultivated in warm climates throughout the world [2,
The leaves are the medicinal part of the plant and have been used extensively in traditional aboriginal herbal remedies [2]. Though native to Australia, its therapeutic uses have been introduced and integrated into traditional medicine systems, including Chinese, Indian, Ayurvedic, and Greco-European [29].

The young leaves of the tree are heart-shaped, bluish-green and sticky whereas the mature leaves are lance-shaped, green and smooth in appearance [19]. The leaves of the tree can be harvested at any time of the year and undergo steam distillation, followed by rectification to obtain the essential oil [2, 23, 29]. The essential oil consists of a volatile oil rich in 1,8-cineole, specifically p-cymene, alpha-pinene, limonene, geraniol and camphene [23]. This volatile oil, eucalyptol, is the active ingredient of the eucalyptus oil and is responsible for its various pharmacological actions [29].

Eucalyptus is used to treat many different ailments. Taken as a tea, it is used to loosen phlegm in the chest and helps open clogged nasal passages [2]. It is also used in ointments to ease muscle soreness due to the oil’s ability to increase blood flow to muscle tissue [2, 23]. Eucalyptus oil has antibacterial and fungicidal activity; in particular, it has been shown to kill *Bacillus subtilis* and several strains of *Streptococcus* [2]. Studies conducted by Trivedi et al. [29], demonstrated that eucalyptus oil had antimicrobial activity against many antibiotic-resistant species including *Klebsiella* spp., *E. coli*, *Proteus* spp., and *Pseudomonas* spp. Because of its antiseptic properties, eucalyptus can be used as a topical antiseptic for minor cuts and scrapes [2].
**Bee Pollen**

Bee pollen consists of the dustlike, air- or insect-borne male reproductive cells of flowering plants [2]. Often, the marketed product is designated bee pollen, which implies that a mixture of pollens from various plants was collected by bees; however this is not always the case [22]. A meshlike pollen trap has been developed where upon reentry to the hive; some of the pollen is removed from the bee’s back legs [22]. However, since it is impossible to determine if a particular pollen grain was originally collected by a bee or not, the marketed product is usually referred to simply as pollen [2].

While pollen is a plant product, it is not technically an herb, but it has been called the miracle food [2]. The chemical constituents of pollen have been extensively investigated and although the different components vary greatly in quantity among pollens of different species, some general ranges have been determined [2, 22]. Polysaccharides particularly starch and cell-wall constituents constitute up to 50% of typical pollen [22]. Low molecular weight carbohydrates make up another 4 to 10 percent and lipids, such as waxes, fats and oils, are extremely variable ranging from 1 to 20% [22]. Protein exists to the extent of 5.9 to 28.3% and approximately 6% of free amino acids are present [22]. Other constituents include about 0.2% of carotenoid and flavonoid pigments plus small amounts of terpenes and sterols [22]. In addition, pollen had also been found to contain vitamins including the B Vitamins and Vitamins A, C, E and K and minerals, such as calcium, copper, iron, magnesium, phosphorous, potassium, silicon and zinc [2].
Pollen, as well as other apicultural products, has gained increased attention for its therapeutic properties. In studies, pollen was reported to both protect the prostate gland and stimulate the production of testosterone [2]. For patients undergoing radiation therapy, pollen helps to protect the liver from depletion of antioxidant stores, which fight free radicals that can harm healthy tissues during the treatments [2]. It has been suggested that pollen has positive effects in treating rheumatoid arthritis and disorders of the stomach, intestines, gallbladder and liver [2, 22].

Many studies have also demonstrated that pollen contains antimicrobial substances that act against bacteria, including *Colibacilli* and certain strains of *Salmonella* [2]. The main antimicrobial action has been attributed to several phenolic compounds with antioxidant activity [30]. Studies conducted by Carpes et al. [30], showed that pollen has strong antimicrobial effects against *B. subtilis*, *P. aeruginosa*, *Klebsiella* spp., *B. cereus* and *S. aureus*. An additional study conducted by Knazovicka et al. [32], demonstrated antimicrobial activity against *P. aeruginosa*, *S. aureus*, and *E. coli*, as well as *Listeria monocytogenes* and *Salmonella enterica*.

**Prickly Ash**

Prickly ash, *Xanthoxylum americanum*, also referred to as toothache tree, yellow wood and angelica tree, is a member of the Rutaceae (citrus) family [2, 15, 23]. It grows throughout China, especially in the Szechuan province and in North America, including New Jersey [2, 15].
The plant is an aromatic shrub or small tree that grows to about 3 meters in height [23]. The branches are prickly, with leaves divided into 5 to 11 toothed leaflets that are lemon-scented when crushed [15]. Flowers, which bloom in April shortly before the leaves appear, are small and green in color and grow in auxiliary clusters [15]. The shiny red berries, which first appear in July, and the root bark are the parts harvested and used for their medicinal qualities [2, 15, 23]. The medicinal parts have shown to contain active compounds including pyranocoumarins, isoquinoline alkaloids, volatile oils and resins [23].

Prickly ash has many medicinal applications. Poultices and infusions of the tree are used to treat colds, coughs and pulmonary problems [15]. A combination of the bark and berries was used to make expectorants and cough syrups [15]. Along with the prickly ash’s products pain-relieving and anti-inflammatory properties, the products also showed to have strong antimicrobial effects [2, 15]. The chemical constituents that give prickly ash its “heat” also kill many foodborne bacteria and parasites [2]. Also, studies by Borchardt et al. [32], have found that prickly ash has potent antimicrobial activity against \textit{Staphylococcus aureus}.

