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ERIC - LENN MICHAEL PAIGE

POTENCIAL ANTITUMORAL DE ESPÉCIES DE *Lippia* (Verbenaceae) DO ESTADO DA BAHIA

Feira de Santana - Bahia

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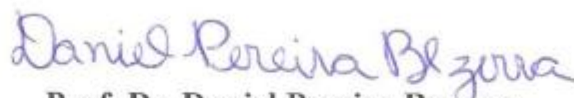

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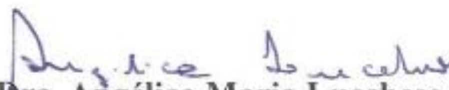

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"Love is never any better than the lover. Wicked people love wickedly, violent people love violently, weak people love weakly, stupid people love stupidly…"- **Toni Morrison**, **The Bluest Eye**

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My father, Ernest Paige, once told me, “Eric, don't sweat the small stuff.” For better or worse my nature is to be a man that lives in an ocean of details, but keeping this advice in mind from time I remind myself to make sure to look at the big picture. In this instance I would say the big picture is this, I am blessed to be doing what I have wanted to since I was 12 years old. In some small way I have the opportunity to contribute to the eradication of cancer. I am blessed to be a scientist with an opportunity to, some small way, make the world a better place. I am truly grateful to everyone who had enough faith in me to risk resources, spend time, care for me, and guide me so I could be a scientist.

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RESUMO

Lippia (Verbenaceae) é um gênero de planta distribuído pela América do Sul, Central e a África Tropical. Aproximadamente 60 espécies são endêmicas do Brasil e 22 são endêmicas do estado da Bahia. *Lippia* tem uma longa história de uso na medicina tradicional, sendo o uso mais comum no tratamento de doenças respiratórias. Apesar da extensa história de *Lippia* no tratamento de doenças, pouco se sabe sobre suas propriedades antitumorais contra tumores cerebrais. Este estudo buscou explorar o potencial antiglioma de óleos essenciais e extratos metanólicos de *Lippia alnifolia, Lippia insignis, Lippia lasiocalycina, Lippia origanoides* e *Lippia thymoides* em células de glioma da linhagem C6. Para avaliar a viabilidade celular foram utilizados o ensaio do MTT. Os efeitos das amostras na morfologia de células cancerosas foram avaliados utilizando-se um microscópio de contraste de fase Nikon Ts-100 e fotos com uma Nikon E4300. Os óleos essenciais foram avaliados nas concentrações de 0,1 $mg.mL^{-1}$, 0,3 $mg.mL^{-1}$, 0,5 $mg.mL^{-1}$ e 1 $mg.mL^{-1}$ e os extratos de metanol a 1 $\mu g.mL^{-1}$, 10 $\mu g.mL^{-1}$, 100 $\mu g.mL^{-1}$, 500 $\mu g.mL^{-1}$ e 1000 $\mu g.mL^{-1}$. A capacidade de inibir a migração celular foi avaliada pelo ensaio de cicatrização da ferida, na qual os extratos foram avaliados a 500 $\mu g.mL^{-1}$ e os óleos essenciais a 0,1 $mg.mL^{-1}$. O ciclo celular foi avaliado na citometria de fluxo nas concentrações de 1000 $ug.mL^{-1}$ para os extratos e 0,03 $mg.mL^{-1}$ para os óleos essenciais. Os óleos essenciais obtidos de *Lippia alnifolia, Lippia insignis, Lippia lasiocalycina* e *Lippia origanoides* reduziram drasticamente a viabilidade celular C6 de uma maneira concentracão-dependente. Já o óleo essencial de *Lippia thymoides* aumentou a viabilidade celular de uma maneira concentracão-dependente. Todos os extratos de metanol parecem ter a capacidade de diminuir abruptamente a viabilidade celular em 500 $\mu g.mL^{-1}$ ou 1000 $\mu g.mL^{-1}$. *Lippia alnifolia* e *Lippia insignis* parecem ter a capacidade de alterar significativamente a morfologia da linha celular C6. Os extratos metanólicos de *Lippia alnifolia*, *Lippia insignis*, *Lippia lasiocalycina*, *Lippia origanoides* e *Lippia thymoides* inibem significativamente a migração de células C6 a uma concentração de 500 $\mu g.mL^{-1}$. Os óleos essenciais de *Lippia alnifolia, Lippia insignis, Lippia lasiocalycina, Lippia origanoidese Lippia thymoides* inibem significativamente a migração de células C6 a uma concentração de 0,1 $mg.mL^{-1}$. Todos os extratos metanólicos e óleos essenciais testados demonstraram a capacidade de induzir algum grau de fragmentação nuclear, vacuolização e inchaço citoplasmático.Estes resultados indicam que os constituintes dos óleos essenciais e extratos metanólicos devem ser isolados e seu potencial anticancerígeno deve ser explorado.

Palavras-Chave: *Lippia*, glioma, óleos essenciais, extratos.

ABSTRACT

Lippia (Verbenaceae) is a plant genus distributed throughout all Southern and Central America, and Tropical Africa. Approximately 60 species are endemic in Brazil and 22 are endemic in the state of Bahia. *Lippia* has a long history of use in traditional medicine, the most common use being in the treatment of respiratory diseases. Despite *Lippia's* extensive history in treating diseases, little is known about its anticancer properties against brain and central nervous system tumors. This paper explores the anticancer potential of 5 species of *Lippia* from the state of Bahia (Brazil),*Lippia alnifolia*, *Lippia insignis*, *Lippia lasiocalycina*, *Lippia origanoides* and *Lippia thymoides.* To assess the cell viability of essential oils and methanol extracts derived from these species MTT assay was utilized. To assess the effects of the essential oils and methanol extracts on morphology of cancer cells a Nikon Ts-100 phase contrast microscope was used, and photos were taken with a Nikon E4300. All essential oils were evaluated at 0.1 mg.mL^{-1}, 0.3 mg.mL^{-1}, 0.5 mg.mL^{-1}, and 1 mg.mL^{-1}. All methanol extracts were evaluated at 1 µg.mL^{-1}, 10 µg.mL^{-1}, 100 µg.mL^{-1}, 500 µg.mL^{-1}, and 1000 µg.mL^{-1}. The ability to inhibit cell migration was assessed by wound heal assay. All methanol extracts were evaluated at 500 µg.mL^{-1}. All essential oils were evaluated at 0.1 mg.mL^{-1}. To access the effects of essential oils and methanol extracts on the cell cycle flow cytometry was used. All methanol extracts were evaluated at 1000 µg.mL^{-1}. All essential oils were evaluated at 0.03 mg.mL^{-1}. The essential oils derived from *Lippia alnifolia*, *Lippia insignis*, *Lippia lasiocalycina*, and *Lippia origanoides* all drastically decreased the cell viability of the C6 cell line in a concentration dependent manner. The essential oil derived from *Lippia thymoides* increased cell viability in a concentration dependent manner. All of the methanol extracts appear to have the ability to abruptly decrease cell viability at either 500 µg.mL^{-1} or 1000 µg.mL^{-1} it. *Lippia alnifolia* and *Lippia insignis* both have the ability to significantly alter the morphology of the C6 cell line. Methanol extracts derived from *Lippia alnifolia*, *Lippia insignis*, *Lippia lasiocalycina*, *Lippia origanoides* and *Lippia thymoides* all significantly inhibit the migration of C6 cells at a concentration of 500 µg.mL^{-1}. Essential oils obtained from *Lippia alnifolia*, *Lippia insignis*, *Lippia lasiocalycina*, *Lippia origanoides* and *Lippia thymoides* significantly inhibited the migration of C6 cells at a concentration of 0.1 mg.mL^{-1}. All methanol extracts and essential oils tested demonstrated the ability to induce some degree of nuclear fragmentation, vacuolization, and cytoplasmic swelling. These results indicate that the constituents of both the essential oils and methanol extracts should be isolated and their anticancer potential should be further explored.

Keywords: *Lippia*, glioma, essential oils, extracts

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1. General Introduction

Gliomas are brain and spinal cord tumors that form from glial cells. Malignant gliomas are heterogeneous, highly invasive brain tumors. Low grade tumors, grade I and grade II, generally grow more slowly and can sometimes be treated with surgery alone. High grade tumors, grade III and grade IV tumors are fast growing and difficult to treat. Glioblastomas are especially aggressive forms of gliomas (LOUIS *et al*, 2007). Most patients diagnosed with glioblastomas die within one year of diagnosis and only 5% survive more than five years (MRUGALA, 2013).

Lippia (Verbenaceae) is a genus of plant that is distributed throughout South and Central American countries, and Tropical Africa. The genus composed of approximately 200 species of shrubs, herbs, and small trees (PASCUAL*et al*, 2001). Of those 200 species, approximately 60 are endemic to Brazil, and 22 are endemic to the state of Bahia. *Lippia* has an extensive history rooted in traditional medicine. Terpenes (some sesquiterpenes, di- and triterpenes), flavonoids, phenols, iridoid glycosides, phenylpropanoids, and naphthoquinones are generally considered to be the most significant non-volatile secondary metabolites common to the genus (OMBITO *et al*, 2014).

Our group has already begun to analyze the bioactive potential of the chemical constituents in *Lippia alnifolia*, *Lippia origanoides* and *Lippia thymoides.* Methanol extracts derived from *L. alnifolia* demonstrated antimicrobial activity against two strains of *S. aureus*, and *C. parapsilosis.* Methanol extracts derived from *L. origanoides* showed the greatest antimicrobial potential of all three species analyzed, demonstrating antimicrobial activity against two strains of *S .aureus, C. albicans, and C. parapsilosis.* The methanol extracts derived from *L. thymoides* analyzed for their antimicrobial activity inhibited the growth of two strains of *S. aureus* (SILVA *et al*, 2015). Essential oils derived from *L. thymoides* have demonstrated antimicrobial activity against various species of gram positive bacteria and antifungal activity against yeast. When applied to rat aorta, rat uterus, and guinea pig trachea the essential oil demonstrated the ability to cause the contracted muscles to relax in vitro (SILVA *et al*, 2015). Little is known about the chemical and biological potential of *L. insignis* and *L. lasiocalycina.*

Despite the genus *Lippia*'s extensive history treating diseases in traditional medicine and recent studies indicating the aforementioned species' pharmaceutical potential, little is known

about their anticancer properties against brain and central nervous system tumors. This study sought to evaluate the anticancer potential of essential oils and methanol extracts of *L. alnifolia*, *L. insignis*, *L. lasiocalycina*, *L. origanoides* and *L. thymoides* on the glioma cell line C6. Specifically, the effects of the essential oils and methanol extracts derived from leaves on cell viability, morphology, cell migration, and cell cycle were examined.

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2. Literature Review

2.1 Glioma

Glioma is a broad category of brain and spinal cord tumors that come from glial cells. There are three types of gliomas. Ependymomas are tumors that come from ependymal cells, oligodendrogliomas are derived from oligodendrocytes, and astrocytomas are tumors that are derived from astrocytes. Two out of every ten brain cancers are astrocytoma. Astrocytomas are usually classified as grade I, grade II, grade III, or grade IV (AMERICAN CANCER SOCIETY, 2017). Grade I, pilocytic astrocytoma, are astrocytoma that are more commonly found in younger patients. Grade II, diffuse astrocytoma, are neoplasms that are infiltrative in nature and display cytological atypia. Grade III, anaplastic astrocytoma, are tumors that display anaplasia and mitotic activity in addition to the characteristics of diffuse astrocytoma. Grade IV tumors, glioblastomas multiforme, that show microvascular proliferation and/or necrosis receive this designation (LOUIS, 2007). Malignant gliomas are heterogeneous, highly invasive brain tumors. Low-grade tumors, grade I and grade II, generally grow more slowly and can sometimes be treated with surgery alone. High-grade tumors, grade III and grade IV tumors are fast growing and difficult to treat. Glioblastomas are especially aggressive forms of gliomas. Most patients diagnosed with this tumor die within one year of diagnosis and only 5% survive more than 5 years (MRUGALA, 2013).

Standard treatment for Glioblastoma multiforme patients is surgery, followed by radiotherapy with concurrent temozolomide (TMZ) and six monthly cycles of adjuvant TMZ. Radiotherapy generally lasts 6 weeks, with the typical concentration being around 60 Gy. TMZ is generally administered concurrently with radiotherapy at a dosage of 75 mg/m2 daily. This is followed by a minimum of 6 months of adjuvant TMZ treatment (150-200 mg/m2 for 5 days every 28 days) (MRUGALA, 2013).

Surgery remains the first and most important treatment modality for patients suffering from brain tumors (VELIZ, 2015). However, surgery is frequently inadequate because of the fluid nature of the disease. In addition, the inability of surgery to remove tumors entirely without causing harm to the healthy brain presents another challenge. In newly diagnosed patients the intensity of the surgery potentially serves as an indicator of survivability. Certain tumor locations,

such as the eloquent cortex, basal ganglia, or brainstem are not ameliorated by surgical intervention and typically patients with tumors in these areas can expect worse prognosis (MRUGALA, 2013).

The most fundamental ability of cancer cells is their sustained chronic proliferation. This trait can be attributed to two factors. Cancer cells develop self-sufficiency in growth signals. Many oncogenes mimic mitogenic growth signals, essentially allowing cancer cells to create their own growth signal. This creates a positive feedback loop, with autocrine stimulation, minimizing dependence on exogenous signaling in the normal cell microenvironment. An example of this is platelet derived growth factor (PDGF) created by glioblastomas (HANAHAN, 2001). Cancer cells develop insensitivity to antigrowth signals. In order to achieve this feat, the cells evade powerful programs, dependent on negative tumor suppressor genes, which negatively regulate cell proliferation (HANAHAN, 2011).

TMZ is an alkylating agent applied to malignant glioma. TMZ works by causing cytotoxicity by spontaneously converting to the reactive methylating agent 5-(3-methyltriazen-1-yl) imidazole-4-carboxamide (MTIC). MTIC then degrades to the methyldiazonium cation. The methyldiazonium cation reacts with DNA to form methyl adducts, such as N3 -methyladenine, N7 -methylguanine, and O6 -methyl-guanine. These methyl adducts result in DNA strand breaks which potentially lead to cell-cycle arrest and delayed cell death. Removal of the O6 – methylguanine by methyltransferase (MGMT) causes resistance to TMZ. Therapeutic agents that suppress MGMT expression and that methylate MGMT promoters have been shown to extend survival of GMB patients by years. So it would be ideal to find new small molecules with the ability to inhibit MGMT (VELIZ, 2015).

The metabolic switch cancers cells make from depending on pyruvate and to lactic acid to in the hypoxic microenvironments is thought to allow cancer cells to a greater availability of building blocks like amino acids and nucleosides. Glutamate and α-ketoglutarate are important sources for of energy for cancer cells. In the tumor microenvironment glutaminolysis and reductive carboxylation are needed to sustain cancer cell growth and proliferation. High amounts of glutamate also aid in the expansion of gliomas. The presence of high amounts of glu can lead to astrocytic swelling and subsequently apoptosis (Maus, 2017). Oxidative stress and its agents have been shown to play an important role in several hallmarks of cancer. Reactive oxygen species (ROS) are key agents driving this process. They have been shown to play key roles in cell

proliferation and anchorage independent cell growth, causing insensitivity to apoptosis, sustaining new angiogenesis, and by altering the migration/invasion program through metabolic and epigenetic mechanisms. Cancer cells displaying high levels of ROS increase basal metabolic activity, mitochondrial dysfunction due to hypoxia or mitophagy, peroxisome activity, and uncontrolled growth factors of cytokine signaling (FIASCHI, 2012)

Angiogenesis is a mechanism by which tumors acquire nutrients and evacuate metabolic waste. During tumor progression an angiogenesis switch is activated, initiating sustained angiogenesis. This switch to sustained angiogenesis is followed by ongoing neovascularization controlled by a biological rheostat. Blood vessels produced with tumors are typically aberrant. Neovasculature is often defined by mature capillary sprouting, convoluted and excessive vessel branching, distorted and enlarged vessels, erratic blood flow, microhemorrhage, leakiness, and abnormal levels of endothelial cell proliferation and apoptosis (HANAHAN, 2011).

Angiogenesis has been explored as a possible avenue in the treatments of various cancers. The result of this form of treatment is often suboptimal. It is thought that ineffectiveness of blocking pro-angiogenic signaling could be linked to the variable proteins associated with the high active endothelial cells that activate growth and motility. However, the effectiveness of drug delivery systems could be improved by employing anti-angiogenic drugs because they create a more normalized vasculature (RAJABI, 2017).

Recently, bevacizumab was adopted as a potential anti-glioma treatment. Bevacizumab is human monoclonal antibody that inhibits vascular endothelial growth factor (VEGF). It has been used in phase III trials in conjunction with chemotherapy to treat metastatic colorectal cancer and non-small lung cancer, and persistent progression free survival in the treatment of metastatic breast cancer and renal cancer. A one-time study was done examining the effects of this drug in patients with glioblastoma resulting in a 77% survival rate (ANJUM, 2017).

The overall inefficacy of the current standard treatment means there is a need for more effective drugs to treat people diagnosed with gliomas. Natural products, especially plant derived natural products, look to be a promising source of novel molecules.

2.2 Natural Products

Natural product discovery has been driven by the realization that they are valuable agents.

Some of their uses include herbicides, insecticides, and pharmaceuticals. Natural products are a complex and chemically diverse group of molecules with varying molecular weights. Many contain significant numbers of stereospecific carbon centers. This makes them of great interest to a wide variety of scientists (KATZ, 2015).

Since the 1960's approximately 50% of new chemical entities have been natural products or natural products inspired. As 2013 the number of new chemical entities approved by the Food and Drug Administration was 1453. Approximately 40% those entities were natural products or natural product inspired. When specifically examining the new chemical entities targeting cancer the number natural products or natural products inspired new chemical entities raises from 50% to 70% (KATZ, 2015).

Natural products have been the chief source of novel molecules since the 1970's. Between 1960 and 1982 the National Cancer Institute began a screening of 35,000 plant samples against the L1210 and P388 leukemia cell lines. The most prominent drug to be discovered during this search was paclitaxel (taxol) (MANN, 2002). The molecule is extracted from the Taxus brevifolia (Pacific yew tree). Paclitaxel is a complex isoprenoid derivative containing a diterpene skeleton (taxane) (BONFILL, 2006). Taxol works by inducing mitotic arrest at the mitotic check a point. This is the major cell cycle control mechanisms and stopping it prevents chromosome missegregation. This ultimately leads to cell inhibition of mitosis (WEAVER, 2014). Vinblastine and vincristine belong to the family of vinca alkaloids and were first introduced in the 1960's. They have been responsible for the long term remission of childhood leukemia, testicular teratoma, Hodgkin's disease and many other cancers. Both of these alkaloids are extracted from the flowers of Catharanthus roseus G. Don (Madagascar periwinkle.) Vinca alkaloids cytotoxicity is due to their microtubule disruption and their interactions with tubulin. When they bind to tubulin they disrupt microtubule congregation. This disruption at the end of mitotic spindle causes cells to enter in mitotic arrest. In addition, vinca alkaloids have been shown to inhibit angiogenesis, one of the hallmarks of cancer, by blocking endothelial proliferation, chemotaxis, and spreading on fibronectin (MOUDI, 2013).

