

Article

In-Vitro Evaluation of 52 Commercially-Available Essential Oils Against *Leishmania amazonensis*

Lianet Monzote ¹, Isabel Herrera ¹, Prabodh Satyal ² and William N. Setzer ^{2,3,*} 

¹ Parasitology Department, Institute of Tropical Medicine “Pedro Kouri”, Havana 10400, Cuba; monzote@ipk.sld.cu (L.M.); isa@ipk.sld.cu (I.H.)

² Aromatic Plant Research Center, 230 N 1200 E, Suite 100, Lehi, UT 84043, USA; psatyal@aromaticplant.org

³ Department of Chemistry, University of Alabama in Huntsville, Huntsville, AL 35899, USA

* Correspondence: wsetzer@chemistry.uah.edu or wsetzer@aromaticplant.org; Tel.: +1-256-824-6519

Academic Editors: Valeria Patricia Sülsen and Albertina Moglioni

Received: 5 March 2019; Accepted: 29 March 2019; Published: 30 March 2019



Abstract: Leishmaniasis is a neglected tropical disease caused by members of the *Leishmania* genus of parasitic protozoa that cause different clinical manifestations of the disease. Current treatment options for the cutaneous disease are limited due to severe side effects, poor efficacy, limited availability or accessibility, and developing resistance. Essential oils may provide low cost and readily available treatment options for leishmaniasis. In-vitro screening of a collection of 52 commercially available essential oils has been carried out against promastigotes of *Leishmania amazonensis*. In addition, cytotoxicity has been determined for the essential oils against mouse peritoneal macrophages in order to determine selectivity. Promising essential oils were further screened against intracellular *L. amazonensis* amastigotes. Three essential oils showed notable antileishmanial activities: frankincense (*Boswellia* spp.), coriander (*Coriandrum sativum* L.), and wintergreen (*Gualtheria fragrantissima* Wall.) with IC₅₀ values against the amastigotes of 22.1 ± 4.2, 19.1 ± 0.7, and 22.2 ± 3.5 µg/mL and a selectivity of 2, 7, and 6, respectively. These essential oils could be explored as topical treatment options for cutaneous leishmaniasis.

Keywords: leishmaniasis; frankincense; coriander; wintergreen; birch

1. Introduction

Leishmaniasis is a collection of parasitic diseases caused by several *Leishmania* species [1,2]. The disease is transmitted by several members of Phlebotominae sand flies. The genera *Lutzomyia* (New World) and *Phlebotomus* (Old World) are the only hematophagous vectors of the diseases. Leishmaniasis is considered by the World Health Organization to be a neglected tropical disease [3]. Depending on the *Leishmania* species, the disease can manifest itself in three main forms: cutaneous, mucocutaneous, and visceral. There are currently around 12–15 million people worldwide infected by *Leishmania* spp. with an estimated 350 million people at risk of acquiring leishmaniasis. Unfortunately, at the present time, there are no vaccines or prophylactic medicines available to prevent the disease. Current chemotherapeutic treatment options include sodium stibogluconate, meglumine antimoniate, conventional (deoxycholate) and liposomal amphotericin B, pentamidine isethionate, miltefosine and paromomycin. However, these agents suffer from severe side effects, poor efficacy, limited availability, high cost, or developing resistance [2,4]. Furthermore, treatment of leishmaniasis is often hampered by limited access to medicines and treatment options in developing countries where the disease is prevalent [5,6].

Essential oils are complex mixtures of volatile phytochemicals with numerous and varied biological properties [7,8]. Essential oils, particularly those that are readily available, may provide low cost treatment options for leishmaniasis. The antiparasitic activities of essential oils and their

components have been demonstrated and previously reviewed [9,10]. Commercially available EOs have been screened for activity against a variety of human pathogens [11–17] as well as for cytotoxic activity [12,16]. In this work, we carried out in-vitro antileishmanial and cytotoxic screening of a selection of 52 commercially available essential oils against the promastigote form of *Leishmania amazonensis* and peritoneal macrophages from BALB/c, respectively. Promising antileishmanial essential oils were further screened against intracellular amastigotes.

2. Results and Discussion

A total of 52 commercially-available essential oils were screened for in-vitro antileishmanial activity against the promastigote form of *L. amazonensis*, as well as for cytotoxic activity against mouse peritoneal macrophages, the host cells for the amastigote form of the parasite. The antileishmanial and cytotoxic activities of the essential oils are summarized in Table 1. Of the essential oils tested, three showed notable antileishmanial activity and selectivity, frankincense oil (from the oleogum resin of *Boswellia* spp.), coriander oil (from the seeds of *Coriandrum sativum* L.), and wintergreen oil (from the leaves of *Gualtheria fragrantissima* Wall.). These three products were also active against the intracellular amastigote form of *L. amazonensis* (Table 2).

The cluster analysis revealed four clusters based on biological activities (Figure 1). Cluster 1 is made up of essential oils that showed good antileishmanial activity with little or no cytotoxicity. The very large cluster 2 can be described as essential oils that showed relatively weak antileishmanial activity and relatively weak cytotoxic activity, cluster 3 is composed of essential oils with both moderate antileishmanial activity but also with moderate cytotoxic activity, and cluster 4 is made up of essential oils that were very cytotoxic.

The active cluster (Cluster 1) can be subdivided into two sub-clusters made up of coriander (*Coriandrum sativum*) and frankincense (*Boswellia* spp.), Cluster 1a, and Cluster 1b, wintergreen (*Gualtheria fragrantissima*) and birch bark (*Betula lenta* L.). Frankincense is the oleogum resin from several *Boswellia* species. Commercial frankincense sources include *B. sacra* Flueck., *B. carteri* Birdw., *B. frereana* Birdw., *B. papyrifera* Hochst. and *B. serrata* Roxb. Commercial frankincense essential oil from doTERRA International is a proprietary blend of largely *B. carteri*, followed by *B. sacra*, *B. papyrifera*, and *B. freriana*. Analysis of the essential oil used in this investigation revealed that it is composed largely of monoterpenes, including α -pinene (41.2%), α -thujene (15.7%), limonene (9.4%), sabinene (4.5%), β -pinene (3.5%), and *p*-cymene (3.3%), as well as octyl acetate (5.4%) and β -caryophyllene (3.1%). Frankincense essential oil showed activity against promastigotes and amastigotes of *L. amazonensis* with IC₅₀ values of <12.5 and 22.1 ± 4.2 μ g/mL, respectively. Consistent with the antiparasitic activity of frankincense essential oil, Fujisaki and co-workers screened *B. carteri* essential oil against *Plasmodium falciparum* and the oil was shown to have an IC₅₀ of 10 μ g/mL [18]. α -Pinene itself has shown activity against both promastigotes (IC₅₀ 19.7 μ g/mL) and amastigotes (IC₅₀ 16.1 μ g/mL) of *L. amazonensis* [19].

