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WOUND HEALING EFFECTS OF *COPAÍFERA* SP ESSENTIAL OILS AND *CYRTOPODIUM FLAVUM* ETHANOLIC EXTRACTS

EFEITOS DE CICATRIZAÇÃO DE FERIDAS POR ÓLEOS ESSENCIAIS DE *COPAÍFERA* SP E POR EXTRATOS ETANÓLICOS DE *CYRTOPODIUM FLAVUM*

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Abstract
In this study we tested antimicrobial, cytotoxic, and wound healing effects of *Copaifera langsdorffii* Desf. and *Copaifera multijuga* Hayne essential oils, as well as root or rhizome ethanol extracts from *Cyrtopodium flavum* Link & Otto ex Rchb. All these plants have been traditionally used as Brazilian natural products for wound healing. Antimicrobial activity was tested using diffusion assays and minimal inhibitory concentration (MIC) tests. All antimicrobial tests were performed in *S. aureus*, *E. coli*, and *C. albicans* cultures. Cytotoxicity was tested in Hepa-1C1C7 cell cultures (MTT incorporation test) and primary cell cultures (Trypan blue viability cell counting). Wound healing was tested *in vitro* by fibroblast cell migration to scratched areas in cell cultures and *in vivo* tested by skin wound closing in mice. Despite their common use to treat wounds, this study shows that these plant extracts had different capacities to kill microorganisms, to induce cell proliferation, and to close open wounds. *C. langsdorffii* essential oil was the only plant extract with antimicrobial, migration, and cellular activation capacity, with very low cytotoxicity. Despite the traditional use of these plant extracts in crude preparations for wound healing purposes, our results supported the use of *C. langsdorffii* only. Therefore, more studies are needed to isolate the chemical compounds responsible for each of *C. langsdorffii* effects to guarantee its safer use.

Resumo
Neste estudo, testamos os efeitos antimicrobianos, citotóxicos e de cicatrização de óleos essenciais de *Copaifera langsdorffii* Desf. e *Copaifera multijuga* Hayne, bem como extratos etanólicos de raiz ou rizoma de *Cyrtopodium flavum* Link & Otto ex Rchb. Todas essas plantas têm sido tradicionalmente utilizadas como produtos naturais brasileiros para cicatrização de feridas. A atividade antimicrobiana foi testada utilizando ensaios de difusão e testes de concentração inibitória mínima (MIC). Todos os testes antimicrobianos foram realizados em culturas de *S. aureus*, *E. coli* e *C. albicans*. A citotoxicidade foi testada em culturas de células Hepa-1C1C7 (teste de incorporação de MTT) e culturas de células primárias (contagem de viabilidade celular com azul de tripan). A cicatrização de feridas foi testada in vitro através da migração de células de fibroblastos para áreas arranhadas em culturas de células e *in vivo* através do fechamento de feridas cutâneas em camundongos. Apesar do uso comum no tratamento de feridas, este estudo mostra que esses extratos vegetais tiveram capacidades diferentes de matar microorganismos, induzir proliferação celular e fechar feridas abertas. O óleo essencial de *C. langsdorffii* foi o único extrato vegetal com capacidade antimicrobiana, de migração e de ativação celular, com citotoxicidade muito baixa. Apesar do uso tradicional desses extratos vegetais em preparações brutas para cicatrização de feridas, nossos resultados apoiam o uso apenas de *C. langsdorffii*. Portanto, mais estudos são necessários para isolar os compostos químicos responsáveis por cada um dos efeitos de *C. langsdorffii* a fim de garantir seu uso mais seguro.

Keywords: antimicrobial; cytotoxicity; immunomodulation; traditional plant medicine

Palavras-chave: antimicrobiano; citotoxicidade; imunomodulação; medicina tradicional à base de plantas.

Introduction

The use of medicinal plants constitutes one of the oldest methods for the treatment for numerous diseases and they continue to play an important role in culture-specific traditional medicinal systems (MEHTA et al., 2015).

From essential oils to total extracts, plant products from the Brazilian flora have been used to treat different types of diseases; essential oils are aromatic substances present in a variety of vegetal organs where chemical compounds with biological activity are concentrated (SANTOS, A. C. A. Dos et al., 2009). Many essential oils were described and they contain antibacterial, antifungi, antiviral, antioxidant, antiparasitic, and insecticide substances (BENOIT-VICAL et al., 2001; MARTÍNEZ et al., 1996). Inside the Brazilian flora, essential oils from *Copaifera langsdorffii* Desf. and *Copaifera multijuga* Hayne have been used as medicinal products.

C. multijuga (Fabaceae family) oil resin was capable to kill tumor cells *in vitro* (LIMA, S. R. M. et al., 2003), to induce a peripheral antinociceptive effect (GOMES et al., 2007), and to act as an anti-inflammatory compound (KOBAYASHI et al., 2011). However, this oil resin can induce pleurodesis (WESTPHAL et al., 2014) and, therefore, it should be used with caution.

Pharmacological studies have confirmed the efficiency of *C. langsdorffii* oil resins as anti-inflammatory (PAIVA, L. A. et al., 1998; PAIVA, L. A. F. et al., 2004), antinociceptive (SOUSA, João Paulo B. et al., 2011), and antimicrobial (SOUZA, A. B. et al., 2010).

Besides Copaifera species, for many centuries orchid extracts have also been used to improve human health. These ethnopharmacological properties from orchids are used to treat skin, lung, and infectious diseases, as well as ailments of the gastrointestinal, reproductive, circulatory, and neurological systems (GUTIÉRREZ, 2010).

