Original Article

# Identification of dysregulated microRNA expression and their potential role in the antiproliferative effect of the essential oils from four different *Lippia* species against the CT26.WT colon tumor cell line

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## ARTICLE INFO

## ABSTRACT

In spite of advances in colorectal cancer treatments, approximately 1.4 million new global cases are estimated for 2015. In this sense, Brazilian plant diversity offers a multiplicity of essential oils as prospective novel anticancer compounds. This study aimed to evaluate the antiproliferative effect of the essential oils from four *Lippia* species in CT26.WT colon tumor cells, as a measurement of cell cycle phase distribution and microRNA expression. CT26.WT showed cell cycle arrest at G2/M phase after treatment with 100 µg/ml of *Lippia alba* (Mill.) N.E.Br. ex Britton & P. Wilson, *Lippia sidoides* Cham., and *Lippia lacunosa* Mart. & Schauer, Verbenaceae, essential oils and, at the same concentration, *Lippia rotundifolia* Cham. essential oil caused an augment of G0/G1 phase. The miRNA expression profiling shows change of expression in key oncogenic miRNAs genes after treatment. Our findings suggest growth inhibition mechanisms for all four essential oils on CT26.WT cells involving direct or indirect interference on cell cycle arrest and/or oncogenic miRNAs expression.

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## Introduction

According to the International Agency for Research on Cancer, colorectal cancer (CRC) was the third most common cancer in men and the second most common in women worldwide in 2012 (Ferlay et al., 2015). Approximately 1.4 million new global CRC cases and more than 750,000 deaths were projected for 2015 (Ferlay et al., 2015). Colorectal cancer treatments involve the standard techniques of surgery, radiation and chemotherapy and a few derivative procedures like cryosurgery, radiofrequency ablation or targeted therapy. Despite technological advances, CRC demonstrates great resistance and resilience against therapy. Approximately 40% of all patients treated for local CRC will have recurrence (Siegel et al., 2012) and thus, the search for new anticancer agents remains essential.

Plant compounds feature important sources of therapeutic compounds for cancer treatment. Countries with rich flora biodiversity as Brazil have a wide range of plant species and there has been a global effort to prospect for biomolecules with pharmacological properties in these regions. Among these, monoterpenes have been suggested as a relevant class of agents that are found in several plant species, including species of the *Lippia* genus, whose pharmacological properties have been related to secondary metabolites, specifically to their essential oils (Pascual et al., 2001). Among the best studied *Lippia* species are *L. alba* (Mill.) N.E.Br. ex Britton & P. Wilson, and *L. sidoides* Cham., and for both of them previous studies reported antioxidant activity indicating that these plants might be potential targets to search for antitumorogenic biomolecules (Ramos et al., 2003; Monteiro et al., 2007). Recently, our group investigated the antiproliferative effect of five *Lippia* species on tumor cells, as determined by MTT assay. The results of this study demonstrated that *L. sidoides* and *L. salviifolia* essential oils had an antiproliferative effect on CT26.WT colon tumor cells (Gomide et al., 2013). Monoterpenes like geraniol found in vegetal essential oils had already been showed to reduce the growth of leukemia

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and melanoma cells (Shoff et al., 1991). Others have also demonstrated that synthetic geraniol is effective *in vitro* and *in vivo* against a variety of cancer types, including hepatoma, pancreatic and even colon cancer, which is highly resistant to chemotherapy (Yu et al., 1995; Burke et al., 1997; Carnesecchi et al., 2001, 2002; Duncan et al., 2004; Ong et al., 2006; Wiseman et al., 2007). The monoterpene limonene is another that has exerted antitumor activity, specifically against breast, skin, liver, lung and stomach cancers in rodents (Elegbede et al., 1986; Wattenberg and Coccia, 1991; Crowell and Gould, 1994; Mills et al., 1995; Kawamori et al., 1996; Crowell, 1999). Additionally, anti-tumor activity has been reported to monoterpenes like carvone, carveol, mentol and perillyl alcohol (He et al., 1997).

The molecular driving forces of CRC can be categorized into genomic instability, genomic modifications and epigenetic alterations (Kanthan et al., 2012). More recently, several studies have observed that an imbalance in miRNA regulating cell cycle oncogenes could also be linked to cancer development. In CRC, studies have demonstrated an association of aberrant miRNA expression and cancer development where some miRNA have been reported to be consistently dysregulated in this disease (Huang et al., 2010).

The aim of the present study was to evaluate the antiproliferative effect of the essential oils extracted from four different *Lippia* species in CT26.WT colon tumor cells as a measurement of cell cycle phase distribution and miRNAs expression.