Another essential oil rich in α -pinene was cypress (*Cupressus sempervirens* L.) with 49.7% α -pinene. Cypress essential oil gave an IC₅₀ of 40.0 μ g/mL against *L. amazonensis* promastigotes, but was too cytotoxic (CC₅₀ 27.2 μ g/mL) against mouse peritoneal macrophages to be considered for further evaluation. Other major components in cypress essential oil were δ -3-carene (27.0%) and terpinolene (4.2%). Juniper (*Juniperus communis* L.) berry essential oil was also dominated by α -pinene (34.9%), with lesser quantities of myrcene (11.9%), sabinene (11.4%), β -pinene (7.9%), and terpinen-4-ol (4.5%). Juniper berry essential oil showed good activity against *L. amazonensis* promastigotes (IC₅₀ 27.4 μ g/mL) and reduced cytotoxicity against mouse peritoneal macrophages (CC₅₀ 103.0 μ g/mL). Thus, the presence of α -pinene seems to impart antileishmanial activity, but the activity is apparently enhanced or attenuated by other components in the essential oil.

Table 1. Screening of 52 essential oils against *Leishmania amazonensis* promastigotes and BALB/c mouse peritoneal macrophages.

Essential Oil	Commercial Source	IC ₅₀ ± SD (µg/mL) Promastigotes <i>L. amazonensis</i>	CC ₅₀ ± SD (µg/mL) Macrophage from BALB/c mice	Selectivity Index (SI)	Comments
<i>Abies sibirica</i> Ledeb. (Siberian fir)	dōTERRA	58.2 ± 8.5	42.5 ± 2.4	1	Unspecific
<i>Anthemis nobilis</i> L. (Roman chamomile)	dōTERRA	27.9 ± 4.5	85.7 ± 6.0	3	Unspecific
<i>Betula lenta</i> L. (birch)	dōTERRA	32.2 ± 6.6	136.8 ± 9.2	4	Unspecific
<i>Boswellia Roxb. ex Colebr. spp. (frankincense)</i>	dōTERRA	<12.5	59.3 ± 1.8	5	Active/Follow up
<i>Cananga odorata</i> (Lam.) Hook. f. & Thomson (ylang ylang)	dōTERRA	36.6 ± 3.9	55.6 ± 0.5	2	Unspecific
<i>Cinnamomum zeylanicum</i> Blume (cinnamon bark)	dōTERRA	<12.5	<12.5	-	Too Toxic
<i>Cistus ladanifer</i> L. (cistus)	Albert Vielle	19.2 ± 4.6	56.9 ± 8.1	3	Unspecific
<i>Citrus aurantium</i> L. (pettitgrain)	dōTERRA	56.9 ± 1.8	77.4 ± 0.9	1	Unspecific
<i>Citrus aurantium</i> L. (wild orange)	dōTERRA	34.8 ± 2.2	35.2 ± 1.7	1	Unspecific
<i>Citrus aurantifolia</i> Swingle (lime)	dōTERRA	<12.5	<12.5	-	Too toxic
<i>Citrus × bergamia</i> Risso & Poit. (bergamot)	dōTERRA	59.2 ± 6.3	44.9 ± 1.6	1	Unspecific
<i>Citrus limon</i> (L.) Osbeck (lemon)	dōTERRA	71.2 ± 1.9	71.1 ± 1.6	1	Unspecific
<i>Citrus × paradisi</i> Macfad. (grapefruit)	dōTERRA	35.5 ± 4.4	<12.5	-	Too Toxic
<i>Citrus reticulata</i> Blanco (tangerine)	dōTERRA	70.7 ± 7.8	54.3 ± 1.0	1	Unspecific
<i>Commiphora myrrha</i> (T. Nees) Engl. (myrrh)	dōTERRA	<12.5	<12.5	-	Too Toxic
<i>Coriandrum sativum</i> L. (coriander)	dōTERRA	<12.5	141.7 ± 4.1	>8	Active/Follow up
<i>Coriandrum sativum</i> L. (cilantro)	dōTERRA	34.4 ± 4.8	45.9 ± 1.3	1	Unspecific
<i>Copaifera</i> L. spp. (copaiba)	dōTERRA	17.2 ± 0.1	23.3 ± 7.1	1	Unspecific
<i>Cupressus sempervirens</i> L. (cypress)	Améo	40.0 ± 2.3	27.2 ± 0.7	1	Unspecific
<i>Cymbopogon flexuosus</i> (Nees) Will. Watson (lemongrass)	dōTERRA	27.8 ± 0.4	30.0 ± 2.0	1	Unspecific
<i>Elettaria cardamomum</i> (L.) Maton (cardamom)	dōTERRA	109.5 ± 1.3	60.5 ± 6.0	1	Unspecific
<i>Eugenia caryophyllata</i> Thunb. (syn. <i>Syzygium aromaticum</i> (L.) Merr. & L.M. Perry) (clove)	dōTERRA	>200	143.1 ± 11.9	-	Inactive
<i>Eucalyptus radiata</i> Sieber ex DC. (eucalyptus)	dōTERRA	164.7 ± 8.3	100.2 ± 8.4	1	Unspecific
<i>Foeniculum vulgare</i> Mill. (sweet fennel)	dōTERRA	>200	66.9 ± 7.9	-	Inactive
<i>Gualtheria fragrantissima</i> Wall.(wintergreen)	dōTERRA	20.7 ± 1.6	135.4 ± 5.7	7	Active/Follow up
<i>Helichrysum italicum</i> G. Don f. (helichrysum)	Améo	42.8 ± 2.2	55.2 ± 2.4	1	Unspecific
<i>Juniperus communis</i> L. (juniper berry)	Améo	27.4 ± 0.5	103.0 ± 9.3	4	Unspecific
<i>Juniperus virginiana</i> L. (cedarwood)	dōTERRA	53.2 ± 8.4	<12.5	-	Too Toxic
<i>Lavandula angustifolia</i> Mill. (lavender)	Améo	70.7 ± 5.0	82.3 ± 2.4	1	Unspecific
<i>Melaleuca alternifolia</i> Cheel (tea tree)	dōTERRA	70.7 ± 6.2	42.5 ± 1.4	1	Unspecific
<i>Melissa officinalis</i> L. (melissa)	dōTERRA	24.6 ± 0.7	37.3 ± 1.4	2	Unspecific
<i>Mentha piperita</i> L. (peppermint)	dōTERRA	172.8 ± 4.1	67.3 ± 4.4	0	Unspecific
<i>Mentha spicata</i> L. (spearmint)	dōTERRA	79.8 ± 3.0	90.4 ± 5.6	1	Unspecific
<i>Myristica fragrans</i> Houtt. (nutmeg)	Améo	133.5 ± 2.6	40.0 ± 1.1	0	Unspecific
<i>Nardostachys jatamansi</i> (D. Don) DC. (spikenard)	dōTERRA	49.8 ± 1.4	22.1 ± 4.0	0	Unspecific
<i>Nepeta cataria</i> L. (catnip)	Mountain Rose	54.0 ± 4.4	81.6 ± 0.8	2	Unspecific