Cyrtopodium is a Neotropical genus from Orchidaceae family distributed from South Florida to North Argentina. The highest number of species from this genus is found in Brazil. Apart from the biological interest, many local authors have described the use of the *Cyrtopodium* species for medicinal purposes: to stop bleeding and improve wound healing; to treat eyelid inflammation; to treat abscesses, folliculitis, or even as syrup to treat coughs; and to treat lung and urinary tract infections. Some

Cyrtopodium sp are even used as an anti-inflammatory and analgesic. Despite all these local reports, however, no systematic studies have been carried out to quantify these plants *in vitro* and *in vivo* effects.

In our work, we evaluated two different plant products: essential oils from *C. langsdorffii* and *C. multijuga* as well as ethanolic extracts from roots and zhizomas of *Cyrtopodium flavum*. These evaluations provide a better understanding of the biological effects of these plants used in traditional medicine.

Material and methods

1. Plant material

Oil resins were obtained from *Copaifera langsdorffii* Desf. and *Copaifera multijuga* Hayne located at “Castelo” city, “Espírito Santo” state (coordinates 20º30’45’’S and 41º11’20’’W) and at “Ariquemes” city, “Rondônia” state (coordinates 9º52’50’’S and 63º04’27’’W), respectively.

Roots and rhizomes of *Cyrtopodium flavum* Link & Otto ex Rchb. were harvested at “Morro do Moreno”, region located at “Vila Velha” city, “Espírito Santo” state (coordinates 20°19'32.75"S and 40°16'36.91" W).

Botanical identification of Copaifera sp. was performed in the *Herbarium* of “Universidade Vila Velha” and voucher specimens were deposited under numbers UVV ES2164 and UVV ES2165 (*C. langsdorffii* and *C. multijuga*, respectively). *C. flavum* was deposited at “Herbário Central da Universidade Federal do Espírito Santo” under number VIES 845.

2. Essential oils from *C. langsdorffii and C. multijuga*

Essential oil extraction was carried out by hydrodistillation in a Clevenger apparatus. Oil resins samples (50 g/extraction) from these trees were boiled heat for 1 h and the essential oils harvested. These aliquots were transferred to glass flasks for freezing at -20ºC. Finally, the frozen material was separated from the final essential oil extract. Each essential oil density (Table 1) was measured by weighting 1 mL samples at 20ºC.

Chromatographic analysis of essential oil components was performed by high-resolution gas chromatography, coupled to mass spectrometry (GC-MS). The injection

volume was 2 µL, containing 1.8 µL of essential oil (30 mg/ml) and 0.2 µL solution of a series of C7-C30 hydrocarbons, as an internal standard in n-hexane. The system used in CG-MS consisted of a gas chromatograph, Ultra GC Thermo Scientific coupled to a mass spectrometer Thermo Scientific. Stationary phase of chromatographic column was fused silica (DB-5 J & W Scientific, 30 m × 0.25 mm × 0.25 mm). Helium was the carrier gas, and the column temperature was increased by 3°C per minute from 60 °C to 240°C. Mass spectra were obtained at 70 eV with a scan rate of 0.84 scan/sec, in the m/z range of 40-500 (ADAMS, 2007). The retention time of the sample components and a mixture of n-alkanes of C7-C30, co-injected into the GC-MS system at the same temperature program, were used to calculate the Kovats Retention Index - KI (ADAMS, 2007) and the van Den Dool and Kratz retention index 1963 (DOOL, VAN DER; KRATZ, 1963).

Files with mass spectra were used to identify the components of essential oils by using the Xcalibur software, version 2.0.7. Identification was based on the spectral similarity made by comparing spectra obtained with those in the spectral library and literature available, and comparing the retention indices calculated with those available in literature (ADAMS, 2007).

3. Ethanolic extracts from *Cyrtopodium flavum*

Root and rhizome parts were dried for 4 days at 50ºC. Subsequently, each plant part was minced into small pieces and kept in maceration with ethanol 92.8º GL, for 24 h, by percolation extraction. Later, exhaustion leaching was performed with ethanol until no color was visible. Next, this raw ethanolic extract was concentrated in a rotary evaporator at 75ºC, 100 rpm. Finally, these extracts were submitted to lyophilization, their densities calculated, and each sample was diluted in DMSO.

4. Diffusion assay

Initially, agar plates were poured with sterile agar Müller-Hinton media and inoculated with 10^8 UFC/mL (according to 0.5 of McFarland scale) of *Staphylococcus aureus* (ATCC #25923), *Escherichia. coli* (ATCC #8739), or *Candida albicans* (ATCC #10231). Then, sterile Whatman 6 mm paper disks were impregnated with 10 µL of essential oils or ethanolic extracts, and each disk was placed on top of those plates.

Final ethanolic extract concentrations were 125 mg/mL, while essential oil concentrations were 181.0 mg/mL and 160.8 mg/mL, *C. langsdorffii* and *C. multijuga*, respectively. Appropriate positive controls containing antibacterial chloramphenicol (10 µg/disk) or antifungal nystatin (10 U/disk) were used as well as negative controls containing 10% DMSO or mineral oil + 5% DMSO. Each plate was incubated for 24 h at 37ºC. Diffusion halos were measured by a caliper rule. Results are expressed as the average plus standard deviation of triplicates of halos.