DNA topoisomerase I (Topo I) is another established target used in the treatment of cancer. DNA topoisomerase I is responsible for the breaking and relaxing, and regulation of DNA chains during replication, transcription, and the remodeling of chromatin. In the 1970's an alkaloid, camptothecin, was isolated from Camptotheca acuminata Decne. Camptothecin has the

ability to inhibit TOPO I. Inhibitors of TOPO I primarily active during the intermediate cleavage step. This leaves the DNA strand undetectable cut for extended periods of time. Inhibition of large amounts of TOPO I lead to modifications in DNA and apoptosis (BRANDÃO, 2010)

Cannabinoids are a diverse group of bioactive chemical compounds with the ability to interact with CB1 and CB2 receptors. There are generally considered to be three different types of cannabinoids. The first being phytocannabinoids, which are produced by different species of plants. The second classification is endocannabinoids, are produced by animals. The last classification is synthetic cannabinoids, which are modified versions of naturally occurring cannabinoids. CB1 receptors are found in great quantities in the central nervous system and in peripheral nerves. CB2 receptors are abundantly found in cells and organs in the immune system. Studies have found high expression of the CB2 receptor in glioblastoma multiforme. Cannabinoids have been tapped as agents responsible for inhibition of tumor cell proliferation, gliomagenesis, and impairment of angiogenesis (ANJUM, 2017).

2.3 Biodiversity in Brazil

Brazil is the most biodiverse country on earth. With more than 56,000 different species of plants, it represents about 19% of the global flora. Current estimates put the number of species of gymnosperms between 5 and 10. It is also estimated that there are between 55,000 and 60,000 species of angiosperms, 3,100 species of bryophytes, between 1,200 and 1,300 species of pteridophytes, and around 525 species of marine algae. Studies have shown that the Atlantic rainforest houses a particularly high level of biodiversity (GIULETTI *et al*, 2005).

The state of Bahia is home to several ecosystems, among them Atlantic Forest, Restinga, Dunes, Mangues, Cerrado, and Caatinga, according to variations of soil, relief, and climate. Bahia also houses one the largest amount of preserved Atlantic Forest area, around 1,200,000 ha. In the south, coastal forests occupy an area of about 100 ± 200 km wide along the Atlantic coast. As they move inland, the forests gradually become drier, shifting from the coastal restinga forest to the dense ombrophilous forest, to the semi-deciduous forest, and to the deciduous jungle (MORI, 1983). Variations in topography and soils produce several microhabitats, which results in high diversity and endemism (THOMAS *et al*, 1998).

2.4 Genus *Lippia*

Lippia (Verbenaceae) is a genus of plant that is distributed throughout South and Central American countries, and Tropical Africa. The genus composed of approximately 200 species of shrubs, herbs, and small trees (PASCUAL et al, 2001). Of those 200 species, approximately 60 are endemic to Brazil, and 22 are endemic to the state of Bahia (SAM, 2017). Terpenes (some di- and triterpenes), flavonoids, phenols, iridoid glycosides, phenylpropanoids, and naphthoquinones are generally considered to be the most significant non-volatile secondary metabolites common to the genus (OMBITO, 2014).

Lippia has an extensive history rooted in traditional medicine. The most common use has been to treat respiratory disorders. In central and south America it has also been used to treat colds, bronchitis, coughs, and asthma. *Lippia alba* and *L. dulcis* Trevor are used as pectoral remedies in Mesoamerica. In Africa, milk and water infusions of *L. javanica* are used to relieve colds, and their leaves and roots are used for shortness of breath and chest. *L. alba, L. dulcet* Trevir., *L. geminata* H.B.K., *L. graveolens* H.B.K., *L. javanica*, *L. nodiflora* (L.) Michx, and *L. triphylla* L'Hér. have been used as an analgesic, anti-inflammatory, and/or antipyretic remedies (PASCUAL et al, 2001).

Work on various *Lippia* species has begun to show promise in demonstrating anti-proliferative properties. The essential oils of *L. multiflora* have been shown to have anti-proliferative activity on LNCaP (prostate), PC-3 (prostate), SF-767(glioblastoma), and SF-763 (glioblastoma). The essential oils were most active on the PC-3 (IC_{50} 0.3 mg·mL^{-1}) and SF-767 cell line (IC_{50} 0.3 mg.mL^{-1}). It was also suggested that there is a relationship between essential oils from *L. multiflora* with anti-proliferative activity and its anti-inflammatory and antioxidant properties. However, these mechanisms aren't very well understood (BAYALA , 2014).

IC_{50} values have been established with the species *L. sidoides* Cham (19.05 µg.mL^{-1}), *L.salviifolia* (30.20 µg.mL^{-1}), and *L. rotundifolia* (36.30 µg.mL^{-1}) when tested on CT26.WT (colon carcinoma) cell line. IC_{50} values have been achieved with a *L. alba* carvone chemotype essential oil (47.80 µg.mL^{-1}) on the A549 (Lung carcinoma) cell line. Thymol was identified as the major component of the *L. sidoides* essential oils. Thymol has also been identified to as having a suppression effect on B16F10 mouse melanoma cells. The major component, nerolidol, of *L.salviifolia* essential oils has demonstrated anti-proliferative action on human leukemia, HL-

60 (GOMIDE et al, 2013).

2.5 Major Constituents of Essential oils

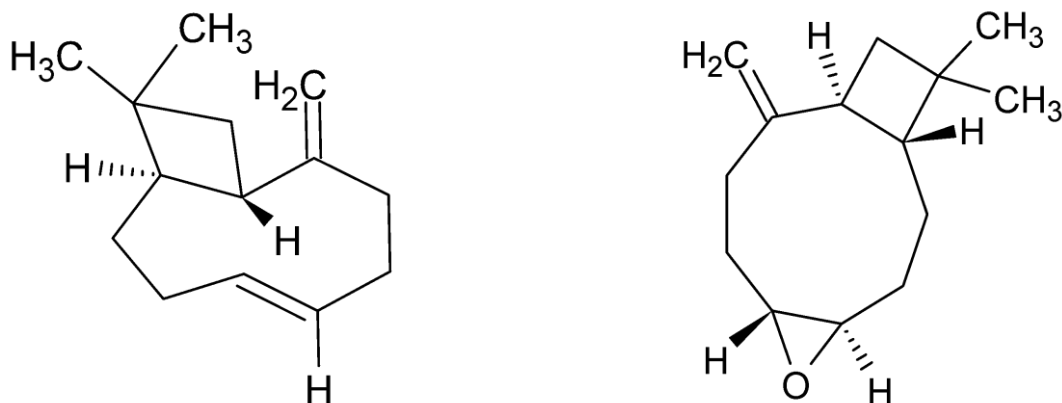

Figure 1. Structures of E-caryophyllene ($C_{15}H_{24}$)and caryophyllene oxide ($C_{15}H_{24}O$)

E–Caryophyllene is a bicyclic sesquiterpene found in the essential oils of several plants. E–Caryophyllene oxide is an oxidation derivative of the E–Caryophyllene. E–Caryophyllene is also classified as a phytocannabinoid. Cannabinoids have to be the ability to activate cannabinoid receptors (CB1 and CB2), however, E–Caryophyllene is only able to activate CB2. This ability to activate CB2 and inability to activate CB1 indicates that E–Caryophyllene could be an effective medicine because it would lack the psychoactive side effects associated with CB1. Cannabinoid receptors are G-proteins associated with energy balance, metabolism, neurotransmission, and immune response (FIDYT et al, 2016).

Currently, it believed three mechanisms are associated with the anticancer properties of E–Caryophyllene and caryophyllene oxide, induction of apoptosis, repression of the cell cycle, and inhibition of angiogenesis and metastasis. Both compounds have demonstrated cytotoxic activity in several different cell lines. Caryophyllene oxide has been shown to have a cytotoxic effect on HeLa (human cervical adenocarcinoma cells), HepG2 (Hepatocellular carcinoma), AGS (human lung cancer cells), SNU-1(human gastric cancer cells), and SNU-16 (human stomach cancer cells). E–Caryophyllene has been shown to have strong anti-proliferative effects on two colon cancer cell lines, HCT-116 and HT-29, and a pancreatic cancer cell line, PANC-1. E–Caryophyllene tested on the intestinal cancer cell line, CaCo-2, did not affect cell viability at the concentrations tested. However, α-humulene (E–Caryophyllene isomer) exhibited strong anti-

proliferative effects on the same cell line. Both compounds have shown to increase the effectiveness of classical cancer drugs like paclitaxel. Caryophyllene oxide was shown to increase the effectiveness of the arrest of microtubules of paclitaxel. E–Caryophyllene was shown to increases the accumulation paclitaxel in the cell, possibly through altering the permeability of the cell membrane (FIDYT et al, 2016).

E–Caryophyllene has been shown to have the ability to negate glutamate induced excitotoxicity in glioma cells. The primary mechanism used to achieve this feat is the reduction of the presence of ROS and mitochondrial dysfunction. C6 cells treated with E–Caryophyllene in concentrations as low as 0.5 $\mu g.mL^{-1}$ and 1 $\mu g.mL^{-1}$, 24 hours before being exposed to glutamate were fully protected from the effects of excitotoxicity. Pretreatment of C6 with E–Caryophyllene also completely prevented the production of glutamate induced ROS production and maintain the integrity of the mitochondrial membrane. Excess levels of glutamate induced significant downregulation of GSH activity and GPx activity. E–Caryophyllene has also demonstrated the ability to upregulate the GSH antioxidant system through a CB2/Nrf2 pathway, negating the effects of this downregulation. This increase in GSH level was accompanied by an increase in GPx activity, implying an indirect activation of C6 redox enzyme system. When cells were treated with glutamate there was a 23% decrease in GSH levels when compared to the control and a 24% decrease in GPx activity when compared to the control. When cells were pretreated with E–Caryophyllene for 24 hours before being exposed to glutamate a reduction in the effects of glutamate on GSH levels and GPx activity were seen. When cells were exposed to only E–Caryophyllene GSH levels and GPx activity increased compared to the control (ASSIS et al, 2014).

Investigations into the anticancer properties caryophyllene oxide have shown it has the ability to inhibit constitutive STAT3 activation in myeloma, breast cancer, and prostate cancer cell lines. This was achieved through the mediation of c-Src and Jak 1/2. Caryophyllene oxide also induces the transcription of tyrosine phosphatase SHP-1 (BAYALA, 2014). Tyrosine phosphatase SHP-1 regulates STAT3 activation in both the nucleus and the cytoplasm via dephosphorylation (BÖHMER, 2014).

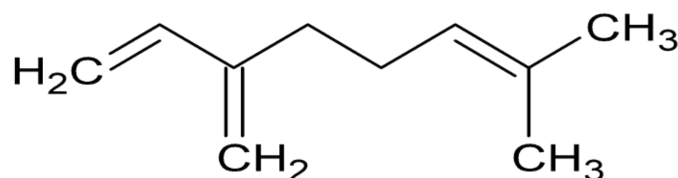

Figure 2. Structure of myrcene ($C_{10}H_{16}$)

Myrcene is naturally occurring olefinic hydrocarbon and is classified as a monoterpene. Recently, a study investigated the inhibitory effects of myrcene derived from the essential oils of *Pinus koraiensis* cones in MDA-MB-231 human breast cancer cells. What was found is that myrcene exhibited to the ability to influence several functions associated with TNFα. Myrcene inhibited TNFα activation of NF-κβ of the enzyme Iκκ. It prevented TNFα induced expression of MMP-9 expression. Myrcene also blocked TNFα induced tumor cell invasion (LEE et al, 2015).

Nuclear Factor - κβ (NF-κβ) is a transcription factor implicated in the regulation of cell proliferation, motility, and survival among a variety of other functions. Aberrant activation of NF-κβ is a common occurrence in glioblastoma multiforme. NF-κβ has also been linked to a developmental regulatory program used by glioblastoma multiforme to acquire a phenotype used to invade other tissue, a proneuronal-to-mesenchymal phenotype. Glioblastomas take this form in response to factors in the microenvironment and cytotoxic treatments. Relb is a transcription factor involved in the expression of mesenchymal genes in a glioma subgroup. Relb mediated NF-κβ signaling has been shown to affect cell migration and invasion (SOUBANNIER, 2017).

Aberrant activation of NF-κβ has also been linked to EGFR through different mechanisms (SOUBANNIER, 2017). Oncogenic EGFR is integral to tumor growth and invasion to glioblastomas the upregulation of EGFR correlates with the upregulation proteases. These enzymes are linked to the degradation of the extracellular matrix, and subsequent alterations in cell to cell and cell to extracellular matrix adhesion that serve as a barrier to invasion and metastasis. Serine proteases are an example of this. Urokinase-type plasminogen activator receptor is associated with tyrosine kinase and is expressed in high amount in glioblastomas, promoting cell invasion and growth. It has been demonstrated in vitro that the upregulation of plasminogen activator inhibitors (PAI) upregulates Urokinase-type plasminogen activator and urokinase-type plasminogen activator receptor increasing cellular matrix degradation (KELLER, 2017).

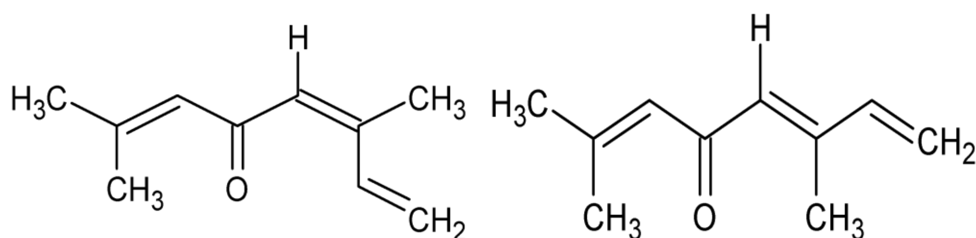

Figure 3. Structure of *cis*–ocimenone ($C_{10}H_{14}O$) and *trans*–ocimenone ($C_{10}H_{14}O$)

Ocimenone is classified as a monoterpene and alkene. Synonyms for cis–ocimenone include Z-ocimenone, cis-tagetenone, (Z)-tagetenone. Synonyms for trans–ocimenone include E-ocimenone, trans-tagetenone, (E)-tagetenone (PUBCHEM, 2017). There doesn't appear to be a lot written in the literature about this molecule and its pharmacological potential. However, there are several studies that have identified both E–ocimenone and Z-ocimenone as chemical constituents present in several essential oils in significant quantities and several papers have begun exploring the pharmacological potential of those essential oils, including their anticancer potential. Essentials oils derived from the fruits of *Dorema ammoniacum* D. Don were tested on human colon adenocarcinoma cell line (SW480) and human breast cancer cell lines (MCF7). Approximately 22.3% of the essential oil was composed of Z-ocimenone and E-ocimenone composed approximately 18.1%. The IC_{50} for MCF7 cells treated with the essential oil was found to be 312 $\mu g.mL^{-1}$, and the IC_{50} for sw480 cells treated with the essential oil was to be 625 $\mu g.mL^{-1}$ (YOUSEFZADI *et al*, 2011). Studies have analyzed the anti-cancer potential of essential oils derived from the leaves of *Tagetes erecta*. IC_{50} values of 0.50 $\mu g.mL^{-1}$, 0.23 $\mu g.mL^{-1}$, and 0.25 $\mu g.mL^{-1}$ against MO59J, U343, and U251 cell lines respectively. E-ocimenone accounted for about 13% of the overall composition of the essential oil (OLIVEIRA *et al*, 2015).

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3. Objectives

3.1 General Objectives

- To evaluate the anticancer potential of essential oils and methanol extracts of *L. alnifolia*, *L. insignis*, *L. lasiocalycina*, *L. origanoides,* and *L. thymoides* on C6 glioma cells.

3.2 Specific Objectives

- To evaluate the effects of essential oils and methanol extracts of leaves *L. alnifolia*, *L. insignis*, *L. lasiocalycina*, *L. origanoides,* and *L. thymoides* on viability of C6 glioma cells.
- To investigate the effects of essential oils and methanol extracts of leaves of *Lippia* species on invasion and proliferation of C6 glioma cells.
- To evaluate relationship between antiglioma effects and composition of essential oils from *Lippia* species.

4. Materials and Methods

4.1 Methanol Extracts and Essential Oils

Methanol extracts and essential oils were provided were provided by the Laboratório de Química de Produtos Naturais e Bioativos (UEFS). The principal constituents of the essential oils used were: β-myrcene (18.90%), myrcenone (15.41%), and E-ocimenone ;(23.56%) for *L. alnifolia*; β-myrcene (12,43%), limonene (14,73%) and E-ocimenone (26,11%) for *L. insignis*; β-myrcene (31.17%), p-cymene (7.17%) and E-ocimenone (24,11%) for *L. lasiocalycina*; carvacrol (53.89%), linalool (5.84%), and E-caryophyllene (5.86%) for *L. origanoides;* E-caryophyllene (29.55%), caryophyllene oxide (8.17%), and germacrene D (6.59%) according to the tables in the appendix.

4.2 Cell Lines and Cell Cultures

Gliomas derived from glial cells of *Rattus norvegicus* (C6) (Benda, 1968) were cultured in 100 mm polystyrene plates (KASVI) in modified Dulbecco's medium (DMEM, Cultilab, SP, Brazil) supplemented with 1% penicillin, 1%, and 10% fetal bovine serum (Cultilab, SP, Brazil REF) and maintained in greenhouses at 37 ° C and 5% CO2 atmosphere.. Cultures were maintained in a humidified atmosphere composed of 95% air and 5% carbon dioxide. After reaching 70% confluency, the adherent cells were detached from the plates and collected using trypsin / EDTA solution (0.05% trypsin, 0.02% EDTA in Ca2 + / Mg2 + free PBS) for five minutes, and plated in new plates required for experimental analysis.

4.3 Morphological and Structural Analysis

Cells were exposed to methanol extracts and essential oils at concentrations between 10 $\mu g.mL^{-1}$ and 1000 $\mu g.mL^{-1}$ and 0.1 $mg.mL^{-1}$, and 1 $mg.mL^{-1}$ respectively. After 48 h the effects of each drug on the morphology were analyzed and photographed a Nikon Ts-100 phase contrast microscope and photos were taken with a Nikon E4300.

