Niobium pentachloride as a stoichiometric reagent in the sequential transesterification-esterification reactions for the synthesis of methyl salicylate and the study of its antimicrobial activity.

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Abstract: Methyl Salicylate (MS), the principal constituent of Wintergreen oil (WO) was obtained from acetyl salicylic acid (ASA) by sequential transesterification-esterification reaction promoted by NbCl5for the first time. The reagents were added simultaneously, and the reaction process involved the transesterification and esterification reactions, which were accompanied by thin layer chromatography and gas chromatography. The conversion rate via GC was 100%, and the MS yield was 94%. A cytotoxicity of 50% and 64% for cultured *S. aureus* and metastatic melanoma cells, respectively, was observed for a concentration of 0.6 mg/mL, whereas no cytotoxicity for non-tumor cells was observed for this concentration, and it is considered to be the optimum concentration.

Keywords: Wintergreen oil; niobium pentachloride; sequential reactions; antimicrobial activity; cytotoxicity; *S. aureus*

1. Introduction

Gaultheria procumbens L (Wintergreen) is a small ericaceous plant cultivated for use in the landscape industry, and it is the source of the essential oil from wintergreen (WO) [1,2]. WO is obtained commercially by steam distillation; however, the most commonly used form of WO is synthetic. Wintergreen oil is now commonly used as a flavoring agent, but its leaves were historically used by North American natives for the treatment of aches

 doi:10.20944/preprints202305.0545.v1

and pains because of their analgesic activity. In fact, MS, the most common salicylate in commercial wintergreen preparations, is routinely used in topical ointments for the treatment of inflammation [2]. It has been demonstrated that some plants produce salicylic acid (SA) as a response to the infection by tobacco mosaic virus (TMV) [3]. Neighboring plants also develop resistance to TMV because the infected plants convert the SA to MS, which, being more volatile, is released into the air and signals the neighboring plants to increase their resistance to TMV.

Oloyede [4] demonstrated the antimicrobial activity of the essential oil from *Laportea aestuans* (Gaud). The principal constituents in the oil were MS (54.50%), fenchol (10.59%), 1,2-cyclohexanedione dioxime (9.40%), 1,4-octadiene (8.86%) and linalool (3.26%). The oil exhibited activity against *Escherichia coli, Staphylococcus aureus (S. aureus), Bacillus subtilis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhi, Candida albicans, Rhizopus stolon, Aspergillus niger and Penicillium nonatum*.

MS can be ingested from various sources, including chewing gum, baked goods, syrups, candy, beverages, ice cream, and tobacco products. A review of the literature regarding the toxicity of MS when ingested orally arrived at an allowable daily intake of 11 mg/kg/d [5]. Vlachojannis et al. [6] explored the antimicrobial activity of Listerine, which is a popular mouthwash that contains MS as one of its components. In the past century, its recipe was changed from an essential oil mouthwash to a five-component mixture (thymol, menthol, eucalyptol, and methyl salicylate dissolved in 27% ethanol). They studied the antimicrobial activities of individual Listerine® components and their mixtures. They tested activity against the bacterial strains *Streptococcus mutans, Enterococcus faecalis, and Eikenella corrodens* and the yeast *Candida albicans.* The established minimum inhibitory concentration (MIC) and the minimum bactericidal/fungicidal concentration (MBC/MFC) assays were applied. None of the combinations of two phenols at the concentrations contained in Listerine® were associated with either an additive or synergistic effect. The same degree of activity was observed against the yeast with thymol as with Listerine. A combination of three of the phenols also exhibited the same activity against the bacteria as Listerine. Similar studies have been performed by other authors [7-10].

Essien et al. [11] studied the antimicrobial activity of volatile constituents from fresh fruits of *Alchornea cordifolia* and *Canthium subcordatum. A. cordifolia* oil contained 25.3% methyl salicylate, whereas the oil from *Canthium subcordatum* contained only 4.5%. A potent in vitro antibacterial activity against *Staphylococcus aureus* (MIC = 78 μg/mL) and marginal antifungal activity against *Aspergillus niger* (MIC = 156 μg/mL) were observed for the essential oil from *A. cordifolia.* Antibacterial activity against *Bacillus cereus and S. aureus* (MIC = 156 μg/mL) and notable antifungal activity against *A. niger* (MIC = 39 μg/mL) were observed for the essential oil from *C. subcordatum.* However, no appreciable cytotoxic effects on human breast carcinoma cells (Hs 578T) and human prostate carcinoma cells (PC-3) were observed for either essential oil.