5. Minimal Inhibitory Concentration (MIC) tests

These tests were performed according to the Clinical and Laboratory Standard Institute (Wayne, PA, USA). Essential oils were tested from the higher concentration of 905 mg/mL and 894 mg/mL (*C. langsdorffii* and *C. multijuga*, respectively), or 500 µg/mL for ethanolic extract samples. Chloramphenicol (1 mg/mL) or Miconazole (20 mg/mL) were used as positive controls against bacterial or fungus growths, respectively. Some wells received no microorganism to ensure that no external contamination was carried out during the whole procedure. Each sample was tested in triplicates.

6. Cytotoxicity assessment by MTT assay

To evaluate cytotoxic/hepatotoxic effects from essential oils or ethanolic extracts, we cultured Hepa-1C1C7 liver cell line (ATCC CRL2026) at different concentrations of these plant substances. Cell respiration was used as a marker of cell viability since the amount of formazan reduced from MTT by mitochondria is directly proportional to the amount of viable cells (MOSMANN, 1983). Each well received different concentrations of essential oils or ethanolic extracts, and cells were kept in culture for additional 24 h. After this incubation period, supernatants were removed, each well received 10 µL of MTT, and a new 4 h incubation period at 37ºC started. MTT absorbance was red at 570 nm. Results were expressed as a percentage relative to control (solvent treated) samples, and dose-response curves were constructed for determination of IC_{50} values. IC_{50} values were generated from results of five serial dilutions of samples and they are the average of two independent experiments.

7. Mice

Adult male BALB/c mice (20 - 38 g) were used as donors of spleens to perform tests with primary cell cultures and to test curative effects of essential oil in wound healing. These animals were kept inside cages containing water and food *ad libitum*, with 12-by-12 h light-dark periods. Each room was kept at 22ºC.

8. Ethics statement

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the "Universidade Vila Velha" and were in agreement with the "Conselho Nacional de Controle sobre a Experimentação Animal" (Concea) directives. The approval ID numbers of this project are #334/2014 and #288/2013.

9. Effect of plant substances in primary cell cultures

Spleens from BALB/c mice were harvested, cells mechanically dispersed by slide friction in phosphate buffered saline (PBS), and red blood cells (RBC) removed by water osmotic shock. After RBC removal, each cell suspension was counted, centrifuged (400X g, 5 min, 10ºC), and resuspended in complete RPMI 1640 medium (supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-mercaptoethanol, 2 mM L-glutamine, and 1 mM sodium pyruvate; Life Technologies, USA). These leukocytes were cultured at 10^7 cell/mL, 24 well-plates (Greiner Bio-One, Germany), for 24 to 48 h, in a humidified chamber containing 5% CO_2 at 37ºC. Each triplicate of cell cultures was counted in trypan blue to exclude dead cells. As controls, we used DMSO (at 2%) or medium (Control).

10. *In vitro* cell migration assay

In vitro wound healing properties can be estimated by cell migration into scratches produced on a monolayer of fibroblasts or epithelial cells (FRONZA et al., 2009; LIANG; PARK; GUAN, 2007). Fibroblast monolayers were divided into groups and treated for 14 h with 100 µg/mL of essential oils or ethanol extracts. Platelet-derived Growth Factor (PDGF, 2 ng/mL) was used as positive control since it induces proliferation/migration of these cells. After treatment, cells were fixed and stained with DAPI (Sigma-Aldrich Inc, USA). Pictures of each scratched area were taken using an

Axio Zeiss Vert.A1 microscope. This AxioCam MRC Zeiss camera camera was controlled by Zen life 2012 SP1 software. All images were processed by Cellprofiler software and results expressed as percentage of cells that migrate and/or proliferate into scratched area in comparison with the untreated control group.

11. Gel preparation

To test *in vivo C. langsdoffii* healing properties, we prepared a carbopol gel containing 1% *C. langsdorffii* essential oil or 5% *Copaifera officinalis* resin (Positive Control). These preparations were applied topically to animal skin wounds each day for 14 days. Negative control groups only received carbopol gel.

12. *In vivo* wound healing test

Each BALB/c mouse was anesthetized with ketamin/xylazin (80 mg/kg and 15 mg/kg, respectively). After this, dorsal-cervical hair was shaved, and two 8 mm full-thickness excision wounds were performed. Each mouse received their respective treatments as previously described. We used 5 animals per group (*C. langsdorffii* 1%, Negative control, Positive control) and they were housed in individual cages after inducing wounds. After surgery, each animal received dipyrone analgesic at 50 mg/kg by intraperitoneal route. Pictures were taken on days 0, 2, 5, 7, 10, and 14 after induced wounds to the skin, at 30 cm distance from each wound. All wound areas were measured, and the percentage of area reduction was evaluated according to the formula:

$$\text{Area reduction (\%)} = ((\text{initial area} - \text{final area})/\text{initial area}) \times 100$$

13. Statistical analysis

One-way ANOVA plus Tukey correction was applied to our results. These analyses were performed by GraphPad Prism, version 5.0 (GraphPad Software Inc., USA). To compare essential oil chemical compounds diversity, Shannon (H') diversity index and Pielou (J) evenness. p values < 0.05 were considered significant.

Results

1. Characteristics of essential oils from two species of *Copaifera* sp

After extraction, yield of essential oils and their densities were similar (Table 1). However, the diversity of chemical compounds and their evenness were higher in *C. langsdorffii* essential oil than *C. multijuga* essential oil (Table 1). Chromatographic profile of essential oils from *C. langsdorffii* and *C. multijuga* was composed of 47 and 54 components, respectively, mostly mono- and sesquiterpenes. There were 11 shared substances between both species: Z-β-ocimene, alo-ocimene, α-cubebene, α-copaene, β-elemene, γ-elemene, α-humulene, α-bulnesene, viridiflorol, 1-epi-cubenol, and epi-α-bisabolol (Table 2). β-duprezianene was the major component (43.70%) in *C. langsdorffii* essential oil, while E-cariofilene was the major substance (33.08%) in *C. multijuga* essential oil (Table 2).