For further morphological evaluation of C6 cell cultures, the cells were analyzed by

staining with the May-Grünwald reagent, which shows an affinity for chromatin, staining acidic and basic nuclear structures. The methodology was carried out according to the manufacturer's technical specifications. Thus, C6 cells were seeded in 24-well plates at a density of 50 x 103 cells/well. The cells were exposed to the essential oils and methanol extracts in the concentrations of 0.1 $mg.mL^{-1}$ and 500 $\mu g.mL^{-1}$ respectively for 72 h. After the treatment period, the cells were washed three times with PBS at pH 7.4 and fixed with cold methanol for 20 min at 0 ° C. The excess cold methanol was discarded, the plates were washed with PBS three times, and the plates allowed to dry at room temperature. Next, 20 drops of the May Grünwald reagent were added, which was incubated for 3 min. After this time 20 drops of distilled water were added. The plate was gently shaken and stood for 2 min. After this period the mixture was discarded, cells washed with distilled water and left at room temperature to be dried and analyzed under Olympus® model AX70 fluorescence microscopy.

4.4 Cell Viability Assay

The test of MTT, (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium, is based on the principle of converting the yellow color substrate (MTT) by using mitochondrial dehydrogenases of living cells into formazan crystals, to a violaceous color (Hansen et al., 1989). C6 cells were grown in 96-well culture plates at a density of 7.5 x 10^3 cells per well, and exposed to the methanol extracts at concentrations of 1, 10, 100, 500, and 1000 $\mu g.mL^{-1}$ and essential oils at concentrations of .1 $mg.mL^{-1}$, .3$mg.mL^{-1}$ .5 $mg.mL^{-1}$ and 1 $mg.mL^{-1}$. Cultures were analyzed after 24 and 48 h. The MTT test was performed after 48 h. 2 h before the expiration of the exposure time, the culture medium containing the oils was removed and replaced with the MTT solution (100 µl/well) at a final concentration of 1 $\mu g.mL^{-1}$ diluted in DMEM (Cultilab). For complete dissolution of the formazan crystals, after 2 h of contact with the MTT, a volume of 100 µl/well of a 20% sodium dodecyl sulfate (SDS), 50% dimethylformamide (DMF) buffer was added (pH 4.7), the plates being maintained for 12 h at 37°C. The optical absorbance of each sample was measured using a spectrophotometer (Varioscan Thermo, Finland) at wavelength 595 nm. The experiments were performed at least in octuplicates in three independent experiments and the results presented as the percentage of viability (mean and standard deviation) in relation to the control.

4.5 Wound Heal Assay

The potential inhibitory ability on cell migration of the essentials oils and methanol extracts derived from 5 different species belonging to the genus *Lippia* was investigated by using a monolayer single lesion test. The assay was performed in 24-well plates. C6 glioma cells (density of 5×10^4 cells/well.) were allowed to attach and reach confluency. A wound (2mm) was made through the center of the plate. The cells were washed twice with phosphate-buffered saline (PBS) to remove detached cells for injury before their subsequent incubation with DMEM in the absence (control) or presence of the essential oils (0.1 $mg.mL^{-1}$) and methanol extracts (500 $\mu g.mL^{-1}$) tested. Cells were photographed at time zero, 24 h, 48 h, and 72 h during treatment to assess the reduction in the length of the lesion areas.

4.6 Cell Cycle Analysis – Flow Cytometry

Flow cytometry analysis of DNA content was performed after essential oils (0.03 $mg.mL^{-1}$) and methanol extracts (1 $mg.mL^{-1}$) were added to logarithmically growing C6 cells. After 48 h exposure, C6 cells were harvested by trypsinization, fixed with cold ethanol and stained with propidium iodide (Biosource, Camarillo, CA). Flow cytometry was performed on a BD FACSCalibur. The software BD Cell Quest TMPro was used to generate histograms and determine the cell cycle phase distribution.

4.7 Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5.0 software. To analyze the results obtained in the cytotoxicity assays we used the ANOVA test followed by the Tukey post-test to analyze the cell viability assays and the Newman-Keuls post-test to analyze cell migration. p-value< .05 *, p-value<.01 **, p-value<.001 ***

5. Results

5.1 Analysis of Morphology

The morphological changes induced in C6 glioma cells by methanol extracts and essentials oils derived from the genus *Lippia* were analyzed under a phase contrast microscope. In figures A – E the results obtained for the methanol extracts are shown. In figures F – J the results obtained for the essentials oils are shown.

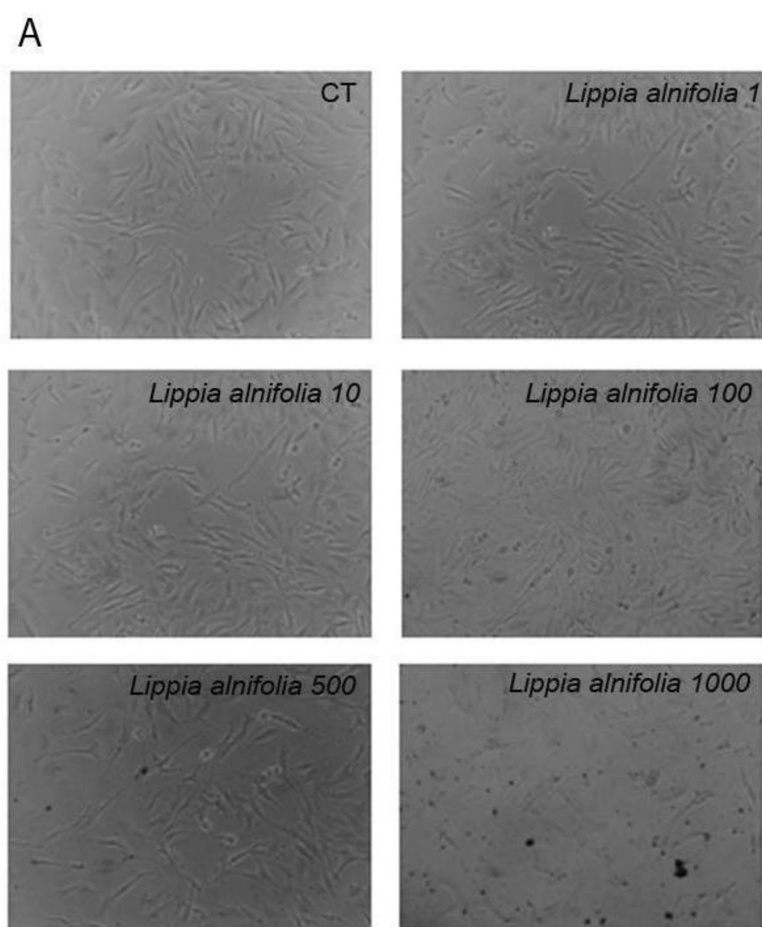

Figure 4. Analysis of C6 cells treated with methanol extracts derived from *L. alnifolia* at 1 $\mu g.mL^{-1}$, 10 $\mu g.mL^{-1}$, 100 $\mu g.mL^{-1}$, 500 $\mu g.mL^{-1}$ e 1000 $\mu g.mL^{-1}$ under a phase contrast microscope after 48 hours.

After 24 h the control displayed a bipolar fusiform morphology indicating the viability of the cells. At 500 $\mu g.mL^{-1}$ cells display bipolar and multipolar fusiform morphology with thin

processes. Some cell bodies appeared to be fragmented. At 1000 $\mu g.mL^{-1}$ cells display multipolar fusiform morphology. Many cell bodies are fragmented. At 500 $\mu g.mL^{-1}$ and 1000 $\mu g.mL^{-1}$ cellularity decreased significantly when compared to the control. After 48 h the control maintained a bipolar fusiform morphology indicating the viability of the cells. At 500 $\mu g.mL^{-1}$ cells display bipolar fusiform morphology and cell bodies are elongated and fragmented. At 1000 $\mu g.mL^{-1}$ cells display spheroid and multipolar fusiform morphology. Cell bodies are fragmented and begin many lose integrity. At 500 $\mu g.mL^{-1}$ and 1000 $\mu g.mL^{-1}$ cellularity decreased when compared to the control.

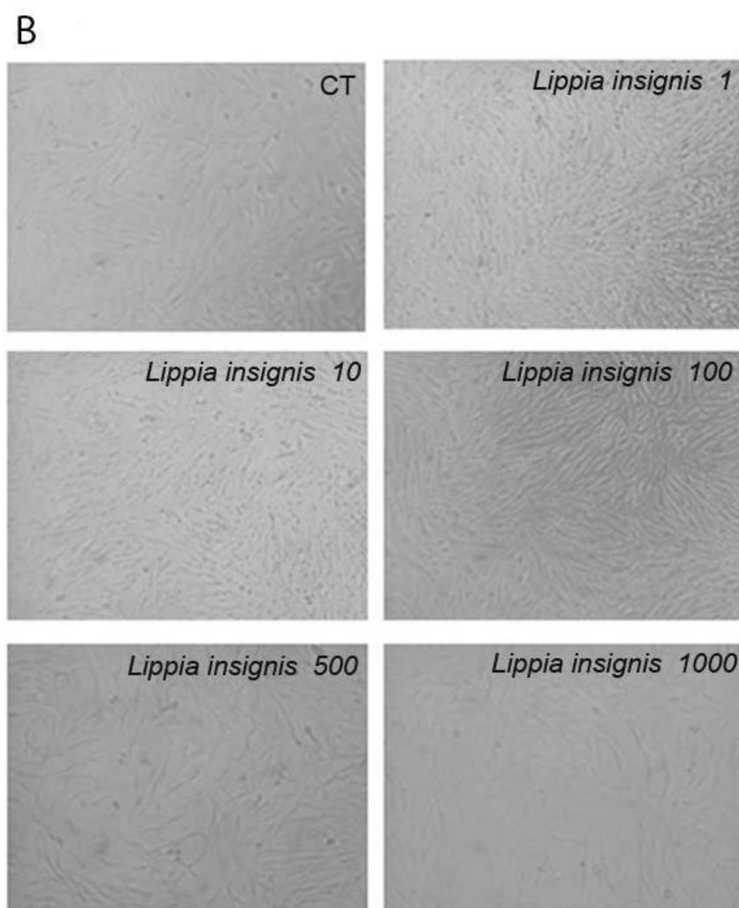

Figure 5.is an analysis of C6 cells treated with methanol extracts derived from *L. insignis* at 1 $\mu g.mL^{-1}$, 10 $\mu g.mL^{-1}$, 100 $\mu g.mL^{-1}$, 500 $\mu g.mL^{-1}$ e 1000 $\mu g.mL^{-1}$ under a phase contrast microscope after 48 hours.

After 24 h the control displayed a bipolar fusiform morphology indicating the viability of the cells. At 500 $\mu g.mL^{-1}$ and 1000 $\mu g.mL^{-1}$ cells displayed bipolar and multipolar fusiform morphology with thin processes. At 500 $\mu g.mL^{-1}$, and 1000 $\mu g.mL^{-1}$ cellularity decreases when

compared to the control. After 48 h the control maintained a bipolar fusiform morphology indicating the viability of the cells. At 500 μg.mL^{-1} and 1000 μg.mL^{-1} cellularity decreased when compared to the control.

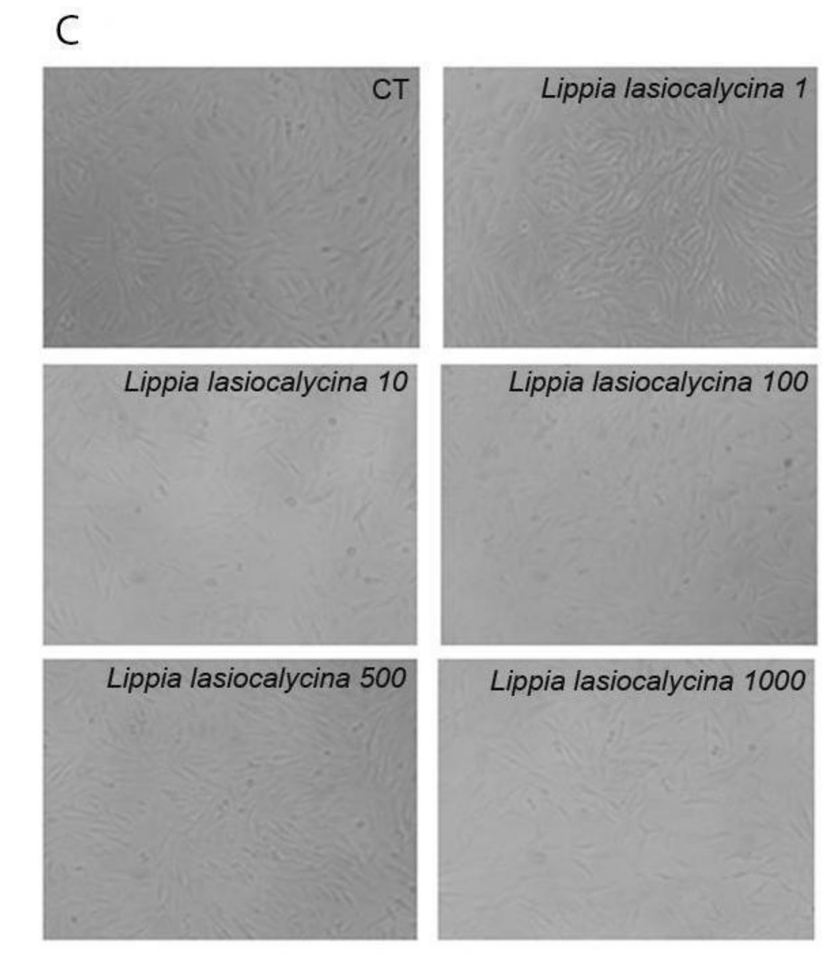

Figure 6. Analysis of C6 cells treated with methanol extracts derived from *L. lasiocalycina* at 1 1 μg.mL^{-1}, 10 μg.mL^{-1}, 100 μg.mL^{-1}, 500 μg.mL^{-1} e 1000 μg.mL^{-1} under a phase contrast microscope after 48 hours.

After 24 h the control displayed a bipolar fusiform morphology indicating the viability of the cells. At 1 μg.mL^{-1}, 10 μg.mL^{-1}, l00 μg.mL^{-1}, 500 μg.mL^{-1}, and 1000 μg.mL^{-1} cells displayed bipolar and multipolar fusiform morphology. At 1000 μg.mL^{-1} cellularity decreased when compared to the control. After 48 h the control maintained a bipolar fusiform morphology indicating the viability of the cells. At 1000 μg.mL^{-1} cellularity is comparable to 24 h.

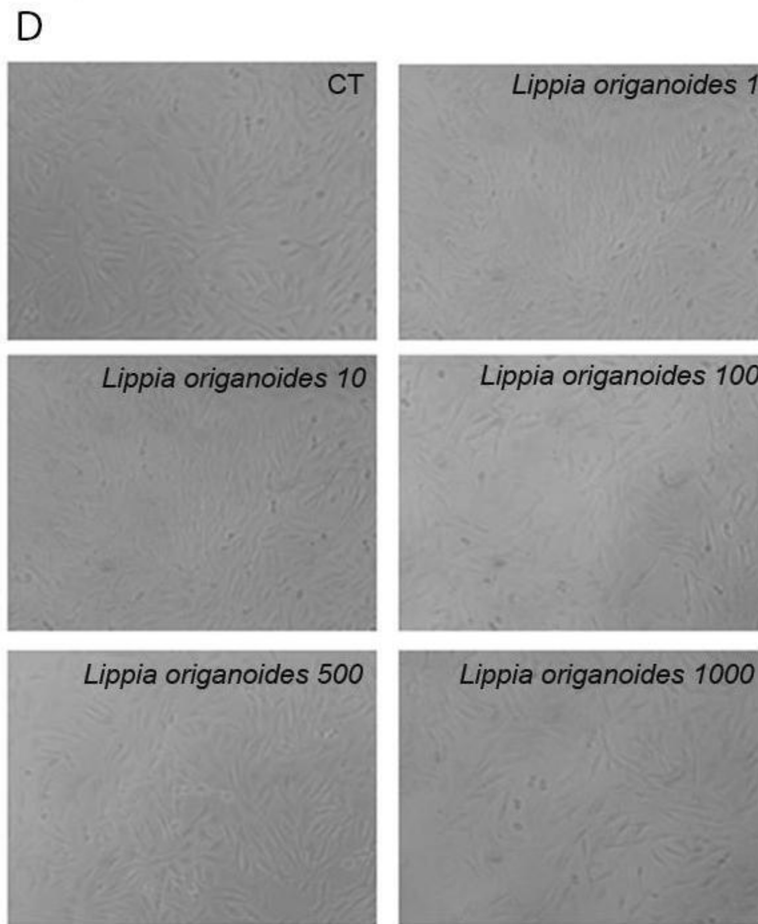

Figure 7. Analysis of C6 cells treated with methanol extracts derived from *L. origanoides* at 1 $\mu g.mL^{-1}$, 10 $\mu g.mL^{-1}$, 100 $\mu g.mL^{-1}$, 500 $\mu g.mL^{-1}$ e 1000 $\mu g.mL^{-1}$under a phase contrast microscope after 48 hours.

After 24 h the control displayed a bipolar fusiform morphology indicating the viability of the cells. At 100 $\mu g.mL^{-1}$ and 500 $\mu g.mL^{-1}$ cells displayed bipolar fusiform and multipolar fusiform morphologies. Cellularity at 100 $\mu g.mL^{-1}$ and 500 $\mu g.mL^{-1}$ were comparable to each other and decreased when compared to the control. At 1000 $\mu g.mL^{-1}$ cells displayed bipolar fusiform and multipolar fusiform morphologies with thin processes. After 48 h the control maintained a bipolar fusiform morphology indicating the viability of the cells. At 1000 $\mu g.mL^{-1}$ cells displayed a bipolar and multipolar fusiform morphology. Cellularity at at 1000 $\mu g.mL^{-1}$remained comparable to cellularity at 24 h.

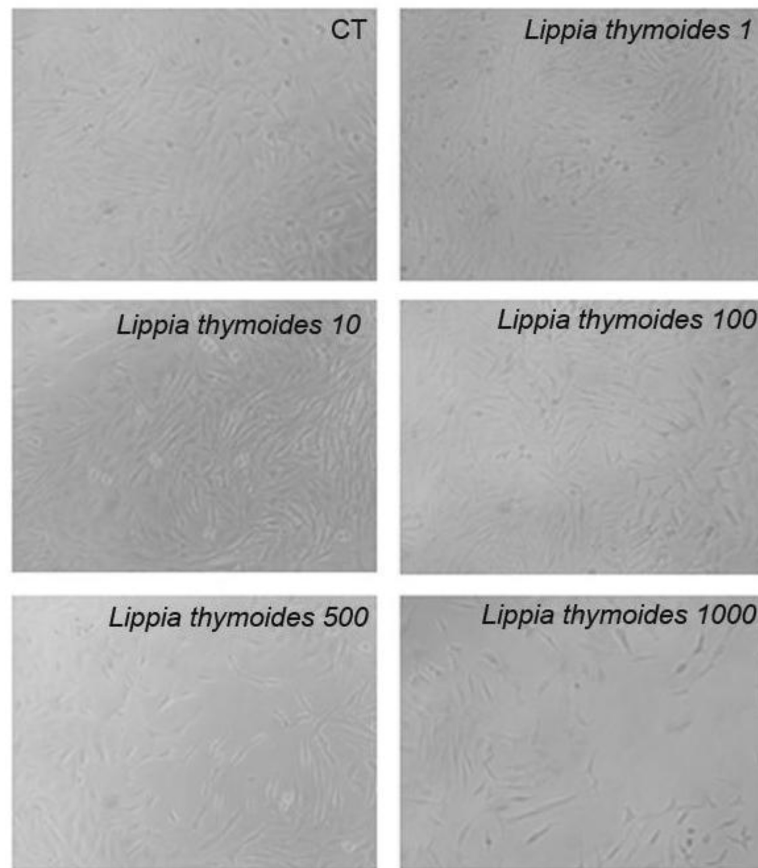

Figure 8. Analysis of C6 cells treated with methanol extracts derived from *L. thymoides* at 1 $\mu g.mL^{-1}$, 10 $\mu g.mL^{-1}$, 100 $\mu g.mL^{-1}$, 500 $\mu g.mL^{-1}$ e 1000 $\mu g.mL^{-1}$ under a phase contrast microscope after 48 hours.

