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Antimicrobial and Anticancer Activity of Essential Oils from Guatemalan Medicinal Plants

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ABSTRACT

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Guatemalan medicinal plants were collected and screened for the presence of essential oils using steam distillation. Oil was found in 63 species from 24 families and was tested in tube dilution assays for activity against *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus mutans*, *Lactobacillus acidophilus* and *Candida albicans*. Several essential oils were highly active with 20 instances of oils inhibiting the microbes at an MIC of 0.31 µl/ml. Oils were also tested against cancerous and established cell lines using a 15% (v/v) agar-media which was developed to improve essential oil solubility. Assays were performed against three cancer lines: Stomach (AGS: CRL-1739), Skin (A375: CRL-1619), Tongue (CAL27: CRL-2095) and an established Monkey Kidney cell line (Vero C 1008: CRL-1586). Assessment of viability was performed using the Neutral Red assay with results indicating that many of the oils significantly inhibited cancer cell lines *in vitro* with 24 individual instances producing an IC_{50} of 0.20 µl/ml or less. Therapeutic indices indicated that many of the highly inhibitory oils were more cytotoxic to cancerous cell lines than to the established cell line.

Key words: Guatemala, medicinal plant, essential oil, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus mutans*, *Lactobacillus acidophilus*, *Candida albicans*, MIC, solubility, cytotoxicity, cancer, stomach, AGS, skin, A375, tongue, CAL27, Vero C 1008, neutral red, IC_{50}, therapeutic index, *Origanum vulgare*, *Lippia graveolens*, *Citrus aurantiifolia*

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CHAPTER I

The Importance of Essential Oils in the Search for New Drugs

Introduction

Successful plant remedies and their preparations as medicinal treatments have been used for thousands of years in indigenous cultures around the world (Balunas & Kinghorn, 2005). Many of these traditionally used plants have been scientifically evaluated with results yielding today's valuable drugs such as asprin, digitoxin, morphine and quinine (Butler, 2004). In most developing countries plants are still relied upon as the primary source of medical treatment due to the cost of prepared medicines. It is estimated that over 65% of the world population relies directly on plants as their main source of medicine (Fabricant & Farnsworth, 2001) with 75-90% of the world's rural communities relying primarily on plants (Fowler, 2006). The WHO reports that 80% of the people of Africa, 40% of the people of China and Asia, and 40% of the people of South America use medicinal plants as their primary care (WHO, 2002). Much of the scientific effort of the past few decades with medicinal plants has focused on documenting the uses of traditional medicine, analyzing the effectiveness of particular remedies, chemically characterizing medicinal plant compounds, and testing plant compounds *in vitro* (Fabricant & Farnsworth, 2001; Butler, 2004; Balunas & Kinghorn 2005; Fowler, 2006; Gertch, 2009). Based on the past history of success in finding new compounds, additional valuable discoveries will be made (Fabricant & Farnsworth, 2001; Butler, 2004; Newmann & Cragg, 2007).

Essential oils are common in plants that are used traditionally as medicinal treatments (Edris, 2007) and currently are more systematically studied (Kalemba & Kunicka, 2003; Lalou, 2004).

Recent literature indicates that essential oils have been tested for activity against many types of organisms known to cause human disease (Boyon et al., 2003; Kalemba & Kunicka, 2003; Lalou, 2004; Anthony, 2005; Edris, 2007) as well as for activity against cancer cell lines (Edris, 2007).

Essential oils are known to be complex mixtures of monoterpenes, sesquiterpenes, and volatile phenolics (Carson & Riley 1995), as well as alcohols, aldehydes, ethers, hydrocarbons and ketones (Kalemba & Kunica, 2003). Synergism has been demonstrated to be an accurate explanation of oil bioactivity (Wittstock & Gershenzon, 2002; Savelev et al., 2003; Burt, 2004). The level of activity is dependent on the combination and ratio of different components as opposed to quantity of the primary constituent (Kalemba & Kunica, 2003; Houghton et al., 2007). Phenols have been credited as being the most active components with the broadest spectrum of antimicrobial activity followed by aldehydes, keytones and alcohols (Kalemba & Kunica, 2003).

Due to high levels of poverty, health care options in rural Guatemala are limited (Goldman et al. 2002). Consequently, rural impoverished individuals choose local experts, who are familiar with traditional healing and plant-based remedies, for treatments (Booth et al., 1993; Goldman et al., 2002; Kufer et al. 2005, Hautecoeur et al. 2007). Many of the plants are prepared for use in teas through decoction or infusion, which are methods shown to extract essential oils and their components (Carnat et al., 1999; Billia et al., 2000; Radulescu et al., 2004). However, most of the commonly used plants have not been thoroughly analyzed, leaving potential bioactivity undocumented (Kufer et al., 2005).

The evidence in these recently published research findings indicate that essential oils and their components have potential to be valuable resources in the production of new drugs useful against human diseases and for cancer chemotherapy. Consequently, this study was undertaken to examine the activity of essential oils from many commonly used Guatemalan medicinal plants against bacteria, a fungus, and several cancer cell lines. The activity displayed in these bioassays will demonstrate the effectiveness of these essential oils through their production of highly inhibitory MIC and IC_{50} values, indicating their potential for development into useful drugs and compounds.

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CHAPTER II

The Antibacterial and Antifungal Activity of Essential Oils Obtained from 64 Guatemalan Medicinal Plants

Introduction

Fragrant and aromatic plants comprise a large portion of the species that have historically been used in traditional medicine (Edris, 2007). These plants typically contain essential oils which have become the focus of many recent studies (Kalemba & Kunicka, 2003; Lahlou, 2004). Essential oils have been tested for bioactivity against bacteria (Edris, 2007), fungi (Kalemba & Kunicka, 2003), parasitic protozoans (Boyom et al., 2003; Anthony, 2005), viruses (Edris, 2007), and cancer cell lines (Edris, 2007), all of which indicate that there is potential for the development of new compounds for drugs.

Due to the level of poverty in Guatemala, health care options in rural areas are limited (Goldman et al., 2002). There is little access to medical heath clinics, and family income can be a major constraint on health care (Goldman et al., 2002; Hautecoeur et al., 2007). As a result, many villagers from rural communities choose to see non-biomedical health practitioners (Goldman et al., 2002) or choose to rely on local knowledge of medicinal plants as solutions to health concerns (Booth et al., 1993; Kufer et al., 2005). Many commonly used plants of Guatemala have been insufficiently studied, leaving an incomplete picture of general phytochemical and pharmacological activities (Kufer et al., 2005).

Consequently, the objectives of this study included the determination, from a selected group of plants, of species containing essential oils and the distribution of these oils across plant families.

Secondly, those plants containing sufficient essential oils were bioassayed to determine their level of activity against *Staphylococcus aureus, Streptococcus mutans*, *Escherichia coli*, *Lactobacillus acidophilus*, and the yeast *Candida albicans*. The resulting MIC (minimum inhibitory concentration) data would be useful in increasing understanding about the medicinal plants that are commonly used by rural villagers and provide preliminary data for future studies.

Materials and Methods

Plant tissue collection

Commonly used medicinal plants were collected in Guatemala from 2007 to 2009. Berny Danilo Gálvez and Carlos Enrique Ardón collected plants in the Chiquimula Department in the villages of Tuticopote Abajo, Salitrón, and Roblarcito of the Torjá River basin, and in San Francisco Chancó of the Chancó River basin in the municipalities of Olopa and San Juan Ermita. Additional collections were made in Guatemala City by Dr. Ivan Rodríguez and Rex Cates at the Museo Odontológico de Guatemala and the Jardín Botánico Maya, by Luis Espinoza and R. Cates in the Pinalito association, and by Alfonso Fuentes and Dany Arbizu in the medicinal plant gardens at the University of San Carlos, Guatemala City, Guatemala.