2. Antimicrobial effect of *Copaifera* sp essential oils and *Cyrtopodium flavum* ethanolic extracts

We evaluated whether these plant extracts could affect the *in vitro* growths of *S. aureus, E. coli,* and *C. albicans* (Figure 1 and Table 3). Regarding the antimicrobial activity against *S. aureus* and *C. albicans*, we could observe halos in the diffusion assays with *C. langsdorffii* and *C. multijuga* essential oils but none in the *C. flavum* ethanolic extracts (Figure 1). Variation of halos sizes was high (Figure 1) which can compromise evaluation of precise antimicrobial effects and doses. Therefore, we decided to perform MIC tests (Table 3). According to these results, *C. langsdorffii* and *C. multijuga* had anti-*S. aureus* effect with MIC of 181.0 mg/mL and 80.4 mg/mL, respectively. However, there was no activity against *C. albicans* in the MIC tests. Also, no activity was seen against the *E. coli* cultures (Table 3). We could also observe another difference in terms of antimicrobial activity: ethanol extract form *C. flavum* rhizome presented anti-*S. aureus* activity in MIC tests at 250 µg/mL (Table 3).

3. Cytotoxic effect of *Copaifera* sp essential oils and *Cyrtopodium flavum* ethanolic extracts

It was clear that *Copaifera* sp essential oils and at least *C. flavum* ethanolic extract from rhizome have antimicrobial activities. However, we needed to determine

their safety by performing toxicity tests. As seen at Figure 2, IC_{50} concentrations for essential oils were around 400 µg/mL, while for ethanol extracts were approaximately 800 µg/mL. These essential oil concentrations are far higher than that observed in antimicrobial tests (Figure 1 and Table 3). In primary cell cultures (Figure 3 and 4), all plant extracts had no deleterious effect up to the final period of time evaluated (48 h). Even more, at the highest concentration tested (500 µg/mL), all plant extracts could induce some cell proliferation at 24 h (Figure 3) and 48 h (Figure 4). In fact, *C. langsdoffii* and *C. multijuga* essential oils induced the highest proliferative response at 24 h (Figure 3), while *C. flavum* ethanolic extracts at 48 h (Figure 4). All plant extracts were not toxic to these primary cell cultures (no decrease in the total amount of cells was observed after *in vitro* stimulation).

4. *In vitro* and *in vivo* wound healing capacity from *Copaifera* sp essential oils and *Cyrtopodium flavum* ethanolic extracts

Since these extracts have been used by local population as treatment to improve wound healing, we tested for their *in vitro* effect in the migration of fibroblast cells (Figure 5) and *in vivo* wound healing (Figure 6). From all plant extracts tested, *C. langsdorffii* essential oil was the only one capable to induce proliferation/migration of fibroblast cells, reaching migration levels similar to the ones observed in positive control cultures stimulated with PDGF (Figure 5). Animals were then treated with carbopol gel containing *C. langsdorffii* at 1% (Figure 6) and a faster wound healing was observed than in untreated wounds (negative control) or observed from wounds treated with gel containing 5% *C. officinalis* resin (positive control).

Discussion

An evenness index less than unity correspond to an asymmetric proportion distribution. Therefore, the evenness indices we measured with respect to essential oils (Tables 1 and 2) indicate asymmetries in the number of substances and their distributed proportional masses. As is typical for essential oils, 40 or more substances may be identified while only a few of them are responsible for the great majority of proportional composition (BAKKALI et al., 2008).

Antibiotic activity was tested by 2 different methods: diffusion disk assays (Figure 1) and MIC (Table 3). Since we observed different result patterns, it the need to evaluate antimicrobial activities with different tests became clear. These differences could be related to intrinsic factors of each test; it is possible that chemical compounds in their own composition diffuse better in a solid surface than water-based mediums, despite both are essential oils. These results also corroborate those from previous experiments that studied the differences between diffusion *vs*. MIC tests and difficulties to standardize different techniques (KRONVALL; GISKE; KAHLMETER, 2011; PFALLER; DIEKEMA; SHEEHAN, 2006).

Antimicrobial activity is one of the plant extract properties that cooperate in the process of wound healing. However, cytotoxic effects have to be evaluated to allow their safer use. In our case, all *Copaifera* sp oils and *C. flavum* ethanolic extract from rhizome were tested in established cell cultures (Figure 2). According to our results, their cytotoxicity was very low, considering that IC_{50} concentrations were very high (Figure 2). Therefore, we could suppose these essential oils could be tested *in vivo* at these concentrations. However, since these are established cell cultures where cellular mechanisms to avoid cell death are in place, it is possible to overestimate cytotoxic effects in primary cell cultures. We also tested these plant extracts in primary cell cultures (Figures 3 and 4) and they were also non-toxic; in fact, at the highest concentrations tested, these plant extracts were able to induce cellular proliferation, indicating stimulatory capacity to lymphoid primary cell cultures.