Esters are produced by a large number of processes. such as heating carboxylic acids with alcohol in the presence of an acid catalyst. In the Fischer or Fischer-Speier Esterification, the Lewis or Brønstedt acid (*p*-TsOH, H_2SO_4) catalyzed esterification of carboxylic acids with alcohols to give esters is a typical reaction in which the products and reactants are in equilibrium [12]. The equilibrium may be influenced by either removing one product from the reaction mixture (for example, removal of the water by azeotropic distillation or absorption by molecular sieves) or by employing an excess of one reactant.

Alternative reactions employ coupling reagents such as N,N′-dicyclohexylcarbodiimide (DCC or DCCD), in the Steglich Esterification, preformed esters (transesterification), carboxylic acid chlorides or anhydrides [13]. These reactions avoid the production of water. Another pathway for the production of esters is the formation of a carboxylate anion, which then reacts as a nucleophile with an electrophile. Esters may also be produced by oxidations, namely by the Baeyer-Villiger oxidation and oxidative esterifications.

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Many other examples of ester synthesis can be mentioned [14-23]. Barbosa et al. [24] demonstrated that $NbCl_5$ and Al_2O_3 catalyzed the esterification and etherification of alcohols under microwave radiation. Gryglewicz [25] also demonstrated that alkaline earth metal compounds act as catalysts for the alcoholysis of oils in the production of esters. Liu et al. [26] observed that red mud, which is an alkaline residue containing various metal oxides. is effective for the production of biodiesel from oils. *C.* Mazzocchia et al. [27] obtained fatty acid methyl esters from triglycerides using heterogeneous catalysis under microwave radiation. However, among all these examples, very little has been reported regarding the use of $NbCl_5$ for promoting the esterification or transesterification reactions.

The aim of the present research work was to use pure methyl salicylate (MS) synthesized in this laboratory for the evaluation of the cytotoxic potential in metastatic melanoma cells, a bacterial culture of *S. aureus,* and in non-tumoral cells of fibroblasts.

2. Experimental

2.1. Raw materials and chemicals

All the reagents (analytical grade), including commercial MS, MeOH and ASA (ASA standard), were supplied by Vetec, São Paulo, Brazil and were used without further purification. Aspirin was donated by a local pharmacy. Dulbecco's Modified Eagle Medium (DMEM) (Gibco) was prepared in deionized water, buffered with sodium bicarbonate (Synth, Brazil) and supplemented with fetal bovine serum (FBS) (Vitrocell Embriolife). Streptomycin, ampicillin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and dimethylsulfoxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mueller Hinton Broth Medium (MHB) (Kasvi) was prepared in deionized water. $NbCl_5$ was donated by Companhia Brasileira de Metalurgia e Mineração.

2.2. Instrumentation

MS content and yield were determined with a GC/MS-QP 2010/AOC 5000 AUTO INJECTOR/Shimadzu Gas Chromatograph/Mass Spectrometer equipped with a 30 m Agilent J&W GC DB-5 MS column. Direct insertion spectra were measured at 70 eV. Quantitative analyses were performed on a Shimadzu GC-2010 gas chromatograph equipped with a flame ionization detector [28]. ^{1}H- and ^{13}C-NMR spectra were recorded on Bruker *Avance* 400 Spectrometers as has been previously described [28]. All the reactions were monitored by TLC using Silica Gel 60 F 254 on aluminum. The chromatograms were visualized by UV light or by using the ethanolic vanillin developing agent [28]. The purification of the products was achieved by flash column chromatography using a mixture of hexane/ethyl acetate in a 9/1 proportion as the eluent [28]. The MW reactions were performed in 10 mL G-10 vials of an Anton Paar single-mode MW Monowave 300 synthesis reactor, powered by an 850 W magnetron, and equipped with temperature sensor and magnetic stirring [28].

