Proliferation of human adipose tissue-derived stem cells stimulated by oil rich in thymol of *Lippia origanoides*[1]

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Abstract

Purpose: To evaluate the effects of this thymol-rich oil in the proliferation of human adipose tissue-derived stem cells.
Methods: Stem cells were isolated from human adipose tissue by liposuction. After the first passage, cells were cultivated in triplicate for three days in control medium and medium supplemented with three oil samples (1.0 µg/mL, 5.0 µg/mL, and 25.0 µg/mL). Cells were analyzed by the MTT assay at passage 1 (P1), and cell proliferation of control and 1 µg/mL groups was determined with a hemocytometer at P2 and P3.
Results: Viability of the essential oil-treated cells was significantly higher than the control group at P1 ($p = 0.0008$). The treatment with the oil, at a concentration of 1 µg/mL, led to increases of 24.8% at P1 and 43.0% at P3 in the rate of cell proliferation compared with control cells.
Conclusion: Supplementing culture medium with essential oil of Lippia origanoides increased cell proliferation, especially at later passages.
Key words: Stem Cells. Adipose Tissue. Cell Proliferation. Thymol.

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■ Introduction

The isolation of mesenchymal stem cell (MSC) from human adipose tissue obtained from liposuction was described in 2001[1]. Liposuction is one of most common esthetic surgical procedures worldwide, and it generates an average of 100–3000 mL of adipose tissue that is routinely discarded, along with its incorporated stem cells[2]. In addition to being easily obtained, adipose-derived stem cells (ADSCs) also have significant therapeutic use[3], as an acute myocardial infarction[4] and bone regeneration[5]. However, according to cellular therapy protocols used in clinical trials, more than one million stem cells are necessary per treatment, necessitating *in vitro* expansion[6].

Repeated passaging of cells *in vitro* incurs the risk of replicative senescence, which primarily occurs due to oxidation caused by free radicals and can be identified by telomere shortening and fibroblast flattening with a concurrent increase in nuclear/nucleolar size that results in stalled cell proliferation[7]. These phenomena limit *in vitro* cell expansion and might hinder their therapeutic applications[8].

Antioxidants are compounds that directly or indirectly inhibit production of reactive oxygen species[9] (ROS). In recent years, there is an increasing interest in finding antioxidant phytochemicals, because they can inhibit the propagation of free radical reactions[10]. Many essential oils have been reported as natural alternative for controlling of oxidative stress such as the essentials oils from *Lippia* species[11].

Lippia origanoides Kunth (Verbenaceae) is an aromatic plant, popularly known in North Brazil as “erva-do-marajó” and is widely found throughout the Brazilian Amazon. The leaves are used as a condiment, as a substitute for oregano and its tea for treating gastrointestinal and respiratory diseases[12]. The antioxidant activity of this essential oil is attributed to the presence of phenol compounds such as thymol, carvacrol, and derivatives[13].

Several biological activities have been reported for thymol. This phenolic compound plays an important role in the scavenging of free radicals such as DPPH (1,1-diphenyl-2-picrylhydrazyl) and ABTS (2,2’-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid). In addition, at lower concentrations, it showed protective effects to cells against damage induced by the H_2O_2 radicals[14,15]. Thus, the aim of this study was to assess the proliferation of human ADSCs in culture medium treated with natural antioxidants present in the *L. origanoides* Kunth essential oil.

■ Methods

The project was approved by the Ethics in Research Committee for Human Beings, Hospital Universitário João de Barros Barreto, Belem-PA, Brazil (Number of process: 487.533).

Adipose tissue was collected from the infra-umbilical region of three patients (27, 29, and 33 years of age; average, 29 years), without comorbidities, treated with liposuction in the private hospital. All patients signed a Free and Informed Consent Form.

Liposuction

Patients were operated on under peridural anesthesia. To collect the lipoaspirate, asepsis and antisepsis were performed in the infra-umbilical region, followed by infiltration with 0.9% saline solution with 1: 500,000 adrenalines at the demarcated site. The sample was aspirated through a syringe with a 3.5-mm diameter cannula and decanted by gravity for 30 minutes. A total of 10 mL of adipose tissue was collected with partial removal of the bloody contents.