## Materials and methods

### Plant material

Fresh leaves were collected from *Lippia alba* (Mill.) N.E.Br. ex Britton & P. Wilson, *L. sidoides* Cham., *L. rotundifolia* Cham. and *L. lacunosa* Mart. & Schauer, Verbenaceae, at the Experimental Station located on the campus of the Federal University of Juiz de Fora, Juiz de Fora, Brazil ($21°46'48.4''$S $43°22'24.4''$ W). Each one of the *Lippia* species was collected from November to December 2010. The voucher specimens of the *Lippia* species evaluated in this study are deposited at the Herbarium of the Botany Department from the Federal University of Juiz de Fora and the voucher specimens numbers are: *L. alba*: 48374, *L. sidoides*: 49007, *L. rotundifolia*: 31376 and *L. lacunosa*: 51920.

### Extraction of essential oils

The essential oils from leaves of the *Lippia* species were obtained by hydrodistillation in a Clevenger-type apparatus for 2 h. The oils were weighed and aliquots of 5 mg of each one of them were stored at $-80°C$ in sealed vials covered with aluminum foil until use. For each one of the assays described one aliquot was thawed and dissolved in 4% dimethyl sulfoxide – DMSO (Sigma, St. Louis, MO, USA) and purified water making up a working solution of 1 mg/ml.

### Gas chromatography/mass spectrometry analysis

The chemical composition of the essential oil of each *Lippia* specie was determined by gas chromatography coupled to mass spectrometry performed on a Shimadzu QP5050A GC/MS instrument, equipped with a PTE-5 Supelco column ($30 \text{ m} \times 0.25 \text{ mm} \times 0.25 \text{ }\mu\text{m}$), as performed by Gomide et al. (2013). Retention indexes (RI) were calculated from retention times generated from the analysis of each oil in comparison with a standard n-alkanes solution, C8-C20, and used to determine the components of each one of the essential oils, according to Adams (1995). The amount of compounds was determined by peaks area integration of the spectra.

### Cell lines and culture condition

Mouse colon carcinoma CT26.WT cells were obtained from ATCC (CRL-2638) and were grown at $37°C$ with 5% $CO_2$ in RPMI 1640 medium pH 7.4 (Cultilab, Campinas, SP, Brazil) supplemented with 10% fetal bovine serum (FBS), 0.1 mg/ml ampicillin, 0.1 mg/ml kanamycin, 0.005 mg/ml amphotericin, 0.2% $NaHCO_3$ and 0.2% HEPES (Sigma, St. Louis, MO, USA).

### Cell cycle analysis

CT26.WT cells were seeded onto 24-well plates at a density of $2 \times 10^4$ cells/well in RPMI supplemented with 10% FBS. After the cells visibly reached around 50% confluence they were exposed for 12 or 24 h with RPMI supplemented with 10% FBS containing the working solution with the essential oils of the four *Lippia* species at the concentrations of 10, 50 and 100 $\mu$g/ml. The negative control samples contained 0.4% DMSO, which is equivalent to the percentage found in the highest concentration evaluated. Then, the cells were collected and resuspended in 300 $\mu$l of HFS solution (0.05% propidium iodide, 1% sodium citrate and 0.5% Triton X-100) (Sigma, St. Louis, MO, USA). Cells were incubated for 2 h at $4°C$. The DNA content of the stained cells was analyzed using FACScan and CellQuest programs (BD Bioscience, San Jose, CA, USA). The histograms showing cell cycle phase distributions in G0/G1, S, G2/M and sub-G1 cells (used as measure of dead cells) were analyzed using FlowJo version 7.6.4 (Treestar, Inc., San Carlos, CA). All assays were performed at least three times, and at least 15,000 events per sample were analyzed. To verify the existence of statistical differences among the samples ANOVA followed by Bonferroni test was performed. Differences bellow 0.05 ($p < 0.05$) were considered significant.

### MicroRNA analysis

CT26.WT cells were seeded in three different 25 cm$^2$ flasks each one at a density of $2 \times 10^4$ cells/well in RPMI supplemented with 10% FBS. After the cells visibly reached around 50% confluence they were treated with RPMI supplemented with 10% FBS containing the essential oil of *L. alba*, *L. rotundifolia*, *L. sidoides* or *L. lacunosa* at a final concentration of 100 $\mu$g/ml. Cells were incubated for a period of 12 h.