Each sample was individually numbered and bagged and placed in a cooler on dry ice. Voucher specimens were collected and are stored in the Natural Products Laboratory at Brigham Young University Provo, UT and the herbarium at the University of San Carlos, CUNORI Campus, Chiquimula, Guatemala (Table 1). Collected samples were then stored in a freezer until shipped on dry ice to the Natural Products Laboratory in Provo, Utah and stored at -80^{o} C until analyzed. Tissue types varied between leaf, root, seed and aerial portions to whole plants. Species were

identified by the taxonomists Jose Vicente Martínez Arvevalo, Mario Esteban Véliz Perez, and Marco Romilio Estrada Muy using voucher specimens and in some instances, vouchers and digital photographs.

Essential oil extraction and preparation

A steam distillation apparatus (Scientific-Glass, Rancho Santa Fe, CA, USA) was used to extract essential oils (Luque de Castro, 1999). In order to determine the general chemical content of these extracts, preliminary samples were extracted and analyzed by GC-MS (HP model 6890/5973, Agilent Technologies Inc., Santa Clara, CA, USA). Commonly found in these extracts were monoterpenes, diterpenes, sesquiterpenes and other volatile compounds routinely extracted by steam distillation. Prior to distillation, fresh plant tissue was weighed and cut into ½ inch sized pieces. 50g of tissue was used for each extraction and steam distillation was conducted at 315^{o} C for 3½ hours. Root and seed samples were first ground in liquid nitrogen using 125g of tissue followed by steam distillation at 315^{o} C for 8 hours.

Essential oils were removed from the distillation apparatus receiver by pipette. To aid in the separation of oils from the water and glass surfaces, 125µl of diethyl-ether (Mallinckrodt-Baker, Phillipsburg, NJ, USA) was added to the receiver. The oil/diethyl-ether mixture was removed, placed in vials and dehydrated using anhydrous sodium sulfate (EMD Chemicals, Darmstadt, Germany). To separate the oils from the sodium sulfate, 200µl of additional diethyl-ether was added. The dehydrated oil/diethyl-ether mixture was evaporated under pressurized nitrogen to remove all traces of diethyl-ether (approx. 35 seconds). The final product of purified essential oil was then placed in an amber vial, weighed and stored at -80^{o} C until bioassayed.

Determination of minimum inhibitory concentration (MIC)

The microbes chosen for bioactivity testing were *Escherichia coli* (ATCC 11229; ATCC, Manassas, VA, USA), *Staphylococcus aureus* (ATCC 6538P; Becton Dickinson Laboratories, Cockeysville, MD, USA), *Streptococcus mutans* (ATCC 33402; ATCC), *Lactobacillus acidophilus* (ATCC 11975; ATCC) and *Candida albicans* (ATCC 90028; ATCC).

The tube dilution assay with slight modification (Donaldson et al., 2005) was selected as the method for determining the minimum inhibitory concentration (MIC). Two percent agar (w/v) added to broth was used to reduce essential oil volatility and increase solubility (Donaldson et al., 2005). *E. coli*, *S. aureus* and *S. mutans* were cultured in Tryptic Soy broth (Becton, Dickinson and Co.), *L. acidophilus* in MRS broth (Becton, Dickinson and Co.) and *C. albican*s in Sabbaraud Dextrose broth (Sigma-Aldrich, St. Louis, MO, USA). *S. mutans* and *L. acidophilus* were incubated in a 5% CO_2 atmosphere at 37^{o} C while *E. coli*, *S. aureus* and *C. albican*s were incubated at 37^{o} C in natural air.

Serial dilutions were used to prepare a series of five borosilicate glass test tubes (13 x 100 mm) for each trial. The initial tube was filled with 4ml of agar-broth and 20µl of essential oil was added. The mixture was vortexed and 2ml was removed and placed into a second tube containing 2ml of agar-broth. This process was repeated to create five dilutions with oil concentrations of 5.00µl/ml, 2.50µl/ml, 1.25µl/ml, 0.63µl/ml and 0.31µl/ml. Each test tube was inoculated with 20µl of microbial broth. Controls for each microbe consisted of two tubes, one receiving no

treatment and the other receiving 20µl of the microbial broth without the addition of oil. All tubes were incubated for 24 hours at 37^{o}C.

After 24 hours, 800µl of iodonitrotetrazolium chloride dye solution (INT) (Sigma-Aldrich) were added to each tube. INT is a colorimetric indicator that changes from clear to purple after exposure to CO_2 indicating bacterial respiration, metabolic activity and growth (Mann & Markham, 1998; Donaldson et al., 2005). A concentration of 20mg/ml was used for *E. coli* and *S. aureus* and 125mg/ml for *C. albicans.* Color change results were observed after 30 minutes. Samples of all tubes that did not exhibit a color change were plated on agar plates to confirm the inhibition of growth. All controls were also plated to confirm the positive results of growth indicated by the INT color change. INT was not used for *S. mutans* or *L. acidophilus* as results were unreliable due to indistinct color changes. Samples of all tubes of *S. mutans* and *L. acidophilus* were plated.

Bacterial plates were incubated for 24 hours at 37^{o}C and then each plate was examined for growth and validation of the INT results. The concentration that inhibited the bacteria was identified and the resultant MIC recorded with the MIC being defined as the lowest concentration of essential oil capable of inhibiting greater than 95% of the growth of the microorganism.

Two positive control drugs were used to verify assay repeatability and provide a comparison for the MIC values derived from the tested essential oils (Hoffmann et al., 1993; McCutcheon et al., 1994; Ritch-Krc et al., 1996). Gentamycin (10mg/ml) was used against *E. coli, S. aureus, S.*

mutans and *L. acidophilus*, and nystatin (1mg/ml in DMSO) used against *C. albicans*. 20µl of these drugs were administered and diluted following the same procedure used for essential oils. All control and experimental groups for all assays were replicated three times.

Results

Number of species with essential oils and their average yield

Of the 141 plant species screened for essential oil content, 63 (45%) produced sufficient oil for collection (Table 1). Forty six (73%) of these produced an average yield of less than 0.2 % (w/w). Nine and five species yielded 0.2% - 0.4% and 0.4% - 0.6%, respectively, two species yielded 0.6% - 1.0%, and one species yielded over 1.0%.

Of the 63 plants that produced collectible oil, 51 (81%) produced sufficient oil quantity to be used in the bioassay. Of those oils tested, 34 (54%) demonstrated activity against at least one organism (Table 2). Thus, 24% of the 141 species collected showed activity against one or more microbes.

Twelve species were not tested due to low oil yield (Table 2). Seven species were not active against the one or two microbes they were tested against, and due to lack of oil were not tested on additional microbes (Table 2).

Essential oil activity as determined by MIC

A highly inhibitory MIC was 0.31µl/ml with no variation among replicates in the range of MIC values. Thirteen species recorded a highly inhibitory MIC as observed in 20 bioassays (25% of

all MIC values) (Table 2). Another 18 species displayed a moderately inhibitory MIC, between 0.31 and 0.63µl/ml, in 20 bioassays (25% of all MIC values). An additional 18 bioassays (22%) from 14 species produced an MIC that was neither highly or moderately inhibitory but had an MIC more inhibitory than the reference drug (Table 2). Overall, 58 (72%) of the recorded MIC values were lower than those of the known reference drugs.