2.3. Typical procedures

2.3.1. Extraction from ASA from Commercial Aspirin Tablets

The crushed aspirin tablets are mixed with MeOH to dissolve the aspirin [29] and insoluble material was removed by filtering through a funnel containing activated charcoal, and the MeOH was eliminated with the help of a rotary evaporator to yield pure ASA.

2.4. Sequential transesterification and esterification of ASA in MeOH using $NbCl_5$ as mediator.

Typically, 1.0000 g of Al_2O_3 was added to a 100 mL one-necked round bottom flask containing AAS acid (4.6852 g; 26.0 mmol) and MeOH (0.5895 g; 18.4 mmol, 2.8:1 mol ratio); to this mixture it was added 0.2700 g $NbCl_5$ (5% mol/mol of $NbCl_5$ in relation to the alcohol). The uncorked flask was immediately heated with the help of a heating mantle for 60 min at 60°C, after which the flask was allowed to cool to room temperature and the reaction components checked by TLC. The reaction mixture was filtered, the solids

 doi:10.20944/preprints202305.0545.v1

washed with chloroform (2 x 10 mL). The combined organic extracts were washed with 10.0 mL of a saturated solution of $NaHCO_3$, dried over anhydrous Na_2SO4 and concentrated under reduced pressure. The resulting residue was subjected to GC/MS analysis, which demonstrated the absence of unreacted SA. The residue was then purified by flash column chromatography on silica gel using hexane: ethyl acetate (9:1) as the mobile phase to yield MS (94%) as a colorless oil.

2.5. Biological activity

2.5.1. Cytotoxicity Test

Mouse Fibroblast L929 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) FBS, sodium bicarbonate (2 g/L), streptomycin (0.1 g/L), and ampicillin (0.025 g/L). Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2. L929 cells were seeded into 96-well polypropylene plates (10^4 cells/well) and incubated for 24 h. The culture medium was removed and replaced with a fresh culture medium containing the various samples. The cells were treated with 0.025 – 3.6 mg/mL of WO. After treatment, the cells were incubated for 24 h. The cells were washed with 100 µL of fresh PBS. The cell viability was evaluated by incubating the cells with 100 µL of an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (0.5 mg/mL) for 3 h. Thereafter, the MTT solution was replaced by DMSO to dissolve the formazan crystals. The final absorbance of formazan was measured in a microplate reader (Synergy H1-Biotek) at 540 nm. The absorbances of cells incubated without samples were considered to be 100% of viability. The cell viability data were analyzed by one-way ANOVA and Tukey's multiple range method ($p < 0.05$) to determine statistical differences between different groups of samples. To evaluate the interaction of WO with the L929 cells by scanning electron microscopy (SEM), L929 cells were seeded on glass slides (10^4 cells/well) and incubated for 24 h. The culture medium was removed and replaced by a fresh culture medium containing the different samples. The cells were treated with 0.600 mg/mL of WO. After treatment, the cells were incubated for 24 h. The glass slides were placed inside Petri dishes and 500 µL of methanol were added to each glass slide and left to rest for 1 h. The liquid was removed and subsequent addition/removal cycles of the ethanol/water solution at increasing ethanol concentrations were performed. Aliquots of 500 µL of solutions containing from 10% to 90% ethanol were added and left to rest on each glass slide for 20 min. Then, 500 µL of absolute ethanol was added to each glass slide and the slide was left to rest for 1 h. After removing the ethanol, the glass slides were dried at room temperature inside the Petri dishes. The slides were sputter coated with gold for 90 s at 20 mA (Sputtering Quorum Q150R ES) before being imaged by SEM (FEI Inspect S50, Hillsboro, OR USA), available at NAPCEM-UNIFESP.

2.5.2. Antitumoral Activity

Murine melanoma B16F10-Nex2 cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS, sodium bicarbonate (2 g/L), streptomycin (0.1 g/L), and ampicillin (0.025 g/L). The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. B16F10-Nex2 cells were seeded into 96-well polypropylene plates (10^4 cells/well) and incubated for 24 h, following the same protocol described for the cytotoxicity test. The interaction between WO and B16F10-Nex2 cells was also evaluated by scanning electron microscopy (SEM), following the same protocol described for the cytotoxicity test.