Isolation and cultivation of MSCs from human adipose tissue

The lipoaspirate tissue (2mL) was washed with 2 mL of phosphate-buffered saline (PBS; Sigma Aldrich, St. Louis, Missouri, USA) and incubated with 200 U/mL collagenase II for 60 min at 37ºC. After incubation, the collagenase was neutralized by adding complete cultivation medium (Dulbecco's Modified Eagle's Medium (DMEM; Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich), 3.7 g/L $NaHCO_3$ (Sigma Aldrich), and 5 µg/mL of gentamicin (Sigma Aldrich). Following centrifugation, the pellet was resuspended twice with DMEM and plated. After 24 hours, any cells still in suspension were removed and the cultivation medium was renewed. The remaining adherent cells, representing MSCs, were kept at 37°C in 5% CO_2 with high humidity, and the cultivation medium was changed every three days until the third passage.

Cellular and histological characterization of cell differentiation

Histology with tissue-specific dyes was used to characterize the ability of MSCs to differentiate into bone tissue, adipose tissue, and cartilage tissue.

To induce osteoblast differentiation, cells at 70% confluence were treated with osteogenic induction medium (DMEM, 0.1 µM dexamethasone (Sigma Aldrich), 50µM ascorbic acid-2-phosphate (Sigma Aldrich), and 10^{-2}M β-glycerophosphate (Mallinckrodt Baker, Phillipsburg, New Jersey, USA)) and cultivated for 21 days.

To induce adipocyte differentiation, nearly confluent cells were treated with adipogenic induction medium (DMEM, 1 µM dexamethasone, and 10µM insulin (Sigma Aldrich)) and cultivated for 14 days.

To induce chondrocyte differentiation, was prepared a cell solution of $1.6x10^7$ viable cells/mL, and after 2 hours, cells were treated with chondrocytic induction medium (DMEM, 6.25 µg/mL insulin, 50µM ascorbic acid-2-phosphate, and 10 µg/mL TGF-β1 (Cell Signaling Technology, Beverly, Massachusetts, USA) and cultivated for 21 days.

After cultivation, the differentiation medium was removed, and the cells were washed once with PBS. Next, cell monolayers were fixed with paraformaldehyde (Electron Microscopy Sciences) for 30 minutes and then washed twice with PBS. To detect osteoblasts, cells were stained with Alizarin Red S (Life Technologies, Carlsbad, California, USA) for 3 minutes. To detect adipocytes, cells were stained with Oil Red O (Life Technologies) for 30 minutes. To detect chondroblasts, cells were stained with Alcian Blue (Life Technologies) for 30 minutes. After staining, cells were washed three times with distilled water and analyzed by an optical microscope (911873 AXIOLAB, Ziss, Germany) – osteoblasts and chondroblasts - and by an inverted microscope (TE–DH100W, Nikon, Japan) – adipocytes.

Plant collection and extraction and analysis of essential oil

The aerial parts of *L. origanoides* were collected in the National Forest of Carajás (Parauapebas-PA, Brazil), which occurs in endemic form in that area. The botanical material was identified by comparison with an authentic voucher (MG 133.921) existing in the Herbarium of Emílio Goeldi Museum (Belem-PA, Brazil). The essential oil was hydrodistilled using a Clevenger-type apparatus (Hanil Lab Tech Ltd, Incheon, Korea), dried with sodium sulfate and stored in a refrigerator at 5ºC. The oil was analyzed by gas chromatography coupled with mass spectrometry (GC-MS) and its volatile constituents identified by comparison with authentic standards existing in the Adams library[16]. The GC-MS analysis was carried on a Thermo Focus DSQ II, under the

following conditions: DB-5ms (30 m x 0.25 mm; 0.25 mm film thickness) fused-silica capillary column; programmed temperature, 60–240°C (3ºC/min); injector temperature, 250 ºC; carrier gas, helium, adjusted to a linear velocity of 32 cm/s (measured at 100°C); injection type, split (1 µL), from 1:1000 hexane solution; split flow was adjusted to yield a 20:1 ratio; septum sweep was a constant 10 ml/min; EIMS, electron energy, 70 eV; temperature of the ion source and connection parts, 200°C. The quantitative data regarding the volatile constituents were obtained by peak area normalization using a FOCUS GC/FID operated under similar conditions of the GC–MS, except the carrier gas, which was nitrogen. The retention index was calculated for all the volatiles constituents using a homologous series of n-alkanes (C8-C32, Sigma-Aldrich)[17].