S. mutans had the most highly inhibitory MIC values and was the microorganism most inhibited based on the number of essential oils showing activity against it (72%) (Table 2). *L. acidophilus* was second most likely to be inhibited with 56% of all tests showing activity, although none of the MIC values recorded reached a highly inhibitory level. *C. albicans* was the third most likely with 48% showing inhibition followed by *S. aureus* with 28%. *E. coli* was the most resistant organism with only a 19% susceptibility rate.

E. coli was the microbe with the most inhibitory MIC value as averaged across all oil trials (0.86µl/ml), indicating that when inhibited, the level of inhibition was high (Table 2). *S. mutans* was the second most inhibited at 0.92µl/ml followed by 0.99µl/ml for *C. albicans*, 1.77µl/ml for *S. aureus*, and 2.05µl/ml for *L. acidophilus.*

Family distribution of species containing essential oil

Oils were collected from 24 individual families with nine families being represented by more than one species (Table 3). 24% of all species with oil were from Asteraceae, 11% from Lamiaceae, 9% from Rutaceae and 8% from Verbenaceae. These four families accounted for 52% of all species that produced an essential oil.

Of the nine families represented by more than one species, six families had multiple species producing MIC values, but only three families had more than one species with a highly inhibitory MIC value (0.31µl/ml) (Table 3). One species was collected from each of the families Bixaceae, Myricaceae and Vitaceae, and each specimen produced a highly inhibitory MIC. Eleven families had MIC values that were moderately inhibitory (between 0.31-0.63µl/ml) to at least one microbe (Table 3). Also, potential specificity of a species against a microbe is indicated since five families produced a species that was highly inhibitory to at least one microbe species.

Discussion

Number of species with essential oils and their average yield

Several species examined in this study have not previously been reported as having essential oils (Table 2). Essential oil has been reported from the fruits of *Spondias purpurea* (Koziol & Macia, 1998), but has not been reported from the leaves which were used in this study. Oils of *Arnica montana, Buddleja americana, Tagetes lucida, Tagetes filifolia, Pluchea odorata, Cissus verticillata, Ilex aquifolium, Cupressus lusitanica, Litsea guatemalensis, Piper auritum* and *Spilanthes americana* were active in this study but are not reported elsewhere as active against the microbes tested here. Additionally, activity of tissue specific oils from the seeds of *Bixa orellana,* the leaves of *Citrus limetta, Citrus aurantium,* and *Citrus aurantiifolia,* and the aerial portions of *Foeniculum vulgare* are new contributions.

Essential oil activity as determined by MIC

Results of this study indicate that many of the oils have good antibacterial or antifungal potential (Table 2). Several species in this study show high potential for future research. *Origanum vulgare* and *Lippia graveolens* produced highly inhibitory MIC values for *E. coli*, *S. mutans*, *S.*

aureus and *C. albicans* and MIC values for *L. acidophilus* lower than those of the reference drugs, indicating the ability of these oils to inhibit a variety of microbes at a high level. The oils of these two species are reported to have similar compositions (Salguiero et al., 2003) which may explain the similarity of results.

Sin Sin was highly inhibitory to *S. mutans* and *S. aureus* but not tested on other microbes due to low yield. *Citrus aurantiifolia* and *Cinnamomum zeylanicum* were highly inhibitory against one microbe and produced MIC values of moderate inhibition and lower than the reference drug for three other microbes, indicating broad spectrum activity. *Tagetes filifolia* shows promise with two MIC values of moderate inhibition and *Mentha piperita* also indicates potential with one MIC of moderate inhibition and three MIC values below those of the reference drugs.

Teloxys ambrosioides exhibited specific activity against *C. albicans*, having one of the lower MIC values but also a consistent range (Table 2). These results are supported by Jardim et al., (2008) who found a high level of inhibition for this oil against a number of fungi.

Family distribution of species containing essential oil

Essential oils have previously been reported from various species of each family tested in this study (Lahlou, 2004; Bakkali et al., 2008). Some families and their species produced a higher average MIC against specific microbes than other families (Table 4). Notable were the average values of species from Lauraceae against *S. mutans* (.31µl/ml), Rutaceae against *C. albicans* (0.36µl/ml), Lamiaceae against *S. mutans* (0.42µl/ml) and Asteraceae against *E. coli* (0.42µl/ml). Oils from species of the same family (e.g. Rutaceae) are known to produce some of the same compounds, increasing their likelihood of inhibiting particular microbes (Edris, 2007).

Conclusion

Results of this study indicate that essential oils are common to plants that are used as traditional medicines in Guatemala and are produced from many families. Most commonly, species with oil are from Asteraceae, Lamiaceae, Rutaceae and Verbenaceae. Highly inhibitory MIC values were reported for *Origanum vulgare***,** *Lippia graveolens, Citrus aurantiifolia,* Sin Sin, *Cinnamomum zeylanicum* and eight other species, many of which show potential for development based on these values. *Arnica montana*, *Buddleja americana, Tagetes lucida, Tagetes filifolia, Pluchea odorata, Cissus verticillata, Ilex aquifolium, Cupressus lusitanica , Litsea guatemalensis, Piper auritum* and *Spilanthes americana* demonstrated activity not previously reported and several were highly inhibitory to the microbes tested. High levels of inhibition were also observed across species from the same family. These and other results indicate that essential oils can be highly active against human microbial pathogens *in vitro*. *In vivo* testing is needed to determine if these whole oils or their components can be developed into resources for the treatment of oral, gastric and dermal infections and opportunistic fungal infections. Cytotoxicity data needs to be collected for these oils to confirm their safety in drug development and in everyday use by those who rely on these plants for traditional medicine.

Essential oils could be responsible for many of the positive health effects reported by users of traditional medicine. A high percentage of traditional preparations involve teas created either by decoction or infusion which are known methods for the extraction of essential oils and their components (Carnat et al., 1999; Bilia et al., 2000; Radulescu et al., 2004). Additional understanding needs to be gained about the role essential oils play in the effectiveness of medicinal teas.

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Table 1. Species, family, common name, tissue type and mean oil yield per species for Guatemalan medicinal plants extracted by steam distillation

Species	Family	Common Name	Tissue Type	$\bar{X}$ Oil Yield (%)
Achillea millefolium L.	Asteraceae	Milenrama	Aerial Portion	0.12
Ageratum sp.	Asteraceae	Violeta	Aerial Portion	0.04
Anacardium occidentale L.	Anacardiaceae	Marañon	Leaf	0.01
Arnica montana L.	Asteraceae	Árnica	Aerial Portion	0.07
Baccharis latifolia Pers.	Asteraceae	Conrrobo Negro	Aerial Portion	0.07
B. trinervis Pers.	Asteraceae	Corrimiento	Aerial Portion	0.11
Bixa orellana L.	Bixaceae	Achiote	Seed and Pod	0.03
Buddleja americana L.[1]	Buddlejaceae	Salvia Santa	Leaf	0.20
B. davidii Franch.	Buddlejaceae	Hoja de Lanza	Aerial Portion	0.07
Bursera simaruba Sarg.	Burseraceae	Palo de Jiote	Leaf	0.02
Casimiroa edulis La Llave	Rutaceae	Matasano	Leaf	0.02
Cinnamomum zeylanicum Blume	Lauraceae	Canela	Leaf	0.92
Cissus verticillata (L.) Nicolson & C.E.Jarvis [1]	Vitaceae	Tabardillo	Aerial Portion	0.03
Citharexylum donnell-smithii Greenm. [1]	Verbenaceae	Coralillo	Leaf	0.03
Citrus aurantiifolia (Christm.) Swingle	Rutaceae	Limón Criollo	Leaf	0.40

Table 1. cont.