Table 1. Cont.

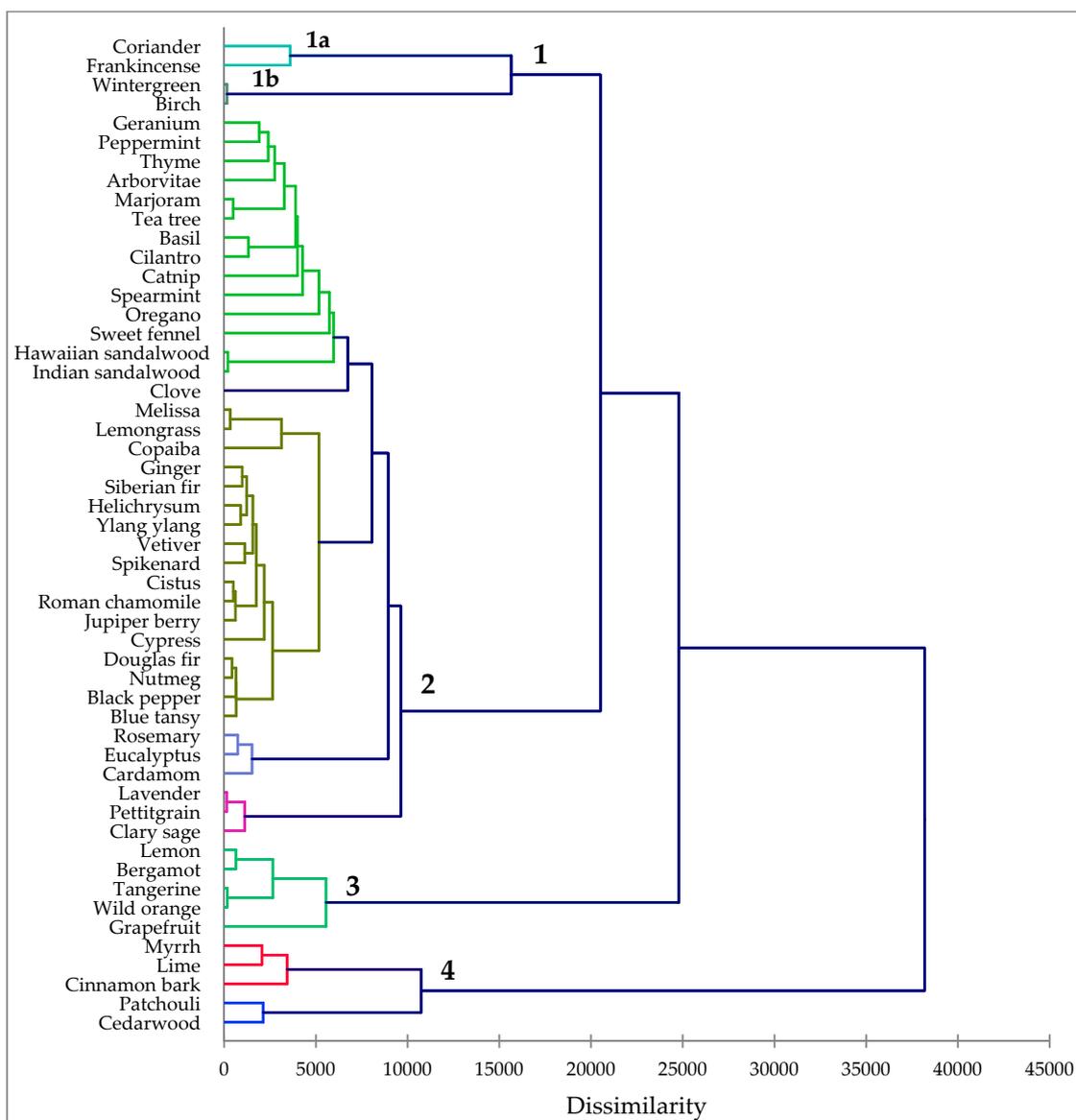
Essential Oil	Commercial Source	IC ₅₀ ± SD (µg/mL) Promastigotes <i>L. amazonensis</i>	CC ₅₀ ± SD (µg/mL) Macrophage from BALB/c mice	Selectivity Index (SI)	Comments
<i>Ocimum basilicum</i> L. (basil)	dōTERRA	>200	69.1 ± 9.0	-	Inactive
<i>Origanum majorana</i> L. (marjoram)	dōTERRA	>200	25.7 ± 1.3	-	Inactive
<i>Origanum vulgare</i> L. (oregano)	dōTERRA	>200	66.5 ± 0.9	-	Inactive
<i>Pelargonium graveolens</i> L'Hér. ex Aiton (geranium)	Améo	>200	57.4 ± 3.6	-	Inactive
<i>Piper nigrum</i> L. (black pepper)	dōTERRA	57.7 ± 3.7	35.6 ± 5.7	1	Unspecific
<i>Pogostemon cablin</i> (Blanco) Benth. (patchouli)	Améo	68.7 ± 7.8	<12.5	-	Too Toxic
<i>Pseudotsuga menziesii</i> (Mirb.) Franco (Douglas fir)	dōTERRA	82.5 ± 4.5	37.7 ± 3.2	0	Unspecific
<i>Rosmarinus officinalis</i> L. (rosemary)	dōTERRA	89.7 ± 2.0	83.4 ± 7.3	1	Unspecific
<i>Santalum album</i> L. (Indian sandalwood)	dōTERRA	105.5 ± 6.0	29.9 ± 6.3	0	Unspecific
<i>Santalum paniculatum</i> Hook. & Arn. (Hawaiian sandalwood)	dōTERRA	43.1 ± 2.2	25.9 ± 5.3	1	Unspecific
<i>Salvia sclarea</i> L. (clary sage)	Améo	>200	58.6 ± 9.0	-	Inactive
<i>Tanacetum annuum</i> L. (blue tansy)	dōTERRA	52.2 ± 2.8	36.6 ± 5.8	1	Unspecific
<i>Thuja plicata</i> Donn ex D. Don (arborvitae)	dōTERRA	67.1 ± 3.1	61.9 ± 6.1	1	Unspecific
<i>Thymus vulgaris</i> L. (thyme)	dōTERRA	>200	30.5 ± 5.5	-	Inactive
<i>Vetiveria zizanioides</i> (L.) Nash (syn. <i>Chrysopogon zizanioides</i> (L.) Roberty) (vetiver)	dōTERRA	19.0 ± 3.3	31.7 ± 2.8	2	Unspecific
<i>Zingiber officinale</i> Roscoe (ginger)	dōTERRA	39.9 ± 3.4	58.3 ± 4.7	1	Unspecific
Pentamidine		0.37 ± 0.01	11.7 ± 1.7	31	Active