For cell treatments, the essential oil was initially solubilized in dimethyl sulfoxide (DMSO; Sigma-Aldrich) at concentration 10.0 µg/mL. This stock solution was diluted to concentrations of 25.0 µg/mL, 5.0 µg/mL and 1.0 µg/mL in DMEM.

MTT assay of cellular growth rate

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed during the first cellular passage (P1) to determine cell viability in the treatment and control groups. Cells were plated at a concentration of $3x10^3$ cells/well in a 96-well plate (TPP, Trasadingen, Schaffhausen, Switzerland) and incubated with different essential oil concentrations (1.0 µg/mL, 5.0 µg/mL, and 25.0 µg/mL) in triplicate. After the third day of cell cultivation, the culture medium was replaced with 100 µL of 5 mg/mL MTT solution in 900 µL in DMEM (without FBS). After 3 hours incubation at 37ºC, the MTT solution was removed and 100 µL of DMSO was added to each well and the plate was shaken for 10 minutes. The percentage of cellular growth rate (GR %) was based on absorbance at 490 nm (A_{490}) measured in spectrophotometer UV/VIS and calculated according to the following equation.

Cell counting

At P2 and P3, $4x10^4$ cells/mL were plated in two 35-mm plates, one with only DMEM and the other with 1.0 µg/mL essential oil (the concentration with the highest rate of cell proliferation). After 72 hours, cells were re-suspended with trypsin and 1 µl of cell were placed and counted in a Neubauer Chamber. All the cells within each medium square were counted using x4 objective and multiplied for a correction factor of 10^4 (cell/ml).

Statistical analysis

Statistical analyses were performed using the GraphPad Prism version 5.0 (GraphPad Software, California, USA). A one-way ANOVA test was performed to assess the rate of cell proliferation using values obtained from the MTT assay followed by Tukey's post hoc test. Values of $p \leq 0.05$ were considered significant.

■ Results

Characterization of tissues differentiated from ASCs are shown in Figure 1. Figure 1A-C exhibit the control of each histological characterization. Numerous fat drops formed in the adipocytic lineage (Figure 1D). We observed glycosaminoglycan accumulation in the chondrogenic lineage, as revealed by Alcian Blue (Figure 1E). The osteogenic lineage stained with Alizarin Red S, indicating an accumulation of extracellular calcium (Figure 1F).

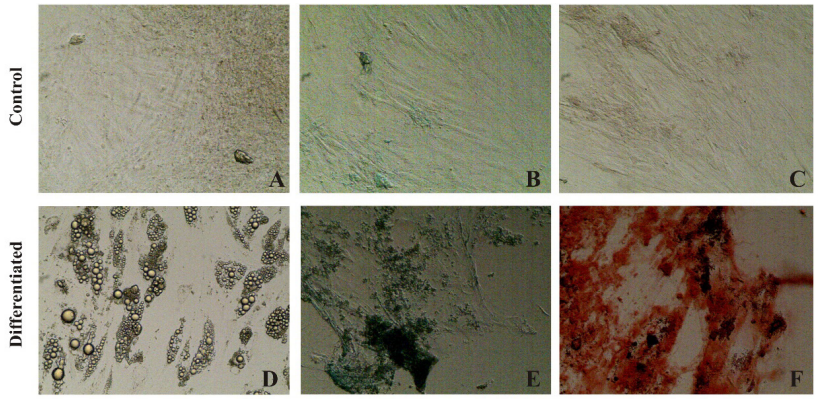

Figure 1 - (**A-C**) ASCs cultured control. (**D**) Adipogenic differentiation of the ASCs. Fat vesicles detected by staining with Oil Red O after 14 days of induction. (**E**) Chondrogenic differentiation of ASCs stained with Alcian Blue after 21 days of induction. (**F**) Osteogenic differentiation of ASCs stained with Alizarin Red S after 21 days of induction.