C. aurantium L.	Rutaceae	Naranja	Leaf	0.06
C. limetta Risso	Rutaceae	Lima	Leaf	0.10
Clematis dioica L. [1]	Ranunculaceae	Bejuco de Cancer	Aerial Portion	< .01
Croton sp.	Euphorbiaceae	Quina	Leaf	0.23
Cupressus lusitanica Mill.	Cupressaceae	Ciprés	Leaf	0.26
Cymbopogon citratus Stapf	Poaceae	Té Limón	Leaf	0.03
Elephantopus spicatus Aubl. [1]	Asteraceae	Oreja de Coche	Aerial Portion	0.03
Eucalyptus sp.	Myrtaceae	Eucalipto	Leaf	0.46
Eupatorium semialatum Benth.	Asteraceae	Venadillo	Aerial Portion	0.04
Foeniculum vulgare Mill.	Apiaceae	Hinojo	Aerial Portion	0.14
Ilex aquifolium L.	Aquifoliaceae	Trueno	Leaf	0.02
Lantana camara L.	Verbenaceae	Cinco Negritos	Aerial Portion	0.05
Lippia dulcis Trevir.	Verbenaceae	Hierba Dulce	Aerial Portion	0.09
L. graveolens Kunth	Verbenaceae	Oregano	Leaf	0.47
Liquidambar styraciflua L.	Hamamelidaceae	Liquidambar	Leaf	0.02
Litsea guatemalensis Mez	Lauraceae	Laurel	Leaf	0.20
Mangifera indica L.	Anacardiaceae	Mango	Leaf	0.02

Table 1. cont.

Mentha piperita L.	Lamiaceae	Menthol Piperita	Aerial Portion	0.93
Mentha sp.	Lamiaceae	Hierba Buena	Aerial Portion	0.07
Murraya paniculata (L.) Jack	Rutaceae	Limonaria	Leaf	0.05
Myryca sp.	Myricaceae	Chiso	Leaf	0.21
Neurolaena lobata R.Br.	Asteraceae	Tres Puntas	Leaf	0.05
Ocimum basilicum L.	Lamiaceae	Albahaca	Aerial Portion	0.45
O. micranthum Willd.	Lamiaceae	Albahaca	Aerial Portion	0.15
Origanum vulgare L.	Lamiaceae	Oregano de Castillo	Leaf	1.05
Persea americana Mill.	Lauraceae	Aguacate	Leaf	0.03
Pimenta dioica (L.) Merr.	Myrtaceae	Pimienta	Leaf	0.26
Pinus maximinoi H.E.Moore [1]	Pinaceae	Pino	Leaf	0.09
Piper auritum Kunth	Piperaceae	Santa Maria	Leaf	0.33
Pluchea odorata Cass.	Asteraceae	Siguapate	Leaf	0.03
Psidium guajava L.	Myrtaceae	Guayabo	Leaf	0.13
Rhus terebinthifolia Schltdl. & Cham. [1]	Anacardiaceae	Sal de Venado	Leaf	0.03
Rosmarinus officinalis L.	Lamiaceae	Romero	Leaf	0.14
Ruta chalepensis L.	Rutaceae	Ruda	Aerial Portion	0.05

Table 1. cont.

Spilanthes americana Hieron. ex Sodiro	Asteraceae	Ixmaramac	Aerial Portion	0.09
Spondias purpurea L.	Anacardiaceae	Jocote	Leaf	0.01
Stevia connata Lag.	Asteraceae	Guapillo	Root	0.08
Stigmaphyllon ellipticum A.Juss. [1]	Malpighiaceae	Contra Hierba	Leaf	< .01
Tagetes erecta L.	Asteraceae	Flor de Muerto	Aerial Portion	0.02
T. filifolia Lag.	Asteraceae	Anís de Monte	Aerial Portion	0.51
T. lucida Cav.	Asteraceae	Pericón	Aerial Portion	0.24
Teloxys ambrosioides (L.) W.A.Weber	Chenopodiaceae	Apasote	Aerial Portion	0.04
Thymus vulgaris L.	Lamiaceae	Tomillo	Aerial Portion	0.02
Verbena litoralis Kunth [1]	Verbenaceae	Verbena	Aerial Portion	0.02
Vernonia leiocarpa DC. [1]	Asteraceae	Suquenay	Leaf	0.11
Vetiveria zizanioides Nash	Poaceae	Valeriana	Root	0.03
Zingiber officinale Roscoe	Zingiberaceae	Jengibre	Rhizome	0.03
Sin Sin[2]		Sin Sin	Aerial Portion	0.36

[1]Species not previously reported to have essential oil
[2]Species not identified

Table 2. MIC (ul/ml)[1] and MIC range data (in parentheses) for essential oils from Guatemalan medicinal plants tested for activity against microbial taxa

Species	***E. coli***	***S. aureus***	***S. mutans***	***L. acidophillus***	***C. albicans***
A. millefolium	NA	NA	1.46 (0.63 - 2.50)	3.75 (1.25 - 5.00)	2.50 (2.50)
Ageratum sp.	NT	NA	0.31 (0.31)	NT	NA
A. occidentale	NA	NT	NT	NT	NT
A. montana	NA	NA	2.71 (0.63 - 5.00)	NA	NA
B. latifolia	NA	NA	0.94 (0.31 - 1.25)	NT	NA
B. trinervis	NT	NA	IN	NT	NT
B. orellana	NA	NA	0.31 (0.31)	NA	NA
B. americana	NA	NA	0.63 (0.63)	NA	NA
B. davidii [2]	NT	NT	NT	NT	NT
B. simaruba [2]	NT	NT	NT	NT	NT
C. edulis [2]	NT	NT	NT	NT	NT
C. zeylanicum	0.83 (0.63 - 1.25)	1.04 (0.63 - 1.25)	0.31 (0.31)	1.46 (0.63 - 2.50)	0.63 (0.31 - 1.25)
C. verticillata	NA	NA	0.31 (0.31)	NA	NA
C. donnell-smithii [2]	NT	NT	NT	NT	NT
C. aurantiifolia	2.50 (1.25 - 5.00)	0.42 (0.31 - 0.63)	0.63 (0.63)	1.25 (1.25)	0.31 (0.31)
C. aurantium	NA	2.92 (1.25 - 5.00)	2.92 (1.25 - 5.00)	NT	0.42 (0.31 - 0.63)

Table 2. cont.