IC₅₀: Median inhibitory concentration. Concentration that inhibits 50% of promastigote growth. CC₅₀: Median cytotoxic concentration. Concentration that causes the mortality of 50% of macrophages. SD: Standard deviation. SI: Selectivity index: CC₅₀/IC₅₀. In bold, essential oils that were selected as active and selective.

Table 2. Screening of frankincense, coriander, and wintergreen essential oils against *Leishmania amazonensis* intracellular amastigotes.

Essential Oil	IC ₅₀ ± SD (µg/mL)	SI
<i>Boswellia</i> spp. (frankincense)	22.1 ± 4.2	2
<i>Coriandrum sativum</i> (coriander)	19.1 ± 0.7	7
<i>Gualtheria fragrantissima</i> (wintergreen)	22.2 ± 3.5	6
Pentamidine	1.3 ± 0.1	9

IC₅₀: Median inhibitory concentration. Concentration that inhibits 50% of promastigote growth. SD: Standard deviation. SI: Selectivity index: CC₅₀/IC₅₀.

**Figure 1.** Dendrogram obtained from the agglomerative hierarchical cluster analysis of 52 commercial essential oil compositions and antileishmanial and cytotoxic activities.

Coriander is the fruit of *C. sativum*. Commercial coriander essential oil used in this study was obtained from dōTERRA International and the major components were linalool (73.5%), α -pinene (5.3%), and γ -terpinene (4.5%). A linalool-rich (73.2%) coriander essential oil was screened by Rondon and co-workers against *Leishmania chagasi* and was shown to be very effective against amastigotes (IC₅₀ 1.51 µg/mL), but much less active against the promastigotes (IC₅₀ 181 µg/mL) [20]. It is

tempting to speculate that linalool might be the compound responsible for the antileishmanial activity. Indeed, Rosa and co-workers have shown linalool to be very active against both promastigotes (IC_{50} 0.0043 $\mu\text{g}/\text{mL}$) and amastigotes (IC_{50} 0.0155 $\mu\text{g}/\text{mL}$) of *L. amazonensis* [21]. However, the presence of linalool does not necessarily translate to a good antileishmanial profile. Pettitgrain (*Citrus aurantium* L.) leaf essential oil (25.4% linalool), lavender (*Lavandula angustifolia* Mill.) essential oil (34.4% linalool), and basil (*Ocimum basilicum* L.) essential oil (55.7% linalool), were only marginally active against *L. amazonensis* promastigotes (IC_{50} 56.9, 70.7, and >200 $\mu\text{g}/\text{mL}$, respectively). There may be other components in these essential oils attenuating the antileishmanial activity of linalool.

Interestingly, wintergreen (*Gualtheria fragrantissima*) essential oil, which was dominated by methyl salicylate (99.7%), showed antileishmanial activity against both *L. amazonensis* promastigotes (IC_{50} 20.7 $\mu\text{g}/\text{mL}$) and amastigotes (IC_{50} 22.2 $\mu\text{g}/\text{mL}$). *Betula lenta* (birch bark) essential oil (99.9% methyl salicylate) was somewhat less active against *L. amazonensis* promastigotes (IC_{50} 32.2 $\mu\text{g}/\text{mL}$). Considering the abundance of methyl salicylate in both wintergreen and birch essential oils, this compound is likely the active component.

