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Potencial antineoplásico e avaliação da composição química dos óleos essenciais de folhas e flores de *Tagetes ostenii* Hicken

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Antineoplastic potential and chemical evaluation of essential oils from leaves and flowers of *Tagetes ostenii* Hicken

Abstract

Cervical cancer ranks fourth in incidence in women worldwide and the third in Brazil. Since conventional treatments have a series of adverse reactions, there is an urgency in the search for new strategies against cancer. In this context, natural products represent a promising source of new active molecules with antitumor potential. The Tagetes genus has been described in the scientific literature with numerous biological effects, but there are few data on the *Tagetes ostenii* Hicken species. In this work, we investigate the chemical composition of essential oils from leaves and flowers of *T. ostenii* and evaluated the effects of these oils in a cervical cancer cell line, SiHa, and non-tumoral cell line, HaCat. The chemical analysis revealed the major components of the leaves essential oil (EO 1) as dihydro-tagetone (65.3%) and (Z)-tagetone (14.9%), while (Z)-β-ocimene (56.3%), (Z)-ocimenone (26.8%) and (E)-ocimenone (11.8%) were the main compounds of the flower's essential oil (EO 2), one week after extraction. The cell viability after treatment was evaluated by the MTT assay and revealed a significant inhibition in tumor cell viability at all concentrations for both tested oils. The IC50 of EO 1 in SiHa cells was 72 ng/mL and 83 ng/mL for EO 2. For HaCat cell line were observed an IC50 of 54.45 ng/mL for EO 1 and 20.83 ng/mL for EO 2. The combined treatment with EO 1 and cisplatin showed a synergistic effect after 48 and 72 hours of treatment, and after 24 and 48 hours for EO 2. Cell migration assessed through the Wound Healing assay revealed that the SiHa cells had their migration process reduced after 48 h of treatment with EO 2. Furthermore, both essential oils were able to significantly inhibit the adhesion process by increasing the number of viable cells in the supernatant about 2.8 times for EO 1 and 7.03 times for EO 2. Clonogenic ability was also reduced markedly by treatment with EO 1 and EO 2 after 24 h at 88.7% and 90%, respectively. Our results also suggest that EO 1 and EO 2 have long-lasting inhibitory activity in tumor cells because only 6,36% of the treated cells with EO 1 and 22,09% with EO 2 were able to recover the viability even after treatment withdrawal. Analysis using Flow cytometer with annexin V/propidium iodide demonstrated that both essential oils induced a cell death through late apoptosis in

most of the tumor cells after 24, 48 and 72 hours of treatment. Together these results suggest a promising antineoplastic effect of essential oils of *T. ostenii* and emphasize the importance and need for additional studies involving samples from plant species.

Keywords: cervical cancer; natural products; essential oils, cytotoxicity.

Introduction

Cancer remains as a major problem of public health worldwide. According to the last estimates of International Agency for Research on Cancer (IARC), the increasing cancer burden is due to several factors including population growth and aging, as well as the changing prevalence of certain causes of cancer linked to social and economic development. Cervical cancer ranks eighth for incidence of all cancers worldwide and fourth for both incidence (6.6%) and mortality (7.5%) in the women population (FERLAY et al., 2018). In Brazil, this neoplasia occupies the third place in incidence and are estimated 16.370 new cases for each year of 2018 and 2019 (INSTITUTO NACIONAL DE CANCER JOSÉ ALENCAR GOMES DA SILVA, 2017)

Actually, is clearly known the association of human papillomavirus (HPV) as a principal cause of cervical intraepithelial neoplasia (CIN) and cervical cancer (SATTERWHITE et al., 2013). Papillomavirus type 16 and human papillomavirus type 18 infections are responsible for 70%–75% of all cervical cancers and 40%–60% of its precursors (CLIFFORD et al., 2006). Furthermore, is also well established that the presence of genetic and epigenetic alterations like DNA methylation, histone modifications, and noncoding RNAs are needed for the development of carcinogenesis (FANG, ZHANG, JIN, 2014; SAAVEDRA, BREBI, ROA, 2012).

Despite the efforts to improve conventional treatments such as radical surgery, radiotherapy, and chemotherapy, it is known that the adverse reactions of these methodologies affect the patients' quality of life. Radiotherapy has been widely used in the treatment of cervical cancer, but this option suffers from a high frequency of acute and chronic complications (GALLAGHER et al., 1986). Patient's most common adverse reactions are abdominal pain, varying degrees of diarrhea, hemorrhage, intestinal obstruction, and granulocytopenia during the therapy, but some of these symptoms can occur months or years after the procedure like intermittent diarrhea;

intolerance to certain foods; malabsorption of vitamins, lactose, and bile acids (DANG et al., 2018; LEE et al., 2017).

The chemotherapy cisplatin has been recognized as the most active cytotoxic agent for treatment of cervical cancer and cisplatin combined with radiation had shown a 30%–50% improvement in survival in patients with locally advanced cervical cancer, but unfortunately with a high level of hematologic and non-hematologic toxicity (KEYS et al., 1999; MORRIS et al., 1999; PETERS III et al., 2000; ROSE et al., 1999; WHITNEY et al., 1999). Moreover, platinum-based antineoplastic resistance is the main reason for the poor prognosis of recurrent or advanced cervical cancer, especially those previously treated with platinum-based chemotherapy or radiosensitizing cisplatin (HISAMATSU et al., 2012; MOORE et al., 2010). Beyond that, it is estimated that approximately 30–35% of patients with invasive cervical cancer will develop recurrent disease after primary treatment (TORRE et al., 2015).

Thus, there is an urgency in the search for new molecules and alternative strategies against cancer. In this context, natural products, especially those from the flora, represent a great source of molecules with biological activities and, also, antitumor potential. Many of the studies that explore plant potential were based on traditional medicine reports from communities and local population (NEWMAN; CRAGG; SNADER, 2003). Within the sphere of cancer, a number of important new commercialized drugs have been obtained from natural sources. Of the 92 drugs commercially available prior to 1983 in the United States, or approved worldwide between 1983 and 1994, approximately 62% can be related to a natural product origin (BRANDÃO et al., 2010; NEWMAN; CRAGG; SNADER, 2000).

Asteraceae is the largest family of angiosperms, comprising 25.000 species (BREMER; ANDERBERG, 1994). In Brazil, the family is represented by approximately 196 genres and about 1,900 species (BARROSO, 1991). Its members are widely used in folk medicine due to its biological activity and chemical composition (SCHIAVON; SCHUCH; FACCIN, 2015).

Tagetes ostenii Hicken is a South American native plant representative of the Asteraceae family, widely distributed in Uruguay and south of Brazil (DEBLE; DE OLIVEIRA; MARCHIORI, 2005). In addition to being popularly known for medicinal indications, species of the genus Tagetes have been scientifically described as

antibacterial, antifungal, larvicidal, insecticidal, antiparasitic, nematicidal, antihyperglycemic and antioxidant (ANDREOTTI et al., 2013; ROMAGNOLI et al., 2005; SALEHI et al., 2018; SCHIAVON; SCHUCH; FACCIN, 2015; VASUDEVAN; KASHYAP; SHARMA, 1997). They are highly available due to their high germination rate and produce flowers and seeds all year round (BARROSO, 1991). In addition, this genus includes different plants producing essential oils of commercial importance already known (SALEHI et al., 2018). Essential oils are low molecular weight compounds derived from secondary plant metabolism and chemical composition characterized by pharmacological activities of great interest (ADAMS, 2007) The species *T. ostenii* also produces essential oils; but very little studied in relation to its biological effects (DEBLE; DE OLIVEIRA; MARCHIORI, 2005).

Thus, through this work, we seek to obtain the results that allow us to evaluate the antineoplastic potential of essential oils obtained from both, leaves and flowers of *T. ostenii* in human cervical cancer cells, as well as to evaluate the possible toxicity in non-tumor cells.