**Black Walnut**

Walnut leaf, commonly referred to as black walnut, \textit{Juglans nigra}, is a member of the Juglandaceae (walnut) family [2, 15]. Walnut trees are native to the dry temperate zones of western Asia, China, India and the United States [23]. The tree can grow to about 120 feet in height. It has pinnate leaves, with 12 to 23 almost alternate, toothed
leaflets and produces spherical fruit [15]. The leaves of the black walnut tree are the parts that are harvested in the spring and summer and dried for medicinal purposes [2].

Walnut leaves have been used in herbal medicine for thousands of years. The Roman naturalist, Pliny the Elder, reported the cultivation of walnut trees in the first century; the trees having reached Rome from the Middle East [2]. The Latin name of the tree is derived from a reference to the god Jupiter [2]. *Juglans* is derived from combining the name *Jupiter* with *glans* (acorn), literally interpreted as “Jupiter’s nuts” [2]. Native Americans made poultices, infusions, and decoctions of the bark and leaves of the walnut tree to treat many different ailments for generations [15]. It was noted that the famed seventeenth-century English herbalist Nicholas Culpepper combined walnut leaf with honey, onion and salt to draw out venom from the bites of snakes and spiders [2].

A number of chemical compounds including fatty oil, tannins and juglone contribute to the medicinal properties of the black walnut [23, 33]. Black walnut is used to treat athlete’s foot and a variety of parasitic infections. Studies have shown that the oil has strong antifungal activity against a variety of yeast species, including *Candida albicans, Saccharomyces cerevisiae*, and others [33]. It has also shown to have potent antimicrobial properties.

Walnut leaves contain astringent tannins, which cross-link skin cells making them impermeable to allergens and infectious microorganisms [2]. In addition, the leaves contain two antimicrobial agents, walnut essential oil and juglone, which act directly on
infectious microorganisms [2]. Juglone is a naphthioquinone, which is found in all plant organs in most members of the family Juglandaceae [33]. Studies by Borchardt et al. [33], showed through the disk diffusion technique that walnut oil has antimicrobial activity against *Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus*. The leaves also contain relatively large concentrations of Vitamin C, which helps fight infection [2].
Hypothesis

When chemical compounds exhibit antimicrobial properties against a microorganism, the microorganism evolves over time, to develop resistance towards the inhibitory substances. In this research, seven different herbal products were tested, as tinctures, essential oils and dilutions of oils, against two different bacterial species. These indicator organisms represented Gram-positive and Gram-negative bacteria. Two different methods, the agar diffusion assay and the MIC determination, were used to determine if the hypothesis is proven or disproven.
Materials and Methods

Nutraceutical Products

The following lists the nutraceutical tinctures, essential oils and other products used in this research along with the manufactures: Bee Pollen capsules, Nature’s Herbs, Wild Countryside Herbal Supplement (Twinlabs, American Fork, Utah); 
Juglans nigra, Black Walnut Liquid Herbal Extract, Fresh green hull (Herb Pharm, Williams, OR); 
Calendula officinalis, Calendula flower herbal tincture (Nature’s Answer, Hauppauge, N.Y.); 
Eugenia caryophyllata, Clove Buds, Aromatherapy Essential Oil, Aroma Vera (American Brand Labs); 
Syzygium aromaticum (flower), Organic Clove herbal tincture, Energique Herbal (Energique, Inc., Woodbine, IA); 
Copaifera officinalis, Amazon Copaiba Oil (Raintree Nutrition, Inc, Austin, TX); 
Eucalyptus globules (leaf), Eucalyptus herbal tincture, Energique Herbal (Energique, Inc., Woodbine, IA); 
Eucalyptus globules, Eucalyptus Aromatherapy Essential Oil, Aroma Vera (American Brand Labs); 
Zanthoxylum americanus (bark), Prickly Ash herbal tincture, Energique Herbal (Energique, Inc., Woodbine, IA).

Indicator Organisms

The organisms used represented both gram-negative and gram-positive species. They were Escherichia coli ATCC 25922 and Staphylococcus aureus ATCC 29213. These ATCC strains were chosen because they demonstrated an acceptable range of sensitivity to a wide range of antimicrobials and antibiotics.
The panel of antibiotics chosen as markers of resistance was as follows: ampicillin (Ampicillin Sodium Salt, Sigma-Aldrich Inc., St. Louis, MO); erythromycin (Sigma-Aldrich Inc., St. Louis MO); kanamycin (Kanamycin Monosulfate, Sigma-Aldrich Inc., St. Louis, MO); norfloxacin (Sigma-Aldrich Inc., St. Louis, MO); vancomycin (Vancomycin Hydrochloride, Sigma-Aldrich Inc., St. Louis, MO); sulfamethazine (Sigma-Aldrich Inc., St. Louis, MO); and tetracycline (Tetracycline Hydrochloride, Sigma-Aldrich Inc., St. Louis, MO). These antibiotics represent many of the common classes of antibiotics used, in both human and veterinary medicine.

The minimum inhibitory concentrations (MICs) for *S. aureus* ATCC 29213 for this marker panel in µg/mL were as follows: ampicillin, 0.4; erythromycin, 0.4; kanamycin, 1.6; norfloxacin, 0.8; vancomycin, 0.8; sulfamethazine, 200; tetracycline, 0.2. For *E. coli* ATCC 25922, the MICs in µg/mL were as follows: ampicillin, 3.1; erythromycin, 0.2; kanamycin, 3.1; norfloxacin, 0.2; vancomycin, 0.8; sulfamethazine, 25; tetracycline, 0.8.