The proliferation results obtained by the MTT assay are shown in Figure 2. The absorbance values passed the Normality Test (p = 0.722) and the Equality of Variance Test (p = 0.24) in all treatments. Compared with the control, cell proliferation was higher in the three experimental groups treated with essential oil from *L. origanoides* (p = 0.0008). Cell proliferation did not significantly differ between the three experimental groups.

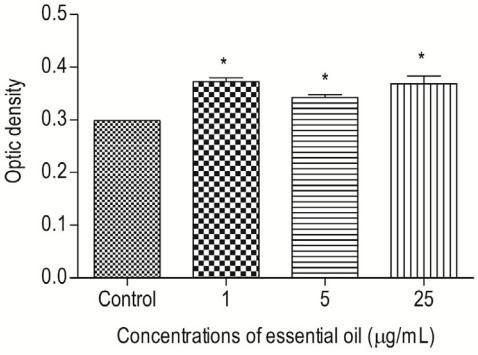

Figure 2 – Averages and standard deviation for absorbance related to cell proliferation (MTT assay) of stem cells derived from human adipose tissue cultivated with essential oil of *Lippia origanoides* in the 1st passage. *indicates a significant difference ($p < 0.05$) in relation to the control group.

The proliferation rates cells treated with 1.0 µg/mL, 5.0 µg/mL, and 25.0 µg/mL of essential oil were 24.8%, 14.7%, and 16.3% greater than control cells, respectively (Figure 3). At P2, control cells and cells treated with 1 µg/mL of essential oil were plated at a concentration of $4x10^4$ cells/mL. After 72 h of cultivation and subsequent trypsin treatment, both groups had a concentration of $18x10^4$ cells/mL (Figure 4). At P3, the cells were again plated at $4x10^4$ cells/mL However, in this case the concentration of the essential oil-treated cells was approximately 43% greater than that of the control cells after cultivation and enzymatic digestion ($20x10^4$ cells/mL vs. $14x10^4$ cells/mL) (Figure 5).

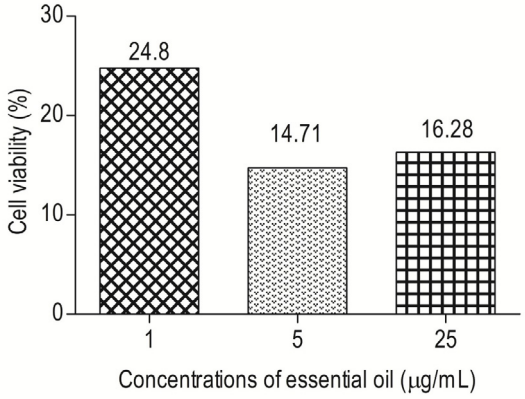

Figure 3 – Growth rate of stem cells derived from human adipose tissue of each group treated (1.5 and 25 µg/mL) in relation to the control in the 1st passage.

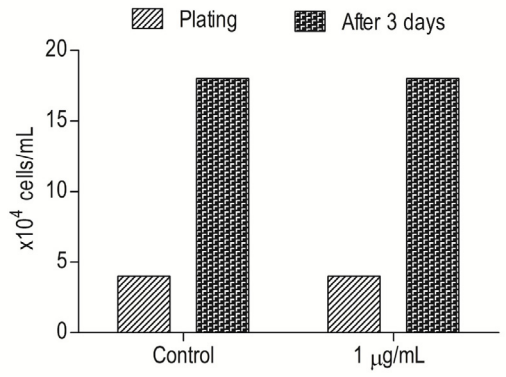

Figure 4 – Effect of the essential oil of *Lippia origanoides* (1 µg/mL) on multiplication of stem cells derived from human adipose tissue in the 2nd passage after 3 days of cultivation.

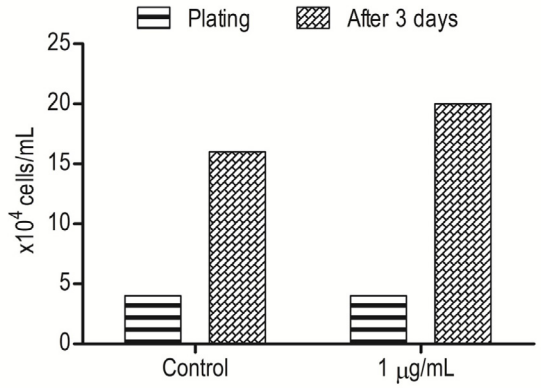

Figure 5 - Effect of the essential oil of *Lippia origanoides* (1 µg/mL) on multiplication of stem cells derived from human adipose tissue in the 3rd passage after 3 days of cultivation.