Copaiba oils (not hydrodistilled essential oils) from several different species of *Copaifera* were screened by Santos and collaborators against *L. amazonensis* [22]. Antileishmanial activities against the promastigotes ranged from 5.0 to 22.0 $\mu\text{g}/\text{mL}$. Consistent with these results, copaiba oil in this study (50% β -caryophyllene) showed an IC_{50} of 17.2 $\mu\text{g}/\text{mL}$. The copaiba essential oil, however, was also too cytotoxic (CC_{50} 23.3 $\mu\text{g}/\text{mL}$) to warrant further consideration. On the other hand, β -caryophyllene was found to be remarkably active against *L. amazonensis* amastigotes (IC_{50} 1.3 $\mu\text{g}/\text{mL}$) but less toxic to murine macrophages (CC_{50} 63.6 $\mu\text{g}/\text{mL}$) [23]. In contrast, antipromastigote activity of β -caryophyllene on *L. amazonensis* was reported to have an IC_{50} of 19 $\mu\text{g}/\text{mL}$ [24]. β -Caryophyllene was also found to show excellent antileishmanial activity against *L. infantum* and *L. major* (IC_{50} 1.06 and 1.33 $\mu\text{g}/\text{mL}$, respectively), with less cytotoxicity against Raw 264.7 mouse macrophage cells [25].

Rosemary (*Rosmarinus officinalis* L.) essential oil from Colombia (composition not reported) showed activity against *L. braziliensis* promastigotes with an IC_{50} of 17.4 $\mu\text{g}/\text{mL}$ [26]. Similarly, rosemary essential oil from Tunisia (43.8% 1,8-cineole, 12.0% camphor, 11.5% α -pinene, 8.6% β -pinene, 4.8% camphene) was active against *L. infantum* (IC_{50} 16.3 $\mu\text{g}/\text{mL}$) and *L. major* (IC_{50} 20.9 $\mu\text{g}/\text{mL}$) promastigotes [25]. Conversely, a commercial rosemary essential oil obtained in Germany was inactive against *L. major* promastigotes (IC_{50} 282 $\mu\text{g}/\text{mL}$) [11]. In this present study, commercial rosemary essential oil (45.9% 1,8-cineole, 12.0% α -pinene, 10.9% camphor, 6.3% β -pinene) was only marginally antileishmanial (IC_{50} 89.7 $\mu\text{g}/\text{mL}$), but also marginally cytotoxic (CC_{50} 83.4 $\mu\text{g}/\text{mL}$). Rosemary essential oils have shown great variation in composition with at least five different chemotypes, which likely result in different biological activities [27].

Mikus and co-workers have screened several essential oils and essential oil components for antileishmanial activity against *L. major* promastigotes [11]. These workers found commercial *Melissa officinalis* L. essential oil to show excellent activity against *L. major* promastigotes (IC_{50} 7.0 $\mu\text{g}/\text{mL}$) with less toxicity against HL-60 (human leukemia) cells (CC_{50} 25.5 $\mu\text{g}/\text{mL}$). Andrade and co-workers screened a commercial *M. officinalis* essential oil (37.2% neral, 52.0% geranial) and found marginal activity against *L. amazonensis* promastigotes with an IC_{50} of 132 $\mu\text{g}/\text{mL}$ [17]. In contrast, *M. officinalis* essential oil from Colombia was inactive against *L. braziliensis* promastigotes [26]. In this present work, *M. officinalis* essential oil showed antileishmanial activity (IC_{50} 24.6 $\mu\text{g}/\text{mL}$) but also cytotoxicity (CC_{50} 37.3 $\mu\text{g}/\text{mL}$) giving it an unfavorable selectivity index. The major components in *M. officinalis* essential oil were β -caryophyllene (13.4%), geranial (30.2%), and neral (23.1%). Citral, a mixture of geranial and neral, has shown antileishmanial activity against *L. infantum*, *L. tropica*, and *L. major* promastigotes with IC_{50} values of 42, 34, and 36 $\mu\text{g}/\text{mL}$, respectively [28]. However, citral has demonstrated in-vitro cytotoxic activity against several cell lines [29–31]. There are several chemotypes of *M. officinalis* based on essential oil composition, which likely account for the differences in biological activities. Nevertheless, the citral chemotype of *M. officinalis* has also shown in-vitro cytotoxicity [32].

Likewise, the unfavorable bioactivity profile of lemongrass (*Cymbopogon flexuosus* (Nees) Will. Watson) essential oil in this study is likely due to the abundance of geranial (49.9%) and neral (23.4%). However, Machado and co-workers found that citral-rich *C. citratus* as well as citral did not exhibit cytotoxic effects on either primary bovine aortic endothelial cells or RAW 264.7 macrophage cells [28]. Similarly, Santin and co-workers found both *C. citratus* essential oil and citral to be antileishmanial against promastigotes (IC₅₀ 1.7 and 8.0 µg/mL, respectively) and amastigotes (IC₅₀ 3.2 and 25.0 µg/mL, respectively) of *L. amazonensis*, but with lower cytotoxicity against J774G8 macrophage cells [33].

As has been appreciated, many pure components present in the tested EOs have exhibited some level of antileishmanial activity. In fact, it is known that the compounds present in the EOs can act synergistically in mixtures, increasing the intrinsic effects of the purified components. Therefore, we suggest the potential use of EOs as mixtures. In addition, we have also taken into account that the tested EOs are commercially available in their present forms, reducing the cost of a therapeutic alternative, which constitutes one of main drawbacks of current antileishmanial treatments. The notable antileishmanial effects and moderate cytotoxicities in the case of mixtures of compounds could be further explored in animal models of cutaneous leishmaniasis by intralesional or topical routes.

3. Materials and Methods

3.1. Essential Oils

The essential oils were obtained from commercial sources, dōTERRA International (Pleasant Grove, UT, USA), Ameo Essential Oils (Zija International, Lehi, UT, USA), Mountain Rose Herbs (Eugene, OR, USA), or Albert Vielle (Vallauris, France) and were previously analyzed by gas chromatography–mass spectrometry.

3.2. Parasites

Leishmania amazonensis parasites (Reference strain MHOM/77/BR/LTB0016) were kindly provided by the Department of Immunology, Oswaldo Cruz Foundation (FIOCRUZ), Brazil. The parasites were routinely isolated from mouse lesions (BALB/c mice) and maintained as promastigotes at 26 °C in Schneider's medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (HFBS) (Sigma-Aldrich, St. Louis, MO, USA) and 100 U of penicillin and 100 µg streptomycin per mL as antibiotics. Parasite cultures were passaged every 3–4 days, but no longer used after the tenth passage after isolation from mice.