C. limetta	NA	3.33(2.50 - 5.00)	1.67 (1.25 - 2.50	4.16 (2.50 - 5.00)	0.42 (0.31 - 0.63)
C. dioica [2]	NT	NT	NT	NT	NT
Croton sp.	NT	NT	IN	NT	NT
C. lusitanica	NA	NA	0.42 (0.31 - 0.63)	1.25 (1.25)	NA
C. citratus	IN	0.63 (0.31 - 1.25)	IN	IN	IN
E. spicatus	NT	NT	IN	NT	NT
Eucalyptus sp.	NA	5.00 (5.00)	2.50 (2.50)	1.25 (1.25)	5.00 (5.00)
E. semialatum	NA	NA	IN	NT	NT
F. vulgare	NA	NA	2.50 (2.50)	5.00 (5.00)	0.63 (0.63)
I. aquifolium	NT	NT	0.42 (0.31 - 0.63)	NT	NT
L. camara [2]	NT	NT	NT	NT	NT
L. dulcis	NT	NT	IN	NA	NT
L. graveolens	0.31 (0.31)	0.31 (0.31)	0.31 (0.31)	0.83 (0.63 - 1.25)	0.31 (0.31)
L. styraciflua [2]	NT	NT	NT	NT	NT
L. guatemalensis	NA	NA	0.31 (0.31)	2.08 (1.25 - 2.50)	NA
M. indica	NA	NT	NT	NT	NT
M. piperita	1.25 (1.25)	1.04 (0.63 - 1.25)	0.42 (0.31- 0.63)	1.67 (1.25 - 2.50)	1.04 (0.63 - 1.25)

Table 2. cont.

Mentha sp.	NT	IN	NT	NT	NT
M. paniculata	NT	NA	IN	NT	NT
Myryca sp.	NA	NA	0.31 (0.31)	NA	NA
N. lobata	NA	NT	NT	NT	NT
O. basilicum	NA	NA	NA	NA	NA
O. micranthum	NA	NA	0.42 (0.31 - 0.63)	2.92 (1.25-5.00)	NT
O. vulgare	0.31 (0.31)	0.31 (0.31)	0.31 (0.31)	1.04 (0.63 - 1.25)	0.31 (0.31)
P. americana	NT	NT	NA	NA	NT
P. dioica	NT	NT	0.42 (0.31 - 0.63)	0.83 (0.63 - 1.25)	NT
P. maximinoi	NA	NA	0.52 (0.31 - 0.63)	1.04 (0.63 - 1.25)	NA
P. auritum	NA	NA	2.08 (1.25 - 2.50)	2.08 (1.25 - 2.50)	0.83 (0.63 - 1.25)
P. odorata	NA	NA	0.31 (0.31)	NT	NT
P. guajava	NA	NA	0.42 (0.31 - 0.63)	NA	NA
R. terebinthifolia [2]	NT	NT	NT	NT	NT
R. officinalis	NA	NA	0.52 (0.31 - 0.63)	2.08 (1.25 - 2.50)	NA
R. chalepensis	NA	NA	2.50 (2.50)	2.08 (1.25 - 2.50)	0.31 (0.31)
S. americana	NT	NT	IN	NT	NT

Table 2. cont.

S. purpurea [2]	NT	NT	NT	NT	NT
S. connata	NA	NT	NT	NT	NT
S. ellipticum [2]	NT	NT	NT	NT	NT
T. erecta	NT	NA	IN	NT	NT
T. filifolia	0.52(0.31 - 0.63)	NA	1.04 (0.63 - 1.25)	NA	0.52 (0.31 - 0.63)
T. lucida	0.31 (0.31)	4.17 (2.50 - 5.00)	NT	NT	NT
T. ambrosioides	NA	NA	NA	NA	0.63 (0.63)
T. vulgaris [2]	NT	NT	NT	NT	NT
V. litoralis	NA	NA	NT	NT	NT
V. leiocarpa	NT	NA	NT	NT	NT
V. zizanioides	NT	NT	0.42 (0.31 - 0.63)	NT	NT
Z. officinale [2]	NT	NT	NT	NT	NT
Sin Sin	NA	0.31 (0.31)	0.31 (0.31)	IN	NA
Gentamycin	2.50 (2.50)	0.83 (0.63 - 1.25)	0.83 (1.25-2.50)	3.33 (2.50 - 5.00)	NT
Nystatin	NT	NT	NT	NT	2.50 (2.50)

[1]NA = activity not observed; NT = not tested on microbes; IN = activity observed but not enough oil for three replicates
[2]Species not tested due to low oil yield

Table 3. Family distribution of Guatemalan medicinal plant species containing essential oil, number of species producing an MIC (ul/ml) against a microbe, and mean MIC values for species in a family

Family	**No. of sp. with oil**	**No. of sp. with MIC**	**Sp. with MIC (%)**	**No. of sp. with highly inhibitory MIC**	**Sp. with highly inhibitory MIC (%)**	**$\bar{\bar{X}}$ family MIC against *E. coli***	**$\bar{\bar{X}}$ family MIC against *S. aureus***	**$\bar{\bar{X}}$ family MIC against *S. mutans***	**$\bar{\bar{X}}$ family MIC against *L. acidophillus***	**$\bar{\bar{X}}$ family MIC against *C. albicans***
Anacardiaceae	4									
Apiaceae	1	1	100					2.50*	5.00*	0.63*
Aquifoliaceae	1	1	100					0.42*		
Asteraceae	15	7	47	3	20	0.42	4.17*	1.13	3.75	1.51
Bixaceae	1	1	100	1	100			0.31*		
Buddlejaceae	2	1	50					0.63*		
Burseraceae	1									
Chenopodiaceae	1	1	100							0.63*
Cupressaceae	1	1	100					0.42*	1.25*	
Euphorbiaceae	1									
Hamamelidaceae	1									
Lamiaceae	7	4	57	1	14	0.78	0.68	0.42	1.93	0.68
Lauraceae	3	2	67	2	67	0.83*	1.04*	0.31	1.77	0.63*

Table 3. cont.

Malpighiaceae	1									
Myricaceae	1	1	100	1	100			0.31*		
Myrtaceae	3	3	100				5.00*	1.11	1.04	5.00*
Pinaceae	1	1	100					0.52*	1.04*	
Piperaceae	1	1	100					2.08*	2.08*	0.83*
Poaceae	2	2	100				0.63*	0.42*		
Ranunculaceae	1									
Rutaceae	6	4	67	2	33	2.50*	2.22	1.93	2.50	0.36
Verbenaceae	5	1	20	1	20	0.31*	0.31*	0.31*	0.83*	0.31*
Vitaceae	1	1	100	1	100			0.31*		
Zingiberaceae	1									

*Not an average because value represents the results of only one species

CHAPTER III

Essential Oils Obtained from 22 Guatemalan Medicinal Plants Evaluated *in vitro* for Activity Against Cancerous and Established Cell Lines

Introduction

Essential oils are complex mixtures of chemicals, and include monoterpenes, sesquiterpenes and phenolics (Carson & Riley, 1995). Their compositions are known to be unique among species of the same family and often among tissues of an individual plant (Cates, 1996; Kalemba & Kunicka, 2003; Bakkali et al., 2008). This uniqueness in essential oil composition is related to both environmental and genetic factors (Valladares et al., 2002; Lahlou, 2004; Bakkali et al., 2008; Hussain et al., 2008; Barra, 2009).

Essential oils are known to have biological activity against a variety of organisms (Edris, 2007), including bacteria (Bakkali et al., 2008), fungi (Kalemba & Kunicka, 2003), protozoans (Boyom et al., 2003; Anthony et al., 2005) and viruses (Edris, 2007). The effectiveness of whole essential oils as well as individual components against cancer cell lines has also been demonstrated (Edris, 2007). Their activity has been shown to be a sum of the effects of the individual components based on the ratio of the different constituents and not necessarily on the quantity of one component (Kalemba & Kunicka, 2003; Houghton et al., 2007). This synergism indicates the potential for individual oils to result in specific mechanisms of action toward a particular organism or cancer cell line (Wittstock & Gershenzon, 2002; Rajesh & Howard, 2003; Savelev et al., 2003; Salminen et al., 2008).