The dried aerial parts of *L. origanoides* provide oil yield of 3.3%. Individual components were identified by comparison of both mass spectrum and GC retention data with authentic compounds, which were previously analyzed and stored in the data system. Seventeen volatile components were identified in the oil, comprising nearly 100% of its composition (Table 1). Thymol (88.2%) was the main constituent, followed by *p*-cymene (6.4%), its biogenetic precursor.

Table 1 - Chemical composition of the essential oil of *Lippia origanoides*.

Compound	RI[a]	RI[b]	Oil[c] (%)
α-Thujene	916	924	0.2
α-Pinene	922	932	0.3
β-Pinene	966	974	0.1
α-Phellandrene	999	1002	0.1
p-Cymene	1014	1020	6.4
E-β-Ocimene	1034	1044	1.1
Terpinolene	1074	1086	0.1
Linalool	1088	1095	0.5
Umbellulone	1158	1167	0.1
Terpinen-4-ol	1168	1174	0.3
Thymol, methyl ether	1217	1232	0.1
Thymol	1279	1289	88.2
Carvacrol	1285	1298	1.0
α-Copaene	1360	1374	0.1
β-Caryophyllene	1402	1417	0.3
α-Humulene	1438	1452	0.7
δ-Amorphene	1500	1511	0.1
Monoterpene hydrocarbons			8.3
Oxygenated monoterpene			90.2
Sesquiterpene hydrocarbons			1.2
Total			99.7

[a]Retention index calculated using the homologous series of *n-alcanes*; [b]Retention index in the literature (ADAMS, 2007); [c]Percentage of compounds obtained by integrating the area in the flame ionization detector.

■ Discussion

Essential oils are one among the most valuable plant products used in medicine and complementary treatment strategies. However, this is the first study, which reports the use of essential oil from *L. origanoides* as a supplement in human stem cell culture.

Our results indicated that the cultivation medium supplemented with essential oil showed a proliferative effect, with the most significant result found at P3. The principal constituent of the oil of *L. origanoides* using in this study was thymol (88.2%), It is classified chemically as monoterpene. This compound

exhibit different mechanisms of action because the presence of a phenolic ring in its structure enhance the antioxidant activity due to its ability for scavenging free radicals, donation of hydrogen atoms or electron, or chelation of metal cations[16]. We have hypothesized that the cell proliferation was due to Thymol's cytoprotective effect, as another monoterpene (1,8-Cineole, Camphor, α-Pinene and β-pinene) presents in oil of *Salvia lavandulifolia* Vahl showed a protective effect in cultured human astrocytoma too[18].

Tavakolinejad *et al*.[19] cultivated human adipose tissue-derived stem cells in medium containing platelet-rich plasma (PRP) instead of FBS or *L. origanoides*. Cellular growth rates in the work of them (40%) were similar to this study at P3 (40 *vs*. 43%). However, even automated methods for obtaining PRP can result in variable product quality. On the other hand, essential oil from *L. origanoides* function as antioxidants, allowing extended cell cultivation *in vitro* without probable cell damage.

Cells become senescent with increasing passage number, thus reducing their growth[8]. Previous studies have found stem cells derived from senescent human adipose tissue at P2 based on a β-galactosidase assay. By P5, almost 20% of cells were derived from senescent tissue[20]. In our study, the antioxidant function of essential oil from *Lippia origanoides* may have inhibited the oxidation of free radicals produced *in vitro*, enabling cell growth even at P3. Future studies should be done to verify the maximum passage number that benefits from essential oil-treatment, as well as examine other basic cell characteristics such as cell senescence and telomere shortening.

■ Conclusions

Supplementing culture medium with essential oil of *Lippia origanoides* increased the proliferation of stem cells derived from human adipose tissue *in vitro*, especially at later cell passages. This essential oil has the potential to become a cheap and efficient mean for optimally expanding adult stem cells *in vitro* for cell therapy.

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