**Culture Preparation**

To grow and maintain the cultures, tryptic soy broth (TSB) and tryptic soy agar (TSA) (Difco Laboratories, Detroit, MI) were used. Tryptic Soy Broth (TSB) was used for the MIC determinations and TSA was used for the agar diffusion assays. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were purchased from the American Type Culture Collection as a lyophilized form. The organisms were prepared by reconstituting the lyophilized cultures and then growing the organisms in TSB, at
37°C overnight with gentle shaking. *E. coli* cultures were streaked onto eosin methylene blue (EMB) agar for single colony isolation and purity confirmation. Likewise, *S. aureus* cultures were streaked onto TSA containing 5% NaCl for single colony isolation and purity confirmation. Single colonies were picked and inoculated into tubes containing TSB and grown as previously described. Stock cultures were streaked on TSA slants and stored at 4°C. Fresh stocks were prepared and stored every two weeks; however, on experiment days, overnight cultures were used.

**Preparation of Nutraceuticals for Experimentation**

The three forms of nutraceutical products used were commercially available tinctures, tinctures prepared in laboratory from capsule or tablet form and commercially produced essential oils. All commercially produced tinctures were used directly from the bottles with no alterations or dilutions. To make tinctures from a capsule or tablet form, a daily dose (according to the manufacture’s specifications) of the nutraceutical was extracted in 50mL of 50% sterile ethanol at room temperature with shaking for a 24 hour period. The essential oils were used at both 100% concentration and at 50% concentration (diluted 1:1 with sterile 50% ethanol). Before experimentation, all herbal products were sterilized, using a syringe and a 0.22 µm filter disk attachment. Sterile 50% ethanol, prepared by filtering through a 0.22 µm filter, was used as the control throughout all exposures.
Agar Diffusion Assay

The agar diffusion technique is a routine, economical and easy screening method used to determine the susceptibility or resistance of a bacterial strain to antibacterial agents. In the assay, the antimicrobial agents were placed into cylinders on top of seeded agar. Following an overnight incubation at 37°C, the agar is examined. If bacterial growth is continuous to the reservoir, then the bacterial strain is deemed to be either insensitive to or resistant to that substance. However, if there is a circular clearing around the well or cylinder, the bacteria were inhibited by the agent. The size of the inhibition zone can be measured.

In this study, the cylinder-plate diffusion assay was used to determine the response of *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 to eleven nutraceutical tinctures and essential oils. For this assay, 20mL of seeded TSA (cooled to 50°C before the inoculum) was added to Petri dishes. When the seeded agar solidified, four 8mm OD stainless steel cylinders were placed evenly on the surface of all the seeded plates at 90° intervals. Three of these wells were charged with 100uL of the nutraceutical being studied; one was a control well charged with 100uL of 50% sterile ethanol. The plates were incubated overnight at 37°C, and were examined the next day. Clear zones of inhibition and zones displaying regrowth were measured using a millimeter ruler and calipers.

In order to study the development of bacterial resistance after the exposure to the nutraceuticals, bacteria found in any of the clear zones of inhibition or the regrowth zones were aseptically removed and transferred to sterile TSB tubes. These isolated organisms were used as the inoculum for the second exposure, again using the agar diffusion cylinder technique. Each strain of the isolated bacteria was exposed to the nutraceutical
tinctures a total of three times, as previously mentioned for a view of developing resistance, if any.

**Antibiotic Preparation and MIC Determination**

*E. coli* ATCC 25922 and *S. aureus* ATCC 29213 cultures were grown in TSB overnight at 37°C with shaking. Based on turbidity measurements, correlated with plate counts, the cultures were diluted to approximately $10^5$ CFU/mL in sterile saline. From the $10^5$ CFU/mL dilution, 200 µL were added to 19.8mL of tryptic soy broth (TSB) to yield approximately $10^3$ CFU/mL of organism. Using a multichannel pipettor, 125 uL of seeded TSB was added to each well of a 96-well microtiter plate. To the first well of each of the next seven rows in the 96-well microtiter plate, 125 uL of the antibiotic stocks were added to the 125 uL of seeded TSB. The first well of the last row in the 96-well microtiter plate will contain 125 uL of 50% ethanol as a control. The wells were mixed well using the multichannel pipettor. After mixing, 125 uL of nutratechical/seeded TSB from column 1 was transferred to the 125 uL of seeded broth in column 2. These wells were mixed and the process continued in the same manner to the column 11, changing pipettor tips for each dilution. For column 12, 125 uL were transferred from column 11 to column 12, the contents of these wells were mixed, and then 125 uL were discarded from column 12, which assured that every well in the 96-well microtiter plate had only 125 uL.

After incubating overnight at 37°C, the absorbance of the medium in the wells of the microtiter plates was measured using an ELISA reader (SLT Lab Instruments,
Research Triangle Park, N.C.). The absorbance of each well was measured at 620nm [34]. The MIC was defined as the concentration of the last well where no growth occurred within 24 hrs [34]. A substantive increase in the absorbance was at least twice the baseline absorbance [34].
Results and Discussion

All of the products used in these studies were chosen based on the availability of the products commercially and the purported antimicrobial activity found in the literature. In this research, a total of 7 products were studied for their antimicrobial activity and the effect they had on the MICs of the test organisms, *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922. The products were Calendula, Clove, Copaiba, Eucalyptus, Bee Pollen, Prickly Ash, and Black Walnut. Calendula was reported to have antibacterial activity [22]; Clove was reported to have antiseptic properties along with antibacterial, antifungal and antiviral properties [23]; Copaiba is reported to have antiseptic properties [26]; Eucalyptus has been reported to have antiseptic properties and strong antibacterial activity, including activity against some antibiotic resistant organisms [2, 29]; Bee Pollen was reported to possess antibacterial properties against some species that cause food borne illnesses [2]; Prickly Ash was used for its anti-inflammatory and antimicrobial properties [32]; and, Black Walnut was purported to have antifungal, anti-parasitic and potent antimicrobial activity [33].

