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# Chemical composition and antifungal and anticancer activities of extracts and essential oils of *Schinus terebinthifolius* Raddi fruit

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## Abstract

In this study, we isolated and characterized dichloromethane and hexane extracts, complexed with hydroxypropyl-β-cyclodextrin (HP-β-CD), of *Schinus terebinthifolius* fruits. Such complexation may be useful in the formulation of herbal medicines, by improving solubility, or increasing stability by reducing the loss of volatile compounds. The cytotoxicity in osteoblasts, antifungal against *Malassezia furfur* and antitumor activities were evaluated in Caco-2 cells. The antifungal activity of the extracts and the essential oil *in natura* or complexed with HP-β-CD against the fungus *M. furfur* was evaluated by agar diffusion methods and quantitatively by inhibitory concentration (IC). All samples inhibited fungus growth and, when complexed with HP-β-CD, the reduced IC was by 50%. Osteoblast cell viability was not affected by the presence of extracts and oil. The antitumor activity of the extract in dichloromethane was evaluated using Caco-2 cells. The results demonstrated significant reduction in cell viability in the presence of the extract, which makes it a promising candidate for cancer treatment.

**Keywords:** *Schinus terebinthifolius*, antifungal, antitumor, cyclodextrin, *Malassezia furfur*.

## Introduction

Brazil has some of the best prospects for economic exploration of biodiversity, with the largest number, estimated between 10 and 20%, of animal and plant species in the world. About 25,000 plant species used worldwide for the production of medicines, including those obtained by synthesis from natural products, as well as herbal medicines (1). The fruits of *Schinus terebinthifolius* Raddi, known as “aroeira-vermelha”, used as a condiment (2). However, besides food use, several parts of the plant have been subject of drug development research due to pharmacological properties such as antimicrobial, anti-inflammatory and antioxidant.

Fungal infections are more prominent due to the increased number of immune compromised are patients, who are more likely to get infections. Accordingly, antimicrobial resistance considered a major public health problem [3]. The genus *Malassezia* comprises yeasts associated with various pathological conditions, including infections such as tinea versicolor, or dermatoses such as seborrhea dermatitis and atopic dermatitis. The most commonly used antifungal agents in the treatment of these infections are triazoles, such as ketoconazole, itraconazole and fluconazole. However, there are *Malassezia* strains resistant to these drugs. Besides, resistance is not the only problem; many of these products are cosmetically unpleasant, while others requires strict regular application, and are associated with recurrences of ringworm and other various adverse effects to the patient [4].

In addition to the antifungal activity associated with active substances from *S. terebinthifolius*, there are some reports about their mutagenic activity in cancer cells [5-6]. Although cancer incidence is growing worldwide, anticancer drugs currently available have several limitations. Most drugs act on both cancer and healthy cells throughout of the organism, causing a series of adverse effects, such as leukopenia, renal failure, and liver, kidney, and lung toxicity. Another major problem in anticancer therapy is acquired drug resistance [7]. The increasing number of infections and the emergence of microorganisms resistant to existing drugs, as well as low specificity and efficiency, make the search for new antifungal and antitumor agents important in improving the therapeutic arsenal.

Complexing plant extracts with cyclodextrins can be useful in the formulation of herbal medicines, by improving solubility, facilitating the incorporation of the extracts, or increasing stability by reducing the loss of volatile compounds [8,9,10]. The cyclodextrins and their derivatives can bind a wide variety of molecules, resulting in improved bioavailability, stability and solubility. The aim of this study was to evaluate the antitumor and antifungal activity of extracts and essential oil from *Schinus terebinthifolius* fruits, isolated or associated/included in hydroxypropyl-β-cyclodextrin (HP-β-CD), and to determine the chemical composition of the extracts and essential oil.

## Materials and methods

### Materials

Hydroxypropyl-β-cyclodextrin was purchased from Cerestar (Milwaukee, WI, USA). Nystatin (NYS) disks (100 IU) were purchased from CECON (São Paulo, Brasil). Dulbecco's phosphate buffered saline, trypsin-ethylene diamine tetraacetic acid (EDTA), fetal bovine serum (FBS) and the 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) kit were purchased from Gibco (NY, USA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit was purchased from Invitrogen (USA). Sodium dodecyl sulfate (SDS)-10% HCl was purchased from American Bioanalytical (Natick, MA, USA). Dulbecco's Modified Eagle Medium (DMEM) for cell culture and Neutral Red solution were purchased from Sigma (St. Louis, MO, USA). High Glucose DMEM was purchased from LGC (São Paulo, Brasil). *Malassezia furfur* (ATCC 14522) was obtained from the American Type Culture Collection (Manassas, VA, USA). The human intestinal Caco-2 cell line was obtained from the American Type Culture Collection (CCL-1 NCTC clone 929). All other materials and solvents were of analytical grade.