After 24 h the control displayed a bipolar fusiform morphology indicating the viability of the cells. At 500 $\mu g.mL^{-1}$, and 1000 $\mu g.mL^{-1}$ cells displayed bipolar and multipolar fusiform morphologies with thin processes. Cellularity at 10 $\mu g.mL^{-1}$, 100 $\mu g.mL^{-1}$, 500 $\mu g.mL^{-1}$, and 1000 $\mu g.mL^{-1}$ decreased when compared to the control. After 48 h the control maintained a bipolar fusiform morphology indicating the viability of the cells. At 1000 $\mu g.mL^{-1}$ cells displayed bipolar and multipolar fusiform morphology with thin processes. Cellularity at 500 $\mu g.mL^{-1}$ and 1000 $\mu g.mL^{-1}$ was comparable to cellularity at 24 h and decreased when compared to the control.

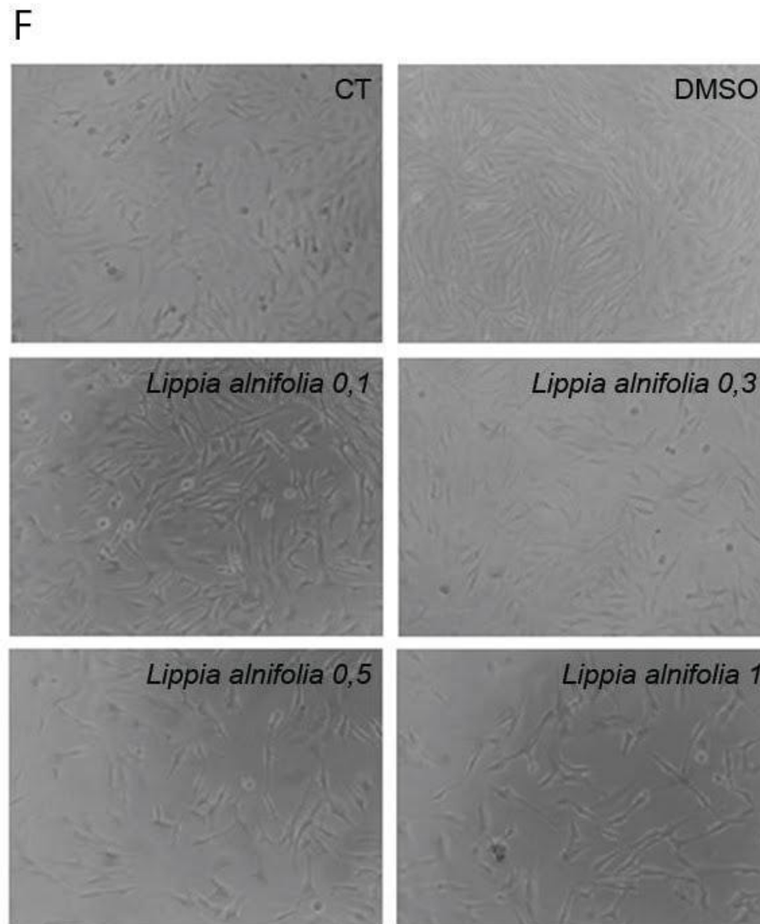

Figure 9. Analysis of C6 cells treated with essential oil derived from *L. alnifolia* at 0.1 $mg.mL^{-1}$, 0.3 $mg.mL^{-1}$, 0.5 $mg.mL^{-1}$ e 1 $mg.mL^{-1}$ under a phase contrast microscope after 48 hours.

After 24 h the control displayed a fusiform bipolar morphology indicating the viability of the cells. When compared with both controls cellularity decreased at 0.1 $mg.mL^{-1}$, 0.3 $mg.mL^{-1}$, 0.5 $mg.mL^{-1}$ e 1 $mg.mL^{-1}$ in a concentration dependent manner. At 0.1 $mg.mL^{-1}$ displayed a bipolar fusiform morphology with thin processes. A few cells display a multipolar fusiform morphology. At 0.3 cells displayed multipolar fusiform morphology and thin processes. At 0.5 $mg.mL^{-1}$ cells displayed a fusiform morphology with thin processes. At 1 $mg.mL^{-1}$ cells displayed multipolar fusiform morphology with thin processes. After 48 h the controls maintained a bipolar fusiform morphology indicating the viability of the cells. Cells at 0.1 $mg.mL^{-1}$, 0.3 $mg.mL^{-1}$, and 0.5 $mg.mL^{-1}$ displayed a bipolar fusiform morphology. At 1 $mg.mL^{-1}$ cellularity drastically decreased. Cells displayed multipolar fusiform morphology with fine processes, indicating activated cells.

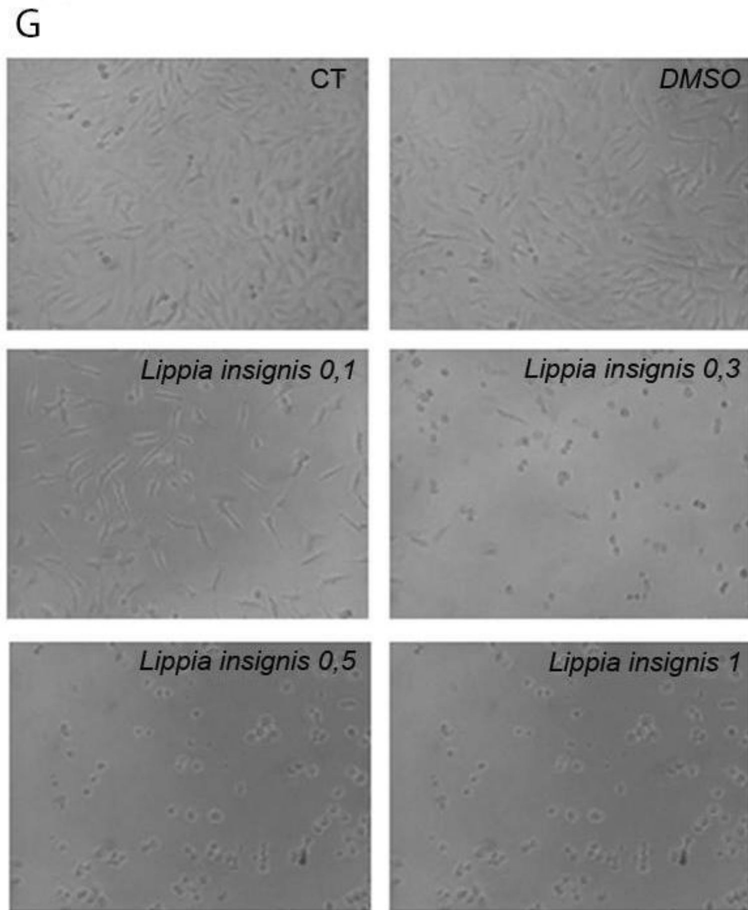

Figure 10. Analysis of C6 cells treated with essential oil derived from *L. insignis* at 0.1 mg.mL^{-1}, 0.3 mg.mL^{-1}, 0.5 mg.mL^{-1} e 1 mg.mL^{-1} under a phase contrast microscope after 48 hours.

After 24 h the controls displayed a fusiform bipolar morphology indicating the viability of the cells. When compared with both controls cellularity decreases drastically at 0.1 mg.mL^{-1}. Cells displayed a bipolar and multipolar fusiform morphology with thin processes indicating the cells have been activated. At 0.3 mg.mL^{-1} cell took on fusiform and spheroid morphologies. There was a minimal lose to cell body integrity. A few cells displayed thin processes. At 0.5 mg.mL^{-1} all cells took on spheroid morphology. When compared to 0.3 mg.mL^{-1}, 0.5 mg.mL^{-1} displayed a slight increase in loss of integrity to cell bodies. At 1 mg.mL^{-1} all cells take on spheroid morphology and display a loss of integrity to cell bodies comparable to that at 0.5 mg.mL^{-1}. After 48 h under the control maintained a bipolar fusiform morphology indicating the viability of the cells.At 0.3 mg.mL^{-1} cellularity greatly decreased when compared to the control. Most cells displayed spheroid morphology. Some cells displayed a bipolar fusiform or multipolar fusiform

morphology with thin processes. At 0.5 mg.mL^{-1} all cells displayed spheroid morphology and cell bodies are fragmented. At 1 mg.mL^{-1} all cells displayed spheroid morphology. Many of the cell bodies are fragmented and many others display a loss of integrity.

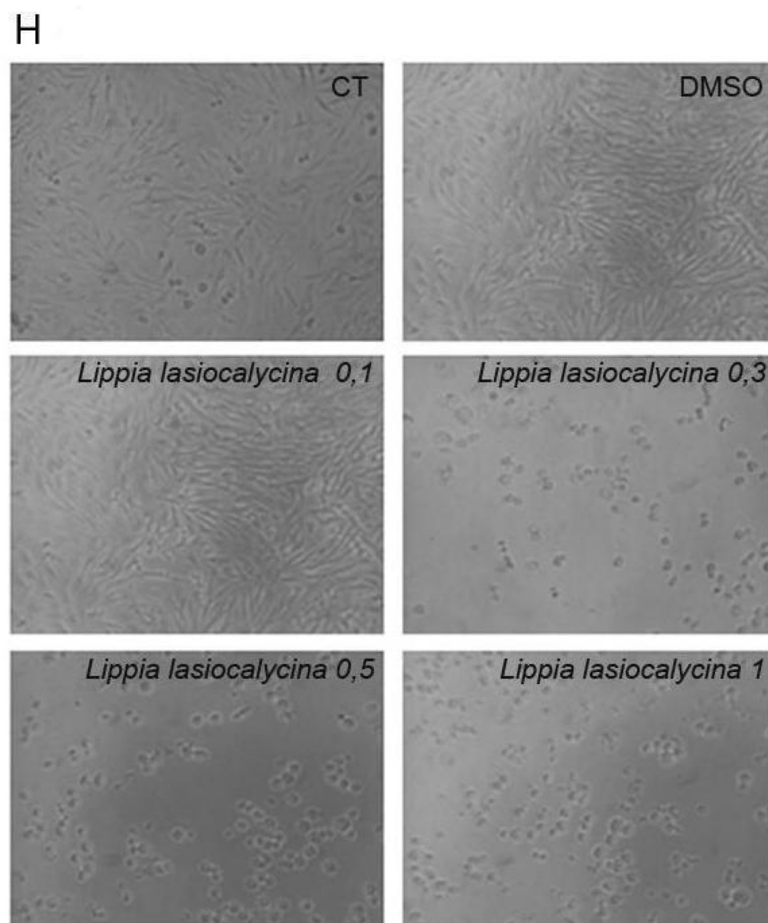

Figure 11. Analysis of C6 cells treated with essential oil derived from *L. lasiocalycina* at 0.1 mg.mL^{-1}, 0.3 mg.mL^{-1}, 0.5 mg.mL^{-1} e 1 mg.mL^{-1} under a phase contrast microscope after 48 hours.

After 24 h the control conditions cells displayed a fusiform bipolar morphology indicating the viability of the cells. When compared with the controls cellularity decreased drastically at 0.1 mg.mL^{-1}, 0.3 mg.mL^{-1}, 0.5 mg.mL^{-1}, and 1 mg.mL^{-1}. At 0.1 mg.mL^{-1} some cells retained a bipolar fusiform morphology. Most cells became spheroid. At 0.3 mg.mL^{-1} all cells have taken on a spheroid morphology. After 48 h the control maintained a bipolar fusiform morphology indicating the viability of the cells. At 0.1 mg.mL^{-1}a number have spheroid morphology. A significant number of cells took on bipolar fusiform morphology or multipolar fusiform

morphology with thin processes. These thin processes indicate reactivity of the remaining cells that have not taken on spheroid shapes. At 1 $mg.mL^{-1}$ cells retained a spheroid shape and cell bodies lost integrity.

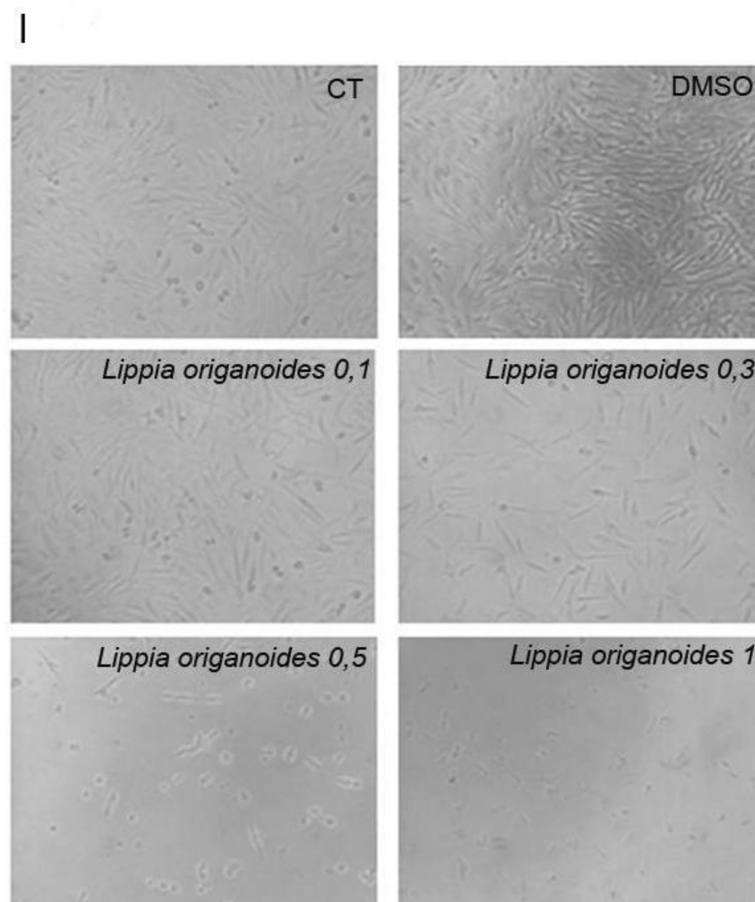

Figure 12. Analysis of C6 cells treated with essential oil derived from *L. origanoides* at 0.1 $mg.mL^{-1}$, 0.3 $mg.mL^{-1}$, 0.5 $mg.mL^{-1}$ e 1 $mg.mL^{-1}$ under a phase contrast microscope after 48 hours.

After 24 h the control displayed a fusiform bipolar morphology indicating the viability of the cells. When compared with the controls cellularity began to decrease at 0.1 $mg.mL^{-1}$ and then drastically decreased at 0.3 $mg.mL^{-1}$. At 0.5 $mg.mL^{-1}$ cell bodies retracted and took on spheroid morphologies. At 1 $mg.mL^{-1}$ there was a complete loss of integrity to the cellular bodies. After 48 h the control maintained a fusiform bipolar morphology indicating the viability of the cells. At 0.1 $mg.mL^{-1}$ cellularity increased when compared to 24 h. At 0.3 $mg.mL^{-1}$ cellularity and morphology remained largely unchanged. A few cells exhibited thin processes. A few of the cells

begin to take on fusiform morphologies. At 0.5 mg.mL^{-1} cells maintained spheroid morphologies. At 1 mg.mL^{-1} there continued to be a complete loss of integrity of the cell bodies.

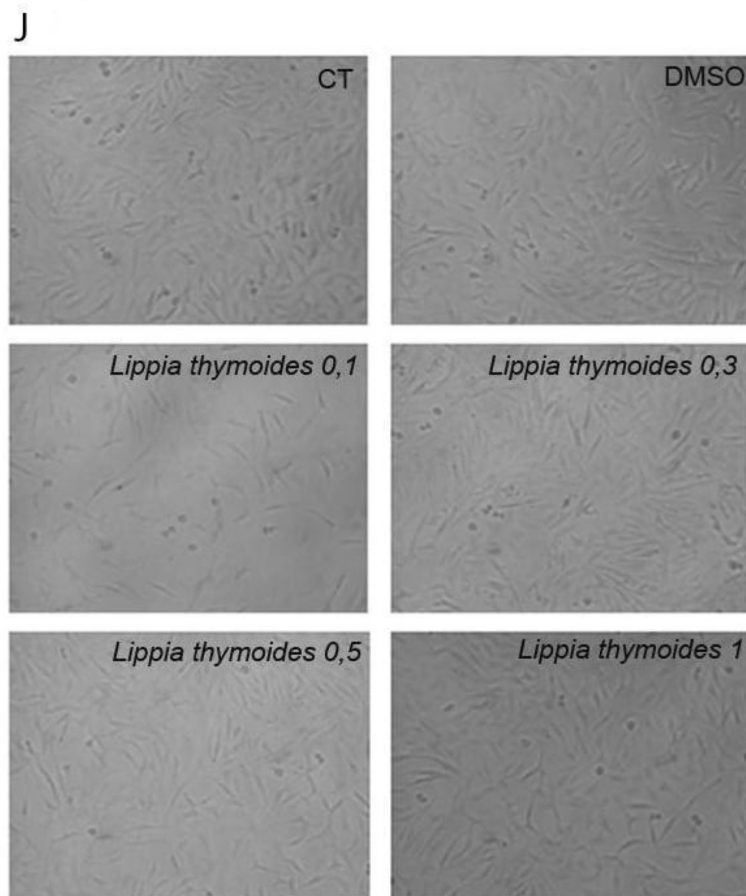

Figure 13. Analysis of C6 cells treated with essential oil derived from *L. thymoides* at 0.1 mg.mL^{-1}, 0.3 mg.mL^{-1}, 0.5 mg.mL^{-1} e 1 mg.mL^{-1} under a phase contrast microscope after 48 hours.

After 24 h the control displayed a fusiform bipolar morphology indicating the viability of the cells. When compared with the controls cellularity decreased at 0.1 mg.mL^{-1}, 0.3 mg.mL^{-1}, 0.5 mg.mL^{-1} e 1 mg.mL^{-1}. After 48 h the control maintained a bipolar fusiform morphology indicating the viability of the cells. At 0.1 mg.mL^{-1}, 0.3 mg.mL^{-1}, 0.5 mg.mL^{-1} e 1 mg.mL^{-1} cellularity drastically increased when compared to 24 h. When compared to each other cellularity is comparable. At 0.1 mg.mL^{-1}, 0.3 mg.mL^{-1}, 0.5 mg.mL^{-1} e 1 mg.mL^{-1} cells display a bipolar fusiform morphology.