Extracts from *C. langsdorffii* seeds were capable of inducing macrophage migration to the mouse peritoneal cavity and their activation with production of superoxide, nitric oxide (ROSÁRIO, M M T et al., 2008), and cytokines like TNF-α, IL-1β, and IL-6 (ROSÁRIO, Marianna Maia Taulois DO et al., 2011). However, it was demonstrated anti-inflammatory activity of oleoresins from *C. multijuga* where macrophage migration to peritoneal cavity and production of nitric oxide were reduced (VEIGA JUNIOR et al., 2007). To the best of our knowledge, there is no previous publication that discusses the effect of extracts from these *Copaifera* species in lymphocyte proliferation. Therefore, it is possible that *Copaifera langsdorffii* and *Copaifera multijuga* have different effects in macrophages but similar effects in lymphocytes. Alternatively, they do have different effects on lymphocytes but similar

effects in healing lesions, such as skin cuts. These hypotheses should require further studies in the future.

At this stage, our results support the beginning of *in vitro* and *in vivo* tests to evaluate some of our plant extracts. *In vitro* experiments (Figure 5) show that *C. langsdorffii* essential oil only had potential to be *in vivo* tested; it was capable to induce proliferation/migration of fibroblast cells (Figure 5). When *in vivo* tested (Figure 6), *C. langsdorffii* confirm our expectative: it induced a faster wound healing than untreated wounds or even with other plant extract already tested by others. Taken together, these results indicate that *C. langsdorffii* essential oil could have synergistic effects in epidermal and dermal compartments to improve wound healing: i) it could activate epidermal/dermal progenitor cells to proliferate and differentiate into corresponding cells (therefore, sealing the wound); ii) at the same time, it could activate dermal immune system to improve tissue reshuffling, and iii) it could promote killing of microorganisms present inside the wound.

Finally, this work addressed predominant biological effects from plant extracts used in South American traditional medicine. We could observe antimicrobial activity for some of them and this can contribute *per se* to their curative capacities. However, *C. langsdorffii* essential oil was the only with antimicrobial and cellular activation capacity, with very low cytotoxicity. Further studies are needed to isolate chemical compounds from these plant extracts and to allow their safer use.

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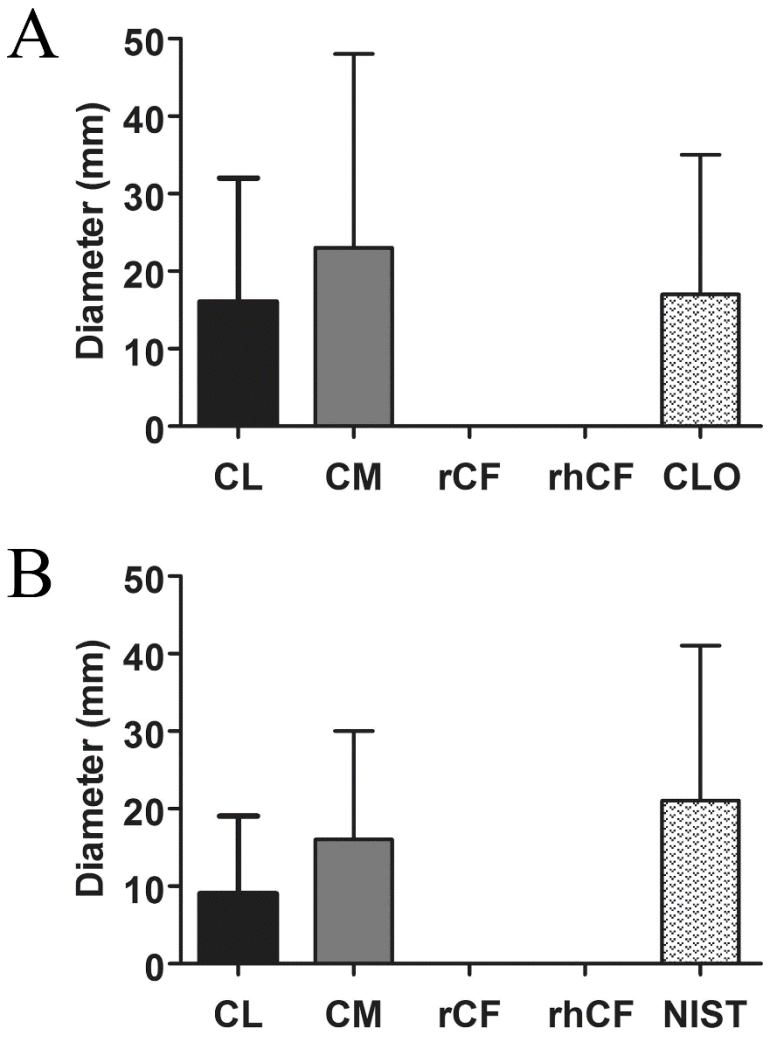

Figure 1 – Microbicidal effect from *Copaifera* sp essential oils and ethanol extracts from *C. flavum*. Essential oils and ethanol extracts were applied in paper discs and inhibitory halos were measured after their incubation in agar plates containing (A) *S. aureus* or (B) *C. albicans*. These plant extracts were tested: essential oils from *C. langsdorffii* (CL) or from *C. multijuga* (CM); ethanolic extracts from roots (rCF) or rhizome (rhCF) from *C. flavum*. Each column represents the average ± SE from triplicates of halos.