**Plant materials and preparation of the crude extracts**

The dried ripe fruits of *S. terebinthifolius* were crushed in order to obtain smaller particles (almost a powder), whereby 100 g were macerated sequentially with 500 mL of hexane and dichloromethane. Each extraction was performed for 7 days at room temperature. All extracts filtered through filter paper and concentrated under vacuum using a rotary evaporator. The extracts produced yields of 5.5% (hexane) and 7.6% (dichloromethane). The essential fruit oil, obtained through the technique of steam distillation, was purchased commercially (LAZLO).

**Microencapsulation process**

Preparations containing HP-β-CD, the essential oil and extracts were made by freeze-drying. Ratios of 5:95 w/w HP-β-CD and the samples were dispersed on top of 0.5% Tween 80 in distilled water, agitated by magnetic stirring for 15min, frozen and lyophilized. The products obtained were oily. Analyses were recorded for cyclodextrins and supramolecular complexes in order to evaluate the complexation allowing comparison between different systems [11].

**Gas chromatography–mass spectrometry (GC–MS)**

The chemical compositions of *S. terebinthifolius* extracts obtained from different extraction solvents were analyzed using a Shimadzu 14 B Chromatograph (model QP5050A). The samples were dissolved in chloroform and injected in a BP-5 column (dimensions: 30 m x 0.25mm), at initial temperature of 50°C for 1 min and, subsequently, increasing 3°C/min to a final temperature of 200°C, with the following parameters: injector split 1/50, equipped with Flame Ionization Detector (FID) detector, an injection volume of 1 µL, and H2 flow of 2.7 mL/min. Identification of volatile components was performed by comparison of their of mass spectra to the spectra existing in the database of equipment, literature data and the Kovats Index. To determine the Kovats index, a mixture of alkanes (C10-C18) was used under the same conditions [12].

**Gas chromatography (GC)**

Identification of the volatile constituents of essential oil before and after the microencapsulation process was accomplished by GC using a HP5890 Chromatograph. The samples were dissolved in chloroform and injected in a column BP-1 (dimensions: 30m x 0.32mm), initial temperature of 50°C for 1min and subsequently gradient of 3°C/min to a final temperature of 200°C, injector split 1/50, FID detector, injection volume 1 µL and $H_2$ flow of 2.7mL/min.

**Antifungal activity determination by disk diffusion test**

To evaluate the antimicrobial potential of the extracts, antimicrobial assays were performed in triplicate using the disk diffusion test [13]. The inoculum was obtained from recent cultures of microorganisms (between 20 - 24 h at 30ºC) by preparing standardized suspensions at 0.5 McFarland scale ($10^5$ UFC/mL). The fungal suspension (0.5 mL) was added to 10mL sterile culture medium (36g/L malt extract, 36g/L peptone, 15g/L agar, 10g/L Tween 80,5g/L glycerol). Subsequently, the medium was poured in Petri dishes under a laminar flow hood. Simultaneously, 20 µL of each sample were added to 6 mm diameter paper disks. The disks containing the samples were placed on the solidified medium. In addition, disks containing the antifungal drug nystatin (100 IU) were placed on plates as a positive control. The susceptibility of the fungus was

determined after 18 h incubation at 30°C. Results were obtained by measuring the diameter of inhibition zones formed around the disks. Analysis of antifungal activity of all extracts are expressed in millimeters and analyzed by Analysis of variance (ANOVA) and post hoc Tukey test ($p < 0.05$).

**Broth Microdilution Test**

The inhibitory concentration (IC) was determined by broth micro dilution [14]. Initially, each sample was diluted two-fold with dimethyl sulfoxide (DMSO) and later held the same serial dilutions (from 31.25 to 4000 µg/mL) in appropriate medium (36g/L malt extract, 36g/L peptone, 10g/L Tween 80,5g/L glycerol) using sterile 96 well microtiter plates, and then inoculated with 100 µL *M. furfur*, including controls for growth, sterility, and DMSO. The plates were incubated at 30°C for 24h. After the incubation period, the absorbance was read in a UV-visible spectrophotometer at a wavelength of 595nm.