Material and Methods

Materials: Penicillin/gentamicin, fungizone (amphotericin B) and fetal bovine serum (FBS) were purchased from Gibco (Gibco BRL, Grand Island, NY). Dulbecco's modified Eagle's medium (DMEM), trypsin/EDTA solution, Trypan Blue dye and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) were purchased from Sigma Aldrich (St. Louis, MO, USA). Annexin V Apoptosis Detection Kit was purchased from QuatroG. Cisplatin chemotherapeutic was kindly donated by Dr. Guido Lenz. All other chemicals and solvents used were of analytical grade.

Plant Material and oil extraction: Flowers and leaves of *T. ostenii* were collected in Santo Antônio da Patrulha, Rio Grande do Sul, Brazil and botanically identified. Once at the laboratory, the plant material was washed with distilled water and the essential oils was obtained by hydrodistillation process for 3 h using a Clevenger type apparatus in accordance with the instructions in the Brazilian Pharmacopoeia (BRASIL, 2010). For the cell's treatment, the essential oils were solubilized in propylene glycol at 1:5.

Chemical analysis: The quantitative and qualitative analysis of the components present in both essential oils were performed in Gas Chromatograph coupled with Mass Spectrometer (GC-MS), Shimadzu QP5000-quadrupole MS system, operating at an ionization energy of 70 eV and an interface temperature of 250 °C. A DB-5 fused silica capillary column (30 m, 0.25 mm; film thickness 0.25 µm; Supelco) was used. Injector and detector temperatures were set at 220 °C and 250 °C, respectively; oven temperature was initially maintained at 60 °C for 3 min and then programmed to 60–300 °C at 3 °C/min (total of 88 min). Helium was used as carrier gas (1 mL/min). Percentages of compositions were obtained from electronic integration measurements by peak area normalization. Scan time and mass range were 1 s and m/z 40–550, respectively, and the injection volume was 1 µL. Four analyzes were performed with both essential oils, the first one at extraction time (0 h), 24 h, 48 h and one week after this procedure. The identification of the components was based in comparison of their retention indices and their mass spectral fragmentation pattern, with data taken from the literature (ADAMS, 2007) and by comparison with mass spectra recorded in the NIST 62 and NIST 12 (National Institute of Standards and Technology) library of mass spectra.

Cell lines: The human cell line derived from invasive cervical carcinoma, SiHa (HPV 16-positive) was obtained from American Type Culture Collection (ATCC - Rock-Ville, MD) and the immortalized human keratinocytes, HaCaT, were kindly donated by Dr. Silvya S. Maria-Engler (Faculty of Pharmaceutical Sciences, University of São Paulo). Cell lines were cultured in DMEM High Glucose supplemented with 10% of fetal bovine serum (FBS), 100 µg/mL streptomycin and 0.5 µg/mL fungizone. Cell culture was maintained at 37 °C in 5% of CO_2 atmosphere.

Cell Viability Analysis: To verify SiHa and HaCat cell viability after treatment, the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay was used (MOSMANN, 1983). The cells were seeded in a density of 3.0×10^3 cells/well in a 96-well plate and incubated for adhesion at 37 °C in 5% CO_2 atmosphere. After cell adhesion the supernatant was aspirated, and the cells were treated with the essential oils at different concentrations (0.01, 0.02, 0.03, 0.04, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 1.5, 2.5, 5, 10, 15, 20, 25 and 30 µg/mL) for 24 hours. Controls were prepared using only DMEM medium and vehicle control using propylene glycol and DMEM. After

withdrawn treatment, MTT solution (0.5 mg/mL) was added to each well and plates were incubated in the dark for 3.5 hours at 37 °C. Formazan crystals formed by tetrazolium cleavage were dissolved with Dimethyl Sulfoxide (DMSO) and quantified at 545 and 630 nm using an EnVision Multilabel Plate Reader (PerkinElmer). The results were expressed as percentage of control, which was considered as 100% of cell viability. IC50 values were calculated from log dose-response curves using GraphPad Prism 5 software.

To evaluate synergistic effect, the chemotherapeutic cisplatin was used in combination with leaf or flower essential oil. SiHa cells were seeded in 96 well plates and incubated with IC50 value of essential oils and IC50 value of cisplatin (80 µM) (NG; YAZAN; ISMAIL, 2011), alone or in combination. After 24, 48 and 72 hours of treatment, cell viability was assessed by the MTT assay described above. For all the assays (excluding the cell viability assay) we used the concentration of propylene glycol present in the IC50 of the flower's oil as a vehicle control because it have the higher concentration of the solvent.

Wound Healing Assay: Cell migration was measured using the wound healing assay as described by (RODRIGUEZ; WU; GUAN, 2005). Briefly, SiHa cells were seeded in 24-well plates (2.8×10^4 cells/well) and incubated to grow to confluence. The confluent cell monolayer was carefully wounded using a pipette tip and then, the cellular debris was removed by washing with PBS. The wounds were photographed before and after treatment with essential oils at IC50 concentration of 83 ng/mL for the flower and 72 ng/mL for the leave. Cell migration into the scratched region was recorded using an Olympus CK40 microscopy system coupled to an UCMOS 03100 KPA digital camera before, 24 and 48 hours after treatment. The results were calculated by comparing the wound width before and after treatment in treated and control cells.

Adhesion Assay: To determine the ability of the essential oils to interfere in the adhesion cell's capacity, SiHa cells were plated (1×10^4 cells/well) in 96-well plates. Immediately the cells were treated with essential oils at IC50 concentration for 8 hours. After this time, the supernatant of each concentration was collected, and the cells were washed with PBS 1x and this solution was collected and mixed with the initial supernatant. Then, the total collected was centrifuged at 1000 rpm for 5 min and the

pellet was mixed with trypan blue dye exclusion (1:1) for counting of viable cells using hemocytometer. Results were expressed as the number of viable treated cells which did not adhere in comparison with the control. We also quantified the adherent viable cells after treatment by fixing the cells with paraformaldehyde 4% and stained with crystal violet 0.5% (diluted in methanol 20%). After this, the cells were washed with distilled water to remove the excess of dye and eluted with acetic acid 10%. The cells were quantified at 570 nm using an EnVision Multilabel Plate Reader (PerkinElmer). The results were expressed as percentage of control, which was considered as 100% of cell adhesion.

Clonogenic Assay: The clonogenic ability following treatment with essential oils of *T. ostenii* in cancer cells was performed by clonogenic assay as described by FRANKEN et al., 2006. For this purpose, SiHa cells (2.8×10^4 cells/well) were seeded in a 24-well plate and after adhesion, subconfluent cultures were treated with essential oils at IC50 concentration for 24 hours. Then the cells were washed with PBS to remove the treatment, trypsinized, counted in a hemocytometer, and replated in 24-well plates (150 cells/well) and incubated at standard conditions for 5 days. After this period the colonies formed were fixed with paraformaldehyde 4% and stained with crystal violet 0.5% (diluted in methanol 20%) for manually counting. Results were expressed as percentage of control, which was considered 100% of colony forming.

Washout Assay: The recovery of cell viability after withdrawal of treatment was determined by Washout Assay. SiHa cells (2.8×10^4 cells/well) were seeded in a 96-well plate and after adhesion, subconfluent cultures were treated with essential oils at IC50 concentration for 24 hours. Then, adherent cells were washed with PBS 1x to remove entire treatment, trypsinized, counted and replated in a 96-well plate (2.800 cells/well). After, the cells were incubated for 4 days at standard conditions, and the MTT assay was used to measure cell viability as described before (Cell Viability Analysis section).

Annexin V /Propidium iodide staining: Phosphatidylserine externalization was determined by the annexin fluorescence signal of an annexin V–fluorescein isothiocyanate conjugate (QuatroG) according to the manufacturer's protocol. SiHa cells were treated for 24, 48 and 72 hours with essential oils at IC50 concentration of 83 ng/mL for the flower and 72 ng/mL for the leaf. After this time, the cells were washed,

trypsinized and centrifuged at 1500 rpm for 5 min. The pellet was resuspended with annexin binding buffer 1x (0.1 M Hepes/NaOH pH 7.4, 1.4 M NaCl, 25 mM $CaCl_2$) and incubated with annexin V and PI at room temperature in the dark for 15 minutes. The cells were analyzed on a BD FACSVerse cytometer equipped with a 488 nm excitation laser and the software Facsuite was used for results analysis.