Subsequently, for the microRNA analysis, the CT26.WT cells were submerged in RNAlater (Invitrogen, Carlsbad, CA, USA) for 24 h at $4°C$, and transferred to $-80°C$. MicroRNA was isolated using mirVana miRNA isolation kit (Applied Biosystems, Foster City, CA, USA), according to manufacturer's directions. Total RNA was quantified by NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the extraction quality was evaluated by Agilent Small RNA kit in Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Cancer MicroRNA qPCR array with Quantimir kit, a panel of 95 cancer-related microRNAs (System Biosciences, Mountain View, CA, USA), was used to examine miRNA differential expression on the two pools formed by each essential oil-treated and untreated (negative control) CT26.WT cells collected from three independent 25 cm$^2$ flasks. The miRNAs were tagged and reverse transcribed using QuantiMir-cDNA technology. The miRNA profiling was performed according to the manufacturer's instructions. Forward primers used in this study were designed to be the exact sequences of the miRNA, and are listed in the miRBase database (http://www.mirbase.org). Real-time PCR was performed using standard run conditions (40 cycles, $60°C$ anneal/extension) on a ABI Prism 7300 Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's directions. Samples were normalized to U6 transcript and analyzed using the REST 384 software by pair-wise

fixed reallocation randomization test (Pfaffl et al., 2002) and ΔΔCt method (Livak and Schmittgen, 2001). Relative expression values are shown as mean ± standard error (SEM) and differences bellow 0.05 ($p < 0.05$) were considered significant.

## Results and discussion

### Composition of the essential oils obtained from four Lippia species

Since previous studies have demonstrated that the quantity of the main components of the essential oils obtained from *Lippia* species varies according to the sampling period, gas chromatography–mass spectrometry analysis (GC–MS) was performed to quantify each oil composition. The most concentrated compounds (above 6% of total composition) were identified and are shown in Table 1. A total of nine main compounds were found for all species. In *L. alba* oil (Alb), geranial and citral predominate. For *L. sidoides* oil (Sid), thymol and *o*-cymene were the most concentrated. The major constituent of *L. rotundifolia* essential oil (Rot) was β-myrcene and finally, β-myrcene and myrcenone were the most abundant in *L. lacunosa* oil (Lac). This data agrees with previous results from Gomide et al. (2013), and the observed chemotypes validate the identities of the *Lippia* species used in this study.

### The antiproliferative effect of essential oils from Lippia species as determined by the distribution of CT26.WT colon tumor cell cycle phases

Several studies have shown that terpenes present chemopreventive and therapeutics properties against human cancers (Kinghorn et al., 2003). Among the terpenes, the class of monoterpenes has emerged as an advantageous agent to be used as an anticancer drug for treatment of tumors that are resistant to chemotherapy and to minimize the side effects of current treatments (Shoff et al., 1991; Yu et al., 1995; Burke et al., 1997; He et al., 1997; Crowell et al., 1999; Duncan et al., 2004; Wiseman et al., 2007; Paduch et al., 2007). Several monoterpenes are identified in *Lippia* species Brazil being one of the largest centers of diversity of this genus, comprising 70–75% of all known species.

A previous study showed potent antiproliferative effects in CT26.WT cells treated with *Lippia* essential oils (Gomide et al., 2013). In this study, CT26.WT cells were treated for 12 and 24 h with the essential oils extracted from *L. alba*, *L. sidoides*, *L. rotundifolia* and *L. lacunosa*. It was observed that all four *Lippia* essential oils affected the CT26.WT cell line by inducing cell cycle arrests either in G0/G1 or in G2/M phases. The representative histograms of the cell cycle phase distribution of CT26.WT cells are shown in Fig. 1. Table 2 presents the percentages of sub-G1, G0/G1, S and

G2/M CT26.WT treated cells as measured by flow cytometry after PI staining.

At 50 and 100 μg/ml, results show that treatment with Alb lead to a significant increase of G2/M phase after 12 and 24 h. Decreasing the concentrations to 10 μg/ml still shows an increase of G0/G1 phase cells for the same times (Table 2 and Fig. 1). In agreement with our results, the antiproliferative effect of geranial, the major compound of Alb (Table 1), had already been demonstrated. Carnesecchi et al. (2001) and Wiseman et al. (2007) observed geraniol (its oxidize form is geranial) cell cycle arresting effects. The first reported geraniol affected progression through the S phase of the cell cycle on colon cancer cells, while the second reported a G0/G1 cell cycle arrest on pancreatic cancer cells. In addition, Wiseman et al. (2007) reported a role for p21Cip1 and p27Kip1 as mediators of G0/G1 cell cycle arrest in pancreatic adenocarcinoma, and reduced levels of expression of cyclins A, B1 and the CDK2. In agreement with these previous studies, ours results also indicated that the antiproliferative effects of Alb might relate to its ability to affect the cell cycle, specifically at G2/M phase. Similar results were observed by Chaouki et al., 2009 when MCF-7 breast cancer cells were treated for 48 and 72 h with citral, another major compound found in Alb (Table 1).