The study reported here is part of a larger project aimed at gaining a better understanding of the use of medicinal plants by rural Guatemalan villagers (Miller, 2010). Common traditional preparations of medicinal plants involve teas which are created either by decoction or infusion, and are known methods for the extraction of essential oils (Carnat et al., 1999; Bilia et al., 2000; Radulescu et al., 2004). Preliminary screening of 141 medicinal plant species from Guatemala yielded 44 species with sufficient essential oil content for microbial bioassays (Miller, 2010). Of these 44 species, 22 were selected for this study and their activity against several cancer cell lines and their level of cytoxicity are reported.

Materials and Methods

Plant selection and tissue collection

Plants from Guatemala were selected for this study based on quantity of essential oil yielded from previous steam distillation extractions (Miller, 2010). All plants chosen are utilized by Guatemalans in traditional medicine, although none of the plants are reported as traditional treatments for cancer. Commonly used medicinal plants were collected in Guatemala from 2007 to 2009. Berny Danilo Gálvez and Carlos Enrique Ardón collected plants in the Chiquimula Department in the villages of Tuticopote Abajo, Salitrón, and Roblarcito of the Torjá River basin, and in San Francisco Chancó of the Chancó River basin in the municipalities of Olopa and San Juan Ermita. Additional collections were made in Guatemala City by Dr. Ivan Rodríguez and Rex Cates at the Museo Odontológico de Guatemala and the Jardín Botánico Maya, by Luis Espinoza and R. Cates in the Pinalito association, and by Alfonso Fuentes and Dany Arbizu from the medicinal plant gardens at the University of San Carlos, Guatemala City Guatemala.

Each sample was individually numbered and bagged and placed in a cooler on dry ice. Voucher specimens were collected and are stored in the Natural Products Laboratory at Brigham Young University Provo, UT and the herbarium at the University of San Carlos, CUNORI Campus, Chiquimula, Guatemala (Table 1). Collected samples were then stored in a freezer until shipped on dry ice to the Natural Products Laboratory in Provo, Utah and stored at -80^{o} C until analyzed. Tissue types varied between leaf, seed and aerial portions to whole plants. Species were identified by the taxonomists Jose Vicente Martínez Arvevalo, Mario Esteban Véliz Perez, and Marco Romilio Estrada Muy using voucher specimens and in some instances, vouchers and digital photographs.

Essential oil extraction and preparation

A steam distillation apparatus (Scientific-Glass, Rancho Santa Fe, CA, USA) was used to extract essential oils (Luque de Castro, 1999). In order to determine the general chemical content of these extracts, preliminary samples were extracted and analyzed by GC-MS (HP model 6890/5973, Agilent Technologies Inc., Santa Clara, CA, USA). Commonly found in these extracts were monoterpenes, diterpenes, sesquiterpenes and other volatile compounds routinely extracted by steam distillation. Prior to distillation, fresh plant tissue was weighed and cut into ½ inch sized pieces. 50g of tissue was used for each extraction and steam distillation was conducted at 315^{o} C for 3½ hours. Seed samples were first ground in liquid nitrogen using 125g of tissue followed by steam distillation at 315^{o} C for 8 hours.

After extraction, oil was pipetted from the receiver without the assistance of any additional solvents. All oils were immediately dehydrated with anhydrous sodium sulfate (EMD Chemicals Inc., Darmstadt, Germany), weighed and stored in amber vials at -80^{o} C until tested.

Cell lines and cytotoxicity

Three cancer cell lines AGS (Stomach, ATCC CRL-1739; ATCC, Manassas, VA, USA), A375 (Skin, ATCC CRL-161; ATCC) and CAL27 (Tongue, ATCC CRL-2095; ATCC) were chosen for testing of bioactivity. An establish cell line from Monkey Kidney cells, Vero C 1008 (ATCC CRL-1586; ATCC), was chosen to determine cytotoxicity of the essential oils and for calculating a therapeutic index.

A375, CAL27 and Vero C 1008 cell lines were grown in DMEM (GIBCO, Grand Island, NY, USA) fortified with 10% Fetal Bovine Serum (FBS) (ATCC), 5ml of 1M HEPES (Hyclone, Logan, UT, USA), 2.5ml of 100mM sodium pyruvate (Hyclone) and 5ml of 10mg/ml gentamycin (Sigma-Aldrich, St. Louis, MO, USA). Cells were grown to 90% confluency in 75cm^2 flasks (Sarstedt, Newton, NC, USA) and then seeded into 96-well plates (Sarstedt). AGS cells were grown in Ham's F-12 Kaighn's Modification media (Hyclone) fortified with FBS (10%), 5ml of 1M HEPES and 5ml of 10mg/ml gentamycin. Cells were grown to 90% confluency in 175cm^2 flasks (Sarstedt).

Method adaptation

Assessment of the bioactivity of essential oils can be problematic, due to the highly volatile nature of the oils and their lack of solubility (Donaldson et al., 2005). Volatile components were found to cross-contaminate adjacent wells of 96-well plates (Donaldson et al., 2005) even at low concentrations, thereby leading to inaccurate estimations of Minimum Inhibitory Concentration (MIC) and IC_{50} values.

Donaldson et al. (2005) proposed the addition of 2% biological grade agar (w/v) (Remmal et al., 1993) to the culture media to remedy this problem in microbial tube dilution assays. In order to adapt the method of Donaldson et al. (2005) to allow the use of 96-well plates, 15% biological grade agar (v/v) was added to the cell culture media. The addition of 15% agar (v/v) mixed with cell culture media was consistently shown to have no inhibitory effects on the growth of untreated cells in preliminary trials, and the resulting mixture of inert agar maintained a stable emulsion over a 24 hour period and minimized oil volatility.

Cell culture techniques

DMEM agar-media was prepared by adding melted molecular biology grade agar (Fisher, Fair Lawn, NJ, USA) to incomplete media at a 15% v/v ratio at room temperature, and the mixture was allowed to cool. FBS (10%) was then added followed by 5ml of 1M HEPES, 2.5ml of 100mM sodium pyruvate and 5ml of 10mg/ml gentamycin. Ham's F-12 Kaighn's Modification agar-media was prepared in the same manner with the omission of sodium pyruvate.

Cells were seeded in 96-well plates and incubated for 24 hours in order to reach 90% confluency. AGS cells were seeded at a density of 7.0×10^4, A375 at 6.0×10^4, CAL27 at 5.0×10^4 and Vero C 1008 at 2.0×10^4. Each well was filled with 150µl of complete media and then placed in an incubator with a 5% CO_2 atmosphere.

Seeded plates were removed from the incubator after 24 hours and the media removed. Plate design allowed for two essential oils and controls to be tested on each plate in three replications. Essential oils were serially diluted in agar-media resulting in final concentrations of 7.0µl/ml, 3.5µl/ml, 1.75µl/ml, 0.88µl/ml, 0.44µl/ml, 0.22µl/ml, 0.11µl/ml and 0.05µl/ml. 200µl of diluted

essential oil was then added to each well. Controls consisted of 200µl of agar-media in wells with no additives. All edge wells remained unseeded and were filled with 200µl of sterilized distilled water (DDH_2O). Each plate was returned to the incubator for an additional 24 hours.