Changes in the morphology of C6 induced by extracts derived from *L. alnifolia, L. insignis, L. lasiocalycina, L. origanoides,* and *L. thymoides* were analyzed using a light

microscope and May-Grünwald-Giemsa staining. Cells were exposed to either DMEM cell culture media alone or 500 $\mu g \cdot mL^{-1}$ of one of the extracts and analyzed after 72 h. All cells treated with the methanol extracts displayed some degree of cytoplasmic swelling and nuclear fragmentation. Cells treated with the extract from *L. alnifolia* displayed vacuolization, blebbing, and DNA condensation. Cells treated with the extract derived from *L. insignis* displayed vacuolization, blebbing, rounding of cell bodies, and DNA condensation. Cells treated with the extract from *L. lasiocalycina* display vacuolization, blebbing, and rounding of cell bodies. Cells treated with the extract from *L. thymoides* displayed vacuolization, blebbing, DNA condensation, and rounding and shrinking of the cell bodies. Cells treated with the extract from *L. origanoides* displayed a loss of cellular integrity, vacuolization, and blebbing (See appendix IX).

Changes in the morphology of C6 induced by essential oils from *L. alnifolia, L. lasiocalycina, L. origanoides,* and *L. thymoides* were also analyzed. Cells were exposed to either DMEM cell culture media alone or 0.1 $mg.mL^{-1}$ of one of the extracts and analyzed after 72 h. Cells treated with essential oils derived from *L. alnifolia* displayed vacuolization, blebbing, and DNA fragmentation, DNA condensation, and cellular shrinkage. Cells treated with the extract derived from *L. lasiocalycina* display vacuolization, cytoplasmic swelling, blebbing, DNA fragmentation, and rounding of cell bodies. Cells treated with essential oils from L. *thymoides* displayed vacuolization, blebbing, DNA fragmentation, cytoplasmic swelling, and rounding the cell bodies. Cells treated with essential oils from *L. origanoides* displayed cytoplasmic swelling, rounding of the cell bodies, and blebbing (See appendix X).

5.3 Cell Viability

The cytotoxicity of each methanol extract was assessed using a MTT assay on C6 glioma cells after 48 h (Figure K)

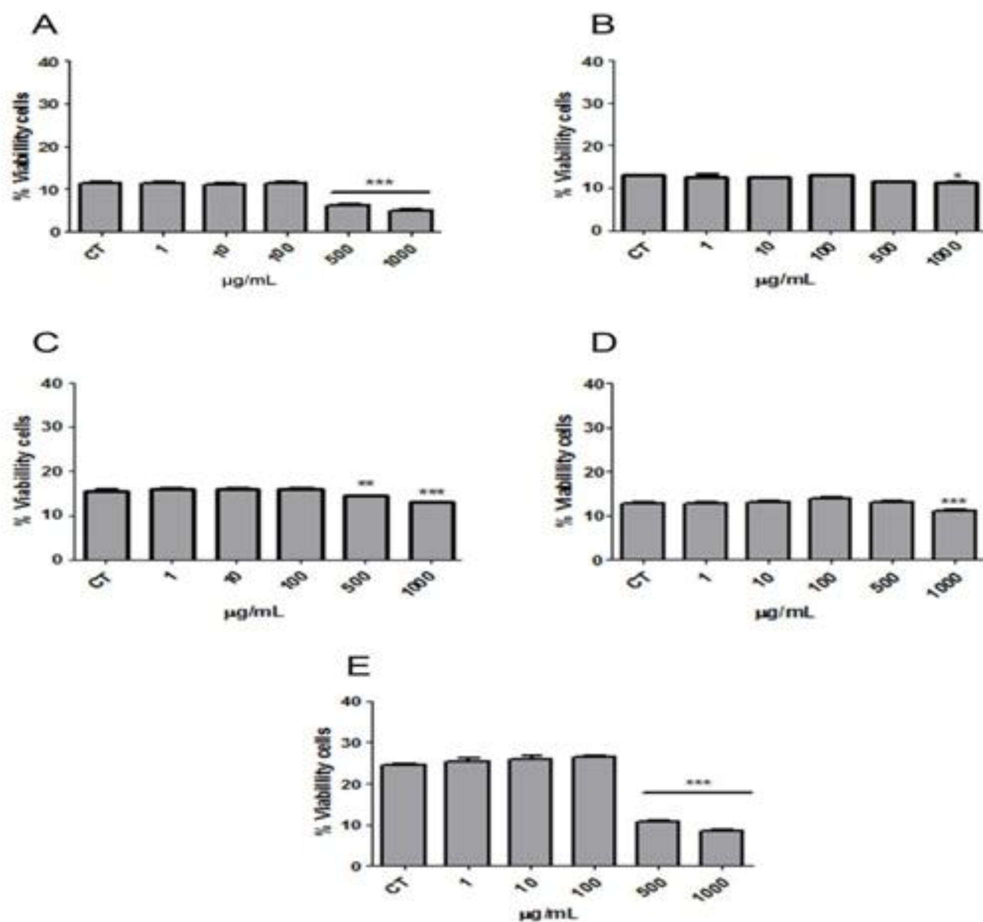

Figure 14. Analysis of C6 cells treated with methanol extracts from *L. alnifolia* (A), *L. insignis* (B), *L. lasiocalycina*(C), *L. thymoides*(D), and *L. origanoides* (E) after 48 hours. p-value< .05 *, p-value<.01 **, p-value<.001 ***

The cytotoxicity of all the methanol extracts were tested in concentrations varying from 1 to 1000 $\mu g.mL^{-1}$. *L. alnifolia, L. lasiocalycina,* and *L. origanoides* demonstrated a significant decrease in metabolic activity at 500 $\mu g{\cdot}mL^{-1}$ and 1000 $\mu g.mL^{-1}$. *L. insignis* demonstrated a significant decrease in metabolic activity at 1000 $\mu g.mL^{-1}$. *L. thymoides* demonstrated a significant decrease in metabolic activity at 1000 $\mu g.mL^{-1}$ when compared to the control.

The cytotoxicity of essential oils derived from *L. alnifolia*, *L. insignis*, *L. lasiocalycina*, *L. origanoides*, and *L. thymoides* were assessed using a MTT assay on C6 glioma cells at 48 h. (Figure L).

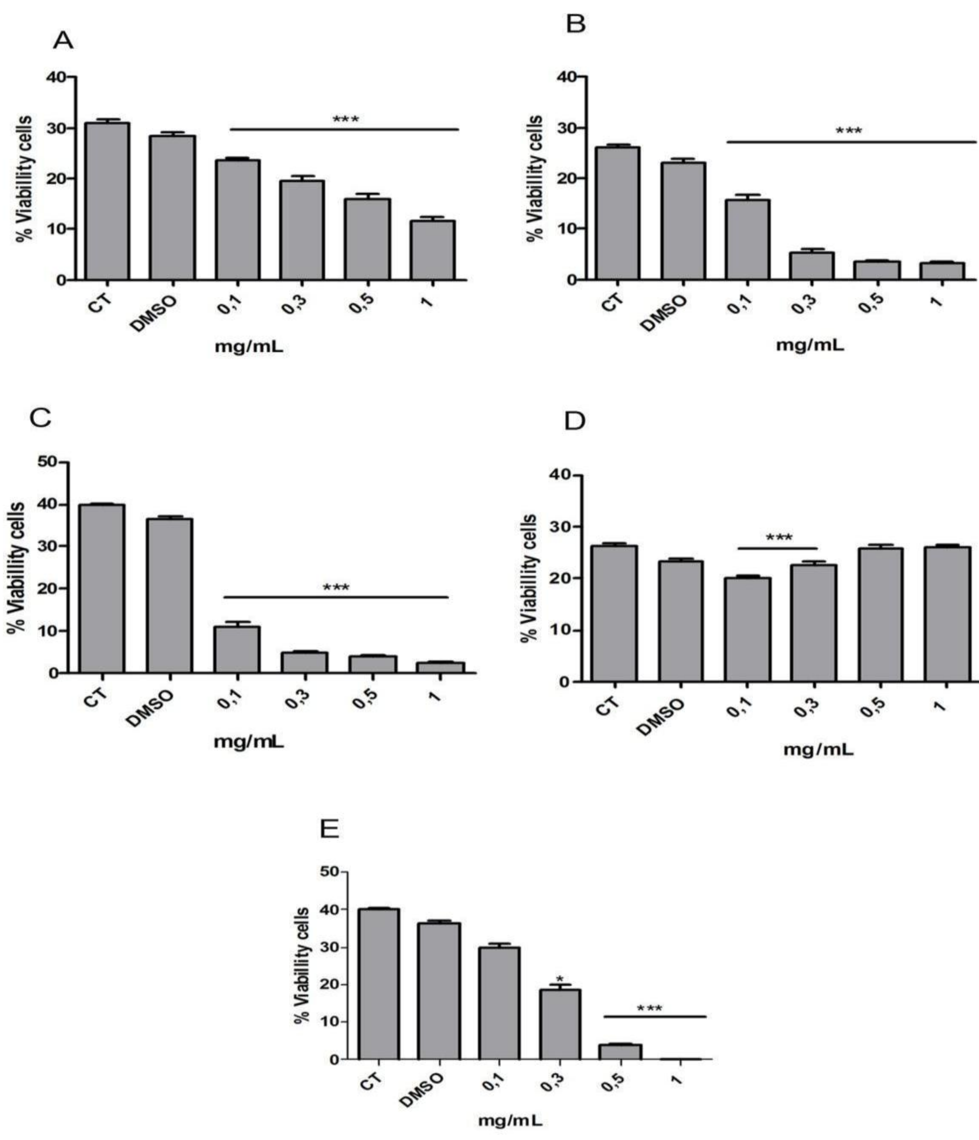

Figure 15. Analysis of C6 cells treated with essential oils from *L. alnifolia*(A), *L. insignis* (B), *L. lasiocalycina*(C),*L. thymoides*(D), and *L. origanoides* (E) after 48 hours. p-value< .05 *, p-value<.01 **, p-value<.001 ***

The cytotoxicity of all the essential oils was tested in concentrations varying from 0.1 $mg.mL^{-1}$ to 1 $mg.mL^{-1}$. Essential oils from *L. alnifolia*, *L. insignis*, *L. lasiocalycina* and *L. origanoides* decreased cell viability in a concentration dependent manner. Essential oils from *L. insignis* and *L. lasiocalycina* produced similar curves, decreasing cell viability in at comparable rates. The exception being at the concentration 0.1 $mg.mL^{-1}$ where *L. lasiocalycina* demonstrates a greater ability to reduce viability than *L. insignis*. The essential oil from *L. thymoides* appeared to increase cell viability in a concentration dependent manner with the highest concentration demonstrating viability comparable to the control.

5.4 Flow Cytometry

The cell cycle distribution was analyzed after exposure to each methanol extract (1000 μg.mL^{-1}) for 48 h. Under control conditions, the proportion of C6 cells in the G0/G1 phase increased with time with a concomitant decrease in S phase. A total of 65.84% of cells in the control remained in the G0/G1 phase. A total of 13.14 percentof the cells in the control arrested in the S phase of the cell cycle and 14.35 percent of the cells in the control arrested in the in G2/M phase of the cell cycle. A total of 70.60 percent of the culture treated with *L. alnifolia* experienced cell death. Of the remaining cells 20.70% of the culture arrested in G0/G1 phase. A total of 22.71 percent of the culture treated with *L. insignis* experienced cell death. Of the remaining cells 48.45% were arrested in G0/G1 phase and 23.21 percent of the cells arrested in G2/M phase.A total of89.86 percent of the culture treated with *L. lasiocalycina* experienced cell death. Of the remaining cells 8.10 percent arrested in G0/G1 phase. A total of 72.12 percent of the culture treated with *L. thymoides* experienced cell death. Of the remaining cells 21.30 percent of the culture arrested in G0/G1 phase. *L. origanoides* arrested 77.39% of the culture in G0/G1 phase. See appendix VI.

The cell cycle distribution was analyzed after exposure to each essential oil obtained from*L. alnifolia, L. lasiocalycina, L. origanoides, L. thymoides* (0.3mg.mL^{-1}) for 48 h. Under control conditions, the proportion of C6 cells in the G0/G1 phase increased with time with a concomitant decrease in S phase. A total of 65.84 percent of cells in the control remained in the G0/G1 phase. A total of 13.14 percent of the cells in the control arrested in the in S phase of the cell cycle and 14.35 percent of the cells in the control arrested in the in M phase of the cell cycle. A total of 76.35 percent of cells treated with the essential oil obtained from*L. alnifolia* arrested in G0/G1 phase. A total of 74.80 percent of cells treated with the essential oil from *L. lasiocalycina* arrested in G0/G1 phase. A total of 75.16 percent of the culture treated with *L. origanoides* arrested in G0/G1 phase. Of the remaining cells 19.51 percent of the culture arrested in G0/G1 phase. A total of 77.31 percent of cells treated with the essential oil from *L. thymoides* arrested in G0/G1 phase. See appendix VI.

5.5 Wound Heal Assay

A wound heal assay was used to investigate the anti-migration potential of the methanol extracts and essential oils from *L. alnifolia, L. insignis, L. lasiocalycina, L. origanoides,* and *L. thymoides*. Each of the methanol extracts inhibited migration to some extent in C6 cells when compared to the control. After 72 h, the control lesion demonstrated a 100% closure. The lesion treated with the methanol extract from *L. alnifolia* closed 30 % after 72 h. The lesion treated with the methanol extract from *L. insignis*closed 44% after 72 h. The lesion treated with the methanol extract from *L. lasiocalycina*closed 64% after 72 h. The lesion treated with the methanol extract from *L. origanoides* closed 19% after 72 h. The lesion treated with the methanol extracts from *L. thymoides* closed 44% after 72 h. All methanol extracts were statistically significant with p-values < .001 See appendix VII

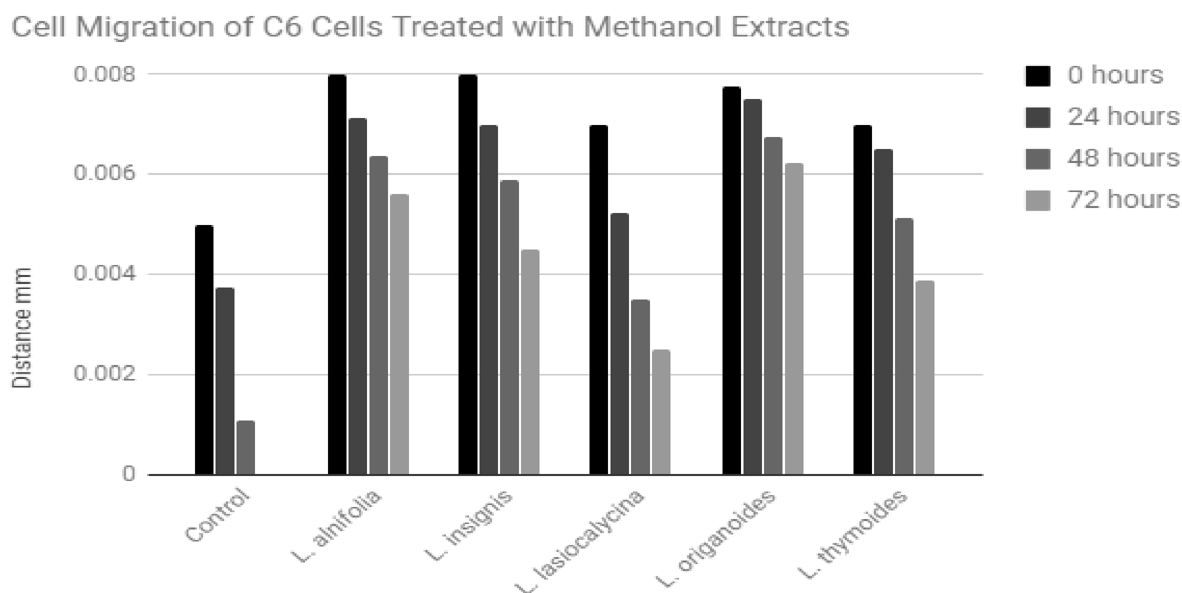

Figure 16. Analysis of the cell migration of C6 cells treated with methanol extracts derived from *L. alnifolia, L. insignis, L. lasiocalycina,L. thymoides* , and *L. origanoides* after 72 hours at a concentration of 500 µg.mL^{-1}.

Each of the essential oils inhibited migration to some extent in C6 cells when compared to the controls. After 72 h, the control lesion demonstrated a 100 % closure. The lesion treated with DMSO demonstrated a 70% closure after 72 h. The lesion treated with the essential oil from *L. alnifolia* closed 64% after 72 h. The lesion treated with the essential oil from *L. lasiocalycina*

closed 40% after 72 h. The lesion treated with the essential oil from *L. origanoides* closed 48% after 72 h.The lesion treated with the essential oil from *L. thymoides* closed 56% after 72 h. All essential oils were statistically significant with p-values <.05 with the exception of *L. lasiocalycina*. See appendix VIII.

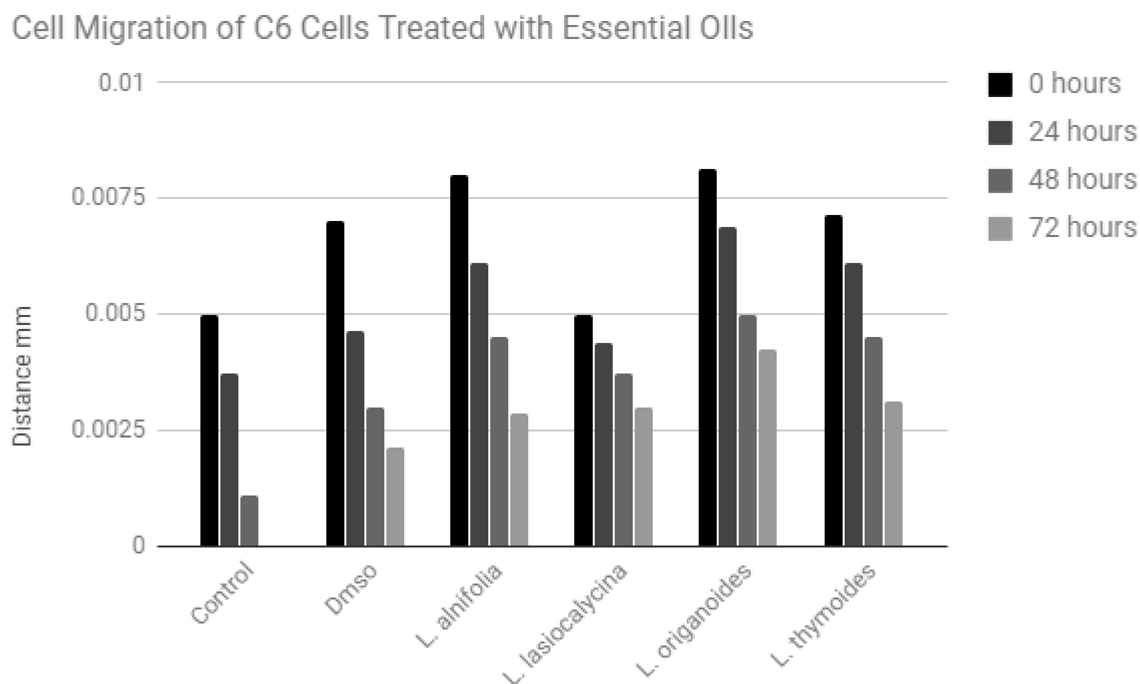

Figure 17. Analysis of the cell migration of C6 cells treated with essential oils derived from *L. alnifolia, L. lasiocalycina,L. thymoides* , and *L. origanoides* after 72 hours at a concentration of 0.1 $mg.mL^{-1}$.