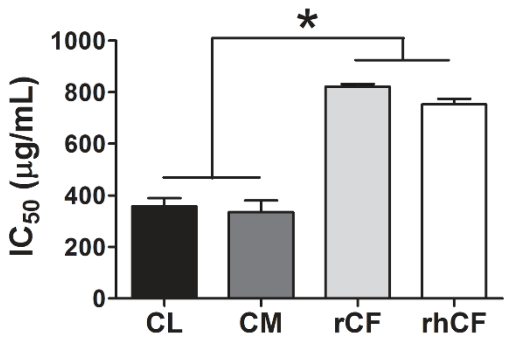

Figure 2 – Cytotoxic effect of essential oils and ethanol extracts on hepatocyte cell line. Hepa-1C1C7 cell line was cultured at different concentrations of the following plant products: essential oils from *C. langsdorffii* (CL) or from *C. multijuga* (CM); ethanol extracts from roots (rCF) or rhizome (rhCF) from *C. flavum*. Each column represents the average ± SE from triplicates of culture.

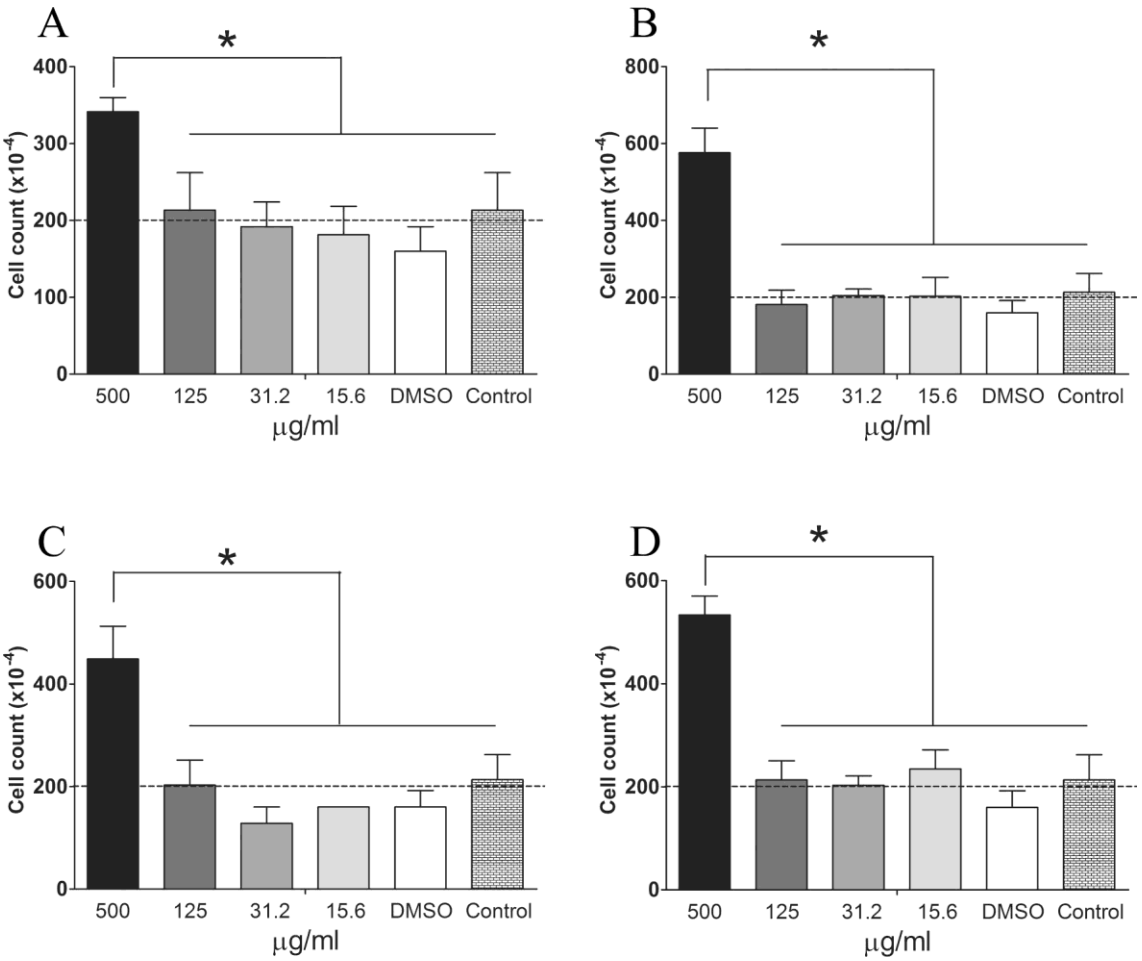

Figure 3 – Effect of essential oils and ethanol extracts in primary cell cultures. Spleen cells from BALB/c mice were harvested and cultured for 24 h in the presence of the following plant products: essential oil from (A) *C. langsdorffii* or from (B) *C. multijuga*; ethanol extract from (C) roots or (D) rhizome from *C. flavum*. Each column represents the average ± SE from triplicates of culture. Dotted line: initial cell concentration ($200x10^4$/well).