2.5.3. Antibacterial Test

The antimicrobial activity was evaluated by using a modified NCCLS broth microdilution method [30]. Antimicrobial activity was monitored using a liquid growth inhibition assay against *S. aureus* (ATCC 6538). The pre-inoculum of the strain was prepared in MHB (Mueller Hinton Broth Medium) for approximately 12 h at 37 °C. The inoculum was standardized as 10^6 cells/mL by measuring the absorbance at 630 nm and plated into 96-well polypropylene plates. Bacterial cells were treated with 0.025 – 3.6 mg/mL of WO. After

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treatment, the cells were incubated for 24 h. The inhibition of bacterial growth was evaluated by measuring the absorbance at 600 nm after 24 h (Synergy H1-Biotek). The *S. aureus* grown in MHB in the absence of any samples was used as a negative control.

3. Results and Discussion

3.1. Synthesis of MS using $NbCl_5$ as stoichiometric reagent.

Recently, we reported that MS or wintergreen oil (WO) from ASA was obtained in excellent yield (94%), using 20% w/w of the catalyst SiO_2-SO_3H, with a surface area of 115 m^2/g, a pore volume of 0.38 cm^3g^{-1} and 1.32 mmol H^+/g in a tandem transesterification-esterification reaction process promoted in a microwave reactor [28]. In other studies, we explored the use of $NbCl_5$ in esterification reactions. This time, we are using $NbCl_5/Al_2O_3$ in the synthesis of the benzyl ester [24] and graphite-coated niobium on silica in the synthesis of phenyl esters [31]. We studied the sequential reaction process because nucleophilic reagents act differently in transesterification and esterification reactions in the synthesis of MS in this new and innovative reaction process (Fig. 1), where the reactivity of the high valence transition metal niobium with ASA was investigated.

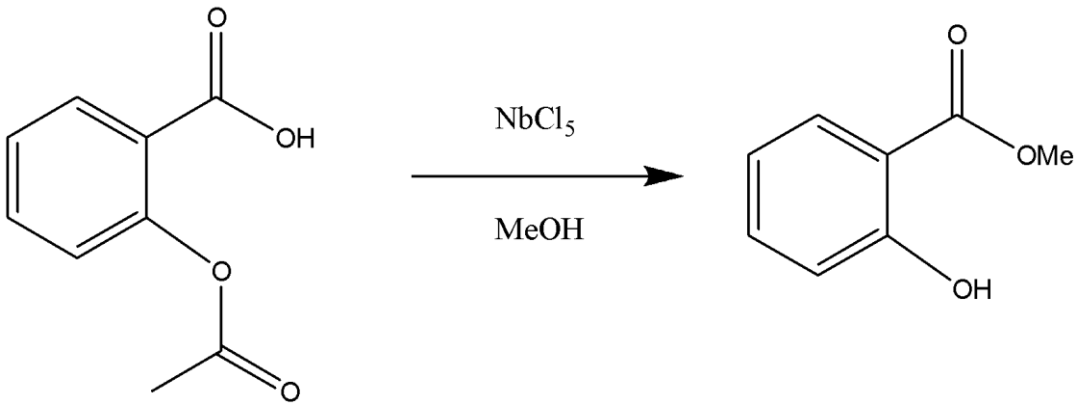

Figure 1. Synthesis of MS from ASA using $NbCl_5$ as a stoichiometric reagent.