6. Discussion

The purpose of this study is to evaluate the anticancer potential of essential oils and methanol extracts of *L. alnifolia*, *L. insignis*, *L. lasiocalycina*, *L. origanoides,* and *L. thymoides* on C6 glioma cells. The effects of methanol extracts and essential oils derived from *L. alnifolia*, *L. insignis*, *L. lasiocalycina*, *L. origanoides,* and *L. thymoides* on cell viability were analyzed using a MTT assay. The methanol extracts all significantly decreased the viability of cells at different concentrations. *L. alnifolia, L. lasiocalycina*, *L. origanoides,* and *L. thymoides* decreased cell viability at 1 $mg.mL^{-1}$. *L. insignis* decreased cell viability at 1 $mg.mL^{-1}$. *L. alnifolia, L. lasiocalycina,* and *L. origanoides* both decreased cell viability at 0.5 $mg.mL^{-1}$. *L. alnifolia* and *L. origanoides* seemed to have the greatest negative impact on cell viability.

The essential oils tested all negatively affected the viability of cells in a concnetration dependent manner, with the exception of the essential oil derived from *L. thymoides.* The essential oils derived from *L. alnifolia, L. insignis* and L. *lasiocalycina* significantlydecreased cell viability in a concentration dependent manner. The principal compounds present in *L. alnifolia* are β-myrcene (18.90%), myrcenone (15.42%), and E-ocimenone (23.56%). The chemical composition of *L. insignis* and L. *lasiocalycina* are similar, with the major constituents of *L. insignis* being β-myrcene (12.34%), limonene (14.73%), and E-ocimenone (26.11%), while the major constituents of *L. lasiocalycina* are β-myrcene (31.17%), and E-ocimenone (24.10%). The curves created by *L. insignis* and *L. lasiocalycina* are extremely similar, with the exception of the decrease in viability demonstrated by cells exposed to the essential oils at the lowest concentration, 0.1 $mg.mL^{-1}$. The viability of cells treated with L. *insignis* seems to be a little bit higher than cells treated with *L. lasiocalycina* at the lowest concentration tested. This possible discrepancy in cell viability could be due to the larger amount of β-myrcene present in *L. lasiocalycina.* The principal constituents of the essential oil derived from *L. alnifolia* appear in similar concentrations as that of *L. insignis*. However, the level of loss in cell viability is less than that of *L. insignis* or *L. lasiocalycina.* This could be due to the presence of p-cymene. The anti-proliferative properties of p-cymene have been established against the U87 (glioblastoma) cell line when used as part of a arene-ruthenium(II) curcuminoid complex. The arene-ruthenium(II) curcuminoid complexes tested achieved an IC_{50} value as low as 9.4 μM (CARUSO, 2016). The terpene p-cymene is the only molecule that appears in the chemical compositions of both *L. insignis* (7.24%)or*L. lasiocalycina* (7.17%)in relatively high and similar concentrations that does not appear in the chemical composition of *L. alnifolia.*

The essential oil derived from *L. thymoides* appears to increase cell viability in a concentration dependent manner. There was a significantly decrease in viability of cells at 0.1 $mg.mL^{-1}$ and 0.3 $mg.mL^{-1}$, the most significant change occurring at 0.1 $mg.mL^{-1}$. Cells treated with 0.5 $mg.mL^{-1}$ and 1 $mg.mL^{-1}$ appear display same level of viability has the control. At least some of the compounds composing the essential oil have been shown to have anti-proliferative effects on cancer cells (FIDYT et al, 2016). In one study the principal compound, E-caryophyllene, was shown to intensify anti-proliferative effects of other drugs, including a naturally occurring constituent found in the essential oil, α-humulene (LEGAULT J, 2007). Another study concluded that E-caryophyllene also has the ability to mitigate excitotoxicity in C6

cells linked to excessive glutamate accumulation. The primary mechanism used to achieve this feat is the reduction of the presence of ROS and mitochondrial dysfunction(ASSIS et al, 2014). The presence of these constituents like α-humulene and or the ability of E-caryophyllene to enhance the anti-proliferative effects of some molecules could be responsible for the decrease in viability in the initial concentrations tested. The ability of E-caryophyllene to reduce mitochondrial damage could explain the cells exposed to higher concentrations of the essential oil returning to control level viability.

The essential oil derived from *L. origanoides* decreased cell viability in a concentration dependent manner with a p-value < 0.05 at 0.3 $mg.mL^{-1}$ and a p-value < 0.001 at 0.5 $mg.mL^{-1}$ and 1 $mg.mL^{-1}$. The principal compound in the essential oil is carvacrol (53.89%). Carvacrol has been shown to have different effects on different cell types, many of them anticancer and closely associated with the regulation of Ca_2+ induction. The effect carvacrol has on the viability of cells has been studied in the human glioblastoma cell lines, U87 and DBTRG-05MG. Carvacrol decreased cell viability of U87 after 24 hours with an IC_{50} value of 561.3±22.2μM. Cell proliferation was also significantly decreased in a dconcentration dependent manner at 0.5 $mg.mL^{-1}$ - 1 $mg.mL^{-1}$ in U87 cells (Chen W-L, 2015). The cell viability of DBTRG-05MG decreased in a concentration dependent manner at concentrations of 0.2 $mg.mL^{-1}$ - 0.8 $mg.mL^{-1}$ and completely lost cell viability at 1 $mg.mL^{-1}$ (Liang WZ, 2012). The effects of *L. origanoides* on C6 cells mirror the results of previous studies conducted using carvacrol, the primary compound in the essential oil. This implies that the ability of *L. origanoides* to affect cell viability is due to its high concentration of carvacrol.

The effects of the essential oils and methanol extracts from *L. alnifolia*, *L. insignis*, *L. lasiocalycina*, *L. origanoides,* and *L. thymoides* on the morphology of C6 cells after 48 h were evaluated under a phase contrast microscope. The concentrations of essential oils examined ranged 0.1 $mg.mL^{-1}$ to 1 $mg.mL^{-1}$ and the concentrations of the methanol extracts examined ranged from 10 $\mu g.mL^{-1}$ to 1000 $\mu g.mL^{-1}$. All methanol extracts displayed decreases in cell density at 1000 $\mu g.mL^{-1}$. Cells treated with the methanol extract derived from *L. alnifolia* displayed elongated and fragmented bodies at 500 $\mu g.mL^{-1}$. At 1000 $\mu g.mL^{-1}$ cells lose celular integrity and become spheroid. All essential oils, with the exception of *L. thymoides,* display decreases in cell density in a concentration dependent manner. Cells treated with the essential oil derived from *L. thymoides* display changes on morphology and population density similar to that

of the control at the highest concentrations tested. Cells treated with the essential oil derived from *L. insignis* and *L. lasiocalycina* both display spheroid morphologies at 0.3 $mg.mL^{-1}$. Cells treated with the essential oil derived from *L. origanoides* begin taking on morphological changes at 0.5 $mg.mL^{-1}$ and completely lose cell integrity at 1 $mg.mL^{-1}$.

Cells undergoing programed cell death display a series of morphological changes. Apoptosis is characterized by cellular shrinkage, membrane blebbing, nuclear chromatin condensation and fragmentation (VERMEULEN K, 2005). Hallmarks of necrosis include swelling of the cell body followed by disruption of the cell membrane (PROSKURYAKOV, 2003). Vacuolization has also been shown to be an indicator of a cytopathological conditions leading to cell death (SHUBIN, 2016). The effect of the essential oils and methanol extracts on the morphology of C6 cells after 72 hours through the use of May-Grunwald staining. All cells treated with the methanol extracts displayed some degree of vacuolization, cytoplasmic swelling, blebbing, and nuclear fragmentation. *L. thymoides, L. insignis* both displayed rounding of cells. *L. thymoides* displayed some degree of cell shrinkage. L. origanoides displayed a loss of cellular integrity. All cells treated with essentials displayed some degree of blebbing. Cells treated with *L. alnifolia*, *L. lasiocalycina*,and *L. thymoides* displayed vacuolization and nuclear fragmentation. Cell treated with *L. lasiocalycina, L. thymoides,* and *L. origanoides* all displayed some degree of cytoplasmic swelling. *L. lasiocalycina* and *L. origanoides* displayed rounding of cell bodies. The morphology changes of all cells treated with essential oils and methanol extracts indicated the possibility of the occurrence of programed cell death, like apoptosis or necrosis.

The multistep invasion metastasis cascade is a process by which cancer cells originating from primary tumors have the ability to travel to other parts of the body and establish new colonies where space and nutrients are initially sparse. This colonization is responsible for 90% of the deaths in human cancers (HANAHAN 2001). Tumor infiltration into surrounding tissue is the first step of the metastasis cascade (HANAHAN 2011). Through the use of a wound heal assay this study analyzed the ability of methanol extracts from *L. alnifolia*, *L. insignis*, *L. lasiocalycina*, *L. origanoides,* and *L. thymoides* to inhibit the migration of C6 glioma cells. However, due to a non-descript volatile toxicity only the essentials oils from *L. alnifolia*, *L. lasiocalycina*, *L. origanoides,* and *L. thymoides* were tested. The methanol extracts all significantly inhibited the migration of cells after 72 h at 0.5 $mg.mL^{-1}$ when compared to the control. *L. alnifolia* demonstrated a 30 % wound closure, *L. insignis* demonstrated a 44% wound

closure, *L. lasiocalycina* demonstrated a 64% wound closure, *L. origanoides* demonstrated a 19% wound closure,and *L. thymoides* demonstrated a 44% wound closure, making *L. alnifolia* and L. *origanoides* the best candidates for further study of migration inhibition.

With the exception of *L. lasiocalycina,* all essential oils significantly inhibited the migration of cells after 72 h at 0.1 $mg.mL^{-1}$ when compared to the control treated with DMSO. *L. alnifolia* demonstrated a 64% wound closure, *L. lasiocalycina* demonstrated a 40% wound closure, *L. origanoides* demonstrated a 48% wound closure, and *L. thymoides* demonstrated a 56% wound closure. Several of the principal constituents in the essential oils have been shown to to have inhibitory effects on glioblastoma or other cancer cells lines. At a concentration of 0.5 $mg.mL^{-1}$ carvacrol was shown to significantly inhibit the migration of U87 glioblastoma cells (Chen W-L, 2015). At a concentration of 100 µM β-myrcene has been shown to possibly promote the inhibition of migration of MDA-MB-231 human breast cancer cells through the downregulation of NF-κB-mediated MMP-9 expression (LEE J-H, 2015). This indicates that carvacrol could also be responsible for the ability of *L. origanoides* to inhibit the cell migration of C6 glioma.

L. alnifolia and *L. lasiocalycina* contain virtually the same percentage of principal molecules in their chemical compositions. *L. lasiocalycina* contains almost twice the amount of β-myrcene as a percentage of its chemical composition. However, the essential oil derived from *L. alnifolia* significantly reduced the migration of the glioma in the experiment and the oil derived from *L. lasiocalycina* did not. The lack of significance demonstrated by the essential oil derived from *L. lasiocalycina* on the inhibition of migration could potentially be attributed to the low concentration used during the experiment. The ability of L. *alnifolia* to significantly inhibit the migration of C6 cells could be due to the presence myrcenone in its chemical composition. It appears in higher concentrations in the oil derived from *L. alnifolia* than in the oil derived from *L. lasiocalycina.*

β-caryophyllene oxide, one of the principal constituents in *L. thymoides,* has demonstrated concentrationdependent anti-migration potential against MG-63 human osteosarcoma cells (PAN, 2016). β-caryophyllene oxide has also been shown to have anti-migration effects against MDA-MB-231 cells at concentrations lower than 40 $\mu g.mL^{-1}$. The phytocannabinoids, tetrahydrocannabivarin (THCV) and cannabidivarin (CBDV) have been shown to be effective in inhibiting the cell migration in U87 cells (ROSS, 2014). The ability of β-caryophyllene oxide to

inhibit cell migration in both osteosarcoma and adenocarcinoma taken into consideration with the effectiveness of other phytocannabinoids' ability to inhibit the migration of glioblastoma indicated that it may responsible for the ability of *L. thymoides* to inhibit the migration of C6 cells.

To examine the effects of the essential oils derived from *L. alnifolia*, *L. lasiocalycina*, *L. origanoides,* and *L. thymoides* and methanol extracts derived from *L. alnifolia*, *L. insignis*, *L. lasiocalycina*, *L. origanoides,* and *L. thymoides* on the cell cycle of C6 glioma cells after 48 h cultures were stained with propidium iodide and analyzed using flow cytometry. Due to a nondescript volatile toxicity the essential oil derived from *L. insignis* was excluded from this experiment. At 0.03 $mg.mL^{-1}$ the essential oils from *L. alnifolia* (76.35%), *L. lasiocalycina* (74.80%), *L. origanoides* (75.16%)and *L. thymoides* (77.31%) all disrupt more cells' ability to leave G0/G1 phase of the cell cycle when compared to the control (65.84%). At 1 $mg.mL^{-1}$ the methanol extracts caused 70.60% percent of the culture treated with *L. alnifolia*experienced cell death, 22.71% of the culture treated with *L. insignis* experienced cell death, 89.86% of the culture treated with *L. lasiocalycina* experienced cell death, and 72.12% of the culture treated with *L. thymoides* experienced cell death. *L. origanoides* (77.39%)arrested more cells in G0/G1 phase than the control (65.84 %). *L. insignis* (23.21%)arrested more cells in G2/M phase than the control (14.35%). The high percentages of cell death seen in cells treated with the methanol extracts imply that they could be inducing apoptosis. The disruption in the cell cycle caused by the essentials indicate that one or more of their constituents could potentially be able to cause cell cycle arrest in C6 glioma cells.

In conclusion all the drugs tested in this study showed some degree of bioactivity against the C6 cell line, albeit some at relatively high concentrations. Because all extracts displayed some degree of cytotoxicity, the efficacy of their fractions should be explored, with priority being given to *L. alnifolia* and *L. origanoides*. The essential oil components E-caryophyllene, carvacrol, p-cymene, and β- myrcene have all been isolated from other plants in similar studies and yielded similar results to those obtained by test done with essential oils containing them in high concentrations from this study. These constituents should be isolated and experiments should be done to start elucidating the mechanisms of their bioactivity. Myrcenoneand E-ocimenone should be isolated and undergo general screenings to assess the degree to which their presence influenced the cytotoxicity and other bioactivity of the essential oils tested in this study.

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Appendix I

Table 1: Chemical composition of the essential oils obtained from the leaves from *Lippia alnifolia*

Compound	IKlit	IKcalc	%±DP
α-pinene	939	940	6.43±0.85
sabinene	975	970	trace
β-pinene	977	977	trace
β- myrcene	990	986	18.90±0.36
limonene	1029	1029	1.00±0.07
1,8-cineol	1031	1032	1.27±0.01
E-β-ocimene	1050	1048	0.54±0.03
ipsdienol	1145	1144	1.16±0.06
myrcenone	1149	1151	15.41±0.62
α -terpineol	1188	1188	0.71±0.11
verbenone	1205	1212	2.69±0.47
Z-ocimenone	1229	1231	3.00±0.40
E-ocimenone	1238	1240	23.56±0.80
α-cubebene	1348	1345	0.62±0.01
α-copaene	1376	1370	2.28±0.01
E-caryophyllene	1419	1415	1.02±0.01
allo-aromadendrene	1460	1457	0.96±0.01
trans-cadina-1(6),4-diene	1476	1471	0.60±0.04
germacrene D	1485	1481	2.45±0.01
biciclogermacrene	1500	1490	2.42±0.03
α-muurolene	1500	1492	0.51±0.05
γ-cadinene	1513	1513	1.19±0.01
trans-calamenene	1522	1520	3.44±0.21
spatulenol	1578	1577	2.38±0.15

Total de compounds identified: 92,47±0,52
IKlit - Kovats Index Literature; IKcalc - Calculated Kovats Index

Appendix II

Table 2: Chemical composition of the essential oils obtained from the leaves from *Lippia insignis*

Compound	IKlit	IKcalc	%±DP
α-thujene	930	927	0.24±0.02
α-Pinene	939	935	trace
sabinene	975	973	0.16±0.00
β-pinene	977	977	trace
β- myrcene	990	989	12.43±0.02
α-terpinene	1017	1016	1.17±0.03
p-cymene	1026	1024	7.24±0.84
limonene	1029	1029	14.73±0.60
E-β-ocimene	1050	1048	1.78±0.04
γ -terpinene	1059	1059	6.99±0.14
cis-sabinene hydrate	1070	1068	trace
terpinolene	1088	1089	0.19±0.01
linalol	1096	1096	2.80±0.79
ipsdienol	1145	1144	0.85±0.12
myrcenone	1149	1151	6.37±1.14
terpinen-4-ol	1177	1179	trace
α -terpineol	1188	1188	0.55±0.06
Z-ocimenone	1229	1231	5.29±0.49
thymol, mthyl ether	1235	1237	trace
E-ocimenone	1238	1240	26.11±1.05
thymol	1290	1290	trace
carvacrol	1298	1297	0.21±0.01
piperitenone oxide	1368	1368	trace
E-caryophyllene	1419	1420	1.90±0.43
α-humulene	1454	1455	0.52±0.12
germacrene D	1485	1481	1.97±0.60
biciclogermacrene	1500	1496	2.55±0.90
β-bisabolene	1505	1504	trace
spatulenol	1578	1577	1.32±0.17
caryophyllene oxide	1583	1583	0.29±0.04

Total de compounds identified: 95,49±1,55
IKlit - Kovats Index Literature; IKcalc - Calculated Kovats Index

Appendix III

Table 3: Chemical composition of the essential oils obtained from the leaves from *Lippia lasiocalycina*