The products were randomly chosen based on their commercial availability. No brand was specifically chosen over another. All nutraceuticals were purchased in either tincture, essential oil or in capsule form and both tinctures and essential oils were used in experimentation. Products in capsule form were made into a tincture by diluting a daily dose, based on the directions of the specific manufacturer, into 50mL of 50% sterile alcohol. Black Walnut, Calendula, and Prickly Ash were purchased in tincture form only; Clove and Eucalyptus were purchased as a tincture, an essential oil, and an
additional tincture was made from the oil by diluting the product 1:1 in 50% sterile alcohol; Copaiba was purchased as an essential oil and was made into a tincture by diluting the oil 1:1 in 50% sterile alcohol; and, Bee Pollen was purchased in capsule form and, for this particular brand, Nature’s Promise (Twinlabs, American Fork, Utah), the daily dose of nine capsules was dissolved in 50% sterile alcohol to make a tincture.

One main concern with these products is the lack of standardization, particularly with dosages and alcohol content in the commercially available tinctures. Only one brand was studied for each herb in these experiments. Research into other brands and their daily dosages and alcohol content could demonstrate that huge variability exists between company products. The same herb may be grown, harvested, and processed differently, and therefore, daily dosages will differ immensely between various manufacturers. Some products may be inherently more or less potent depending upon the alcohol content of the tincture. Since there is no standardization in the nutraceutical market it is very difficult to form generalized conclusions for any of the experimental herbs. The data presented, therefore represents results for these specific brands of herbs only.

To establish whether these specific herbal products possessed antibacterial activity, two analytical procedures were used; the agar diffusion assay and the MIC determination. The agar diffusion assay was used to determine whether the tinctures, oil or dilutions expressed any antibacterial activity. The second analytical system, the MIC
determination, was used to determine the presence of antibacterial activity in the extracts and to titrate this activity after the third exposure of the test culture [34].

Table 3 shows the average results from the three exposures (P1, P2, and P3) in the agar diffusion assays for all products assayed against the *S. aureus* ATCC 29213 and *E. coli* ATCC 25922. The numbers represent the diameter of the zones of inhibition on the agar plate in millimeters. Tables 4 and 5 show the MICs (µg/mL of antimicrobial activity) resulting from the nutraceutical extract against *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 respectively.
Table 3 – Resistance development in test strains over the course of three passages caused by nutraceutical alcohol extracts

<table>
<thead>
<tr>
<th>Product</th>
<th>E. coli ATCC 25922</th>
<th>Resistance Development</th>
<th>S. aureus ATCC 29213</th>
<th>Resistance Development</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P1</td>
<td>P2</td>
<td>P3</td>
<td>P1</td>
</tr>
<tr>
<td>Bee Pollen</td>
<td>23.0</td>
<td>36.7</td>
<td>38.3</td>
<td>Decrease</td>
</tr>
<tr>
<td>Black Walnut</td>
<td>40.3</td>
<td>41.7</td>
<td>41.7</td>
<td>No Change</td>
</tr>
<tr>
<td>Calendula</td>
<td>45.0</td>
<td>44.7</td>
<td>40.3</td>
<td>Increase</td>
</tr>
<tr>
<td>Clove Oil</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NA</td>
</tr>
<tr>
<td>Clove Oil 1:1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NA</td>
</tr>
<tr>
<td>Clove Tincture</td>
<td>31.3</td>
<td>36.0</td>
<td>38.7</td>
<td>Decrease</td>
</tr>
<tr>
<td>Copaiba Oil 1:1*</td>
<td>NZ</td>
<td>NZ</td>
<td>NZ</td>
<td>NA</td>
</tr>
<tr>
<td>Eucalyptus Oil</td>
<td>29.0</td>
<td>26.3</td>
<td>12.7</td>
<td>Increase</td>
</tr>
<tr>
<td>Eucalyptus Oil 1:1*</td>
<td>21.0</td>
<td>15.3</td>
<td>12.3</td>
<td>Increase</td>
</tr>
<tr>
<td>Eucalyptus Tincture</td>
<td>NZ</td>
<td>NZ</td>
<td>NZ</td>
<td>NA</td>
</tr>
<tr>
<td>Prickly Ash</td>
<td>NZ</td>
<td>NZ</td>
<td>NZ</td>
<td>NA</td>
</tr>
</tbody>
</table>

*P indicates passage
* Essential oils were diluted 1:1 using 50% sterile ethanol
- complete sensitivity; no zones of inhibition could be measured
NZ no zones
NA not applicable
Table 4 – MICs (µg/mL) resulting from the nutraceutical extract and antimicrobial using *Staphylococcus aureus* ATCC 29213 as the indicator organism