Sid also affected CT26.WT cell cycle. Treatment with 100 μg/ml showed an increased percentage of G2/M phase and decreased S phase cells after 12 h, while an increase in G0/G1 was observed after 24 h. The antiproliferative effect of thymol, the major compound of Sid, had been previously demonstrated. Recently, Jaafari et al. (2012) and Deb et al. (2011) obtained cell cycle arrest at sub G0/G1 after thymol treatment in leukemic cells.

The Rot caused an increase of CT26.WT cells on G0/G1 phase at 50 and 100 μg/ml after 12 and 24 h of treatment (Table 2 and Fig. 1). Treatment with 50 and 100 μg/ml of Lac lead to a G2/M phase increase after 12 and 24 h as well as a considerable decrease in S phase. At 100 μg/ml there was also an increase in G0/G1 phase after 24 h of treatment (Table 2 and Fig. 1). Abdallah and Ezzat (2011) showed that the essential oil extracted from *Pituranthos tortuosus*, which contains β-myrcene (major compound of Rot and Lac) showed cytotoxicity against colon, liver and breast cancer cell lines. There were very few studies reporting effects of other identified compounds from those *Lippia* oils.

### Identification of differential expression of miRNAs in Lippia essential oil CT26.WT treated cells

Several studies show that miRNAs are master regulators of cell cycle genes in different cancer and therefore, we decided to investigate if miRNAs dysregulation were involved in the observed cell cycle interference caused by essential oils from *Lippia* species. To identify differential expression patterns of miRNAs in treated

**Table 1**
Percentage chemical composition of the majority compounds of the essential oils extracted from leaves of *Lippia alba*, *L. sidoides*, *L. rotundifolia* and *L. lacunosa*, as determined by gas-chromatography followed by mass spectrometry.

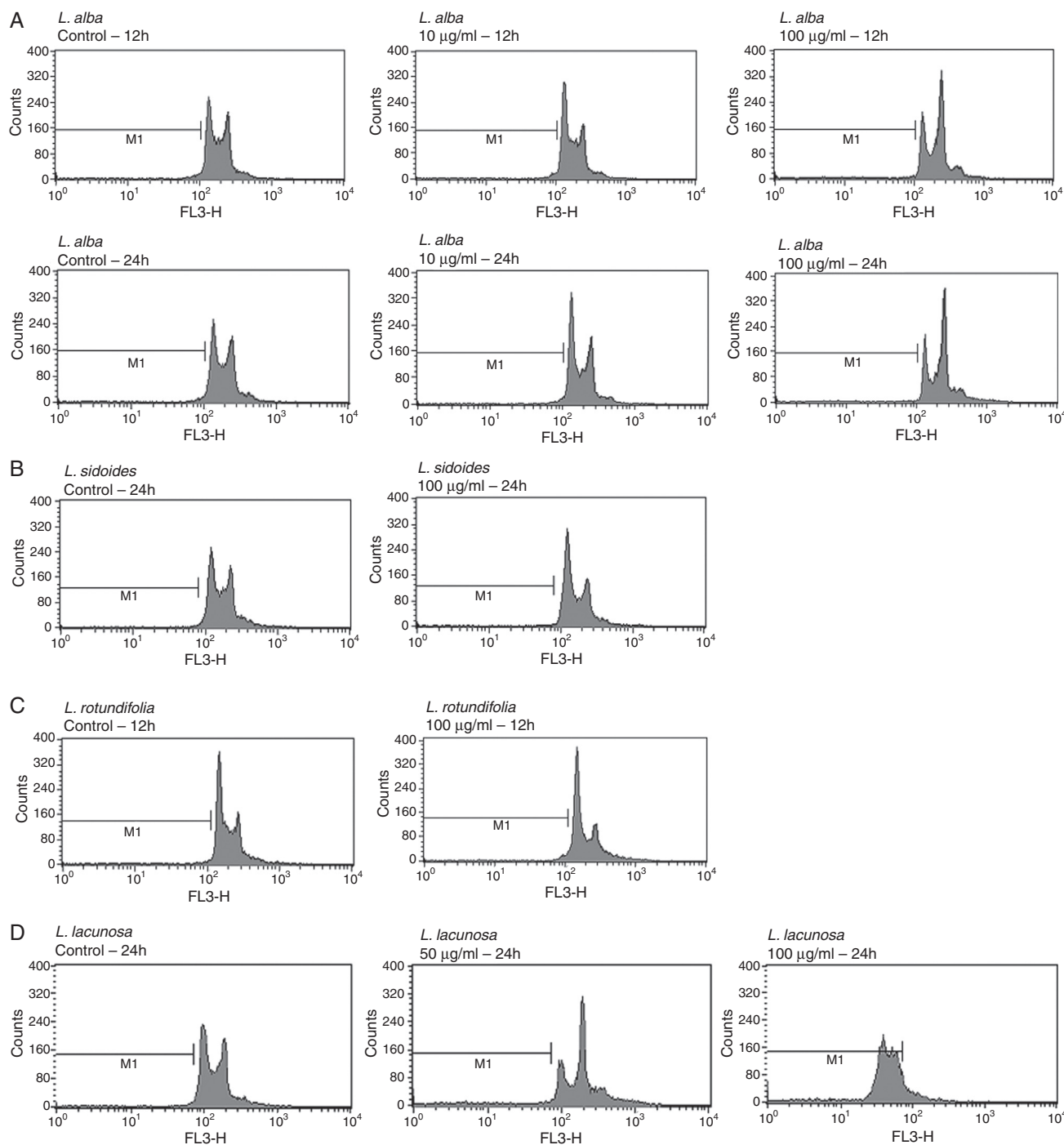| Compounds | RI[a] | L. alba | L. sidoides | L. rotundifolia | L. lacunosa |
|---|---|---|---|---|---|
| β-Pinene | 980 | | | 16.30 | |
| β-Myrcene | 991 | | | 52.39 | 53.52 |
| α-Phellandrene | 1005 | | | 13.27 | |
| o-Cymene | 1022 | | 30.08 | | |
| Limonene | 1031 | 22.43 | | 10.94 | |
| Myrcenone | 1148 | | | | 25.41 |
| Citral | 1240 | 20.54 | | | |
| Geranial | 1270 | 29.24 | | | |
| Thymol | 1290 | | 38.42 | | |
| Total | | 72.21 | 68.50 | 92.90 | 78.93 |
| Number of retained compounds selected | | 7 | 8 | 5 | 4 |

