EVALUATION OF THE ANTITUMOR ACTIVITY OF THE *MELALEUCA ALTERNIFOLIA* ESSENTIAL OIL USING AN *IN VITRO* BREAST CANCER CELL MODEL

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Introduction

The longevity of the human population has been increasing worldwide in the last decades, especially due to an increase in human's quality of life, medical and scientific advances, and decreased rates of human mortality, which directly reflect on life's expectancy. However, human ageing brings a considerable increase in the number of chronic degenerative and chronic non-transmissible diseases, such as cancer, which has a high incidence among elderly people. Among elderly women, the most incident cancer is breast cancer (BENZ & YAU, 2008).

In fact, breast cancer is the most incident neoplasia and the second major cancer-related death cause among women worldwide, after non-melanoma skin cancer. Global statistics show an estimated number of 1.7 million new breast cancer cases in 2012, with a cancer-related death rate of over 500 thousand cases worldwide for the same year (IARC, 2016). Breast cancer tumor progression is classified into four different stages, from 0 to IV. In stage III, cancer cells can spread through the mammary and adjacent tissues, leading to development of Locally Advanced Breast Cancer (LABC). LABC can evolve from swelling and redness of the mammary tissue to the formation of exposed lesions and wounds (CHIA et al., 2008).

Although there are many therapeutic strategies for LABC treatment, patients' survival rates are still very low and topical treatments are still less explored. In this sense, the search for new therapeutic agents is still indispensable. *Melaleuca alternifolia* is an Australian native plant but it is widely cultivated in the world because of its essential oil, known as tea tree oil (TTO). TTO is topically used in folk medicine for several treatments, such as acne vulgaris and wounds related to herpes virus. Nevertheless, several scientific evidences have suggested that TTO presents biological properties, such as antimicrobial, antifungal, anti-inflammatory, and antitumor activity, especially against melanoma cancer cell lines (KOH et al., 2002; HALCÓN & MILKUS, 2004; KIM et al., 2004; CARSON et al., 2006; HAMMER et al., 2006). Moreover, TTO has also been suggest to

reduce skin inflammation and improve healing processes (KOH et al., 2002; HALCÓN & MILKUS, 2004).

Although there are many investigations assessing the effects of TTO or several TTO isolated components, especially on melanoma tumors, most of these studies were restricted to skin cancer cells due to the toxicity of its bioactive compounds (IRELAND et al., 2012). Taking into account the above mentioned complications related to LABC, solutions are still needed to address this health issue including exploratory studies of new antitumor therapeutic alternatives, based in natural products such as TTO, with potential topical activity directed for subjects with LABC. In this context, the aim of this study was to evaluate the antitumor activity of the *Melaleuca alternifolia* essential oil using an *in vitro* model of breast cancer.