<table>
<thead>
<tr>
<th></th>
<th>AMP</th>
<th>ERY</th>
<th>KAN</th>
<th>NOR</th>
<th>VAN</th>
<th>SUL</th>
<th>TET</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bee Pollen</td>
<td>12.5</td>
<td>0.2</td>
<td>1.6</td>
<td>0.2</td>
<td>0.8</td>
<td>100</td>
<td>0.8</td>
</tr>
<tr>
<td>Black Walnut</td>
<td>0.4</td>
<td>0.2</td>
<td>1.6</td>
<td>0.8</td>
<td>0.8</td>
<td>100</td>
<td>0.1</td>
</tr>
<tr>
<td>Calendula</td>
<td>0.4</td>
<td>0.4</td>
<td>1.6</td>
<td>0.8</td>
<td>0.8</td>
<td>200</td>
<td>0.2</td>
</tr>
<tr>
<td>Clove Oil</td>
<td>0.8</td>
<td>0.2</td>
<td>3.1</td>
<td>0.8</td>
<td>0.8</td>
<td>200</td>
<td>0.1</td>
</tr>
<tr>
<td>Clove Oil 1:1</td>
<td>0.8</td>
<td>0.2</td>
<td>3.1</td>
<td>0.4</td>
<td>0.8</td>
<td>100</td>
<td>0.1</td>
</tr>
<tr>
<td>Clove Tincture</td>
<td>0.8</td>
<td>0.4</td>
<td>3.1</td>
<td>0.8</td>
<td>0.8</td>
<td>100</td>
<td>0.1</td>
</tr>
<tr>
<td>Copaiba Oil 1:1</td>
<td>0.8</td>
<td>0.4</td>
<td>1.6</td>
<td>1.6</td>
<td>0.8</td>
<td>200</td>
<td>0.1</td>
</tr>
<tr>
<td>Eucalyptus Oil</td>
<td>0.8</td>
<td>0.2</td>
<td>1.6</td>
<td>1.6</td>
<td>0.8</td>
<td>100</td>
<td>0.1</td>
</tr>
<tr>
<td>Eucalyptus Oil 1:1</td>
<td>0.8</td>
<td>0.2</td>
<td>1.6</td>
<td>0.8</td>
<td>0.8</td>
<td>100</td>
<td>0.1</td>
</tr>
<tr>
<td>Eucalyptus Tincture</td>
<td>0.4</td>
<td>0.4</td>
<td>1.6</td>
<td>0.8</td>
<td>0.8</td>
<td>200</td>
<td>0.2</td>
</tr>
<tr>
<td>Prickly Ash</td>
<td>12.5</td>
<td>0.2</td>
<td>1.6</td>
<td>0.2</td>
<td>0.8</td>
<td>50</td>
<td>0.8</td>
</tr>
<tr>
<td>b Control</td>
<td>0.4</td>
<td>0.4</td>
<td>1.6</td>
<td>0.8</td>
<td>0.8</td>
<td>200</td>
<td>0.2</td>
</tr>
</tbody>
</table>

a AMP, ampicillin; ERY, erythromycin; KAN, kanamycin; NOR, norfloxacin; VAN, vancomycin; SUL, sulfamethazine; TET, tetracycline

b Control virgin culture of *Staphylococcus aureus* ATCC 29213 that has not been exposed to herbal products
Table 5 – MICs (µg/mL) resulting from the nutraceutical extract and antimicrobial using *Escherichia coli* ATCC 29522 as the indicator organism

<table>
<thead>
<tr>
<th></th>
<th>AMP</th>
<th>ERY</th>
<th>KAN</th>
<th>NOR</th>
<th>VAN</th>
<th>SUL</th>
<th>TET</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bee Pollen</td>
<td>12.5</td>
<td>0.2</td>
<td>1.6</td>
<td>0.2</td>
<td>0.8</td>
<td>50</td>
<td>0.8</td>
</tr>
<tr>
<td>Black Walnut</td>
<td>6.3</td>
<td>0.1</td>
<td>1.6</td>
<td>0.2</td>
<td>0.8</td>
<td>50</td>
<td>0.8</td>
</tr>
<tr>
<td>Calendula</td>
<td>3.1</td>
<td>0.2</td>
<td>1.6</td>
<td>0.2</td>
<td>0.8</td>
<td>25</td>
<td>0.8</td>
</tr>
<tr>
<td>Clove Tincture</td>
<td>3.1</td>
<td>0.2</td>
<td>1.6</td>
<td>0.2</td>
<td>0.8</td>
<td>12.5</td>
<td>0.8</td>
</tr>
<tr>
<td>Eucalyptus Oil</td>
<td>6.3</td>
<td>0.2</td>
<td>1.6</td>
<td>0.2</td>
<td>0.8</td>
<td>50</td>
<td>0.8</td>
</tr>
<tr>
<td>Eucalyptus Oil 1:1</td>
<td>12.5</td>
<td>0.2</td>
<td>1.6</td>
<td>0.4</td>
<td>0.8</td>
<td>50</td>
<td>0.8</td>
</tr>
<tr>
<td>b Control</td>
<td>3.1</td>
<td>0.2</td>
<td>3.1</td>
<td>0.2</td>
<td>0.8</td>
<td>25</td>
<td>0.8</td>
</tr>
</tbody>
</table>

*a* AMP, ampicillin; ERY, erythromycin; KAN, kanamycin; NOR, norfloxacin; VAN, vancomycin; SUL, sulfamethazine; TET, tetracycline

*b* Control virgin culture of *Escherichia coli* ATCC 29522 that has not been exposed to herbal products
Bee Pollen

In both the gram negative and gram positive test organisms, gradually increasing sensitivity was observed over three passages using the agar diffusion assay. *E. coli* ATCC 25922, when first exposed to the bee pollen tincture exhibited a zone of inhibition measuring 23.0 millimeters (mm), (Table 3). Surprisingly, increased sensitivity was quickly observed after the second exposure, where the zone of inhibition increased to 36.7 mm and then increased to 38.0 mm upon the third exposure, (Table 3). The greater the zone of inhibition, the more sensitive the organism is to the compound. *S. aureus* ATCC 29213 exhibited a small increase of sensitivity over the three exposures to the bee pollen tincture, with a zone of inhibition first measured at 23.0 mm and finally at 26.0 mm upon the third exposure, (Table 3).