This study involved two reaction processes (transesterification of ASA and esterification of AS reactions). The activity of $Nb(OMe)_5$, a metallic precursor previously formed from $NbCl_5$ and MeOH, in transesterification of ASA (acetyl transesterification; 10 min at room temperature) was excellent. With regard to the esterification reaction involving $NbCl_5$ and regarding the chemistry of niobium alkoxides, many coordination complexes have been identified with monocarboxylic acids, and they are related to different niobium nuclearities. Loiseau et al. describe the formation of a variety of Niobium(V) Polyoxo clusters, obtained from the reaction with aromatic monocarboxylic acids [32]. The use of benzoic acid results in a tetranuclear core $Nb_4(\mu_2\text{-}O)_4(L)_4(OEt)_8]$ (L=benzoate (1)) with four Nb. Brown, Wallbridge and Alcock, in a work entitled "Preparation of some oxoniobium carboxylates. X-Ray crystal and molecular structure of $[\{NbCl_3(O_2CPh)\}_2O]$" [34], obtained the dimer $[Nb_2Cl_6(O_2C\text{-}C_6H_4OH)_2O]$, where the additional μ_2-oxo group linked the two adjacent niobium centers. In the current work, we believe that the formation of the niobium oxo clusters occur in the esterification reaction or that types of niobium-centered coordination complexes have been formed from niobium pentachloride in the reaction process involving ASA, MeOH and $NbCl_5$. Recent works described similar synthetic pathways with $NbCl_5$ combined with derivatives of benzoic acids [33-35]. We believe that the esterification reaction gave rise to the formation of molecular dinuclear complexes, in which the niobium centers are bridged through the carboxylate arm of the monotopic acetyl salicylate molecules [niobium (V) polyoxo clusters; Fig. 2].

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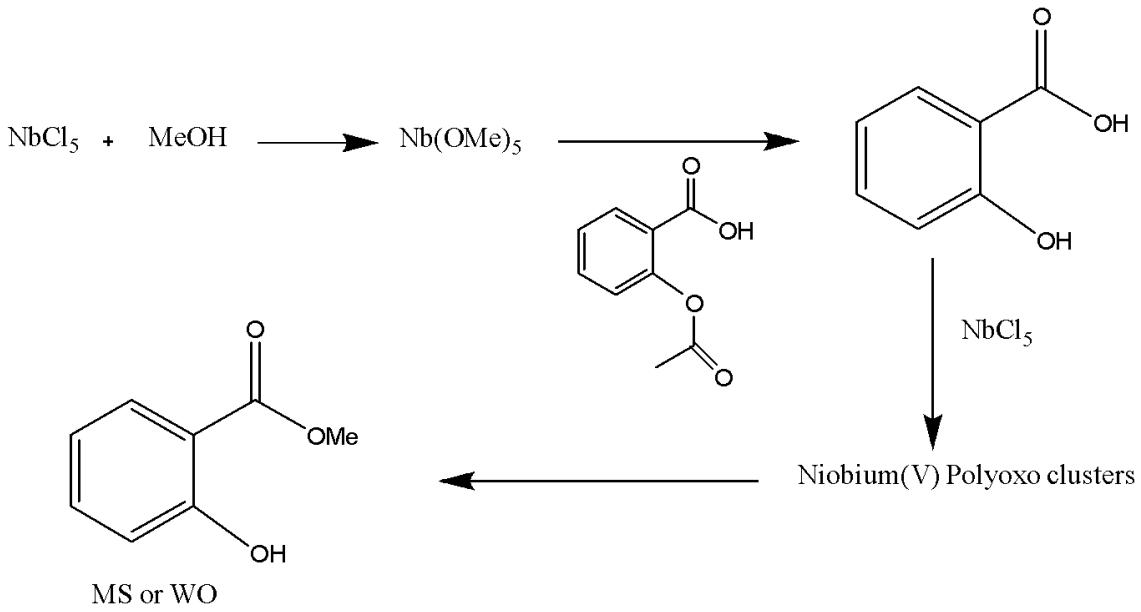

Figure 2. Scheme of synthesis MS.

Addition of MeOH to the initial solution led to the exchange of some chloride anions with methoxy (OMe) groups, which stabilized this specific configuration without any central bridging μ_2-oxo group. Initial niobium alkoxides play a key role in the synthesis of niobium (V) carboxylate coordination complexes in the esterification process that is induced by the alkoxy groups (leaving as alcohols) and the carboxylic groups, which result in the production of water molecules and the creation of μ_2-oxo-bridges between niobium (V) atoms.

According to Loiseau et al. the formation of the niobium oxo clusters in the esterification reaction induces the release of water molecules that further react with niobium atoms through oxolation in different Nb_2O, Nb_4O_4 and Nb_8O_{12} nuclearities [32]. The formation of these oxo clusters is fundamental for the transesterification-esterification reactions (ASA to MS) to occur at room temperature without the need of a solvent to remove the water by distillation as an azeotrope. This observation is due to the fact that the reactions involved in the process reported herein do not involve the traditional Fischer equilibrium.