Compound	IKlit	IKcalc	%± DP
α-thujene	930	927	trace
α-pinene	939	939	trace
sabinene	975	975	1.23±0.07
β- myrcene	990	991	31.17±1.16
p-cymene	1026	1026	7.17±0.62
limonene	1029	1031	0.31±0.00
Z-β-ocimene	1037	1038	trace
E-β-ocimene	1050	1049	1.67±0.06
γ -terpinene	1059	1061	2.29±0.02
linalol	1096	1098	1.29±0.09
crisanthenona	1127	1125	trace
ipsdienol	1145	1146	0.43±0.05
myrcenone	1149	1153	4.05±0.35
borneol	1169	1163	1.34±0.05
α-terpineol	1188	1190	trace
Z-ocimenone	1229	1231	6.51±0.13
E-ocimenone	1238	1241	24.10±0.80
geraniol	1252	1255	0.34±0.02
geranial	1267	1271	0.66±0.08
thymol	1290	1292	trace
β-elemene	1390	1393	0.50±0.05
E-caryophyllene	1419	1423	4.00±0.35
α-guaiene	1439	1442	2.51±0.27
α-humulene	1454	1457	1.43±0.14
germacrene D	1485	1483	0.41±0.04
biciclogermacrene	1500	1498	0.41±0,05
α-bulnesene	1509	1508	1.22±0.20
spatulenol	1578	1580	1.70±0.19
caryophyllene oxide	1583	1585	1.77±0.40

Total de compounds identified: 96,30±0,20
IKlit - Kovats Index Literature; IKcalc - Calculated Kovats Index

Appendix IV

Table 4: Chemical composition of the essential oils obtained from the leaves from*Lippia thymoides*

Compound	IKlit	IKcalc	%±DP
α-thujene	930	928	trace
α-pinene	939	936	1.62±0.04
camphene	954	951	0.19±0.00
sabinene	975	975	1.83±0.55
β-pinene	977	979	0.88±0.04
β- myrcene	990	989	0.38±0.06
α-phellandrene	1002	1004	trace
α -terpinene	1017	1017	trace
p-cymene	1026	1025	0.78±0.51
limonene	1029	1031	2.75±0.16
1,8-cineol	1031	1034	5.17±0.47
E-β-ocimene	1050	1048	trace
γ -terpinene	1059	1060	0.48±0.14
trans-pinocarveol	1139	1140	0.18±0.00
trans-verbenol	1144	1142	trace
ipsdienol	1145	1145	0.20±0.00
borneol	1169	1166	0.43±0.02
terpinen-4-ol	1177	1177	0.56±0.16
α-terpineol	1188	1189	trace
mirtenol	1195	1195	trace
carvacrol	1298	1298	trace
δ-elemene	1338	1339	0.25±0.10
α-Cubebene	1348	1352	0.79±0.09
α-copaene	1377	1379	3.49±0.60
β-bourbonene	1388	1387	0.42±0.11
β-cubebene	1388	1391	0.31±0.07
β-elemene	1388	1392	0.39±0.05
α -gurjunene	1409	1411	0.39±0.02
E-caryophyllene	1419	1426	29.55±0,47
γ -elemene	1436	1435	0.22±0.01
trans-muurola-3,5-diene	1453	1453	0.55±0.15
α-humulene	1454	1457	2.65±0.12
allo-aromadendrene	1460	1464	0.79±0.11
germacrene D	1485	1485	6.59±2.98
trans-muurola-4(14),5-diene	1493	1493	0.99±0.16
α-muurolene	1500	1499	0.63±0.70
cuparene	1505	1507	2.18±0.57
β-bisabolene	1505	1509	0.54±0.09
cubebol	1515	1516	0.43±0.10
cis-calamene	1529	1526	5.59±0.99
trans-cadina-1,4-diene	1534	1535	0.42±0.04
germacrene B	1561	1561	2.53±0.72
caryophyllene oxide	1583	1588	8.17±3.40
α-muurolol	1646	1648	0.70±0.05

Total de compounds identified: 83,80±1,02
IKlit - Kovats Index Literature; IKcalc - Calculated Kovats Index

Appendix V

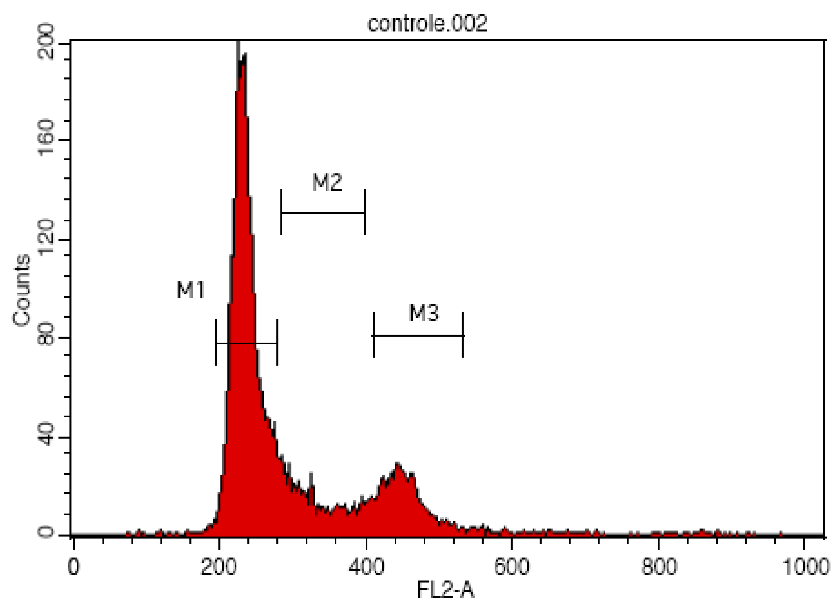
Table 5: Chemical composition of the essential oils obtained from the leaves from *Lippia origanoides*

Compound	IKlit	IKcalc	%±DP
α-thujene	930	928	0.16
α-Pinene	939	939	trace
camphene	954	954	0.14
β- myrcene	990	995	0.51
α -terpinene	1020	1019	0.46
p-cymene	1026	1024	4.03
limonene	1029	1022	0.15
α -terpinene	1059	1063	3.50
linalol	1096	1100	5.84
camphor	1146	1048	1.69
borneol	1169	1067	0.82
terpinen-4-ol	1177	1080	0.63
thymol, mthyl ether	1235	1238	2.87
α-elemene	1390	1393	0.53
thymol	1290	1296	4.00
carvacrol	1299	1311	53.89
carvacrol acetate	1372	1376	0.26
E-caryophyllene	1419	1424	5.86
α -bergamotene	1434	1438	0.28
aromadendrene	1441	1442	1.13
α-humulene	1454	1458	1.80
biciclogermacrene	1500	1500	4.16
7-epi-α-selineno	1522	1521	0.43
espatulenol	1578	1584	1.59
caryophyllene oxide	1583	1588	0.73
viridiflorol	1592	1594	0.18

Total de compounds identified: 83,80±1,02
IKlit - Kovats Index Literature; IKcalc - Calculated Kovats Index

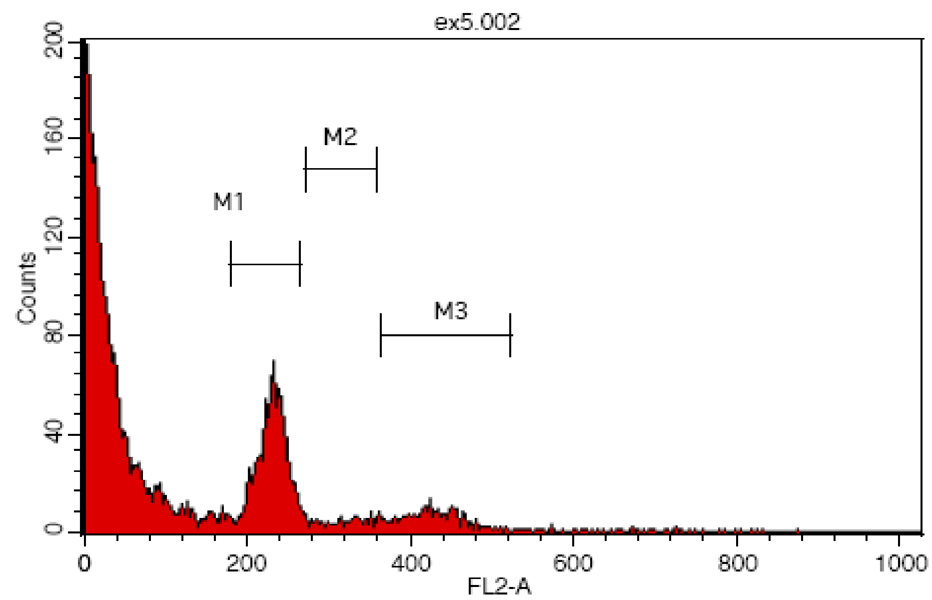
Appendix VI

<u>Control</u>



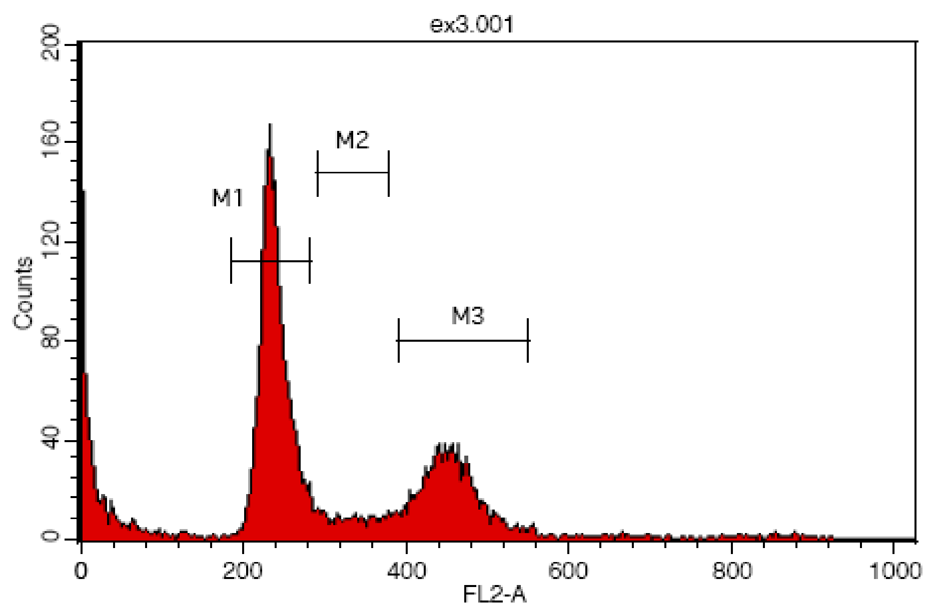
Marker	Left, Right	% Total	Mean	CV
All	0, 1023	98.75	289.39	33.98
M1	195, 277	65.84	234.43	7.10
M2	284, 398	13.14	330.84	10.53
M3	410, 533	14.35	451.79	5.91

L. alnifolia - Methanol Extract



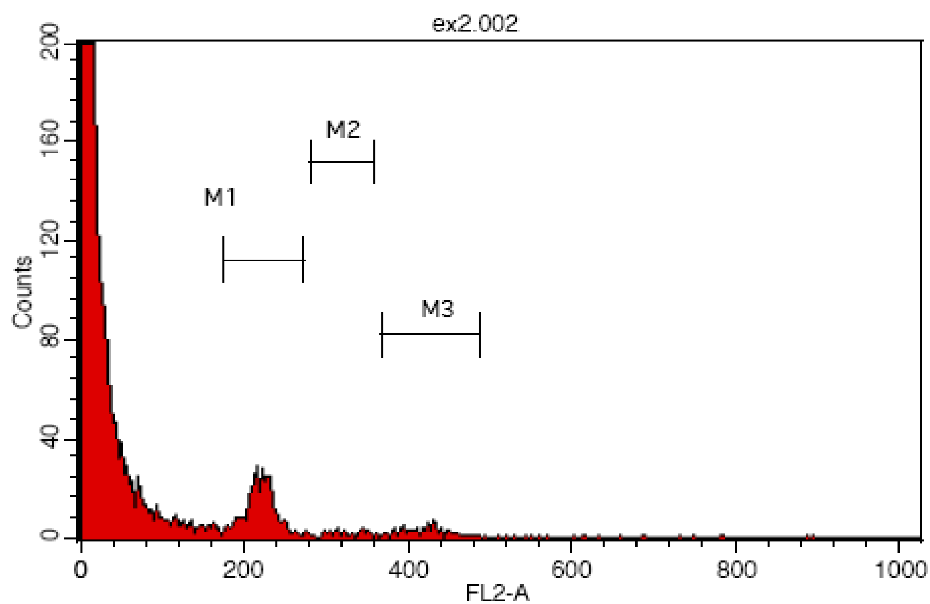
Marker	Left, Right	% Total	Mean	CV
All	0, 1023	99.43	104.56	124.79
M1	179, 265	20.70	229.16	7.33
M2	271, 358	2.58	316.72	8.14
M3	364, 523	5.55	425.81	7.83

<u>*L. insignis* - Methanol Extract</u>



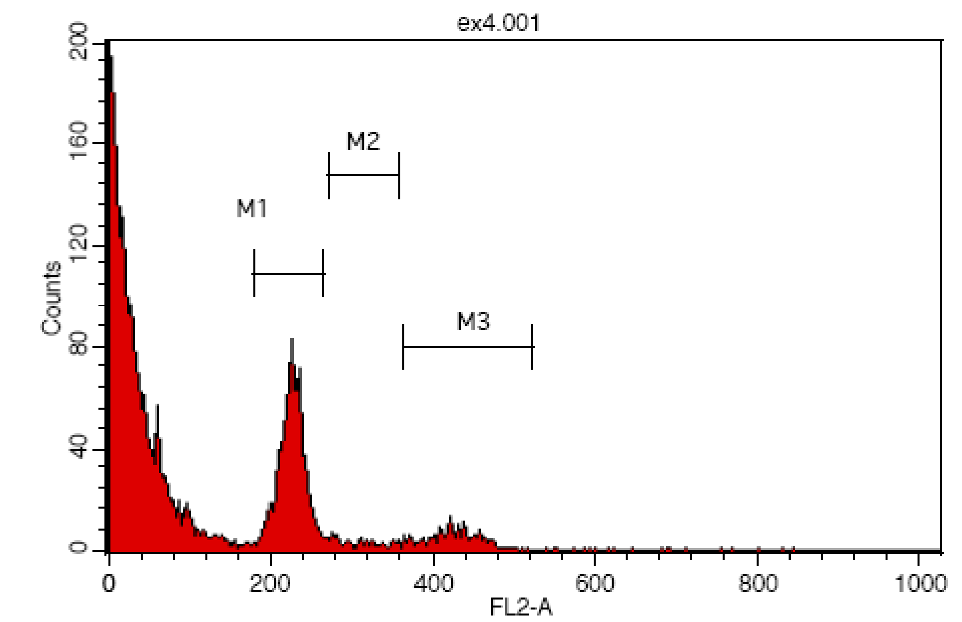
Marker	Left, Right	% Total	Mean	CV
All	0, 1023	99.47	262.86	60.84
M1	185, 281	48.45	236.93	6.81
M2	291, 377	5.10	333.02	7.80
M3	391, 550	23.21	453.93	7.33

<u>*L. lasiocalycina*</u> - Methanol Extract



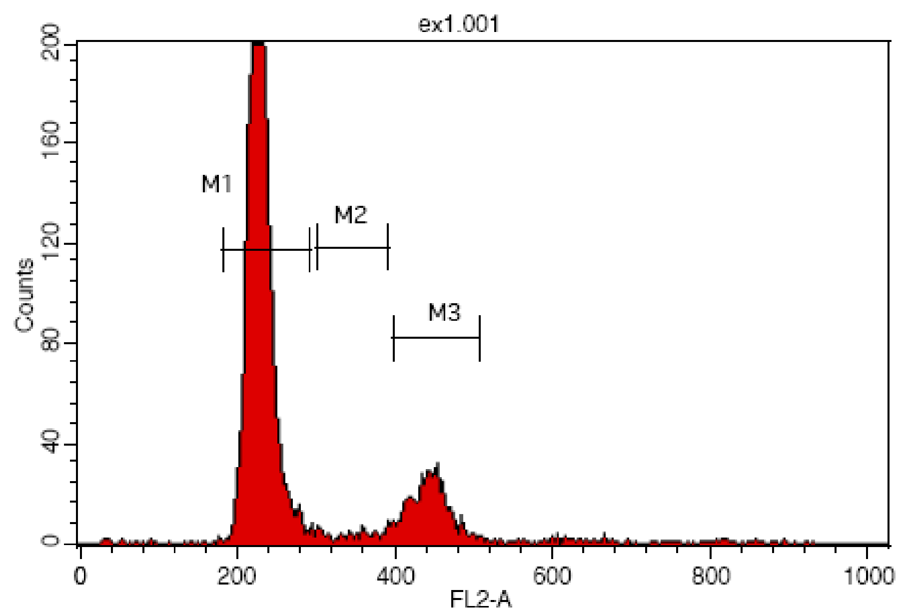
Marker	Left, Right	% Total	Mean	CV
All	0, 1023	99.71	47.83	182.32
M1	175, 271	8.10	218.86	7.70
M2	281, 358	0.71	320.83	6.24
M3	367, 487	1.75	419.36	6.40

<u>*L. thymoides* - Methanol Extract</u>



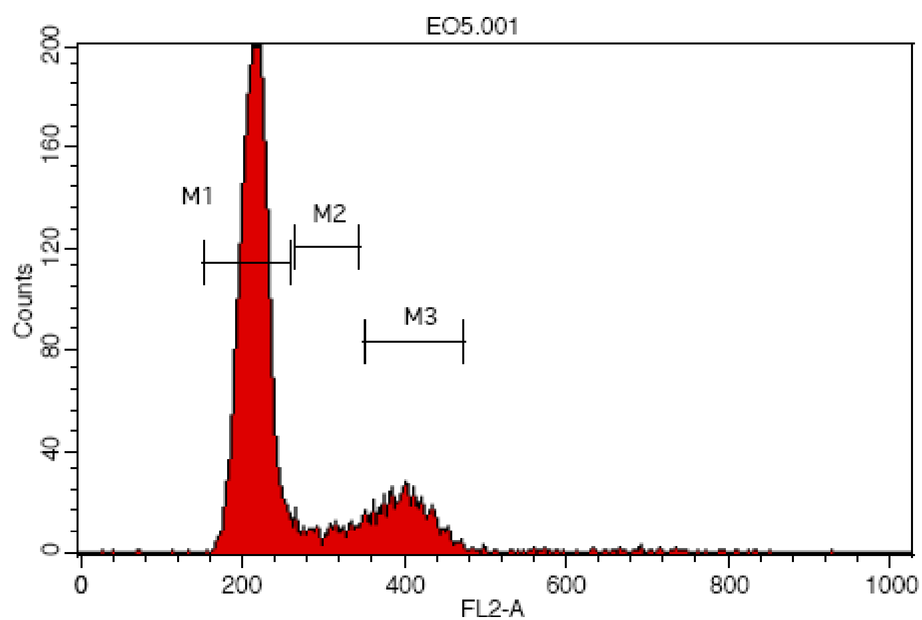
Marker	Left, Right	% Total	Mean	CV
All	0, 1023	99.24	92.81	126.74
M1	179, 265	21.30	224.56	6.64
M2	271, 358	1.59	310.42	8.95
M3	364, 523	4.23	424.45	7.09