**Cell culture**

Osteoblasts (OB) were isolated from the calvaria of 1 - 4 days old neonatal male Wistar rats obtained from the animal facility of the Institute of Biological Science, UFMG. The calvaria was dissected and freed from the soft tissue, cut into small pieces and rinsed in sterile phosphate-buffered saline (PBS) without calcium and magnesium. The calvaria pieces were incubated with 1% trypsin-EDTA for 5 min, followed by four sequential incubations with 2% collagenase at 37ºC for 40 min each. The supernatant of the first collagenase incubation, which contains a high proportion of periosteal fibroblasts, was discarded. Subsequent digestions produced a suspension of cells with a high proportion of osteoblasts. After centrifugation at 200g for 10min, each pellet was resuspended in DMEM supplemented with 10% FBS, 1% antibiotic-antimycotic. Cells were grown in a controlled humidified incubator at 37ºC and 5% $CO_2$. After reaching confluence, cells were used for experiments on the 3$^{rd}$ passage and seeded at a density of $10^5$ cells/mL in 96 well plates.

The human intestinal Caco-2 cell line was cultured in High Glucose DMEM with 4.5g/L glucose, 2mM L-glutamine, 2.2M sodium pyruvate, 10mM HEPES buffer, 2mM non-essential amino acids, 2.0g/L sodium bicarbonate, and 100U/mL amphotericin–gentamicin containing 10% FBS. The medium was changed, and cells sub-cultured after they reached confluence, as seen under the phase contrast microscope. Single cell suspension was obtained after trypsinization, and the cells were counted in a hemacytometer (Reichert, Buffalo, NY, USA). Cells were allowed to attach to the plates for 48h in a humidified atmosphere of 5% $CO_2$ and 95% air at 37°C.

**Cellular viability and cytotoxicity assays**

The assessment of viability and cell proliferation in the presence of extracts and the essential oil was performed using the MTT and Neutral Red (NR) test. Additionally, we assessed the metabolic activity of cells through the BCIP-NBT assay to quantify alkaline phosphatase production. All quantitative results were obtained from hexaplicate samples. Data are expressed as mean ± SD. Statistical analysis was carried out using ANOVA and Bonferroni's post-test. A value of $p < 0.05$ was considered statistically significant.

Aliquots of extracts and essential oil in different concentrations were placed in contact with osteoblasts. The plates were incubated for 24h, followed by a determination of cell viability by the MTT assay. Approximately 10µL MTT (5mg/mL) was added to each well. Four hours later, cell morphology was analyzed by inverted

optical microscopy, and formazan crystals were dissolved with SDS-10% HCl. After incubation for 14h, optical density measurements were performed at 570nm.

The NR Assay was conducted on cells were plated in 96 well plates (1.5 x $10^5$cells/well). After 48h incubation, cells were treated with extracts and essential oil in different concentrations in phenol red-free medium for 24h. After treatment, the cells were rinsed with PBS, and then incubated with Neutral Red solution in serum-free DMEM (100µg/mL) at 37°C for 2h. After the incubation, fixing solution was added for 2min and the dye taken up by the cells was extracted by adding 200µL basic methanol (49% v/v methanol in water, 2% acetic glacial acid) to each well. After 15min, the plate was analyzed with the Multi Skan Spectrum (Thermo Scientific) using an excitation wavelength of 540nm and emission wavelength of 630nm. Data were reported as percent of untreated controls. The values are expressed as mean ± SD obtained from five independent experiments carried out in hexaplicate [15].

Alkaline phosphatase detection was conducted using a BCIP/NBT kit. The osteoblast cells were plated in 96 well plates (1.5 x $10^5$cells/well) and, after 48h of incubation, the cells were exposed to the test suspensions, rinsed with PBS, and then incubated with an alkaline phosphatase substrate (1% BCIP/1.5% NBT solution) at 37°C for 2h. After the incubation, SDS-10% HCl was added for overnight incubation, and then the plate was analyzed with the Multi Skan Spectrum UV-Vis spectrophotometer (Thermo Scientific) using an excitation wavelength of 595nm. Data is reported as percent of untreated controls. The values are expressed as mean ± SD obtained from five independent experiments carried out in hexaplicate [16].