There were also some increases in both antibiotic resistance and sensitivity in both test cultures exposed to bee pollen tincture. When *E. coli* ATCC 25922 was exposed to bee pollen tincture, an increase in the MIC values for ampicillin and sulfamethazine were observed. The MIC against ampicillin increased dramatically from the control observation of 3.1µg/mL to 12.5µg/mL, (Table 5). An increase from 25µg/mL to 50µg/mL was observed in sulfamethazine, (Table 5). A minor increase in sensitivity from 3.1µg/mL in the control to 1.6µg/mL was observed in the MIC values for kanamycin, (Table 5). Similar to the results in *E. coli* ATCC 25922, *S. aureus* ATCC 29213 also showed a marked increase in ampicillin MICs from 0.4µg/mL to 12.5µg/mL, (Table 4). In addition, the MIC values for tetracycline increased from 0.2µg/mL, in the control, to 0.8µg/mL in the exposed culture, (Table 4). A small decrease in the
erythromycin MICs was observed from 0.4µg/mL to 0.2µg/mL in the bee pollen tincture exposed culture, (Table 4). It should be reiterated that substantive increases or decreases in resistance or sensitivity is seen as a four-fold shift in MIC values.

**Black Walnut**

In the agar diffusion assay, both test strains of bacteria demonstrated about equal sensitivity to black walnut tincture over the three passages. *E. coli* ATCC 25922 showed a 40.3-41.7 mm zone of inhibition and *S. aureus* ATCC 29213, a 42.3-43.7 mm zone of inhibition, (Table 3). No pattern of sensitivity or resistance however can be inferred from the measurements of the zones of inhibition.

In the MIC determination, increases in the MIC values to ampicillin, and sulfamethazine as compared to the control results were observed in *E. coli* ATCC 25922 exposed to the black walnut tincture. MIC values of 6.3 µg/mL for ampicillin and 50 µg/mL of, sulfamethazine compared to 3.1 µg/mL and 25 µg/mL in the control respectively, were observed (Table 5). A decrease in MICs from the control values to the tincture exposed culture was observed in the values for erythromycin (0.2 µg/mL to 0.1 µg/mL) and kanamycin (3.1 µg/mL to 1.6 µg/mL), (Table 5). No increased rise in the MIC was observed in *S. aureus* ATCC 29213 exposed to the black walnut tincture, (Table 4). However, a slight increase in sensitivity, lower MIC values, was observed to erythromycin, sulfamethazine and tetracycline as compared to the control culture, (Table 4).
Calendula

*E. coli* ATCC 25922 did not demonstrate any increase in sensitivity or resistance to the tincture over three exposures in the agar diffusion assay. Exposures one and two showed zones of inhibition measuring 45.0 mm and 44.7 mm, respectively, however, with exposure three, there was some modest increase in resistance to the calendula tincture with a zone of inhibition measuring 40.3 mm, (Table 3). *S. aureus* ATCC 29213 was exposed to the calendula tincture over three passages, but no pattern of sensitivity or resistance can be inferred from the measurements of the zones of inhibition, (Table 3).

The MIC determination did not show any major resistance or sensitivity increases to the marker antimicrobials. Sensitivity to kanamycin in the calendula-exposed *E. coli* culture was 1.6 µg/mL as compared to the control at 3.1 µg/mL, (Table 5). No changes were observed in the *S. aureus* MICs.

Clove Oil

Clove oil was assayed in the agar diffusion assay as 100% essential oil and as a 1:1 dilution with 50% sterile alcohol. Initial testing using *E. coli* ATCC 25922 demonstrated that the oil and its 1:1 dilution were extremely potent. No organism growth on the assay plates was detected after the initial exposure and therefore no further testing was conducted. It appears that the volatile materials in the clove oil and its 1:1 dilution inhibited all growth.
Clove oil and its 1:1 dilution had observable zones of inhibition against *S. aureus* ATCC 29213 upon first exposure. For the first exposure, the zones of inhibition were measured at 13.7 mm and 12.0 mm for the oil and its 1:1 dilution in 50% sterile alcohol, respectively, (Table 3). A decrease in resistance was observed over the three exposures for the both the oil and its dilution. Zones of inhibition were measured at 18.0 mm and 24.0 mm for exposures two and three to the clove oil, respectively, (Table 3). The diluted oil also exhibited a similar pattern of a modest decrease for exposures two and three, with zones measuring 15.7 mm and 16.3 mm, respectively, (Table 3).