[a] RI – retention index.

**Fig. 1.** Representative histograms of the cell cycle phase distribution of CT26.WT cells after 12 and 24 h treatment with different concentrations of the essential oils extracted from *Lippia alba* (A), *L. sidoides* (B), *L. rotundifolia* (C), and *L. lacunosa* (D). DNA content of the stained cells was analyzed using FACScan and CellQuest program. The negative control samples contained 0.4% DMSO, which is equivalent to the percentage found in the highest concentration evaluated. All assays were performed at least three times, and at least 15,000 events per sample were analyzed.

CT26.WT cells we used real-time PCR-based miRNA expression profiling array with a panel of 95 cancer-related miRNAs and an U6 transcript to normalize signal. Table 3 shows the proportion of miRNAs from CT26.WT cells treated with the essential oils from *Lippia* species that showed differential expression from cells treated with 0.4% dimethyl sulfoxide (DMSO). The results showed that 27.36% of analyzed microRNAs were dysregulated on CT26.WT cells treated with Alb and Lac and 36.84% on cells treated with Sid and Rot.

Fig. 2 shows a heatmap comparing different miRNAs expression after CT26.WT cells treatment with *Lippia* oils. Some genes responded as up or downregulated depending on the essential oil type, but were dysregulated by all four types (miR-142-3p, miR-15b and miR-202), three (miR-22, miR-149, miR-185, miR-21, miR-191, miR-192, miR-181a, miR-132 and miR-296) or at least by one of the oils as shown in Fig. 2. Monzo et al. (2008) and Chen et al. (2009) reported that miR-142-3p and miR-15b were up-regulated in colorectal cancer. Our results show growth inhibition correlated to miR-15b downregulation (in 3 of 4 oils), while miR142-3p expression increased. Ng et al. (2009a, b) identified miR-202 upregulation in colorectal cancer patient plasma, which was also observed after

**Table 2**

Percentages of sub-G1, G0/G1, S and G2/M of CT26.WT cells after 12 and 24 h treatment with the essential oils extracted from *Lippia alba*, *L. sidoides*, *L. rotundifolia* and *L. lacunosa*, as determined by flow cytometry after PI staining. The negative control samples contained 0.4% DMSO, which is equivalent to the percentage found in the highest concentration evaluated. All assays were performed at least three times, and at least 15,000 events per sample were analyzed. Statistical differences were determined with ANOVA followed by Bonferroni test ($p < 0.05$).