## Results and discussion

### Characterization of extracts

The characterization of the dichloromethane and hexane *S. terebinthifolius* extracts were performed by GC, considering as standard the essential oil of the ripe fruits. The hexane and dichloromethane extracts and pure essential oil were characterized using GC/MS. **(TABLE) 1** shows the compounds identified in the essential oil from the *S. terebinthifolius* fruits, and the percentage of these compounds found in hexane (HE) and dichloromethane (DE) extracts.

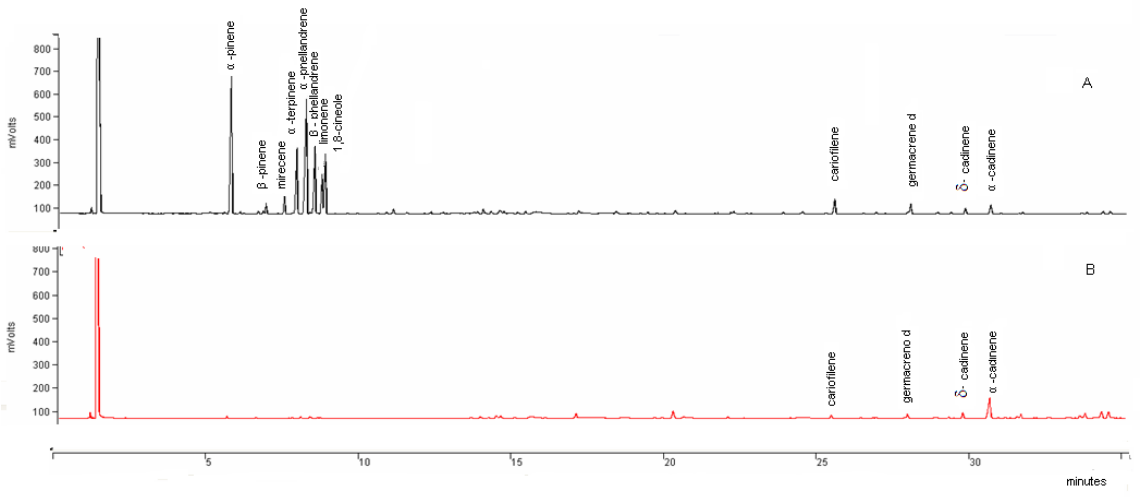
**TABLE 1:** Chemical constituents essential oil (EO), hexane extract (HE), dichloromethane extract (DE) and from Schinus terebinthifolius with the percentage (%) of content and Kovats Index (KI)*.

| Compound | % EO | % HE | % DE | KI* |
|---|---|---|---|---|
| α–pinene | **23.41** | 1.11 | 9.45 | 936 |
| Camphene | **0.28** | - | - | 956 |
| β–pinene | **1.06** | - | 3.69 | 980 |
| Myrcene | **2.08** | - | - | 984 |
| α–terpinene | **20.34** | - | 4.93 | 1022 |
| Limonene | **5.46** | - | - | 1031 |
| α–phellandrene | **9.89** | - | - | 1032 |
| 1,8-cineole | **8.22** | 3.73 | 14.07 | 1033 |
| β–phellandrene | **10.18** | 11.73 | 24.72 | 1050 |
| Terpinolene | **0.65** | - | - | 1086 |
| terpinen-4-ol | **0.26** | - | - | 1177 |
| caryophyllene | **2.43** | 11.90 | 3.61 | 1418 |

| | | | | |
|---|---|---|---|---|
| germacrene D | **1.70** | 13.66 | 4.72 | 1481 |
| δ-cadinene | **0.90** | 1.40 | 1.28 | 1536 |
| α-cadinene | **1.78** | - | - | 1552 |
| Elemol | **0.32** | - | - | 1582 |

The essential oil (EO) contained high percentages of monoterpenes. A similar composition, including a predominance of monoterpene compounds was identified [17]. The characterization of the EO: HP-β-CD microcapsules was performed as described [18] using GC. The volatile constituents of the essential oil before **(FIGURE 1-A)** and after **(FIGURE 1-B)** microencapsulation were identified by GC analysis. When the profile of volatiles of the EO: HP-β-CD microcapsules **(FIGURE 1-B)** were analyzed, several interesting aspects were observed. There was a decrease of peak areas corresponding to α-pinene, β-pinene, myrcene, α-phellandrene, α–terpinene, β-phellandrene, limonene, caryophyllene and 1,8-cineole, compared to the peaks in the free essential oil chromatogram. This decrease suggests that the expected interaction of HP-β-CD with some components of essential oil decreases their availability and consequently, the intensity of corresponding peaks in the chromatogram. On the other hand, for the EO: HP-β-CD microcapsules, an increase in the peak areas for δ-cadinene and α-cadinene was observed. In the pure oil, only α-cadinene was detected. As these aromatic compounds were already separated by enantioselective GC using HP-β-CD derivatives as the stationary phase [19].