Statistical analysis: All values were expressed as averages and standard deviation (SD) from at least two independent experiments performed in duplicate. Data were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey test using the GraphPad Prism 5 (San Diego, USA, 2007). Statistical differences were considered significant when the value was $p < 0.05$.

Results

Chemical analysis of essential oils from leaves and flowers of *T. ostenii*

The chemical analysis of essential oils was performed by Gas Chromatograph coupled with Mass Spectrometer (GC-MS). The essential oils were analyzed at extraction time (0 h), 24, 48 h and one week after this procedure. For leaf's essential oil (EO 1), it was verified a predominance of oxygenated monoterpenes compounds (Table 1), with dihydro-tagetone (64.2%) and (Z)-tagetone (15.9%) as majorities at 0 h. After one week from the extraction, both compounds remained as most abundant with a small variation in the percentage, demonstrating chemical stability.

In relation to flower's essential oil (EO 2), it was observed a predominance of both oxygenated and hydrocarbons monoterpenes. At 0 h of extraction, the compounds (E)-ocimenone (39.9%), (Z)-ocimenone (17.5%) and (Z)-β-ocimene (26.1%) were the majorities. After one week from the extraction these compounds also continuous as predominance but with a significant variation in their abundance. (E)-ocimenone (11.8%) showed a decrease in relation to 0 h while (Z)-ocimenone (26.8%) and (Z)-β-ocimene (56.3%) exhibited an increase in their percentage.

Cell viability analysis of cervical cancer and non-tumoral cell lines after treatment

To investigate cell viability after treatment, the MTT assay was used and different concentrations of both essential oils were tested for 24 hours of treatment. For leaf and

flower essential oil of *T. ostenii*, all tested concentration, from 0.01 to 30 µg/mL, were able to inhibit significantly the viability of cervical cancer cells when compared with control cells (containing only culture medium). While the vehicle (propylene glycol) did not markedly inhibit the viability of these cells in the lower concentrations analyzed (up to 2.5 µg/mL) for both essential oils, demonstrating that the vehicle used did not affect the viability of SiHa cell line (Figure 1 A and B).

At 24 hours of treatment the inhibition of SiHa cells was between 31.3% (at 0.02 µg/mL concentration) to 95.6% (at 1 µg/mL concentration) for EO 1 and between 29.5% (at 0.01 µg/mL) to 93.3% (at 0.5 µg/mL) for EO 2. Based on the cell viability tests it was possible to obtain the half maximal inhibitory concentration (IC50) of 72 ng/mL for the essential oil of the leaf and 83 ng/mL for the flower in the human cervical cancer cell line SiHa after 24 hours of treatment.

We also tested both essential oils of *T. ostenii* to verify cytotoxicity in the non-tumoral cell line HaCat with concentrations between 0.01 to 10 µg/mL. The results from the treatment with EO 1 demonstrated no significant inhibition of cell viability up to 0.05 µg/mL and up to 0.01 µg/mL for EO 2 in comparison to control cells. As shown for the tumor cells, the vehicle tested also did not cause significant inhibition on cell viability of the non-tumoral cell line HaCat in the lower concentrations (up to 1.5 µg/mL) (Figure 1 C and D).

After 24 hours of treatment the inhibition of HaCat cell line was between 2% (at 0.01 µg/mL) to 95.4% (at 1.5 µg/mL) for EO 1 and 3.8% (at 0.01 µg/mL) to 96% (at 0.5 µg/mL) for EO 2. We also calculated the IC50 concentration for both oils in the HaCat cell line and obtained the value of 54.45 ng/mL for the leaf essential oil and 20.83 ng/mL for the flower.

When evaluated the IC50 concentration calculated for SiHa cell line (72 ng/mL for EO 1 and 83 ng/mL for EO 2) in both cell lines, the results for the treatment of SiHa cells with EO 1 showed an inhibition on cell viability of 53.9%, 25% and 64.5%, for 24, 48 and 72 h of treatment, respectively (Figure 2A). For treatment with EO 2, it was observed an inhibition of 47.5%, 68.1% and 92.6% for each time, respectively (Figure 2B). In relation to the non-tumoral cell line HaCat, the treatment with EO 1 and EO 2 inhibited cell viability in 79.6%, 76.7%, and 22.5%, and 41.5%, 18.4% and 21% for each oil respectively for the tree times of treatment (Figure 2C and D).

The selective index (SI) was determined to evaluate the discrimination of treatments between tumoral and non-tumoral cells. The selectivity index was calculated as the ratio IC50 (control cells)/IC50 (tumoral cell line). A selectivity index > 1 indicates that the cytotoxicity on tumoral cells surpassed the one on the healthy non-tumoral cells (CALLACONDO-RIVA et al., 2008). For the results obtained the SI estimated for *T. ostenii* essential oils was 0.75 for EO 1 and 0.25 for EO 2.

Combined treatment with Cisplatin and essential oils of *T. ostenii* results in synergistic effects on cervical cancer cell viability

It is known that polychemotherapy is of proven efficacy and aims to reach cell populations at different stages of the cell cycle and with different phenotypes, to use the synergistic action of drugs, to decrease the development of drug resistance and to promote greater response per dose administered. For this reason, we analyzed the possible interaction between the essential oils studied and the chemotherapy drug cisplatin.

When cancer cells were treated with EO 1 at IC50 concentration and cisplatin simultaneously during 24 hours of incubation the inhibition of SiHa cells was 45.4%. After 48 hours, the combined treatment inhibited the SiHa cells viability in 77.1% and 82.2% after 72 hours. However, a synergistic effect was observed only at 48 and 72 hours of treatment (Figure 2E). In relation to the treatment with EO 2 at IC50 concentration and cisplatin, the inhibition was 62.8%, 92% and 95.6% after 24, 48 and 72 hours of incubation, respectively. A synergistic effect was observed at 24 and 48 hours of treatment.

Effects on cell migration and adhesion capacity after essential oils treatment

Apart from the observed inhibition on the cell viability, the investigation of the essential oils of *T. ostenii* effects in relevant indicators for human cervical cancer cells also demonstrated significant results. The Wound Healing method was performed to determine the rate of cell migration. This method is based on the observation that, upon creation of a "wound" on a confluent cell monolayer, the cells on the edge of this wound will move toward the opening until new cell-cell contacts are established again (LIANG; PARK; GUAN, 2007).

In Figure 3A, we can observe a picture of the wounds in the cell monolayer before (0 h) and after the treatment (24 and 48 h). The tumor cells had their migration process reduced after 48 h of treatment with the essential oil of the flower and we could observe an increase in the wound width in about 7.4% when compared with the initial width (Flower 0h) (Figure 3B). After 48 h of treatment with leaf's essential oil, the cell migration process appears not to be affected and we observed a reduction in the wound width about 22% when compared with the initial width (Leaf 0h). Meanwhile, the control and vehicle control cells had their migration process significantly reduced after 48 h of treatment, with a decrease of 22.5% and 19% when compared with the initial wound width (C 0h and VC 0h), respectively.

The tumor cell adhesion capacity was analyzed after 8 h of treatment and we quantified, simultaneously, the viable cells in the supernatant and the cells that were able to adhere to the plate. Both essential oils of *T. ostenii* were able to significantly inhibit the adhesion process by increasing the number of viable cells in the supernatant about 2.8 times for leaf's oil and 7 times for the flower when compared to control cells (Figure 3C). It was also verified a decrease in adhered viable cells after treatment, with a reduction on cell viability of 84.3% for leaf's treatment and 72.9% for the flower when compared to control cells (Figure 3D).

Clonogenic ability and cell viability recovery after essential oils treatment

Another significant parameter to evaluate the effect of a treatment on cancer cells is the colony forming capacity. The clonogenic assay is a method used to determine the fraction of seeded cells that retain the capacity to produce colonies and for this reason is useful to determine the effectiveness of cytotoxic agents (FRANKEN et al., 2006; MELLO et al., 2014).