| Essential oil (solutions) | sub-G1 | | G0/G1 | | S | | G2/M | |
|---|---|---|---|---|---|---|---|---|
| | 12 h | 24 h | 12 h | 24 h | 12 h | 24 h | 12 h | 24 h |
| *Lippia alba* | | | | | | | | |
| Negative control | | | 25.65 ± 1.58[a] | 27.37 ± 1.30[a] | 40.34 ± 2.82[a] | 39.38 ± 1.38[a] | 24.25 ± 4.03[a] | 23.46 ± 1.56[a] |
| 10 µg/ml | <4 | <5 | 31.12 ± 2.04[b] | 32.35 ± 0.75[b] | 39.91 ± 0.96[a] | 35.29 ± 1.20[a] | 17.96 ± 0.83[b] | 23.11 ± 0.34[a] |
| 50 µg/ml | | | 23.69 ± 0.1[a] | 23.07 ± 0.82[c] | 20.53 ± 1.40[b] | 17.31 ± 2.35[b] | 41.89 ± 0.87[c] | 45.31 ± 2.75[b] |
| 100 µg/ml | | | 21.03 ± 0.61[a] | 21.26 ± 1.17[c] | 30.98 ± 2.50[c] | 27.33 ± 2.27[c] | 32.40 ± 2.34[d] | 39.03 ± 1.32[c] |
| *Lippia sidoides* | | | | | | | | |
| Negative control | | | 28.59 ± 3.44[a] | 29.62 ± 2.00[a] | 38.35 ± 3.32[a] | 39.12 ± 2.48[a] | 18.81 ± 3.93[a] | 18.32 ± 1.40[a] |
| 10 µg/ml | <4 | <2 | 26.82 ± 3.18[a] | 33.82 ± 1.50[ab] | 35.50 ± 3.35[a] | 34.30 ± 1.91[ab] | 18.86 ± 1.69[a] | 19.52 ± 1.77[a] |
| 50 µg/ml | | | 27.84 ± 1.69[a] | 31.35 ± 2.84[a] | 25.98 ± 2.32[b] | 37.04 ± 0.90[a] | 26.15 ± 1.38[ab] | 18.93 ± 1.59[a] |
| 100 µg/ml | | | 23.64 ± 1.76[a] | 38.17 ± 1.92[b] | 19.14 ± 2.66[c] | 30.31 ± 1.15[bc] | 33.25 ± 5.88[b] | 21.23 ± 1.88[a] |
| *Lippia rotundifolia* | | | | | | | | |
| Negative control | | | 29.76 ± 1.87[a] | 36.55 ± 0.47[a] | 38.75 ± 2.44[a] | 29.60 ± 1.17[a] | 15.57 ± 2.36[a] | 20.46 ± 1.19[a] |
| 10 µg/ml | <3 | <1 | 33.77 ± 2.74[a,b] | 37.90 ± 1.80[a,c] | 35.54 ± 2.33[ab] | 28.04 ± 1.58[ab] | 15.93 ± 2.48[a] | 20.98 ± 2.00[a] |
| 50 µg/ml | | | 36.01 ± 1.28[b] | 40.99 ± 1.79[bc] | 33.59 ± 1.26[b] | 24.53 ± 0.42[b] | 15.28 ± 3.06[a] | 20.86 ± 1.91[a] |
| 100 µg/ml | | | 41.65 ± 2.11[c] | 38.30 ± 0.80[ac] | 26.01 ± 1.81[c] | 24.71 ± 0.60[ab] | 16.02 ± 1.35[a] | 21.09 ± 1.77[a] |
| *Lippia lacunosa* | | | | | | | | |
| Negative control | | | 33.49 ± 1.51[a] | 28.27 ± 3.27[a] | 35.30 ± 1.99[a] | 34.75 ± 2.11[a] | 17.79 ± 1.34[a] | 26.27 ± 2.53[ab] |
| 10 µg/ml | <5 | <6 | 33.32 ± 5.53[a] | 32.78 ± 0.87[a] | 24.49 ± 6.19[b,d] | 30.86 ± 0.54[b] | 19.63 ± 1.52[a] | 23.25 ± 1.49[a] |
| 50 µg/ml | | | 19.51 ± 3.21[b] | 19.53 ± 5.35[b] | 9.12 ± 0.44[c] | 20.82 ± 1.00[c] | 33.38 ± 1.76[b] | 29.87 ± 2.32[bc] |
| 100 µg/ml | | | 35.99 ± 2.10[a] | 48.93 ± 1.84[c] | 15.39 ± 7.05[cd] | 4.98 ± 1.93[d] | 33.86 ± 6.82[b] | 34.98 ± 2.70[c] |

The values represent the average of at least three replicates ± SD.
[a,b,c,d] Different letters indicate significant statistical differences in each cell cycle phase and exposure time column by essential oil.

treatment with *Lippia* essential oils. Data indicates Alb, Rot and Sid treatment correlates to reversed miR-15b expression in CT26.WT colon cancer cell line.