**FIGURE 1:** Gas chromatography - mass spectrometry (GC-MS) volatile profile of *S. terebinthifolius* essential oil before (A) and after (B) microencapsulation in hydroxypropyl-β-cyclodextrin (HP-β-CD).




## Antifungal activity

The antifungal activity of essential oil and extracts of *S. terebinthifolius* was evaluated by disk diffusion **(TABLE 2)** and microdilution **(TABLE 3)**. The results obtained in microdilution test correlated with inhibition zone test (DE = 20.6mm, EO = 18.8mm, HE = 17.8mm). The most active extract was that obtained with dichloromethane, followed by the essential oil and hexane extract ($IC_{50}$ = 250, 500, or 1000µg/mL, respectively).

**TABLE 2:** Inhibition zone (mm) of hexane extract (HE), dichloromethane extract (DE) and essential oil (EO) and Nystatin (NYS) of *Schinus terebinthifolius* on *Malassezia furfur.*

| Sample | Inhibition zone (mm) |
|---|---|
| Hexane extract (HE) | 17.8 ± 0.4 |
| Dichloromethane extract (DE) | 20.6 ± 0.8 |
| Essential oil (EO) | 18.8 ± 0.8 |
| Nystatin NYS | 22.3 ± 0.5 |

**TABLE 3:** Inhibitory concentration (IC) of hexane extract (HE), dichloromethane extract (DE), essential oil (EO), hexane extract with 5% HP-β-CD (HE: HP 5%), dichloromethane extract with 5% HP-β-CD (DE: HP 5%), and the essential oil with 5% HP-β-CD (EO: HP 5%) on *Malassezia furfur.*

| Sample | $IC_{50}$ (µg/mL) | $IC_{90}$ (µg/mL) |
|---|---|---|
| HE | 1000 | 4000 |
| DE | 250 | 1000 |
| EO | 500 | 4000 |
| HE:HP 5% | 500 | 2000 |
| DE:HP 5% | 125 | 500 |
| EO:HP 5% | 250 | 2000 |

The dichloromethane extract was the best fungicide in this study, followed by the hexane extract and the essential oil. This was most likely due to the intermediary polarities of dichloromethane, which can extract higher number of intermediary polarity compounds, which may be involved in the fungal inhibition by the sample, compared to hexane which can extract mostly non-polar components. In addition, decomposition of components could occur during the distillation process due to the high experimental temperatures.

As a result, the antifungal properties may decrease slightly compared to the dichloromethane extract. These results are consistent with those obtained [20], in their study of the antifungal activity of essential oil and extracts of flowers of *Melodorum fruticosum*. In that study, the dichloromethane extract of the flowers of *M. fruticosum* also showed higher antifungal activity than the hexane extract and essential oil.

Assessing the antifungal activity of the compounds prepared with HP-β-CD, the dichloromethane extracts, essential oil and hexane extract had $IC_{50}$ of 125, 250 and 500 µg/mL, respectively. This increase in the activity can be attributed to the interaction between HP-β-CD and the components of the extracts or the essential oil, with a possible formation of an inclusion complex, as complex formation alters the physicochemical characteristics of the substrate, such as solubility and stability.

**Cellular Viability**

The cellular viability of osteoblasts in the presence of extracts and essential oil of *S. terebinthifolius* was evaluated by MTT **(FIGURE 2)** and NR **(FIGURE 3)** assays. The MTT assay showed that the essential oil (at 150, 250 and 450 µg/mL; **FIGURE 2**) did not cause reduction on cell viability significantly compared to control. Similar results were obtained with the NR **(FIGURE 3)**.

**FIGURE 2:** Percentage of cellular viability of osteoblasts in the presence of different concentrations (µg/mL) of *S. terebinthifolius* essential oil (EO), hexane (HE) and dichloromethane (DE) extracts by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT).