3.3. In-vitro Anti-promastigote Screening

Screening against *L. amazonensis* promastigotes was carried out as previously described [34–36]. Into each well in a 96-well plate, 50 µL medium (Schneider's medium + HFBS + antibiotics) was added. Into the wells of column 2 and 7, 48 µL medium + 2 µL sample (dimethylsulfoxide solutions of essential oil, 20 mg/mL) were added. Five two-fold serial dilutions were carried out down each column to give final test concentrations of 12.5, 25, 50, 100 and 200 µg/mL. Exponentially growing parasites (4×10^5) in medium were added to each well. Dimethylsulfoxide (DMSO) served as the negative control and pentamidine (Richet, Buenos Aires, Argentina) was used as a positive control. The plates were sealed with Parafilm® and incubated at 26 °C for 72 h. Afterward, 20 µL of a solution (5 mg/mL) of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well. The plates were incubated for an additional 4 h, the supernatant was removed, and the formazan crystals were dissolved with 100 µL DMSO. The absorbance of each well was determined with a plate reader (Molecular Devices, San Jose, CA, USA) with a test wavelength of 560 nm and a reference wavelength of 630 nm from which median inhibitory concentrations (IC₅₀) were calculated [37,38]. The IC₅₀ values were determined from linear dose-response curves fit to the data using a linear equation model. Each test was carried out in triplicate, and the results expressed as means ± standard deviations (SD).

3.4. Mouse Peritoneal Macrophage Cytotoxicity Screening

The median cytotoxic concentrations (CC_{50}) of the essential oils on mouse peritoneal macrophages (the host cells of *Leishmania* parasites) were determined as previously described [34–36]. Macrophages were collected from the peritoneal cavities of normal BALB/c mice and suspended in ice-cold RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with antibiotics. The cells were seeded in 96-well plates (3×10^5 cells/well) and incubated at 37 °C for 2 h. Non-adherent cells were removed. Into the wells of column 2 and 7, 2 μ L of essential oil solutions (as above) and 48 μ L medium (supplemented with 10% HFBS and antibiotics) were also added. Two-fold serial dilutions down each lane were carried out to give final concentrations of 12.5–200 μ g/mL. The macrophages were incubated for 72 h, after which the cytotoxicity was determined using the MTT assay (as above), using 15 μ L/well. The CC_{50} was calculated for each compound in the same manner as the IC_{50} and selectivity indices (SI) for each essential oil were determined (CC_{50} for macrophages / IC_{50} for promastigotes).

Essential oils with an $IC_{50} < 100$ μ g/mL and a $SI \geq 5$ were selected for further evaluation against *L. amazonensis* amastigotes [34].

3.5. In-vitro Intracellular Anti-amastigote Screening

Median inhibitory concentrations (IC_{50}) of the active essential oils on *L. amazonensis* amastigotes were carried out as previously described [34–36]. The peritoneal macrophages, obtained from BALB/c mice (as above), were seeded in 24-well culture plates at 10^6 cells/mL. The plates were incubated at 37 °C under a 5% CO_2 atmosphere for 2 h. Non-adherent cells were removed, and stationary-phase *L. amazonensis* promastigotes were added (4:1 promastigote/macrophage ratio), and incubation continued for an additional 4 h. Free parasites were removed and 1000 μ L RPMI completed medium was added to each well. Into the first well, 990 μ L medium and 10 μ L essential oil solution were added. Four two-fold dilutions were carried out to give final concentrations of 12.5, 25, 50 and 100 μ g/mL. The plate was incubated at 37 °C under a 5% CO_2 atmosphere for 48 h. The cell monolayers were fixed in absolute methanol, stained with Giemsa, and evaluated using a light microscope. The number of intracellular amastigotes was determined by counting 25 macrophages per sample. The results are expressed as percent reduction of infection rate (% infected macrophages \times number amastigotes per infected macrophage) compared to that of the controls. The IC_{50} values were determined from the concentration-response linear curves. Each evaluation was carried out in triplicate and the results expressed as means \pm SD.

3.6. Hierarchical Cluster Analysis

The chemical compositions of the commercial essential oils along with the antileishmanial and cytotoxic activities were used in an agglomerative hierarchical cluster (AHC) analysis. The essential oil compositions of the 52 commercially-available essential oils and the bioactivities were used to determine the associations between the essential oils and their activities using XLSTAT Premium, version 2018.5.53172 (Addinsoft, Paris, France). Dissimilarity was determined using Euclidean distance, and clustering was defined using Ward's method.

4. Conclusions

This study has shown that the essential oils of frankincense, coriander, wintergreen, and birch have notable antiparasitic activities against *Leishmania amazonensis* with an acceptable SI with respect to cytotoxicity against mouse peritoneal macrophages. However, essential oils are complex mixtures of compounds, and the biological activities cannot necessarily be attributed to individual components; there are apparent synergistic and/or antagonistic interactions as well. Nevertheless, essential oils containing appreciable concentrations of α -pinene, linalool, or methyl salicylate should be investigated for antiparasitic activity.

Author Contributions: Conceptualization, W.N.S.; methodology, L.M.; formal analysis, L.M., P.S., and W.N.S.; investigation, L.M., I.H., P.S. and W.N.S.; resources, L.M.; data curation, W.N.S.; writing—original draft preparation, W.N.S.; writing—review and editing, L.M. and W.N.S.; project administration, W.N.S.

Funding: This research received no external funding.