Reversion of expression was observed for miR-92, miR-93, miR-135b, miR-155, miR-191, miR-181a and miR-186 genes which were previously found to be upregulated in colon cancer (Volinia et al., 2006; Monzo et al., 2008; Schepeler et al., 2008; Arndt et al., 2009; Sarver et al., 2009; Earle et al., 2010; Huang et al., 2010) and were downregulated in CT26.WT cells after treatment with Alb, Sid and Rot (Fig. 2). In contrast, miR-143 appeared upregulated on CT26.WT cells treated with Alb, Rot and Lac (Fig. 2), but has been shown to be consistently downregulated in colorectal cancer (Chen et al., 2009; Kulda et al., 2010). Chen et al. (2009) showed that miR-143 acts as a tumor suppressor by inhibiting the KRAS oncogene translation. Alb, Rot and Lac seem to increase expression of miR-143 which could possibly cause a recovery of KRAS tumor suppressor role.

In this study, miR-192 gene was upregulated on CT26.WT cells exposed to Alb and Lac and downregulated by Rot (Fig. 2). Chen et al. (2009) and Earle et al. (2010) demonstrated that miR-192 was downregulated in colorectal cancer. These results indicate that Alb and Lac might be reverting expression of miR-192 in this type of cancer. Braun et al. (2008) also demonstrated that miR-192 is capable of suppressing carcinogenesis by increasing the level of a G1 cell cycle inhibitor p21, leading to cell cycle arrest in G1 phase and in G2/M phase in HCT116 human colorectal cancer cell line. In agreement, cell cycle arrest at these phases was observed on CT26.WT cells treated with Alb and Lac (Table 2 and Fig. 1). Expression of miR-222 was decreased in CT26.WT cells exposed to Alb, Sid and Rot (Fig. 2). Visone et al. (2007) showed that the expression of miR-222 together with miR-221 in human thyroid carcinoma cell line induced cell cycle progression to the S phase and reduced the expression level of the G1 cell cycle inhibitor p27KIP1.

A few miRNA genes showed reversed expression after treatment exclusively with one of the four *Lippia* essential oils. The miRNAs miR-196a, miR-214, miR-149 and miR-30b were shown to be downregulated in colorectal cancer (Monzo et al., 2008; Schepeler

**Table 3**

Total microRNAs differentially expressed after CT26.WT cell line treated with *Lippia* essential oils.

| miRNA | *L. alba* | *L. sidoides* | *L. rotundifolia* | *L. lacunosa* |
|---|---|---|---|---|
| Total | 59 | 59 | 57 | 59 |
| Significants ($p < 0.05$) | 26 | 35 | 35 | 26 |
| Upregulated | 8 | 13 | 12 | 23 |
| Downregulated | 18 | 22 | 23 | 3 |

et al., 2008; Chen et al., 2009; Earle et al., 2010) and our results demonstrated that these miRNA were upregulated after CT26.WT cells were treated with Lac (Fig. 2). On the contrary, miR-17-5p and miR-17-3p were downregulated on CT26.WT cells by Alb and Lac essential oils, respectively (Fig. 2). Bandres et al. (2006) and Volinia et al. (2006) observed miR-17-5p upregulation in colorectal cancer tissue. Monzo et al. (2008) reported that this miRNA is a critical member of a functional group involved in regulating the expression of E2F1, an upstream regulator of TP53 in colorectal cancer cells. They also showed that cells transfected with anti-miR-17-5p, had an increased E2F1 expression, reducing cell growth in a dose dependent manner. In another transfection experiment, Kanaan et al. (2012) transfected miR-17 in colon cancer lines HT-29 and HCT-116 and showed that miR-17 is an E2F1 regulator. Furthermore, Cloonan et al. (2008) have shown that miR-17-5p acts specifically on the transition from G1/S cell cycle phases, interfering with more than 20 genes.