Figure 4B shows that the treatment with both essential oils of *T. ostenii* at IC50 concentration reduced markedly the number of colonies formed in relation to control cells. These results were quantified and revealed that after 24 h of treatment with EO 1 and EO 2 the colony forming capacity was significantly reduced in 88.7% and 90%, respectively when compared to the control (Figure 4C).

To determine the persistence of the effects of treatment exposure in cancer cells we evaluated the cell viability after the removal of the essential oils. Therefore, the

tumor cells were treated for 24 h with each essential oil at IC50 concentration and then the treatment was removed, and the cells were replated and incubated at standard conditions. As shown in Figure 4D only 6.4% of the treated cells with EO 1 and 22.1% with EO 2 were able to recover their viability even after the removal of the treatment in relation to control cells.

Cell death induced by *T. ostenii* essential oils

The data obtained through flow cytometry and double staining for annexin V/ propidium iodide showed that at 24 h of treatment with EO 1 the majority of the cells were marked for apoptosis (16.4%) and late apoptosis (35.9%) (Figure 5 and Table 2). After 48 and 72 h of treatment, we observed an increase in the late apoptosis cell population with values of 84% and 91.3%, respectively. In relation to the treatment with EO 2 at 24 h of treatment, 24.7% of cells were in apoptosis and 60% in late apoptosis. In the treatment of 48 h, the percentage of cells in late apoptosis was 80.3% and 66% after 72 h of treatment.

Discussion

In this article, we analyzed, for the first time, the chemical composition and antitumoral potential of the South American species *T. ostenii,* and this work represents the first biological activity described for this species until now. The essential oils from leaves and flowers of *T. ostenii* were characterized by the presence of oxygenated and hydrocarbons monoterpenes. After one week of the extraction, the major components of the leaves essential oil (EO 1) were dihydro-tagetone (65.3%) and (Z)-tagetone (14.9%), while (Z)-β-ocimene (56.3%), (Z)-ocimenone (26.8%) and (E)-ocimenone (11.8%) were the main compounds for the flower's essential oil (EO 2) (Table 1). When compared with other plants of the genus Tagetes, the species *T. ostenii* characterized in this work showed a similar chemical composition with *Tagetes minuta*. The review by GUPTA; VASUDEVA, (2012) found that EOs from leaves of *T. minuta* were dominated by dihydro-tagetone (2.7–54.2%), (Z)-ocimene (1.4–16.1%), (E)-tagetenone (0.1–19.5%), (Z)-tagetenone (tr–31.4%), (E)-tagetone (0.8–14.5%), (Z)-tagetone (6.6–28.2%), limonene (2–12.4%) and eugenol (16.5%), as more abundance compounds. Another study reported that in the essential oil of the flowering shoots of *T. minuta* was presented β-ocimene, dihydro-tagetone, tagetone and ocimenones

(HETHELYI et al., 1988). CHALCHAT; GARRY; MUHAYIMANA, (1995) reported thirty-seven constituents in the essential oil of the leaves and flowers of *T. minuta* with (Z)-β-ocimene predominating in the oil from flowers and dihydro-tagetone predominating in that from leaves, as was also observed in our analyzes.

The cytotoxicity experiments revealed a significant inhibition of the viability of cervical cancer cells after treatment with leaf and flower essential oils for 24 h. The inhibition of SiHa cells viability was between 31.3% to 95.6% for the treatment with EO 1 and IC50 of 72 ng/mL. The cell viability inhibition induced by EO 2 treatment was between 29.5% to 93.3% and IC50 of 83 ng/mL (Figure 1A and B). It is important to emphasize that inhibition in the viability of SiHa cells were observed at low concentrations and this resulted in low IC50 for both treatments. Studies had shown the antitumor potential of other species of Tagetes genus. As reported by ALI et al., (2014) the essential oil extracted from the leaves of *T. minuta* showed a cytotoxic activity against MCF-7 breast tumor cells, with an IC50 of 54.7 +/- 6.2 ug/mL. Another study also demonstrated cytotoxicity activity of *T.minuta* leaves essential oil against nasopharyngeal cancer cell line (KB) and liver hepatocellular carcinoma cell line (HepG2) with an IC50 of 75 ± 5 and 70 ± 4 µg/mL, respectively (SHIRAZI et al., 2014).

We also evaluated the cytotoxic effect of treatment in a non-tumoral cell line of human keratinocytes, HaCat. After 24 hours of treatment, the viability inhibition of HaCat cells was between 2% to 95.4% for EO 1 and IC50 of 54.45 ng/mL and 3.8% to 96% for EO 2 with an IC50 of 20.83 ng/mL (Figure 1B and C). Other species of the genus, *Tagetes patula*, also demonstrated cytotoxic potential in a murine macrophage strain for ethanolic extract of the aerial parts and ethanolic extract of flowers (POLITI et al., 2016).

We also evaluated the effect of the treatment in SiHa and HaCat cells with the IC50 of essential oils for 24, 48 and 72 h. The treatment of SiHa cells with EO 1 showed an inhibition on cell viability of 53.9%, 25% and 64.5% for 24, 48 and 72 hours respectively (Figure 2A). For treatment with EO 2 was observed an inhibition of 47.5%, 68.1% and 92.7% for each time, respectively (Figure 2B). These results showed that the effects of the treatment with both essential oils are maintained for at least 72 h in the tumoral cell line. In relation to the non-tumoral cell line HaCat, the treatment with

EO 1 and EO 2 inhibited cell viability in 79.6%, 76.7%, and 22.5%, and 41.5%, 18.4% and 21% for each oil respectively for the tree times of treatment (Figure 2C and D). In this case, an increase in cell viability can be observed when the greater the exposure to treatment.

Synergistic effect of the essential oils and cisplatin chemotherapy was also evaluated. For the combined treatment with EO 1 and the mentioned drug a synergistic effect was observed at 48 and 72 hours of treatment (Figure 2E) and for the combined treatment with EO 2, a synergistic effect was observed at 24 and 48 hours of treatment. One of the greatest barriers to the treatment of cancer with chemotherapeutic drugs is the acquisition of drug resistance. For this reason, a strategy combination therapy of drugs aims to achieve synergistic effects and usually acts through different mechanisms to reduce the possibility of drug resistance. Studies also have shown that combinations of chemotherapeutic agents and biologic anticancer agents produce synergistic efficacy and avoid multidrug resistance - MDR (LI; ZHANG, 2016). According to the systematic review and Network meta-analyses (NMA) reported by ROSEN et al., (2017), a cisplatin-paclitaxel-bevacizumab treatment and topotecan-paclitaxel-bevacizumab have a highest probability of being efficacious and demonstrate a trend toward improved overall survival (OS) compared with carboplatin-paclitaxel and other non-bevacizumab-containing therapies.

In this study, we analyze the wound healing capacity of the tumor cells after treatment since cell migration capacity is an important characteristic for cell survival and colonization. Metastasis is the migration of cancer cells away from the primary tumor, a process called tumor invasion, and the leading cause of mortality among cancer patients (CLARK; VIGNJEVIC, 2015; HANAHAN; WEINBERG, 2011). The SiHa cells had their migration process reduced after 48 h of treatment with the essential oil of the flower and we could observe an increase in the wound width in about 7.4% when compared with the initial width (Flower 0h) (Figure 3B). For the treatment with leaf essential oil, the cell migration process appears not to be affected after 48 h. Thus, the treatment with EO 2 was able to reduce the cell migration ability into the scratch and consequently, this result indicates that this treatment can affect parameters such metastasis, cell invasion, and cell grow capacity. The ability to form solid tumors, like

cervical cancer, is also affected by the reduction of cell migration as reported by MEHTA et al., (2015).