Methodology

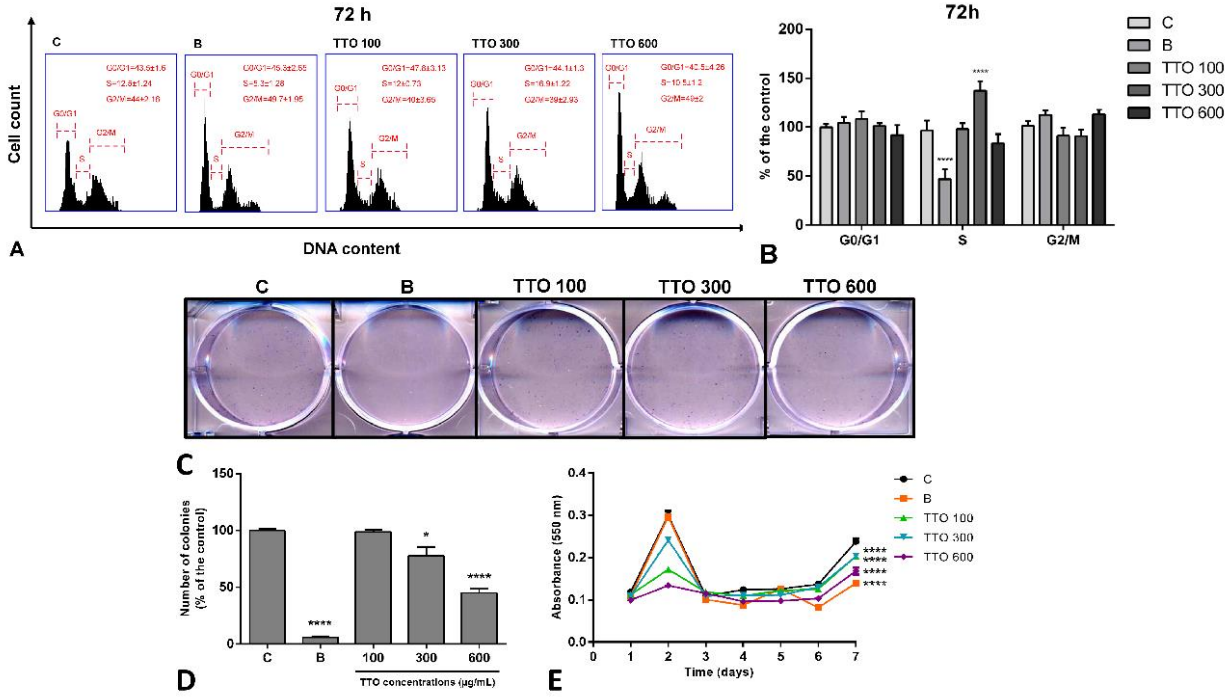
An *in vitro* study was perform using the MCF-7 (ATCC® HTB-22™) breast cancer cell line, purchased from the *American Type Culture Collection* (ATCC, Manassas, VA, USA). Cells were cultivated in *Dulbecco's Modified Eagle Medium* (DMEM) with fetal bovine serum (FBS) to a final concentration of 10% and supplemented with 1% of antibiotics (penicillin/streptomycin) in a humidified incubator with 5% CO_2 until the ideal number of cells was reached to perform all experiments.

Cells were treated with different concentrations of TTO to investigate its antitumor effect (100, 300 and 600 µg/mL). Bleomycin was maintained as positive control of chemotherapeutic agent; untreated cells were maintained as negative control. Cell cycle modulation was performed using flow cytometry analysis with Propidium Iodide (PI) staining reagent following manufacturer's instructions. Colony formation assay and cell growth curve analysis were performed following a similar method to that described previously by Cubillos-Rojas et al. (2014). Apoptosis detection, using flow cytometry analysis, and gene expression, by qRT-PCR analysis, were performed using a similar approach to that described by Cadoná et al. (2017). For gene expression, the *β-actin* gene was used to normalize gene expression. The following sequences of forward and reverse primers were used: *β-actin forward* [TGTGGATCAGCAAGCAGGAGTA] and *reverse* [TGCGCAAGTTAGGTTTTGTCA]; *p53 forward* [TTGGGTCTTTGAACCCTTGCT] and *reverse* [GTGCAGGCCAACTTGTTCAGT]; *Bax forward* [CCCTTTTCTACTTTGCCAGCAA] and *reverse* [CCCGGAGGAAGTCCAATGT]; *Bcl-2 forward* [GAGGATTGTGGCCTTCTTTGAGT] and *reverse* [AGTCATCCACAGGGCGATGT].

Data were statistically analyzed using *GraphPad Prism* software version 6. The results were compared by one- or two-way analysis of variance followed by Dunnett's *post hoc* test. All experiments were conducted in triplicate. Data with $p<0.05$ were considered significant.

Results and Discussion

Figure 1. Cell cycle analysis (A and B), colony formation assay (C and D) and cell growth curve analysis of MCF-7 breast cancer cells treated with 100, 300 e 600 µg/mL of *Melaleuca alternifolia* essential oil (Tea Tree Oil, TTO). *C: Control, B: Bleomycin, TTO: Tea tree oil.*