3.2. Study of the biological activity

A decrease in bacterial growth was observed when *S. aureus* cells were subjected to different concentrations of WO, up to a value of 50% using a concentration of 0.6 mg/mL (Fig. 3). Above this concentration, no significant difference in the decrease in bacterial growth below 50% relative to the optimal concentration of 0.6 mg/mL was observed.

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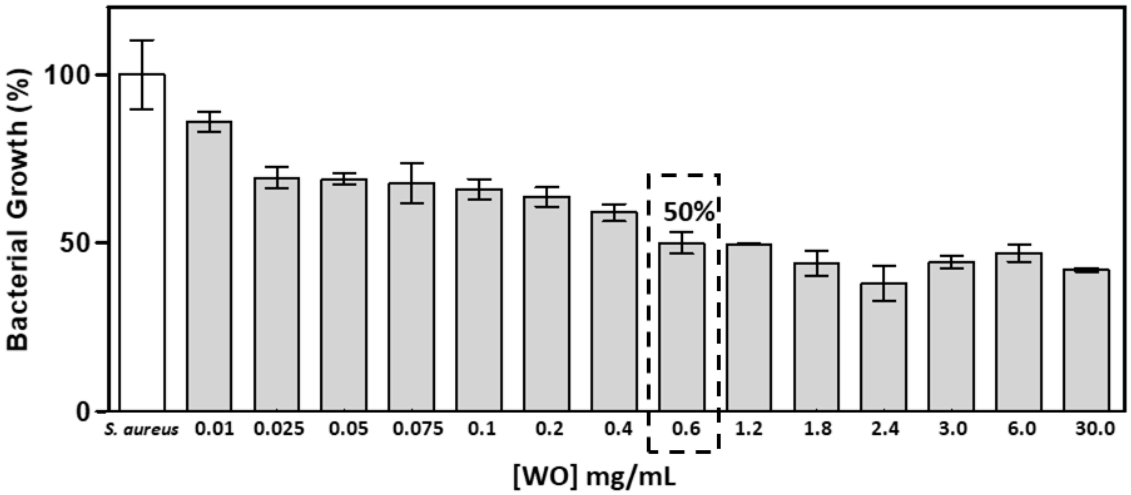

Figure 3. Antimicrobial activity of WO against *S. aureus*.

After observing the antimicrobial effect at concentrations equal to or greater than 0.6 mg/mL of WO, the cytotoxic effect of different concentrations of WO in L929 fibroblast cells (non-tumor) was studied. No cytotoxic effect was observed at concentrations of 0.025 to 0.6 mg/mL of WO, and despite observing a reduction in cell viability, no cytotoxicity at concentrations greater than 0.6 mg/mL (Fig. 4) was observed.