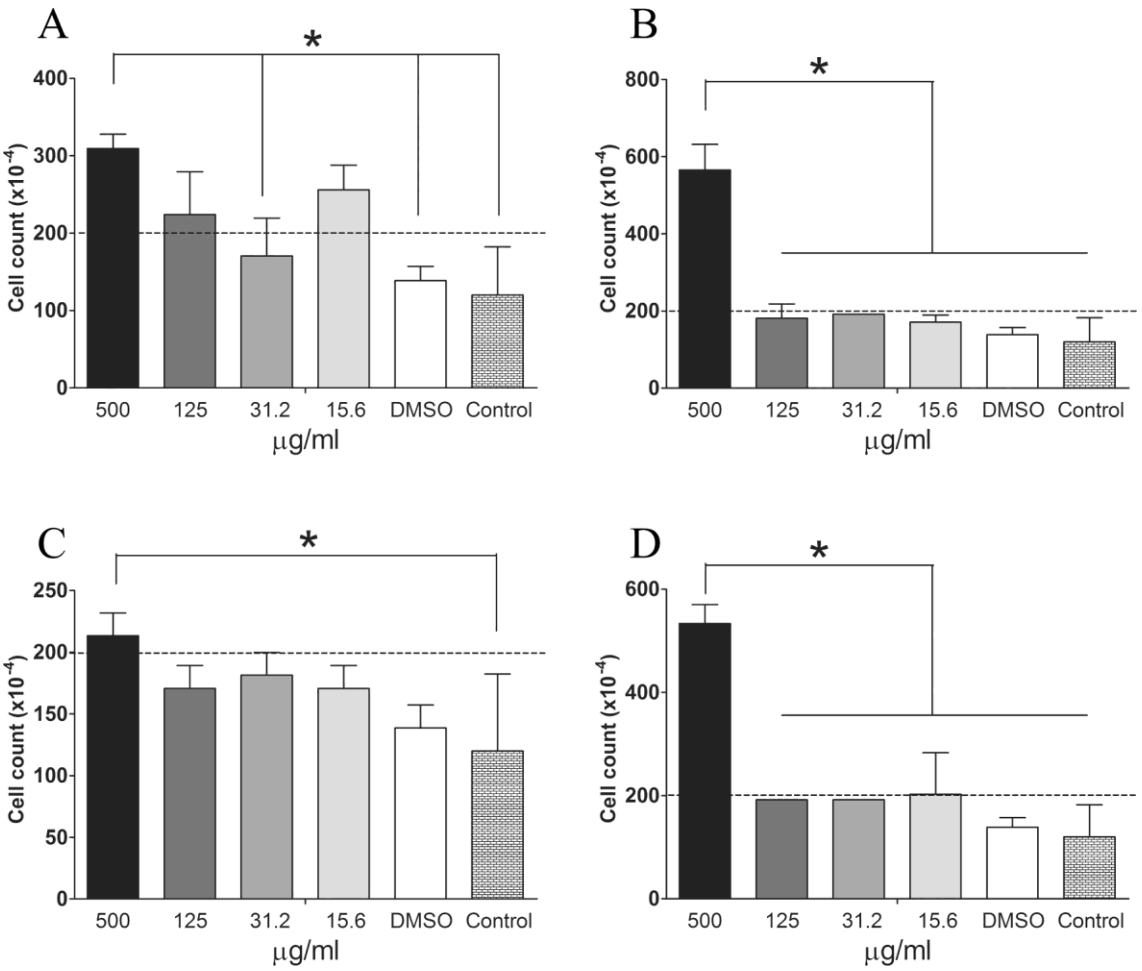

Figure 4 – Effect of essential oils and ethanol extracts in primary cell cultures. Spleen cells from BALB/c mice were harvested and cultured for 48 h in the presence of the following plant products: essential oil from (A) *C. langsdorffii* or from (B) *C. multijuga*; ethanol extract from (C) roots or (D) rhizome from *C. flavum*. Each column represents the average ± SE from triplicates of culture. As controls we added DMSO or medium (Control). Dotted line: initial cell concentration ($200x10^4$/well).