Moreover, both essential oils of *T. ostenii* were able to significantly inhibit the adhesion process by increasing the number of viable cells in the supernatant about 2.8 times for leaf's oil and 7 times for the flower when compared to control cells (Figure 3C). It was also verified a decrease in adhered viable cells after treatment, with a reduction on cell viability of 84.3% for EO 1 treatment and 72.7% for EO 2 when compared to control cells (Figure 3D). The mechanism involving cell adhesion includes the binding of a cell to the extracellular matrix (ECM), other cells, or a specific surface, and it's essential for the growth, survival of the cell and its communication with other cells. This process requires a range of biological events such as three-dimensional re-organization of the cytoskeleton, biochemical reactions and changes in molecules on the surface of the cell (CHEN, 2011). As SiHa cells constitute an adherent cell line, the decrease in cell adhesion capacity consequently reduced the survival ability of these cells. In addition, cell adhesion to extracellular matrix proteins has been well documented as key and general determinant of cancer therapy resistance (DICKREUTER; CORDES, 2017).

Another important factor to be evaluated in tumor cells undergoing treatment is the ability of these cells to form colonies. In order to evaluate the clonogenic ability after treatment clonogenic assay was performed. This assay consists of an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony (FRANKEN et al., 2006). This methodology enables an assessment of the differences in reproductive viability between untreated cells and cells that have undergone various treatments such as exposure to ionizing radiation and various chemical compounds but can also be used to determine the effectiveness of other cytotoxic agents (RAFEHI et al., 2011). The treatment with EO 1 and EO 2 after 24 h reduced markedly the colony forming capacity in 88.7% and 90% respectively, when compared to the control (Figure 4C). The capacity of colony formation is essential to cells to grow and expand in a tumor microenvironment. It is well documented that a reduction of clonogenic capacity is related to a decrease in tumor growth and cancer progression (TOOMEH et al., 2018; ZHANG et al., 2016).

The cell washout assay is very informative since it provides insight into how long the effects of treatment are sustained after its withdrawal. Our results suggest that EO 1 and EO 2 have long-lasting inhibitory activity in tumor cells because only 6.4% of the treated cells with EO 1 and 22.1% with EO 2 were able to recover the viability even after the removal of the treatment in relation to control cells (Figure 4D). These data indicate that the *in vitro* inhibitory activity of both essential oils is durable, probably by regulating specific genes in tumor cells. A prolonged phenotypic response following washout may be due to a higher level of target vulnerability or in addition to long residence time, drug rebinding and/or accumulation of drug in the cell or membrane. In addition, prolonged drug effects might also be due to the slow repair of essential processes that were reversibly damaged by drug treatment (TONGE, 2017).

The investigation of cell death mechanism is pivotal since the type of cell death can influence in the tissue homeostasis and tumor microenvironment. The process of programmed cell death, or apoptosis, is generally characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms. Apoptosis is an organized cell death determines by cell signals that guarantee the integrity of cell membrane and phagocytosis of the death cell (ELMORE, 2007). It is known that this mechanism of cell death may be disrupted in tumor cells, conferring a survival advantage. Correspondingly, a major mode of resistance to antitumor treatments may be insensitivity to apoptosis induction (FISHER, 1994). Some of the mechanisms involved in early apoptotic cell clearance are also involved in the phagocytosis of late apoptotic and even necrotic cells (Poon et al., 2010). For example, the phosphatidylserine externalization on early apoptotic cells was shown recently to promote the ability of macrophages to recognize and phagocytosis late apoptotic and necrotic cells (KRYSKO; D'HERDE; VANDENABEELE, 2006; KRYSKO; VANDENABEELE, 2008). In our study, we can observe that both essential oils tested induced a cell death through late apoptosis in most of the tumor cells studied, at all times of treatments (Figure 5 and Table 2).

In conclusion, the treatment with the essential oils of leaves and flowers of *T. ostenii* were able to significantly inhibit many biological properties in cervical cancer cells, such as cell viability and adhesion ability, migration, clonogenic ability and recovery capacity after withdrawal of treatment. These parameters are important for

the growth of cells derived from solid tumors and reflect the influence of these treatments on metastasis, cancer progression and cell invasion processes.

These findings bring new data on the chemical constitution and the anti-tumor potential of essential oils of *T. ostenii* and highlight the importance of further studies involving plant-derived active for the development of new approaches to cancer treatment.

Conflicts of interest statement

All authors have declared that they have no conflict of interests regarding the publication of this article.