The MIC assay showed increases in both resistance and sensitivity in almost all of the antimicrobial markers. For both the 100% clove oil and its dilution, a non-substantive increase in resistance was shown to ampicillin with values recorded at 0.8 µg/mL, compared to the test sample at 0.4 µg/mL, (Table 4). This modest pattern of resistance was also observed in the kanamycin antimicrobial marker, with an increase from 1.6 µg/mL to 3.1 µg/mL, (Table 4). A similar increase in resistance to tetracycline was observed in both, with the oil-exposed culture showing MIC values of 0.2 µg/mL, compared to the test culture at 0.1 µg/mL, (Table 4). Additional modest increases in sensitivities were observed to norfloxacin. MIC values shifted from 0.8 µg/mL to 0.4 µg/mL for norfloxacin, (Table 4). The MIC values decreased for sulfamethazine from 200 µg/mL to 100 µg/mL, which could be considered substantive because of the large numerical change in concentration.
Clove Tincture

Both *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 exhibited sensitivities to the clove tincture in the agar diffusion assay. Over three exposures, *E. coli* showed an increase in sensitivity to the tincture with zones of inhibition measuring 31.3 mm, 36.0 and 38.7 mm, respectively, (Table 3). Similar increases in sensitivity were found with *S. aureus* over three exposures. Zones of inhibition measuring 27.7 mm, 30.3 mm, and 34.0 were recorded for exposures one, two and three, (Table 3).

For *S. aureus*, the MIC values for ampicillin increased slightly from 0.4 µg/mL in the control culture to 0.8 µg/mL, (Table 4). A modest increase in resistance was also noted to kanamycin. The values increased from 1.6 µg/mL in the control to 3.1 µg/mL in the clove tincture exposed *S. aureus*, (Table 4). The MIC determination assay showed an increase in sensitivity to norfloxacin and sulfamethazine in *E. coli* ATCC 25922 exposed to the clove tincture. The MIC for norfloxacin decreased from 3.1 µg/mL to 1.6 µg/mL as well as a decrease to sulfamethazine from 25 µg/mL, in the control culture, to 12.5 µg/mL in the exposed *E. coli*, (Table 5).

Copaiba

Copaiba oil was diluted 1:1 with 50% sterile ethanol and the resulting tincture was used in the agar diffusion assay, where no zones of inhibition were observed in the *E. coli* ATCC 25922 plates. No further assays were performed. In contrast, measurable zones of inhibition were seen on the plates using *S. aureus* ATCC 29213. Slight resistance developed after the first exposure and the zone of inhibition shrunk from 25
mm at exposure one to 22.3 mm at exposure two, (Table 3). There was no further resistance that developed at exposure three, showing a zone measuring 22.7 mm, (Table 3).

The MIC determination assay showed an increase in resistance in two of the antibiotic markers. The MIC value for ampicillin increased slightly from 0.4 µg/mL in the control strain to 0.8 µg/mL in the exposed *S. aureus* culture, (Table 4). An increase from 0.8 µg/mL to 1.6 µg/mL was also shown in the norfloxacin MIC value. The MIC value of tetracycline decreased from 0.2 µg/mL to 0.1µg/mL, (Table 4). At the very least these increases in resistance are interesting trends, but are not substantive in nature.

**Eucalyptus Oil**

Eucalyptus oil was studied in the agar diffusion assay as the 100% essential oil and as a 1:1 dilution with 50% sterile alcohol. Both the oil and its dilution showed antimicrobial activity in both assay strains. *E. coli* ATCC 25922 showed a development of resistance over the three exposures to both the concentrated oil and the diluted oil. Zones of inhibition at exposure one and two to the oil measured 29.0 mm and 26.3mm, respectively, but decreased in the third exposure to 12.7 mm, (Table 3). The diluted oil exhibited a similar pattern over three exposures, with zones measuring 21.0 mm, 15.3 mm and 12.3 mm, respectively, (Table 3).

*S. aureus* ATCC 29213 did not show an increase in resistance or sensitivity over the threes exposures to the essential oil. Zones of inhibition measured 22.7 mm, 19.3 mm
and 21.3 mm for exposures one, two and three respectively, (Table 3). A slight increase in sensitivity was observed over the three exposures to the diluted oil with zones measuring 17.8 mm and 16.3 mm for exposures one and two and 20.3 mm for exposure three, (Table 3).

Both the oil and diluted oil-exposed *S. aureus* ATCC 29213 cultures showed an increase of resistance to ampicillin. An increase from 0.4 µg/mL in the test strain to 0.8 µg/mL was observed in the MIC assay, (Table 4). Slight increases in sensitivities in both cultures were observed in the MIC values for tetracycline, which shifted from 0.2 µg/mL to 0.1 µg/mL, (Table 4). Against the sulfamethazine marker, there was a decrease from 200 µg/mL to 100 µg/mL, which is probably substantive because of the large concentration change, (Table 4). An increase in sensitivity was also observed in the MIC values for erythromycin. Sensitivity was halved from 0.4 µg/mL in the test strain to 0.2 µg/mL in both exposed cultures of *S. aureus* ATCC 29213, (Table 4). An increase of resistance was seen in the MIC values for norfloxacin, which increased from 0.8 µg/mL in the test strain to 1.6 µg/mL in the culture exposed to the pure essential eucalyptus oil, (Table 4). This increase in resistance was not observed in the *S. aureus* culture exposed to the diluted oil.

The MIC determination showed interesting results in *E. coli* ATCC 25922. Resistance development was evident in the MIC values for ampicillin and sulfamethazine in both the oil and diluted oil exposed tinctures. MIC values for ampicillin increased from 3.1 µg/mL, in the test strain to 6.3 µg/mL and 12.5 µg/mL in the *E. coli* ATCC
25922 exposed to the oil and the dilution, respectively, (Table 5). In both exposed cultures, the MIC values for sulfamethazine increased from 25 µg/mL to 50 µg/mL, possible a substantive increase, (Table 5). Additionally, sensitivity to norfloxacin decreased for the oil dilution exposed strain, with a shift observed from 0.2 µg/mL to 0.4 µg/mL, (Table 5).