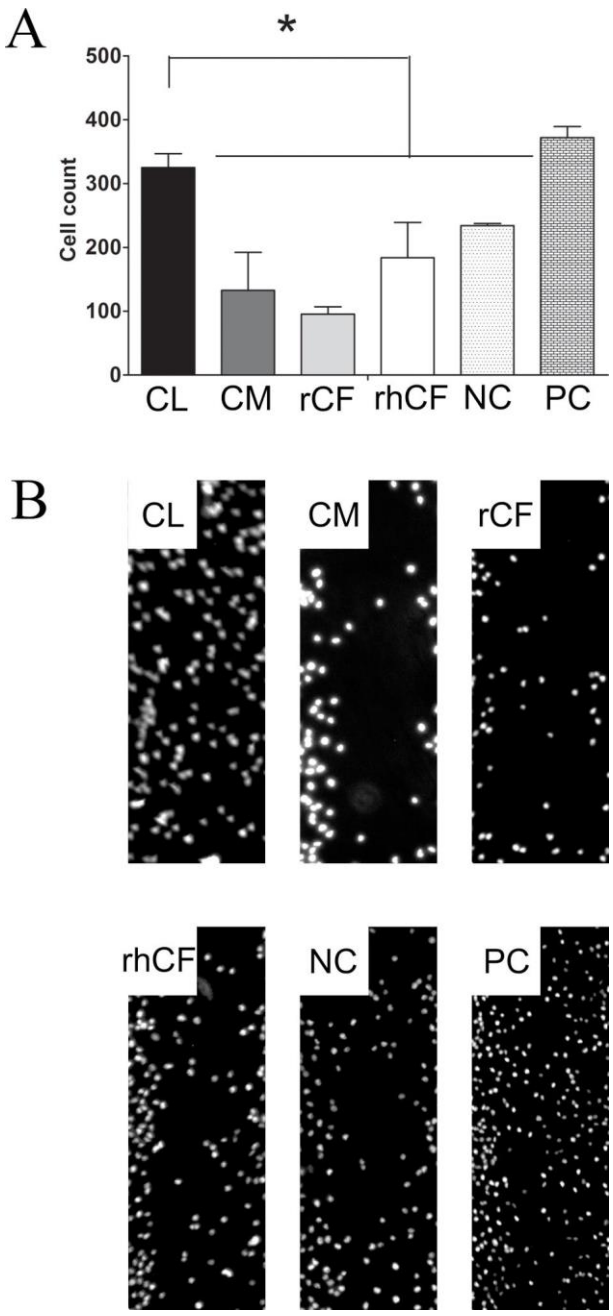

Figure 5 – *In vitro* effect of essential oils and ethanol extracts in cell migration. L929 cells were cultured until almost reach confluence. Then, scratches were performed in these cultures to further evaluate cellular proliferation/migration to scratched areas. (A) Cell counting inside scratched areas. (B) Representative pictures of scratches where cultures were stimulated with different plant extracts: essential oil from *C. langsdorffii* (CL) or from *C. multijuga* (CM); ethanol extract from roots (rCF) or rhizome (rhCF) from *C. flavum*. Cell cultures non-stimulated were used as negative controls (NC) while cultures stimulated with PDGF as positive controls (PC). Each column represents the average ± SE from triplicates of culture.

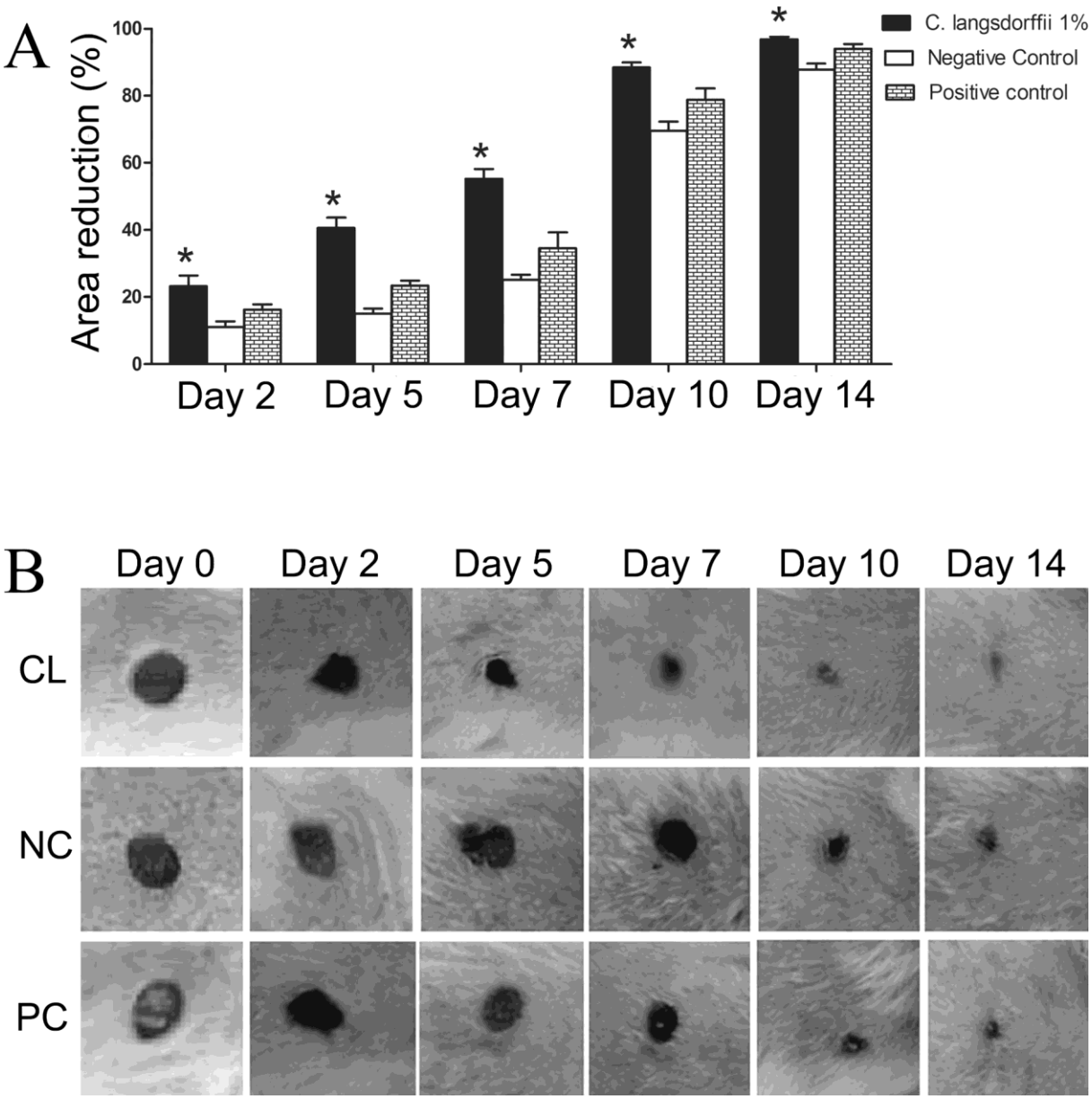

Figure 6 – *In vivo* effect of essential oils and ethanol extracts in wound healing. BALB/c mice were anesthetized and skin punches were performed. Then, these wounds were treated with gel + essential oil from *C. langsdorffii* (CL), gel only (negative control, NC), or treated with gel + 5% *C. officinalis* resin (positive control, PC). (A) Reduction of wound area; (B) Representative pictures from wounds treated or not. Each column represents the average ± SE from duplicates of wound area reductions.