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FIGURES

FIGURE 1

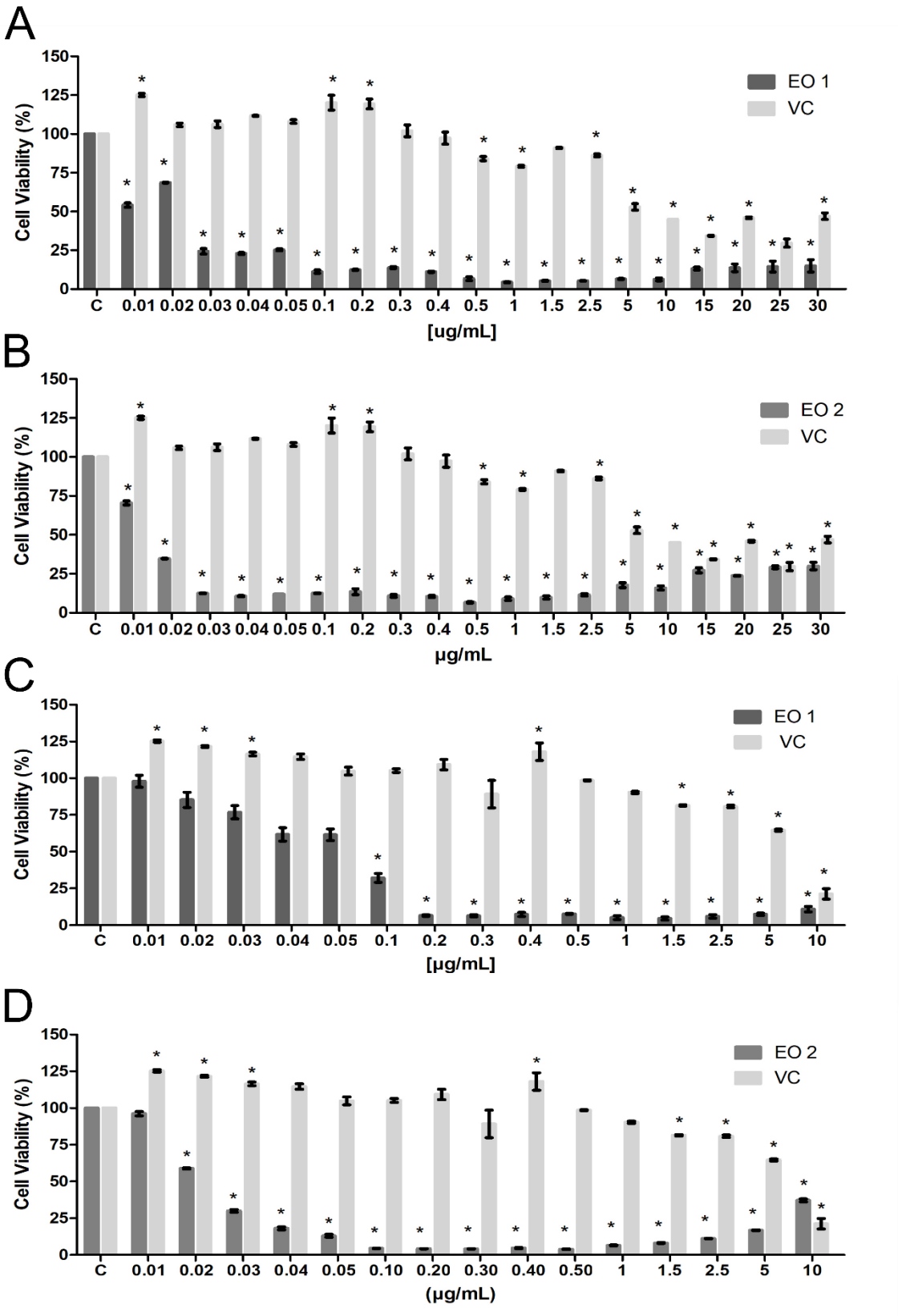


FIGURE 2

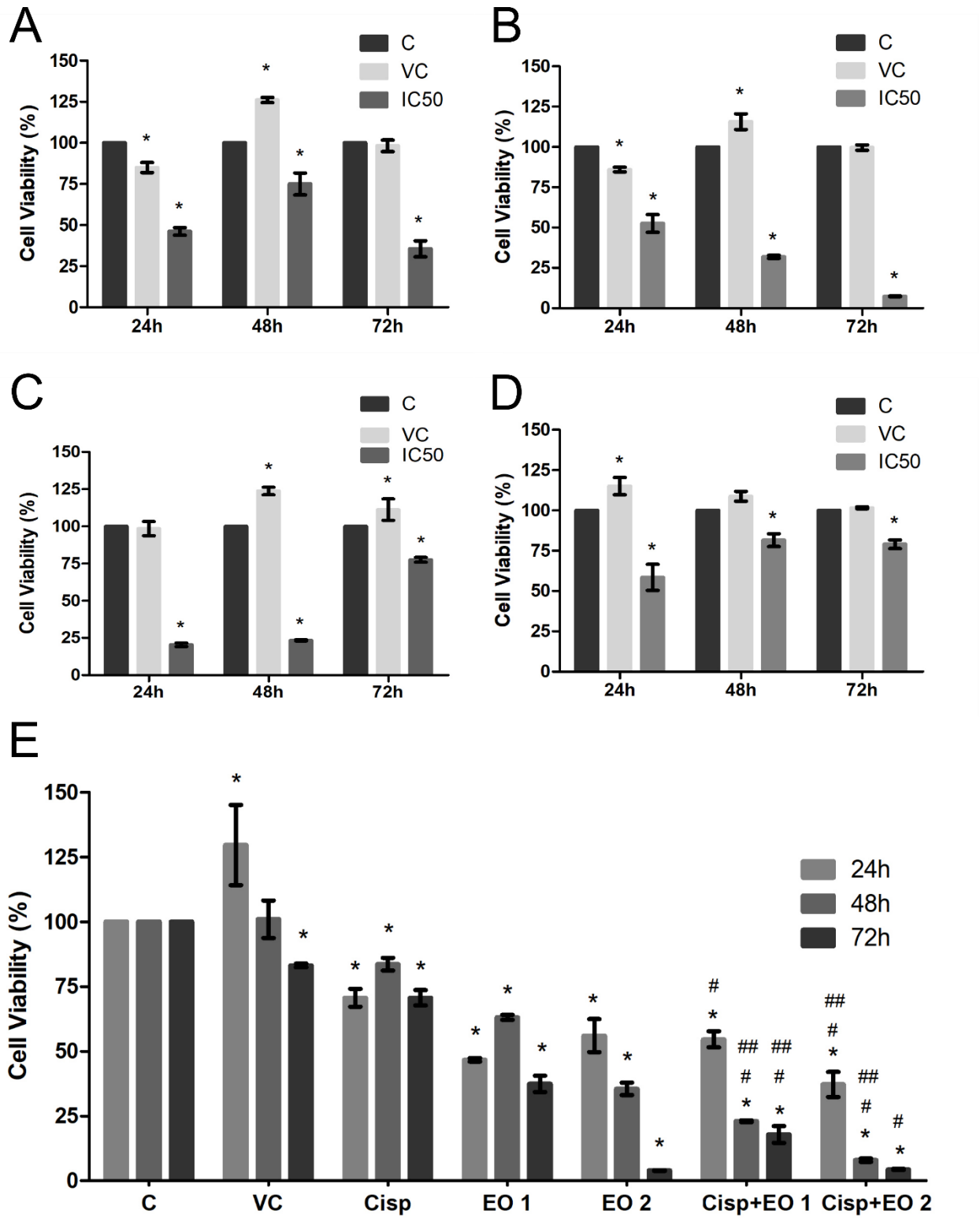

A
B
C
D
E
C
VC
IC50
Cell Viability (%)
150
125
100
75
50
25
0
24h
48h
72h
24h
48h
72h
C
VC
Cisp
EO 1
EO 2
Cisp+EO 1
Cisp+EO 2

FIGURE 3

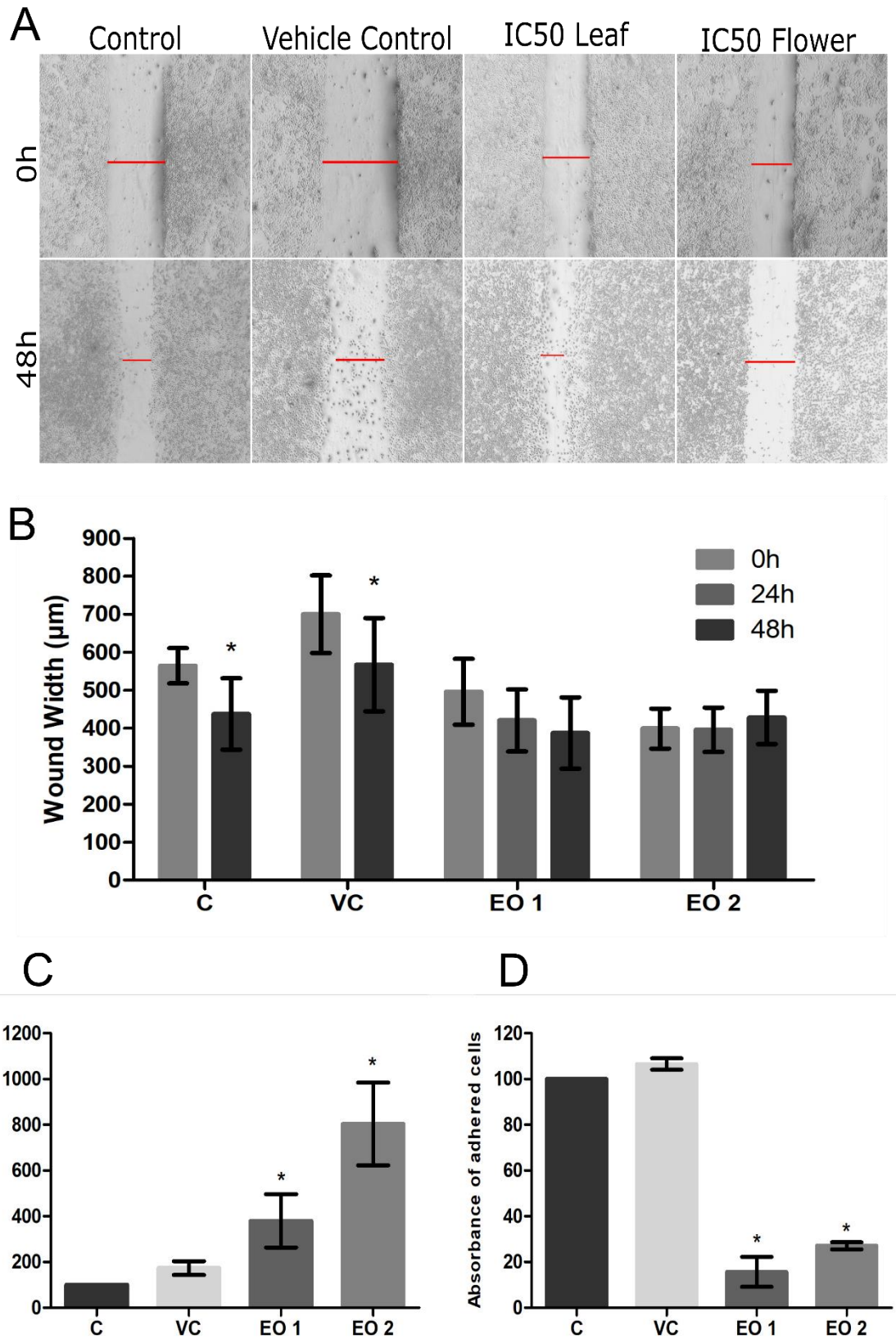

A
Control
Vehicle Control
IC50 Leaf
IC50 Flower
0h
48h
B
Wound Width (µm)
900
800
700
600
500
400
300
200
100
0
0h
24h
48h
*
*
C
VC
EO 1
EO 2
C
D
Number of Unadhered Viable Cells
1200
1000
800
600
400
200
0
*
*
C
VC
EO 1
EO 2
Absorbance of adhered cells
120
100
80
60
40
20
0
*
*
C
VC
EO 1
EO 2