Determination of IC_{50} and CC_{50}

The Neutral Red (NR) assay was chosen for the determination of IC_{50} and CC_{50} because it is a commonly used assay that is sensitive and accurate in the quantitative assessment of *in vitro* cytotoxicity (Borenfreund & Peuner, 1985; Babich & Borenfreund, 1991; Schröterová et al., 2009). Plates were removed from the incubator after 24 hours, and the agar-media with oil was discarded. Phosphate buffered saline (PBS) was used to gently wash and remove all traces of the essential oil and the agar-media from the wells. NR dye solution was made using 0.33mg/ml NR solution (3-aminom-dimethylamino-2-methyl-phenazine hydrochloride in DBPS) (Sigma-Aldrich) and then added to complete media to make a 10% NR media mixture. This solution was added to each well excluding edge wells which were filled with sterilized DDH_2O. Plates were then incubated for three additional hours after which the NR media mixture was removed and discarded. A fixative solution (1% $CaCl_2$; Fisher in 0.5% formaldehyde; Mallinckrodt, Phillipsburg, NJ, USA) was added and then removed after 30 seconds of exposure. This was followed by the addition of a solublization solution (1% acetic acid; EM Science, Gibbstown, NJ, USA, in 50% ethanol; Decon Labs, King of Prussia, PA, USA). Each plate was gently agitated for 10 minutes on a shaker table after which cell viability was measured using a Fusion α-HT Universal Microplate Analyzer (Packard Instruments, Meriden, CT, USA) with a 540 nm filter and a 690 nm reference filter (Babich and Borenfreund, 1991).

Final reading values were generated by subtracting the 690 values from the 540 values followed by correction of the data by subtracting the average value generated from the blank edge wells. The values of three replicate trials were averaged and then graphed using Fathom Dynamic Statistics (Finzer et al., 2001) to determine final IC_{50} and CC_{50} values. A Therapeutic Index was calculated using the ratio CC_{50}/ IC_{50} (Greer et al., 2010).

Results

Essential oil yield

Oil yield from steam distillation indicates that 13 species produced a yield of 0.25% or less, seven species between 0.25 - 0.5% and two species greater than 0.5% (Table 1). The families Asteraceae, Lamiaceae, Myrtaceae, Lauraceae and Rutaceae were represented by multiple species. Yield data shows that oils from Rutaceae consistently yielded 0.25% or less while the other families produced yields with more variation. Single extractions of individual species produced enough oil for all assays to be performed. All species used in this study come from families known to produce oils (Lahlou, 2004; Bakkali et al., 2008), although *Pinus maximinoi* and *Buddleja americana* have not been previously reported to have essential oils (Miller, 2010).

IC_{50} and CC_{50}

All oils assayed showed inhibitory activity against one or more cancer cell lines (Table 2). Highly inhibitory IC_{50} values of 0.10µl/ml or less were observed against cancer cell lines in eight instances from four species (12% of total recorded IC_{50}). Additionally, 28 moderately inhibitory IC_{50} values (between 0.10µl/ml and 0.30µl/ml) were observed from 15 species (42% of total recorded IC_{50}), with a total of 36 instances of an IC_{50} of 0.30µl/ml or less. In total, ten IC_{50} values (45%) for the A375 line, 12 IC_{50} values (54%) for the AGS line and 14 IC_{50} values (64%) for the

CAL27 line were below 0.3µl/ml. The calculation of the average IC_{50} of each cell line shows the CAL27 line having the most inhibitory average IC_{50} with 0.29µl/ml, followed by AGS at 0.32µl/ml and A375 at 0.49µl/ml.

Highly inhibitory IC_{50} values of 0.10µl/ml or less were produced by oils from *Citrus aurantiifolia* (3), *Origanum vulgare* (2), *Teloxys ambrosioides* (2) and *Lippia graveolens* (1). All values from *C. aurantiifolia* were less than 0.05µl/ml, which was the smallest measurable IC_{50} value able to be assessed in this assay. Oils from *C. aurantiifolia, T. ambrosioides, L. graveolens, O. vulgare* and *Pinus maximinoi* were the most inhibitory to the CAL27 line. A375 cells were most effectively inhibited by *C. aurantiifolia, T. ambrosioides, O. vulgare, L. graveolens* and *Cinnamomum zeylanicum,* and AGS was most inhibited by *C. aurantiifolia, Citrus limetta, L. graveolens, Psidium guajava,* and *Eucalyptus* sp.

All essential oils were shown to be cytotoxic to the Vero C 1008 cell line at some concentration (Table 2). Ten oils (45%) produced highly cytotoxic CC_{50} values of 0.10µl/ml or less and nine oils (41%) produced moderately inhibitory CC_{50} values (between 0.10µl/ml and 0.30µl/ml). In total, 19 oils (86%) produced a CC_{50} value below 0.30µl/ml against the Vero C 1008 cells. The most cytotoxic CC_{50} values were produced by the oils of *Cupressus lusitanica, Citrus aurantiifolia, Bixa orellana, Buddleja americana,* and *Teloxys ambrosioides.*

The calculation of the Therapeutic Index (TI) resulted in 14 incidences (21%) where the TI value was greater than 1, indicating higher cytotoxcity to cancer cells over cells from the established cell line (Table 3). All three recorded TI values for *Ruta chalepensis* were over 1, two values

over 1 were recorded for *Citrus limetta, Citrus aurantium, Rosmarinus officinalis* and *Origanum vulgare* and one value over 1 was recorded for *Eucalyptus* sp.*, Pinus maximinoi* and *Lippia graveolens.* Ten TI values were unable to be calculated due to IC_{50} or CC_{50} values below the smallest measurable value able to be assessed in this assay (Table 3)..

Discussion

IC_{50} and CC_{50}

Many of the essential oils used in this study have not previously been tested against human cancer cell lines *in vitro* (Table 2). The IC_{50} values produced here indicate the first known reporting of their activity against cancer cell lines. All of the oils used showed some inhibitory effect on the cancer cells lines (Table 2) and many displayed high inhibition at low concentrations, which is a good evaluator to determine which extracts should be selected for additional research and testing. *Citrus aurantiifolia* was the most effective oil against all three cancer cell lines with an IC_{50} below 0.05µl/ml for each line (Table 2). Oil from *Origanum vulgare* produced highly inhibitory IC_{50} values against the A375 and the CAL27 cell lines and *Lippia graveolens* produced a highly inhibitory IC_{50} value against the CAL27 line. The calculated average IC_{50} for both oils is 0.12µl/ml, indicating potential for broad scale cancer cell inhibition. Both oils have been reported to have similar composition, providing an explanation for their comparable levels of activity (Salgueiro et al., 2003). Oil from *Teloxys ambrosioides* also produced two highly inhibitory IC_{50} values against A375 and CAL27 but the value against AGS was significantly less inhibitory, which suggests more potential for line specific activity. Additional oils with average IC_{50} values of moderate inhibition are *Litsea guatemalensis, Cinnamomum zeylanicum* and *Psidium guajava* with values of 0.19µl/ml, 0.20µl/ml and 0.21µl/ml, respectively.

Results of the TI calculation indicate that a number of oils used in this study show potential based on their relative level of cytotoxicty to cells from the established cell line (Table 3). The TI values of *Citrus limetta, Citrus aurantium, Lippia graveolens* and *Origanum vulgare* indicate the potential of these oils against the CAL27 line. Additionally TI values of *C. limetta, C. aurantium* and *Eucalyptus* sp. indicate potential against the AGS line while oils of *O. vulgare* and *Rosmarinus officinalis* showed similar results towards the A375 line.