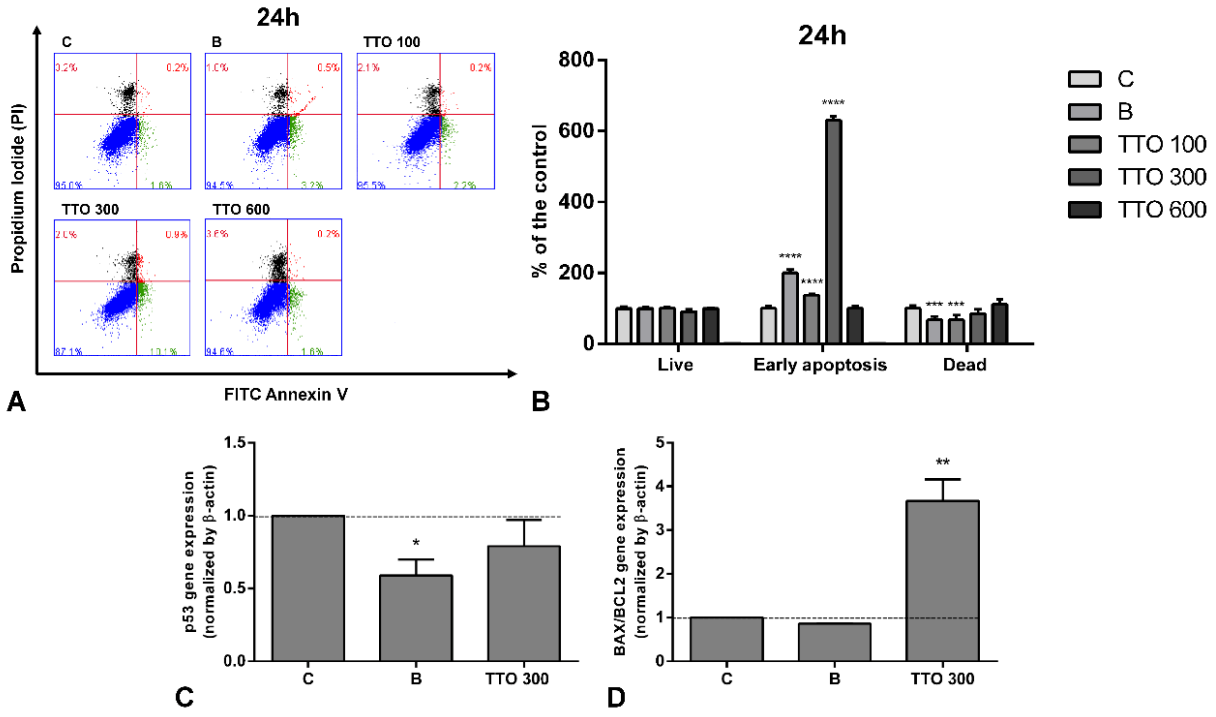


Cell cycle modulation analysis (Figure 1, A and B) revealed a significant increase in the S phase of the cell cycle for cells treated with TTO at the concentration of 300 µg/mL, demonstrating that the oil was able to induce cell cycle arresting. Bleomycin significantly decreased the number of cells in the S phase of the cell cycle. The antitumor effects of bleomycin are related to its capacity of binding to iron and oxygen to produce reactive oxygen species (ROS) that can induce single- and double-strand DNA breaks (CHEN et al., 2008). Some of the main common cellular responses to bleomycin treatment are apoptosis, mitotic cell death and cell cycle arresting (CLOOS et al., 2002).

The colony formation assay (Figure 1, C and D) and cell growth curve (Figure 1, E) displayed an antiproliferative capacity of the essential oil. Bleomycin and the TTO concentrations

of 300 e 600 µg/mL were able to significantly reduce colony formation after 10 days of treatment. For the cell growth curve analysis, all the TTO tested concentrations and bleomycin were able to significantly reduce cell growth after 7 days of treatment.

Figure 2. Flow cytometry (A and B) and gene expression (C and D) analysis of MCF-7 breast cancer cells treated with 100, 300 e 600 µg/mL of *Melaleuca alternifolia* essential oil (Tea Tree Oil, TTO). *C: Control, B: Bleomycin, TTO: Tea tree oil.*



Flow cytometry (Figure 2, A and B) results showed that bleomycin and TTO at low concentrations increased the number of cells in the early stages of apoptosis. Further, gene expression analysis (Figure 2, C and D) were performed with the best TTO concentration. The analysis exhibited that bleomycin significantly decreased the expression of the *TP53* gene, whereas TTO 300 µg/mL significantly increased the expression of the *BAX/BCL2* gene ratio, suggesting some possible antiapoptotic mechanism due to the expression of genes related to programmed cell death.

Conclusion

The results suggest that the *M. alternifolia* essential oil has antitumor activity on breast cancer cells of the MCF-7 cell line. In this sense, the oil could be used for the development of new therapeutic agents, mainly topic for LABC, in order to increase the survival rate and patients' quality of life.

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