FIGURE 4

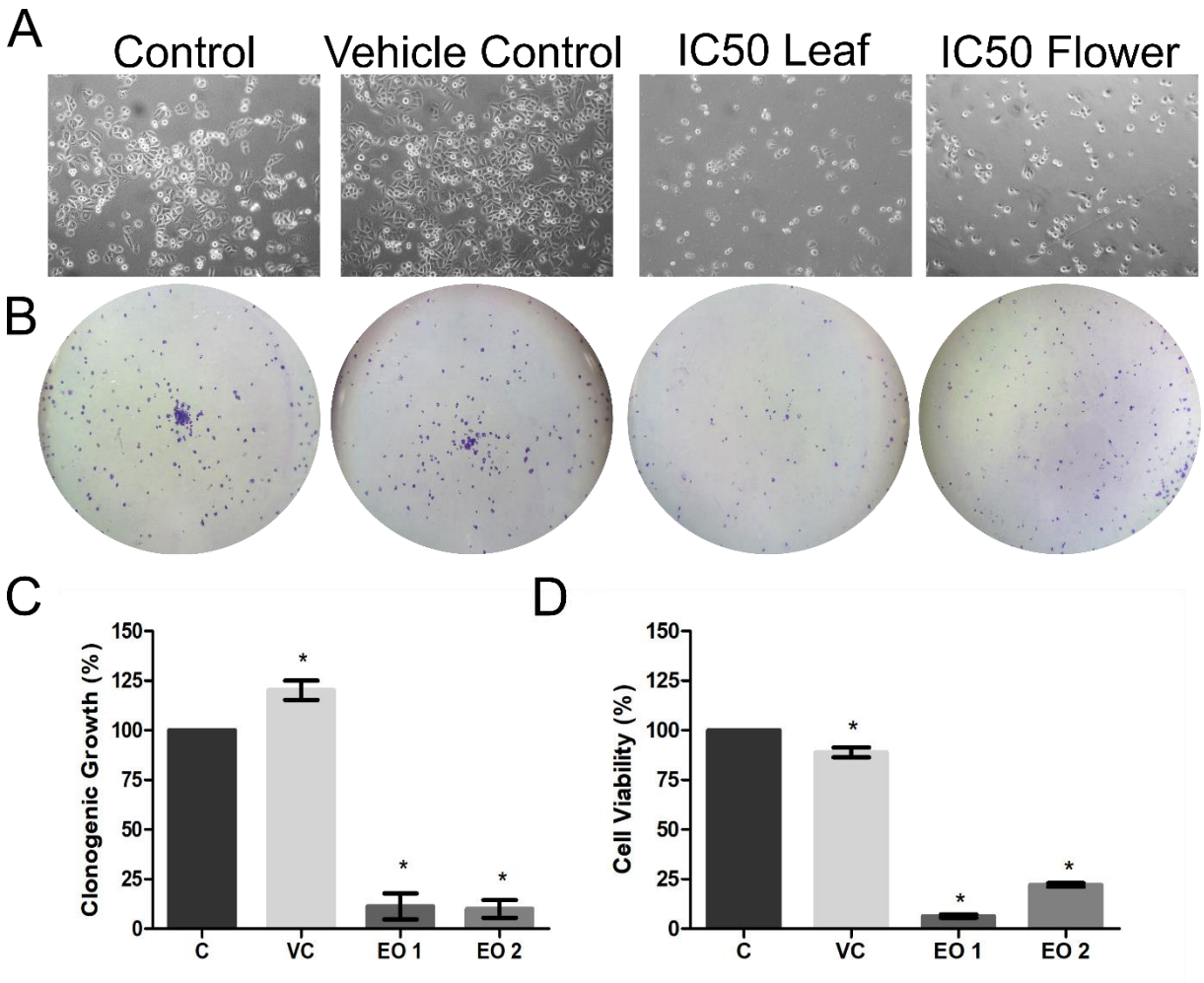
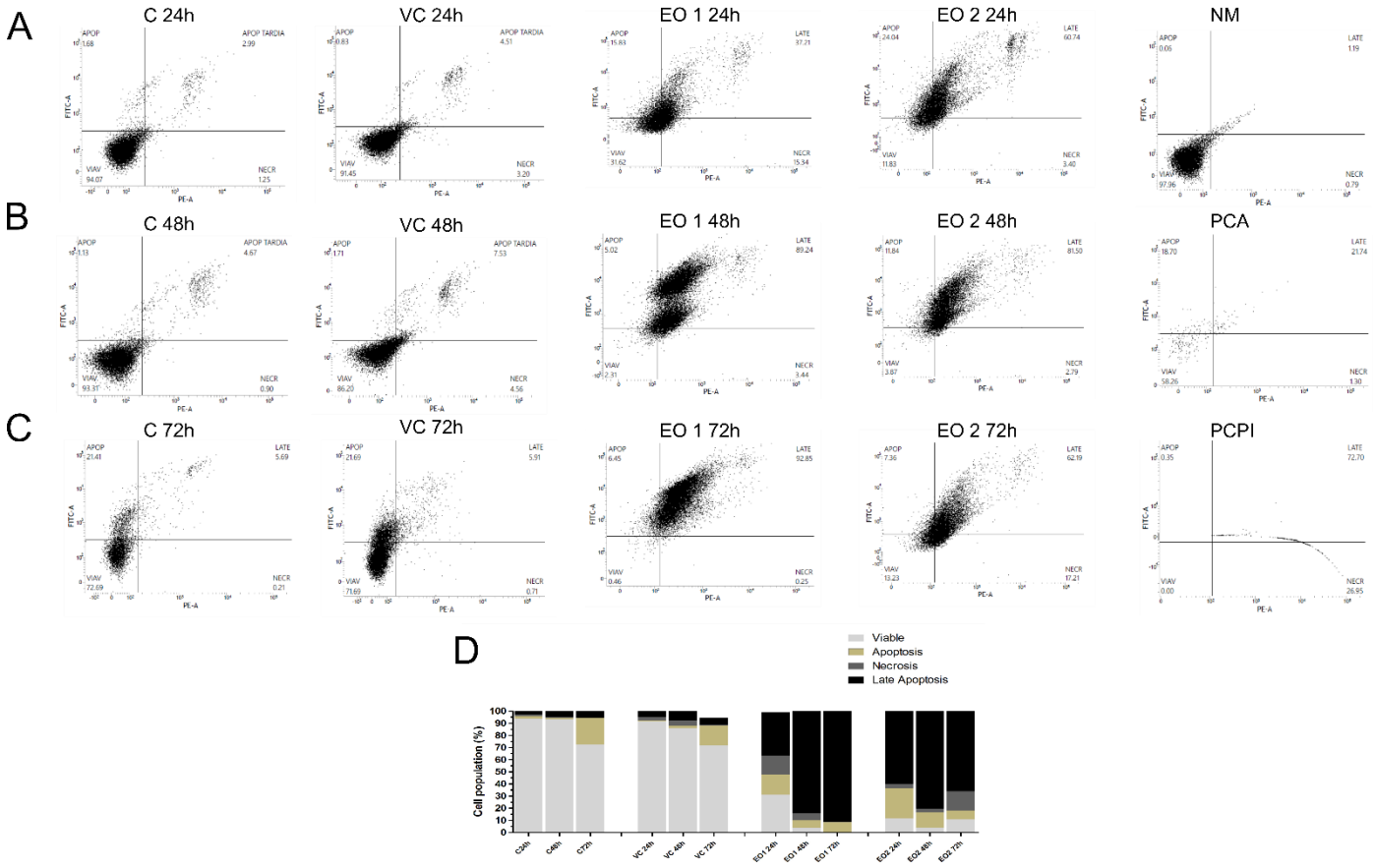