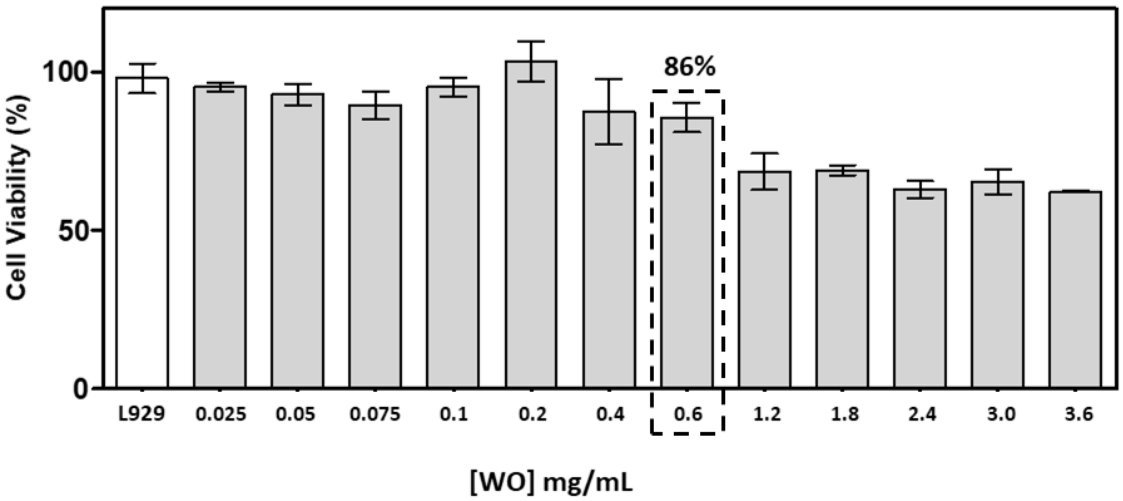

Figure 4. Cytotoxicity of WO in L929 cells.

Once the non-cytotoxic concentration for fibroblast cells was defined and the antimicrobial activity against *S. aureus* was defined, the cytotoxicity of WO in metastatic melanoma cells at the optimal concentration of 0.6 mg/mL was determined. A cell viability of 64% compared to the control without the presence of WO (Fig. 5) was observed. At higher concentrations, no significant difference was observed in the reduction of cell viability compared to the optimal concentration.

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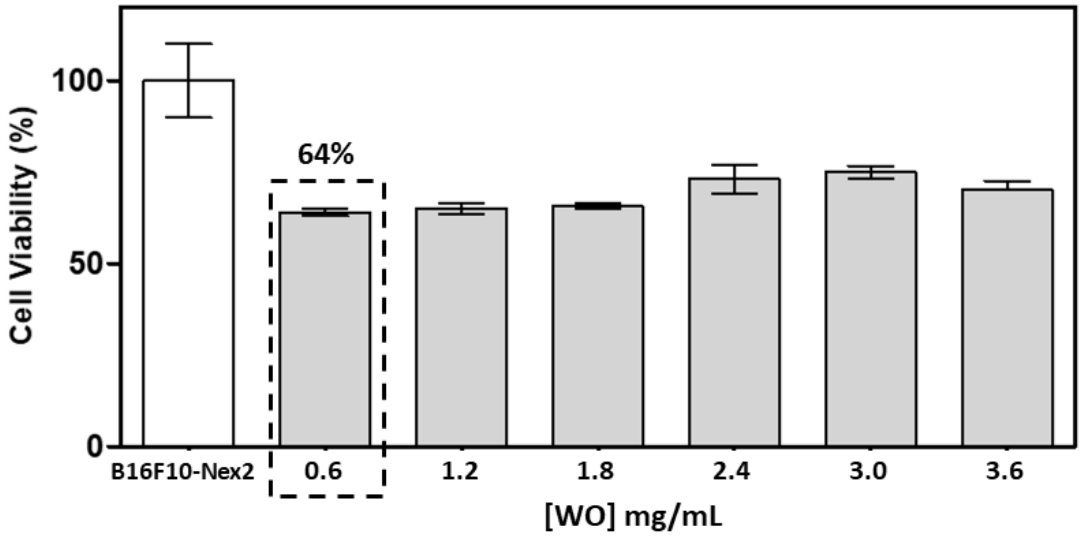

Figure 5. Cytotoxicity of WO in B16F10-Nex2 cells.

4. Conclusions

The use of $NbCl_5$ as a reagent demonstrated that the transesterification and esterification reactions occur at different times, and the transesterification reaction proved to be faster. This reaction process is called sequential because MeOH does not act as the nucleophile in either reaction, but the reactions involve intermediate complexes with niobium. The reaction conditions were mild, and the yield obtained was close to 100%. The concentration of 0.6 mg/mL of WO was considered to be cytotoxic for tumor cells. Lower cell viability was observed in cultured metastatic melanoma cells; and no cytotoxicity for non-tumor fibroblast cells was observed at the same concentration. The synthetic WO successfully obtained in this work by the transesterification reaction using $NbCl_5$ has a potential for antimicrobial and possibly antitumoral applications in the absence of cytotoxicity for non-tumor cells. More studies regarding these activities are desirable.

5. Acknowledgments

The authors acknowledge the support by the SENAI CIMATEC and PRPPG/UFVJM in response to Resolution 15/2019.

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