Taken together, these results suggest that a possible mechanism for *Lippia* oils growth inhibition might be through reversion of expression of miRNAs regulating cell cycle inhibitors.

In conclusion, the four essential oils tested in this study showed an antiproliferative effect on CT26.WT colon cancer cells that lead to a cell cycle arrest on G0/G1 or G2/M phases. This effect might be attributed to the compounds of those essential oils whose mechanism of action potentially involves differential expression of key oncogenic miRNAs.
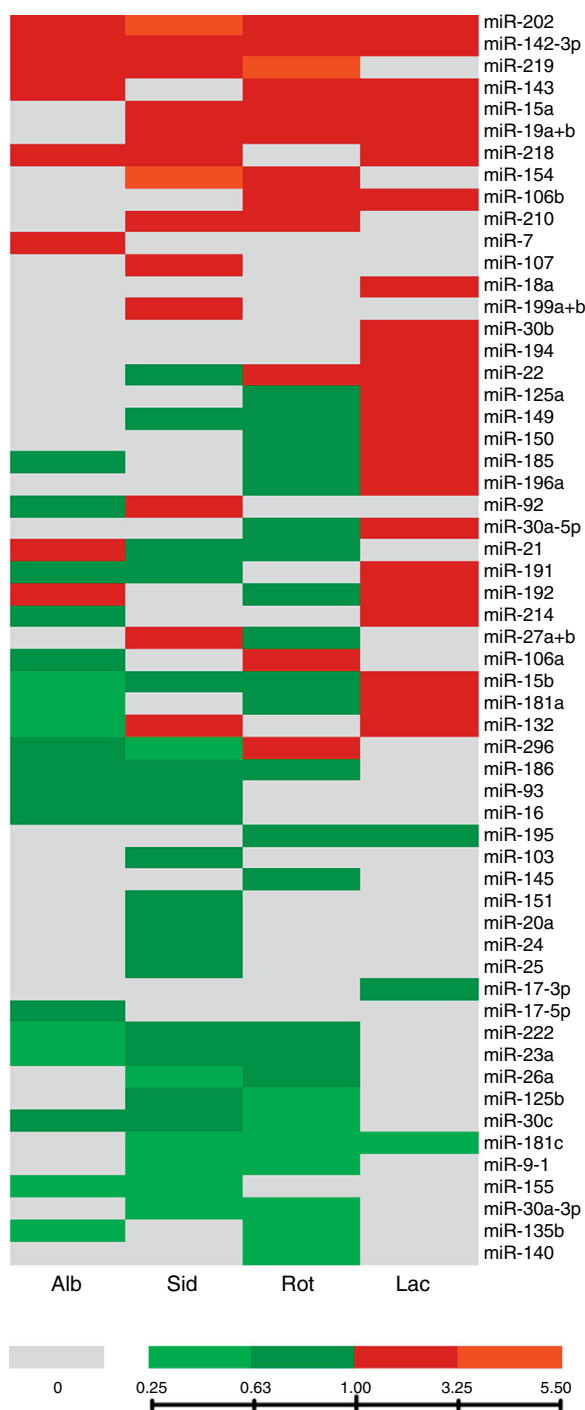
**Fig. 2.** Heatmap of differentially expressed microRNAs in CT26.WT cell line after treatment with *Lippia* oils. CT26.WT cells were treated with 100 mg/ml of *L. alba* (Alb), *L. sidoides* (Sid), *L. rotundifolia* (Rot) and *L. lacunosa* (Lac) essential oils for 12 h. A pool of triplicates of each treatment was subjected to microRNAs analysis by real-time PCR test using a panel of 95 cancer-related microRNA. The Ct values obtained were normalized from the U6 transcribed and analyzed by the REST software (significance level $p < 0.05$). The values of fold change obtained above 1, referred to up-regulated microRNAs, were divided into two groups as well as those between 0 and 1 corresponding to the down-regulated microRNA.

## Ethical disclosures

**Protection of human and animal subjects.** The authors declare that no experiments were performed on humans or animals for this study.

**Confidentiality of data.** The authors declare that no patient data appear in this article.

**Right to privacy and informed consent.** The authors declare that no patient data appear in this article.

## Authors' contributions

MSG (M.Sc. student) contributed in collecting plant sample, extracting and analyzing the essential oil, running the laboratory work, analysis of the data and drafted the paper. FOL and MTPL contributed to biological studies and data discussion. DRLR and MAM contributed to microRNA analysis and to data discussion. GPCJ contributed to critical reading of the manuscript. TMAA contributed in GC/MS analysis. CMC designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

## Conflicts of interest

The authors declare no conflicts of interest.

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