The oil from *Ruta chalepensis* was the only oil that generated three TI values greater than 1, although none of the individual IC_{50} values were highly inhibitory and the average IC_{50} was 0.62µl/ml. This oil shows potential for additional testing and identification of active components to determine if similar compounds are active against both non-cancerous and cancerous cells. Average TI values of 2.05 and 1.52 were calculated for *C. limetta* and *C. aurantium* against CAL27 and AGS, respectively, possibly indicating broad spectrum activity. *O. vulgare* showed a similar result against the CAL27 and A375 lines with an average TI of 1.18. This result is significant due to the relatively high average IC_{50} required to effectively inhibit skin cancer cells throughout this study.

Conclusion

The aims of this study were to provide more understanding about medicinal plants commonly used in Guatemala. Essential oils seem to be found in a large number of these plants and may play a role in their effectiveness. This study has demonstrated an improved method for evaluating the effects of essential oils on cancer cell lines used for *in vitro* screenings. Results

have also shown that essential oils can be highly effective against a variety of cancer cell lines in *vitro* with oils from *Citrus aurantiifolia, Origanum vulgare, Teloxys ambrosioides* and *Lippia graveolens* showing potential for future development. Additional results of the Therapeutic Indices indicate that essential oils can be more toxic to cancerous cells than to cells from the established cell line, with *Citrus limetta, Citrus aurantium, L. graveolens, O. vulgare, Eucalyptus* sp*, Rosmarinus officinalis* and *Ruta chalepensis* showing broad and line-specific potential for development.

Additional tests are needed to determine the extent of effectiveness of these essential oils. Active compounds need to be isolated and tested for their specific levels of cytotoxicity to cancerous and non-cancerous cells. The cancer cell lines used in this study are particular as potential cancers that could be treated directly with essential oils. *In vivo* studies need to be conducted to determine how living organisms metabolize the components of essential oils presented directly in a whole or fractionated form.

The search for new and more effective drugs for cancer should include essential oils as resources. With various methods of action and with a wide variety of compounds found naturally in unique combinations, essential oils show much potential in the development of new drugs and functional products.

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Table 1. Species, family, common name, tissue type and percent yield per species for Guatemalan medicinal plants extracted by steam distillation

Species	Family	Common Name	Tissue	% Yield (w/w)
Achillea millefolium L.	Asteraceae	Milenrama	Aerial Portion	0.11
Bixa orellana L.	Bixaceae	Achiote	Seed	0.12
Buddleja americana L.	Buddlejaceae	Salvia Santa	Leaf	0.09
Cinnamomum zeylanicum Blume	Lauraceae	Canela	Leaf	0.45
Citrus aurantiifolia (Christm.) Swingle	Rutaceae	Limón Criollo	Leaf	0.25
C. aurantium L.	Rutaceae	Naranja	Leaf	0.08
C. limetta Risso	Rutaceae	Lima	Leaf	0.17
Cupressus lusitanica Mill.	Cupressaceae	Ciprés	Leaf	0.93
Eucalyptus sp.	Myrtaceae	Eucalipto	Leaf	0.35
Foeniculum vulgare Mill.	Apiaceae	Hinojo	Aerial Portion	0.07
Lippia graveolens Kunth	Verbenaceae	Oregano	Aerial Portion	0.45
Litsea guatemalensis Mez	Lauraceae	Laurel	Leaf	0.24
Mentha piperita L.	Lamiaceae	Menthol Piperita	Aerial Portion	0.50
Ocimum basilicum L.	Lamiaceae	Albahaca	Aerial Portion	0.33
Origanum vulgare L.	Lamiaceae	Oregano de Castillo	Aerial Portion	0.66

Table 1. cont.

Pinus maximinoi H.E.Moore	Pinaceae	Pino	Leaf	0.04
Piper auritum Kunth	Piperaceae	Santa Maria	Leaf	0.27
Psidium guajava L.	Myrtaceae	Guayabo	Leaf	0.19
Rosmarinus officinalis L.	Lamiaceae	Romero	Leaf	0.23
Ruta chalepensis L.	Rutaceae	Ruda	Aerial Portion	0.07
Tagetes filifolia Lag.	Asteraceae	Anís de Monte	Aerial Portion	0.50
Teloxys ambrosioides (L.) W.A.Weber	Chenopodiaceae	Apasote	Aerial Portion	0.12

Table 2: IC_{50} values (ul/ml) for essential oils of Guatemalan medicinal plants tested for activity on cancerous and established cell lines *in vitro*

Species	Vero C	CAL27	A375	AGS
A. millefolium [1]	0.2	0.28	0.29	0.4
B. orellana [1]	<.05*	0.79	0.65	0.29
B. americana [1]	0.06	0.27	0.44	0.39
C. zeylanicum	0.08	0.16	0.18	0.25
C. aurantiifolia	<.05*	<.05*	<.05*	<.05*
C. aurantium [1]	0.25	0.16	0.37	0.17
C. limetta [1]	0.31	0.18	0.62	0.13
C. lusitanica [1]	<.05*	0.22	0.25	0.36
Eucalyptus sp.	0.17	0.32	0.38	0.16
F. vulgare [1]	0.3	0.42	0.64	0.87
L. graveolens [1]	0.09	0.07	0.14	0.15
L. guatemalensis [1]	0.11	0.17	0.2	0.19
M. piperita	0.09	0.23	0.4	0.35
O. basilicum	0.14	0.34	0.36	0.39
O. vulgare	0.1	0.08	0.09	0.18
P. maximinoi [1]	0.15	0.15	0.6	0.17
P. auritum [1]	0.17	0.43	0.86	0.41
P. guajava	0.07	0.21	0.28	0.15
R. officinalis	0.26	0.41	0.24	0.21
R. chalepensis [1]	0.72	0.51	0.71	0.64
T. filifolia [1]	0.42	0.7	2.6	>7**

Table 2. cont.

T. ambrosioides [1]	0.06	0.07	0.08	0.66

[1]Oils not previously reported to have been tested on cancer cell lines *in vitro*
*IC_{50} values are below the measurable values of this assay
**IC_{50} values are above the measurable values of this assay

Table 3: Therapeutic Index values for essential oils of Guatemalan medicinal plants tested for activity on cancerous and established cell lines *in vitro*

Species	CAL27	A375	AGS
A. millefolium	0.71	0.69	0.50
B. orellana	†	†	†
B. americana	0.22	0.14	0.15
C. zeylanicum	0.50	0.44	0.32
C. aurantiifolia	†	†	†
C. aurantium	1.56	0.68	1.47
C. limetta	1.72	0.50	2.38
C. lusitanica	†	†	†
Eucalyptus sp.	0.53	0.45	1.06
F. vulgare	0.71	0.47	0.34
L. graveolens	1.29	0.64	0.60
L. guatemalensis	0.65	0.55	0.58
M. piperita	0.39	0.23	0.26
O. basilicum	0.41	0.39	0.36
O. vulgare	1.25	1.11	0.56
P. maximinoi	1.00	0.25	0.88
P. auritum	0.40	0.20	0.41
P. guajava	0.33	0.25	0.47
R. officinalis	0.63	1.08	1.24
R. chalepensis	1.41	1.01	1.13
T. filifolia	0.60	0.16	†

Table 3. cont.

T. ambrosioides	0.86	0.75	0.09

†TI unable to calculate due to lack of IC_{50} or CC_{50} value