A
Control
Vehicle Control
IC50 Leaf
IC50 Flower
B
C
Clonogenic Growth (%)
150
125
100
75
50
25
0
*
*
*
C
VC
EO 1
EO 2
D
Cell Viability (%)
150
125
100
75
50
25
0
*
*
*
C
VC
EO 1
EO 2

FIGURE 5


A
C 24h
VC 24h
EO 1 24h
EO 2 24h
NM
B
C 48h
VC 48h
EO 1 48h
EO 2 48h
PCA
C
C 72h
VC 72h
EO 1 72h
EO 2 72h
PCPI
D
Viable
Apoptosis
Necrosis
Late Apoptosis
Cell population (%)
C24h
C48h
C72h
VC 24h
VC 48h
VC 72h
EO1 24h
EO1 48h
EO1 72h
EO2 24h
EO2 48h
EO2 72h

TABLES

TABLE 1

		Leaves				Flowers			
RI	**Compound**	**0h**	**24h**	**48h**	**1 week**	**0h**	**24h**	**48h**	**1 week**
Monoterpenes hydrocarbons									
1026	limonene	3.0	3.1	3.7	3.4	2.4	2.4	2.8	5.1
1036	(Z)-β-ocimene	4.1	4.1	4.4	3.4	26.1	26.1	28.1	56.3
Oxygenated monoterpenes									
1051	dihydro-tagetone	64.2	64.2	62.8	65.3	4.2	4.3	7.4	-
1144	(E)-tagetone	3.9	3.5	4.5	4.2	1.5	1.5	1.9	-
1152	(Z)-tagetone	15.9	15.9	17.2	14.9	4.6	4.6	8.3	-
1230	(Z)-ocimenone	-	-	-	-	17.5	17.6	18.7	26.8
1239	(E)-ocimenone	-	-	-	-	39.9	39.9	31.6	11.8
Sesquiterpenes hydrocarbons									
1478	biciclogermacrene	1.4	1.4	0.9	0.7	2.4	2.4	1.3	-
1504	δ-cadinene	1.3	1.3	1.2	1.3				-
1565	copaen-4-α-ol	0.3	0.3	0.5	0.3				-
Oxygenated sesquiterpenes									
1572	Spathulenol	2.9			0.9				-
1581	epi-globulol	0.3	0.3	0.3					-
1631	epi-α-cubenol		2.4	1.7	1.2				-
1644	cubenol	3.6	3.6	2.7	3.5				-

RI, retention index; percentage of peak area relative to total peak area; Compounds are listed in the order of elution on DB5 column.

TABLE 2

	Viable (%)			**Apoptosis (%)**			**Necrosis (%)**			**Late Apoptosis (%)**		
	24h	**48h**	**72h**	**24h**	**48h**	**72h**	**24h**	**48h**	**72h**	**24h**	**48h**	**72h**
C	94.07	93.31	72.69	1.68	1.13	21.41	1.25	0.9	0.21	2.99	4.67	5.69
VC	91.45	86.2	71.69	0.83	1.71	16.35	3.2	4.56	0.71	4.51	7.53	5.91
EO 1	31.16	3.83	0.39	16.36	6.15	21.69	15.58	5.97	0.27	35.9	84.04	91.28
EO 2	11.9	4.03	10.59	24.73	12.7	7.47	3.47	2.96	16.02	59.9	80.3	65.92

LEGENS OF FIGURES AND TABLES

FIGURE 1

Effect of treatment with different concentrations of *T. ostenii's* leaves (EO 1) and flowers (EO 2) essentials oils. Cell viability of SiHa cell line after 24 h of treatment with EO 1 **(A)** and EO 2 **(B)**. Cell viability of HaCat cell line after 24 h of treatment with EO 1 **(C)** and EO 2 **(D)**. Data show mean and standard deviation of two independent experiments performed in duplicate. * P <0.05 = significant in relation to control (C). (One-way ANOVA followed by Tukey's test).

FIGURE 2

Effect of treatment with IC50 concentration of *T. ostenii's* leaves (EO 1) and flowers (EO 2) essentials oils. Cell viability of SiHa cell line after 24, 48 and 72 h of treatment with EO 1 (72 ng/mL) **(A)** and EO 2 (83 ng/mL) **(B)**. Cell viability of HaCat cell line after 24, 48 and 72h of treatment with EO 1 (72 ng/mL) **(C)** and EO 2 (83 ng/mL) **(D)**. Data show mean and standard deviation of two independent experiments performed in duplicate. * P <0.05 = significant in relation to control (C). (One-way ANOVA followed by Tukey's test)**.** **(E)** Cell viability of SiHa cell line after treatment with EO 1, EO 2 and cisplatin (cisp.) (80 µM), alone or in combination after 24, 48 and 72 h. Data show mean and standard deviation of two independent experiments performed in duplicate. *P < 0.05: significant in relation to control. $^{\#}$P < 0.05: significant in relation to cisplatin alone (Cisp.). $^{\#\#}$ P < 0.05: significant in relation to essential oil alone (EO 1 or EO 2). (One-way ANOVA followed by Tukey's test).

FIGURE 3

Effect of treatment with IC50 concentration of *T. ostenii's* leaves (EO 1) and flowers (EO 2) essentials oils on cell migration and adhesion capacity. Representative pictures **(A)** and measurement of the wound width **(B)** before and after 24 and 48 hours of treatment with *T. ostenii* essential oils at IC50 concentration (OE 1 = 72 ng/mL and OE 2 = 83 ng/mL). Data show mean and standard deviation of two independent experiments performed in duplicate. * P <0.05: significant in relation to time 0 h. Effect of treatment with IC50 concentration (OE 1 = 72 ng/mL and OE 2 = 83 ng/mL) of *T. ostenii* essential oils on the adhesion capacity of SiHa cells for 8 hours. Results were expressed as the number of viable treated cells which were in the supernatant **(C)** and the percentage of cells that could adhere to the plate **(D)**. Data show mean and standard deviation of two independent experiments performed in

duplicate. * P <0.05 = significant in relation to control (C). (One-way ANOVA followed by Tukey's test).

FIGURE 4

Effect of treatment with IC50 concentration of *T. ostenii's* leaves (EO 1) and flowers (EO 2) essentials oils on clonogenic ability and cell viability recovery capacity. Representative pictures of the cells during treatment **(A);** the colonies formed **(B)**; and measurement of the colonies formed **(C)** after 24 hours of treatment with *T. ostenii* essential oils at IC50 concentration (OE 1 = 72 ng/mL and OE 2 = 83 ng/mL) in SiHa cells. Data show mean and standard deviation of two independent experiments performed in triplicate. * P <0.05 = significant in relation to control (C). (One-way ANOVA followed by Tukey's test). **(D)** Analysis of the cell viability through the washout assay after 24 hours of treatment with *T. ostenii* essential oils at IC50 concentration. Data show mean and standard deviation of two independent experiments performed in triplicate. * P <0.05 = significant in relation to control (C). (One-way ANOVA followed by Tukey's test).

FIGURE 5

Flow cytometry analysis of the cell death mechanism by the labeling with annexin V / propidium iodide in SiHa cells treated with *T. ostenii* essential oils at IC50 concentration (OE 1 = 72 ng/mL and OE 2 = 83 ng/mL) after 24 **(A)**, 48 **(B)** and 72 h **(C)**. Graphical abstract of results from cell death analysis **(D).** NM = not marked; PCA = positive control Anexin; PCPI = positive propidium iodide.

TABLE 1

Chemical composition of essential oil obtained from leaves and flowers of *Tagetes ostenii* by hydrodistillation and analyzed in four different days from the extraction moment.

TABLE 2

Percentage of cells from Flow cytometry analysis of the cell death mechanism by the labeling with annexin V / propidium iodide in SiHa cells treated with *T. ostenii* essential oils at IC50 concentration (OE 1 = 72 ng/mL and OE 2 = 83 ng/mL) after 24, 48 and 72 h. C = control and VC = vehicle control.