L. origanoides - Methanol Extract



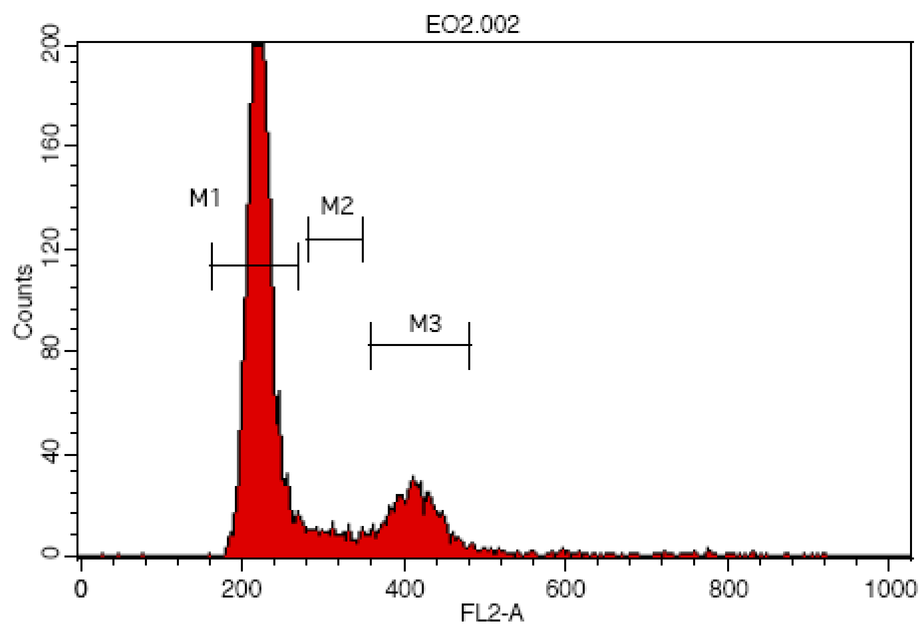
Marker	Left, Right	% Total	Mean	CV
All	0, 1023	95.17	267.44	36.33
M1	182, 291	77.39	227.57	6.38
M2	301, 391	2.33	349.25	8.08
M3	397, 507	12.50	441.31	5.18

<u>*L. alnifolia* - Essential Oil</u>



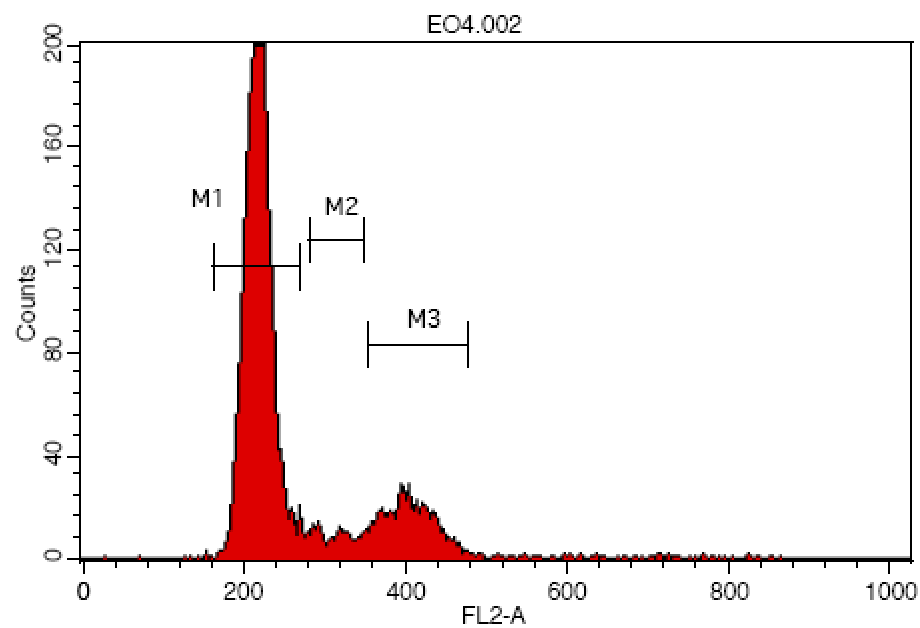
Marker	Left, Right	% Total	Mean	CV
All	0, 1023	98.18	251.55	32.48
M1	152, 258	76.35	213.72	7.20
M2	265, 344	5.20	306.45	7.74
M3	350, 474	14.59	400.21	7.09

<u>*L. lasiocalycina* - Essential Oil</u>



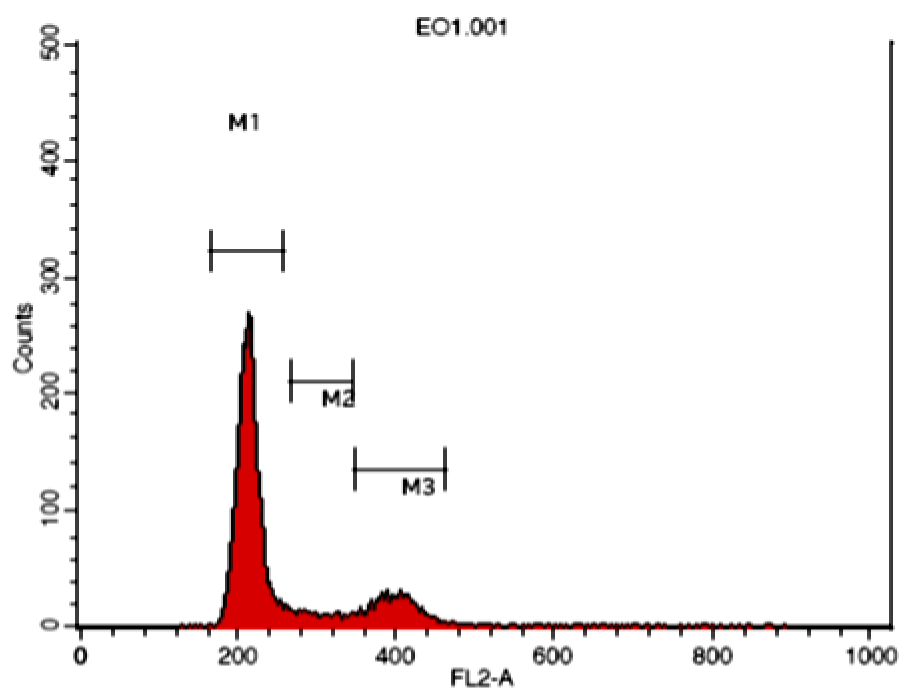
Marker	Left, Right	% Total	Mean	CV
All	0, 1023	98.69	264.02	33.11
M1	162, 268	74.80	221.16	6.35
M2	281, 348	4.68	312.22	6.26
M3	357, 480	16.07	411.86	6.55

<u>*L. thymoides*</u> - Essential Oil



Marker	Left, Right	% Total	Mean	CV
All	0, 1023	98.88	253.65	31.52
M1	162, 268	77.31	216.56	7.24
M2	281, 348	4.73	312.94	6.57
M3	353, 477	14.71	404.40	7.00

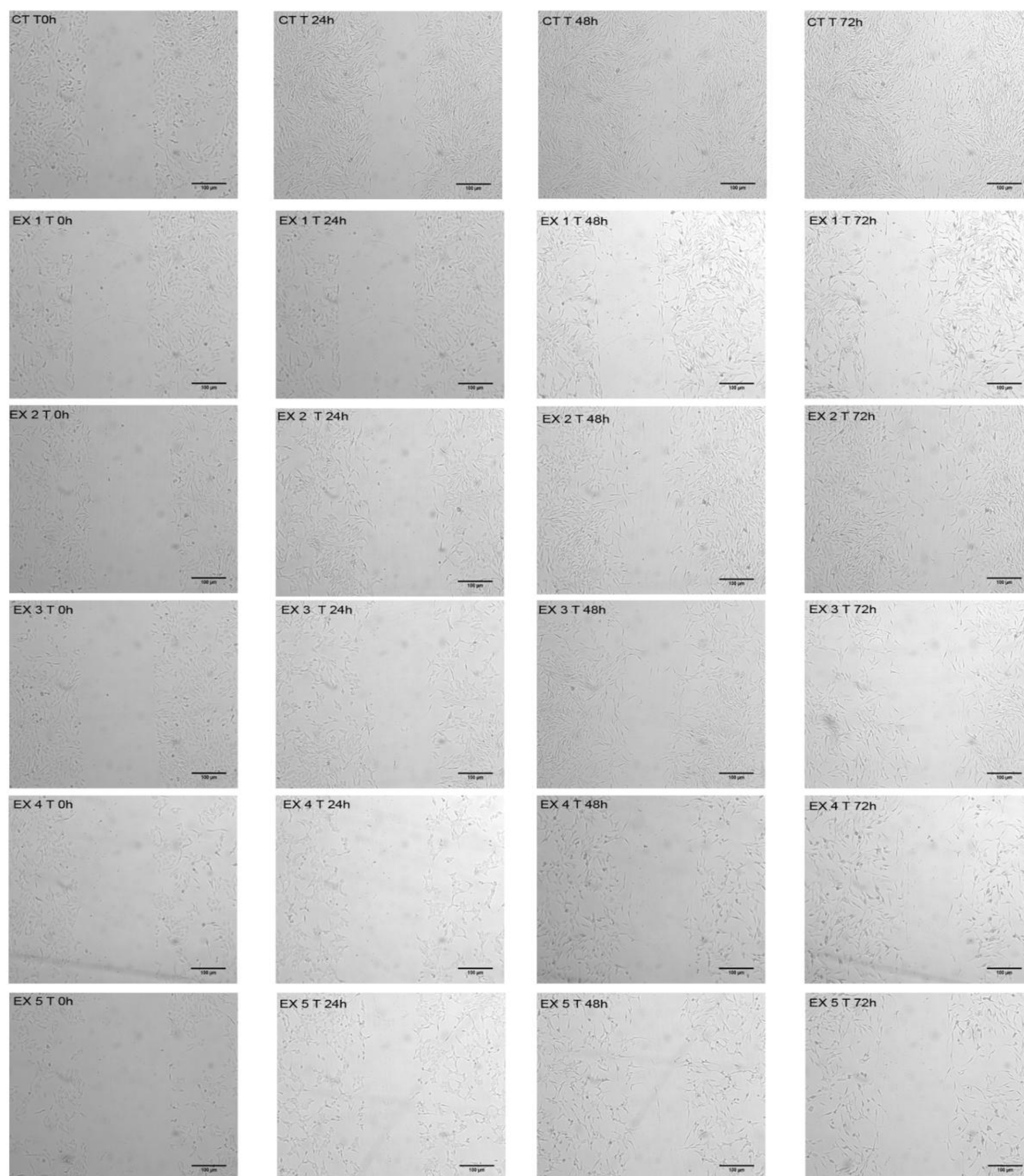
L. origanoides - Essential Oil



Marker	Left, Right	% Total	Mean	CV
All	0, 1023	98.86	251.78	32.43
M1	165, 256	75.16	212.06	6.43
M2	265, 343	5.77	302.29	7.77
M3	349, 462	15.33	397.07	6.08

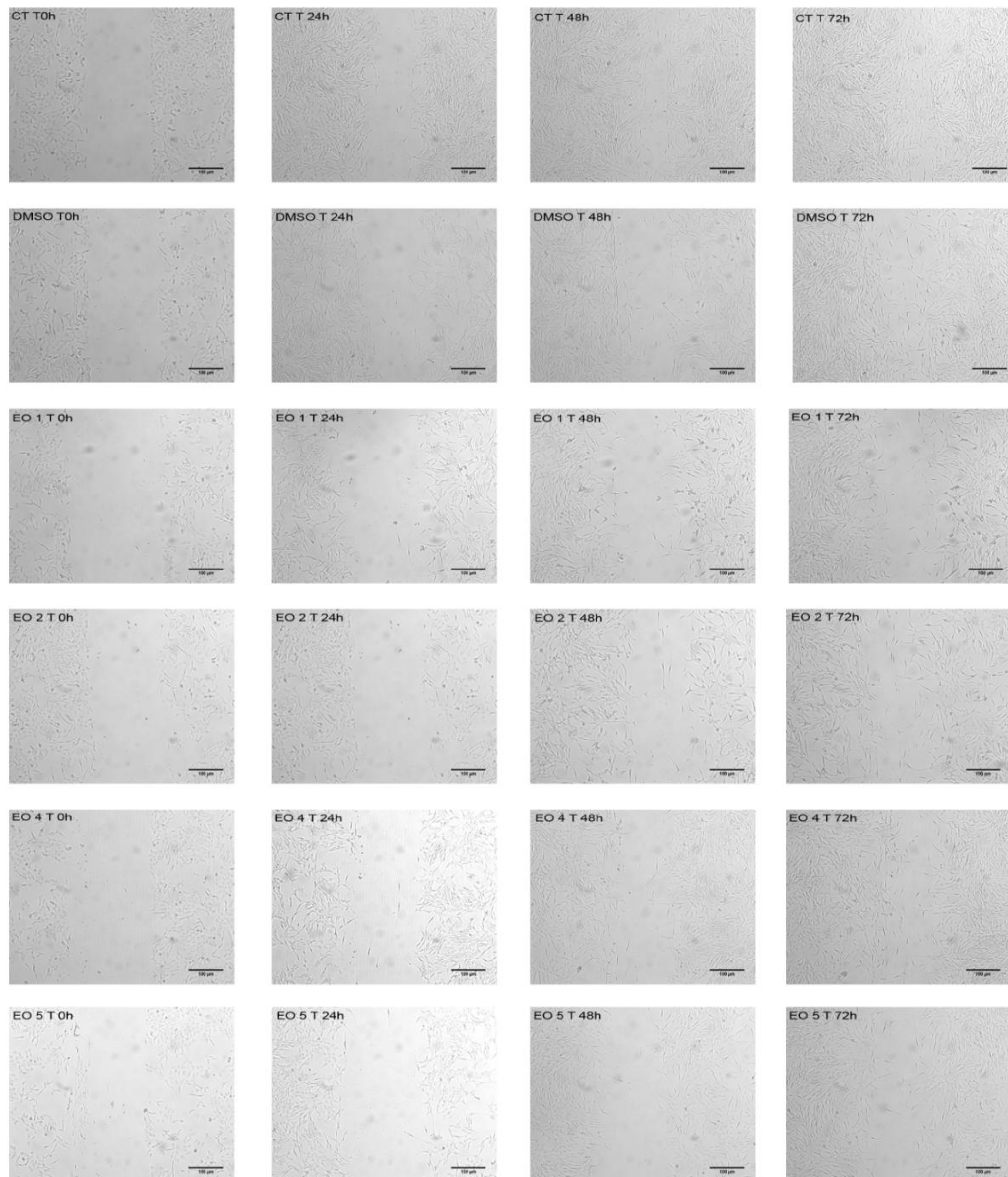
Appendix VII

Methanol Extracts - Wound Heal Assay



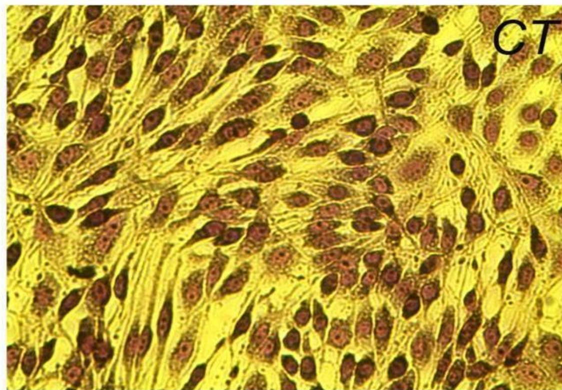
Appendix VIII

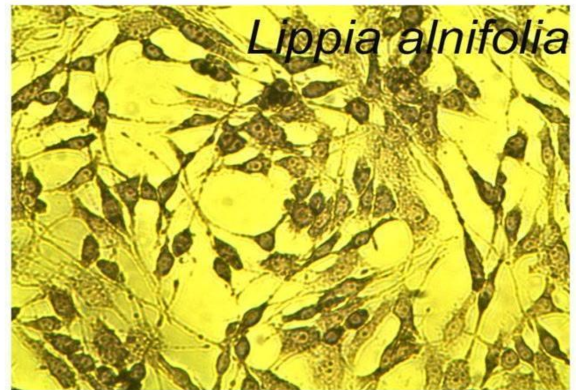
Essential Oils - Wound Heal Assay

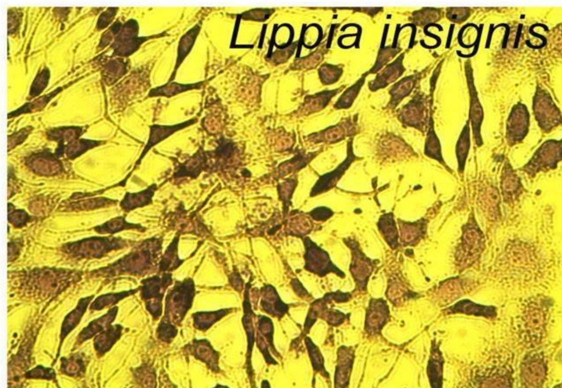


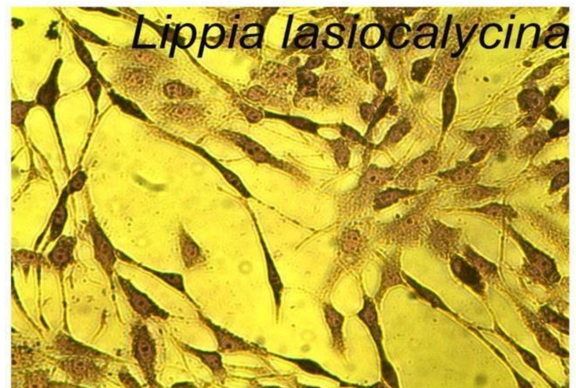
Appendix IX

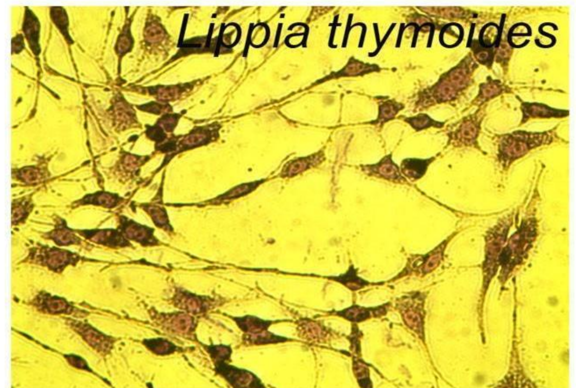
May-Grunwald Methanol Extracts











Appendix X

May-Grunwald Essential Oils