Acknowledgments: W.N.S. is grateful to Zija International and dōTERRA International for generously donating essential oils. L.M. and W.N.S. carried out this work as part of the activities of the Research Network Natural Products against Neglected Diseases (ResNetNPND), (<http://www.resnetnpnd.org/Start/>). P.S. and W.N.S. contributed to this project as part of the activities of the Aromatic Plant Research Center (APRC, <https://aromaticplant.org/>).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Torres-Guerrero, E.; Quintanilla-Cedillo, M.R.; Ruiz-Esmenjaud, J.; Arenas, R. Leishmaniasis: A review. *F1000Research* **2017**, *6*, 750. [CrossRef]
2. Alvar, J.; Arana, B. Leishmaniasis, impact and therapeutic needs. In *Drug Discovery for Leishmaniasis*; Livas, L., Gil, C., Eds.; Royal Society of Chemistry: London, UK, 2018; pp. 3–23.
3. World Health Organization. Neglected Tropical Diseases. Available online: https://www.who.int/neglected_diseases/diseases/en/ (accessed on 1 March 2019).
4. Iqbal, H.; Ishfaq, M.; Wahab, A.; Abbas, M.N.; Ahmad, I.; Rehman, A.; Zakir, M. Therapeutic modalities to combat leishmaniasis, a review. *Asian Pacific J. Trop. Dis.* **2016**, *6*, 1–5. [CrossRef]
5. Hailu, T.; Yimer, M.; Mulu, W.; Abera, B. Challenges in visceral leishmaniasis control and elimination in the developing countries: A review. *J. Vector Borne Dis.* **2016**, *53*, 193–198. [PubMed]
6. Sunyoto, T.; Potet, J.; Boelaert, M. Why miltefosine—a life-saving drug for leishmaniasis—is unavailable to people who need it the most. *BMJ Glob. Heal.* **2018**, *3*, e000709. [CrossRef]
7. Firenzuoli, F.; Jaitak, V.; Horvath, G.; Henri, I.; Bassolé, N.; Setzer, W.N.; Gori, L. *Essential Oils: New Perspectives in Human Health and Wellness*; Hindawi Publishing Corp.: London, UK, 2014.
8. Başer, K.H.C.; Buchbauer, G. *Handbook of Essential Oils: Science, Technology, and Applications*; CRC Press: Boca Raton, FL, USA, 2010.
9. Monzote, L.; Alarcón, O.; Setzer, W.N. Antiprotozoal activity of essential oils. *Agric. Conspec. Sci.* **2012**, *77*, 167–175.
10. Bero, J.; Kpoviessi, S.; Quetin-Leclercq, J. Anti-parasitic activity of essential oils and their active constituents against *Plasmodium*, *Trypanosoma* and *Leishmania*. In *Novel Plant Bioresources: Applications in Food, Medicine and Cosmetics*; Gurub-Fakim, A., Ed.; John Wiley & Sons Ltd.: Oxford, UK, 2014; pp. 455–470.
11. Mikus, J.; Harkenthal, M.; Steverding, D.; Reichling, J. In vitro effect of essential oils and isolated mono- and sesquiterpenes on *Leishmania major* and *Trypanosoma brucei*. *Planta Med.* **2000**, *66*, 366–368. [CrossRef]
12. Powers, C.N.; Osier, J.L.; McFeeters, R.L.; Brazell, C.B.; Olsen, E.L.; Moriarity, D.M.; Satyal, P.; Setzer, W.N. Antifungal and cytotoxic activities of sixty commercially-available essential oils. *Molecules* **2018**, *23*, 1549. [CrossRef] [PubMed]
13. Orchard, A.; Viljoen, A.; van Vuuren, S. Wound pathogens: Investigating antimicrobial activity of commercial essential oil combinations against reference strains. *Chem. Biodivers.* **2018**, *15*, e1800405. [CrossRef]
14. Orchard, A.; van Vuuren, S.F.; Viljoen, A.M. Commercial essential oil combinations against topical fungal pathogens. *Nat. Prod. Commun.* **2019**, *14*, 151–158. [CrossRef]
15. Serra, E.; Hidalgo-Bastida, L.A.; Verran, J.; Williams, D.; Malic, S. Antifungal activity of commercial essential oils and biocides against *Candida albicans*. *Pathogens* **2018**, *7*, 15. [CrossRef]
16. Cannas, S.; Usai, D.; Tardugno, R.; Benvenuti, S.; Pellati, F.; Zanetti, S.; Mollicotti, P.; Cannas, S.; Usai, D.; Tardugno, R.; et al. Chemical composition, cytotoxicity, antimicrobial and antifungal activity of several essential oils. *Nat. Prod. Res.* **2016**, *30*, 332–339. [CrossRef]
17. Andrade, M.A.; Azevedo, S.; Motta, F.N.; Lucilia, M.; Silva, C.L.; De Santana, J.M.; Bastos, I.M.D. Essential oils: In vitro activity against *Leishmania amazonensis*, cytotoxicity and chemical composition. *BMC Complement. Altern. Med.* **2016**, *16*, 444. [CrossRef]