**Eucalyptus Tincture**

Commercially available eucalyptus tincture was assayed using both cultures in the agar diffusion assay. Antimicrobial activity was only detected in *S. aureus* ATCC 29213. In the assay, no increases in sensitivity or resistance developed over the three exposures. Zones of inhibition were measured at 26.7 mm, 24.3 mm and 27.3 mm in exposures one, two and three, respectively, (Table 3). The MIC determination assay also did not show any development of sensitivity or resistance to any of the antimicrobial markers.

**Prickly Ash**

Prickly ash showed no antimicrobial effect against the *E. coli* assay strain in the agar diffusion assay. Zones of inhibition and resistance development were observed in the *S. aureus* ATCC 29213 plates. Prickly ash was not very potent against *S. aureus* at the first exposure yielding a zone of inhibition measuring 9.3 mm, (Table 3). Sensitivity developed in the second exposure and increased in the third exposure with zones of inhibition measuring at 13.7 mm and 19.7 mm, respectively, (Table 3).
A significant increase in the ampicillin MIC values, from 0.4 µg/mL in the control culture to 12.5 µg/mL in the exposed, *S. aureus* strain, was observed, (Table 4). MICs also increased for tetracycline, which increased from 0.2 µg/mL to 0.8 µg/mL, (Table 4). Notable sensitivities were observed for norfloxacin and sulfamethazine and a slight increase in sensitivity was seen in erythromycin. MICs for norfloxacin fell from 0.8 µg/mL in the control to 0.2 µg/mL in the exposed culture, 200 µg/mL to 50 µg/mL for sulfamethazine, and 0.4 µg/mL to 0.2 µg/mL for erythromycin, (Table 4).

Closer examination of the data showed some interesting trends. In the agar diffusion assay, the test organisms *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 showed trends both towards resistance and sensitivity to the nutraceutical tinctures. However, a trend towards increased sensitivity was observed more frequently for both test organisms to the nutraceutical extracts. There appeared to be no correlation between the results in the agar diffusion assay and the MIC assay, and results in one were not indicative of the other.

In the MIC assay, there appeared to be a development of trends and substantive increases and decreases in MIC values. Bee Pollen and Prickly Ash exposed cultures showed both substantive increases and decreases in MIC values. A substantive increase in the MIC values for ampicillin and tetracycline was observed in the Bee Pollen and Prickly Ash tincture exposed cultures. Ampicillin MIC values showed a thirty-fold increase from 0.4 µg/mL in the test strain to 12.5 µg/mL in the tincture exposed cultures and tetracycline MIC values also displayed a four-fold shift from 0.2 µg/mL to 0.8
µg/mL. Alternatively, a four-fold decrease in the norfloxacin MIC values from 0.8
µg/mL to 0.2 µg/mL was observed for the Bee Pollen and Prickly Ash exposed cultures.
Sulfamethazine MIC values showed a substantive decrease from 200 µg/mL to 50 µg/mL
only in the Prickly Ash exposed *S. aureus* ATCC 29213. No substantive changes were
observed in the erythromycin, kanamycin and vancomycin MIC values.

After the initial results from the agar diffusion assay for *E. coli* ATCC 25922,
only six nutraceutical products underwent further testing in the MIC assay. The assay
culture was extremely sensitive to the clove oil and its 1:1 dilution, and no growth
appeared on these plates after the first exposure. Alternatively, no antimicrobial activity
was detected and no measurable zones of inhibition were observed with the Copaiba oil
1:1 dilution, Eucalyptus tincture or the Prickly Ash tincture and therefore no further
testing was conducted. It was interesting to see that Prickly Ash had no effect on *E. coli*
ATCC 25922, but showed antimicrobial activity in *S. aureus* ATCC 29213 and a
development of both substantive increases and decreases in MIC values of four out of the
seven antimicrobial markers. This may be indicative of the innate difference between
gram-negative and gram- positive bacterial cell walls.

A similar trend seen in *S. aureus* ATCC 29213 was observed in the MIC values of
ampicillin for the Bee Pollen exposed *E. coli* ATCC 25922 culture. A substantive four-
fold increase from 3.1 µg/mL in the test culture to 12.5 µg/mL was observed. Likewise
the same four-fold increase in ampicillin MICs was also seen in the Eucalyptus oil 1:1
dilution exposed culture.
Conclusions

The results of this study are indicative of the fact that exposure to nutraceuticals can have a direct impact on the effectiveness of antimicrobials and/or antibiotics. This is important because an increase in resistance can lead to problems in terms of antibiotic efficacy and an increase in resistant organisms. A trend showing increases in sensitivity, which was observed in both test strains more frequently in this study, may be the key to discovering ways to enhance antibiotics used in therapies.

The chemical composition of the nutraceutical tinctures is very complex and it is unsure if it is one particular active ingredient or a combination that is increasing the sensitivity or resistance of the test organisms to the antibiotic markers. Further experiments on the chemical components of the active tinctures would need to be conducted. Chemically separating the active ingredients contained in each extract, particularly the Bee Pollen and Prickly Ash tinctures and Eucalyptus essential oil might yield interesting results. Once separated, each individual active ingredient could be assayed against *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 in both the agar diffusion assay and the MIC assay. It should be noted however, that the MIC assay proved to be a much better indicator of sensitivity and resistance than the agar diffusion assay. Consistently, it was shown in this study that there were no real parallels between the agar diffusion assay and MIC assay.
References