18. Fujisaki, R.; Kamei, K.; Yamamura, M.; Nishiya, H.; Inouye, S.; Takahashi, M.; Abe, S. In vitro and in vivo anti-plasmodial activity of essential oils, including himokitiol. *Southeast Asian J. Trop. Med. Public Health* **2012**, *43*, 270–279. [[PubMed](#)]
19. Rodrigues, K.A.D.F.; Amorim, L.V.; Dias, C.N.; Moraes, D.F.C.; Carneiro, S.M.P.; Carvalho, F.A.D.A. *Syzygium cumini* (L.) Skeels essential oil and its major constituent α -pinene exhibit anti-*Leishmania* activity through immunomodulation in vitro. *J. Ethnopharmacol.* **2015**, *160*, 32–40. [[CrossRef](#)] [[PubMed](#)]
20. Rondon, F.C.M.; Bevilaqua, C.M.L.; Accioly, M.P.; de Moraes, S.M.; de Andrade-Júnior, H.F.; de Carvalho, C.A.; Lima, J.C.; Magalhães, H.C.R. In vitro efficacy of *Coriandrum sativum*, *Lippia sidoides* and *Copaifera reticulata* against *Leishmania chagasi*. *Rev. Bras. Parasitol. Veterinária* **2012**, *21*, 185–191. [[CrossRef](#)]
21. Rosa, M.D.S.S.; Mendonça-Filho, R.R.; Bizzo, H.R.; Rodrigues, I.D.A.; Soares, R.M.A.; Souto-Padrón, T.; Alviano, C.S.; Lopes, A.H.C.S. Antileishmanial activity of a linalool-rich essential oil from *Croton cajucara*. *Antimicrob. Agents Chemother.* **2003**, *47*, 1895–1901. [[CrossRef](#)]
22. Santos, A.O.; Ueda-Nakamura, T.; Dias Filho, B.P.; Veiga Junior, V.F.; Pinto, A.C.; Nakamura, C.V. Effect of Brazilian copaiba oils on *Leishmania amazonensis*. *J. Ethnopharmacol.* **2008**, *120*, 204–208. [[CrossRef](#)] [[PubMed](#)]
23. Soares, D.C.; Portella, N.A.; Ramos, M.F.D.S.; Siani, A.C.; Saraiva, E.M. *trans*- β -Caryophyllene: An effective antileishmanial compound found in commercial copaiba oil (*Copaifera* spp.). *Evidence-Based Complement. Altern. Med.* **2013**, *2013*, 761323. [[CrossRef](#)] [[PubMed](#)]
24. do Carmo, D.F.M.; Amaral, A.C.F.; Machado, G.M.C.; Leon, L.L.; Silva, J.R.D.A. Chemical and biological analyses of the essential oils and main constituents of *Piper* species. *Molecules* **2012**, *17*, 1819–1829. [[CrossRef](#)]
25. Essid, R.; Zahra, F.; Msaada, K.; Sghair, I.; Hammami, M.; Bouratbine, A.; Aoun, K.; Limam, F. Antileishmanial and cytotoxic potential of essential oils from medicinal plants in Northern Tunisia. *Ind. Crop. Prod.* **2015**, *77*, 795–802. [[CrossRef](#)]
26. Arévalo, Y.; Robledo, S.; Muñoz, D.L.; Granados-Falla, D.; Cuca, L.E.; Delgado, G. Evaluación in vitro de la actividad de aceites esenciales de plantas colombianas sobre *Leishmania braziliensis*. *Rev. Colomb. Ciencias Químico-Farm.* **2009**, *38*, 131–141.
27. Satyal, P.; Jones, T.H.; Lopez, E.M.; McFeeters, R.L.; Ali, N.A.A.; Mansi, I.; Al-Kaf, A.G.; Setzer, W.N. Chemotypic characterization and biological activity of *Rosmarinus officinalis*. *Foods (Basel, Switzerland)* **2017**, *6*, 20. [[CrossRef](#)]
28. Machado, M.; Pires, P.; Dinis, A.M.; Santos-Rosa, M.; Alves, V.; Salgueiro, L.; Cavaleiro, C. Monoterpenic aldehydes as potential anti-*Leishmania* agents: Activity of *Cymbopogon citratus* and citral on *L. infantum*, *L. tropica* and *L. major*. *Exp. Parasitol.* **2012**, *130*, 223–231. [[CrossRef](#)]
29. Setzer, W.N.; Schmidt, J.M.; Eiter, L.C.; Haber, W.A. The leaf oil composition of *Zanthoxylum fagara* (L.) Sarg. from Monteverde, Costa Rica, and its biological activities. *J. Essent. Oil Res.* **2005**, *17*, 333–335. [[CrossRef](#)]
30. Wright, B.S.; Bansal, A.; Moriarity, D.M.; Takaku, S.; Setzer, W.N. Cytotoxic leaf essential oils from Neotropical Lauraceae: Synergistic effects of essential oil components. *Nat. Prod. Commun.* **2007**, *2*, 1241–1244. [[CrossRef](#)]
31. Bayala, B.; Bassole, I.H.N.; Maqdasy, S.; Baron, S.; Simpore, J.; Lobaccaro, J.-M.A. *Cymbopogon citratus* and *Cymbopogon giganteus* essential oils have cytotoxic effects on tumor cell cultures. Identification of citral as a new putative anti-proliferative molecule. *Biochimie* **2018**, *153*, 162–170. [[CrossRef](#)] [[PubMed](#)]
32. Sharopov, F.S.; Wink, M.; Khalifaev, D.R.; Zhang, H.; Dosoky, N.S.; Setzer, W.N. Composition and bioactivity of the essential oil of *Melissa officinalis* L. growing wild in Tajikistan. *Int. J. Tradit. Nat. Med.* **2013**, *2*, 86–96.
33. Santin, M.R.; dos Santos, A.O.; Nakamura, C.V.; Filho, B.P.D.; Ferreira, I.C.P.; Ferreira, P.; Ueda-Nakamura, T. In vitro activity of the essential oil of *Cymbopogon citratus* and its major component (citral) on *Leishmania amazonensis*. *Parasitol. Res.* **2009**, *105*, 1489–1496. [[CrossRef](#)] [[PubMed](#)]
34. Monzote, L.; Piñón, A.; Setzer, W. Antileishmanial potential of tropical rainforest plant extracts. *Medicines* **2014**, *1*, 32–55. [[CrossRef](#)]
35. Pastor, J.; García, M.; Steinbauer, S.; Setzer, W.N.; Scull, R.; Gille, L.; Monzote, L. Combinations of ascaridole, carvacrol, and caryophyllene oxide against *Leishmania*. *Acta Trop.* **2015**, *145*. [[CrossRef](#)] [[PubMed](#)]
36. Monzote, L.; Jiménez, J.; Cuesta-Rubio, O.; Márquez, I.; Gutiérrez, Y.; da Rocha, C.Q.; Marchi, M.; Setzer, W.N.; Vilegas, W. In vitro assessment of plants growing in Cuba belonging to Solanaceae family against *Leishmania amazonensis*. *Phyther. Res.* **2016**, *30*.
37. Sladowski, D.; Steer, S.J.; Clothier, R.H.; Balls, M. An improved MTT assay. *J. Immunol. Methods* **1993**, *157*, 203–207. [[CrossRef](#)]

38. Dutta, A.; Bandyopadhyay, S.; Mandal, C.; Chatterjee, M. Development of a modified MTT assay for screening antimonial resistant field isolates of Indian visceral leishmaniasis. *Parasitol. Int.* **2005**, *54*, 119–122. [[CrossRef](#)] [[PubMed](#)]

Sample Availability: Samples of the essential oils reported in this work are available from the authors.



© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).