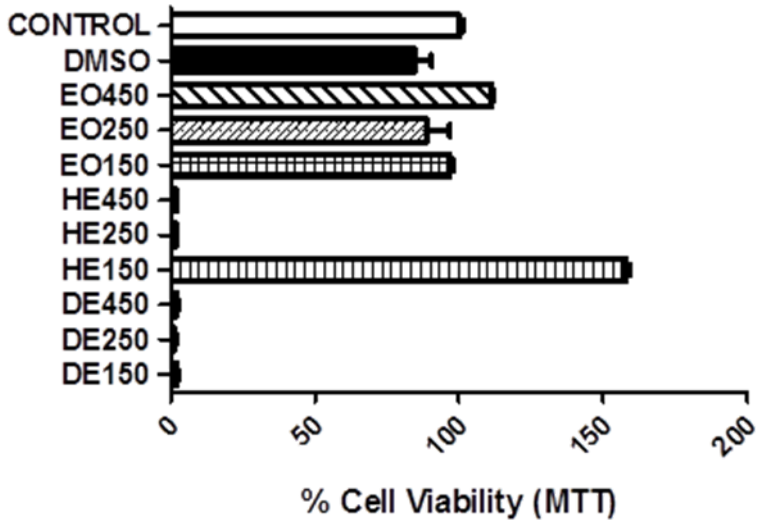
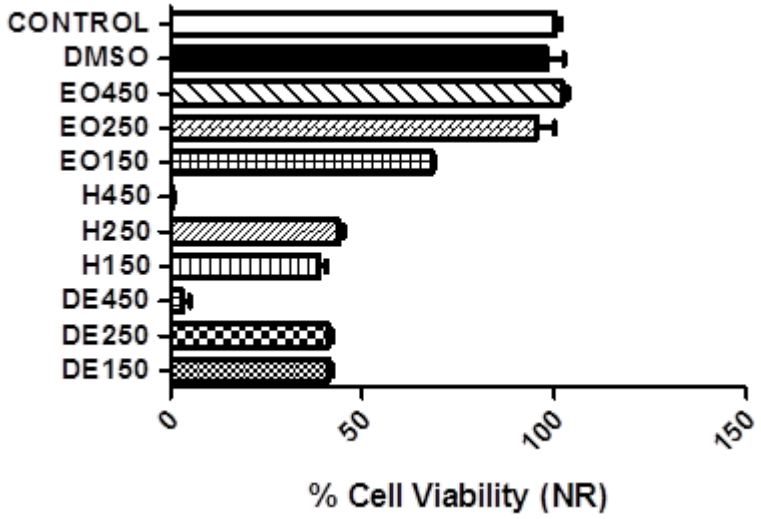
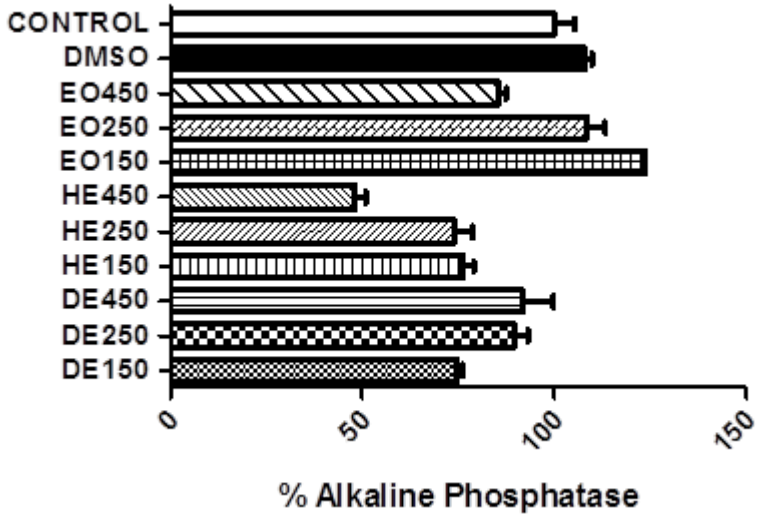



**FIGURE 3:** Percentage of cellular viability of osteoblasts by Neutral Red (NR), in the presence of different concentrations (µg/mL) of *S. terebinthifolius* essential oil (EO), hexane (HE) and dichloromethane (DE) extracts.



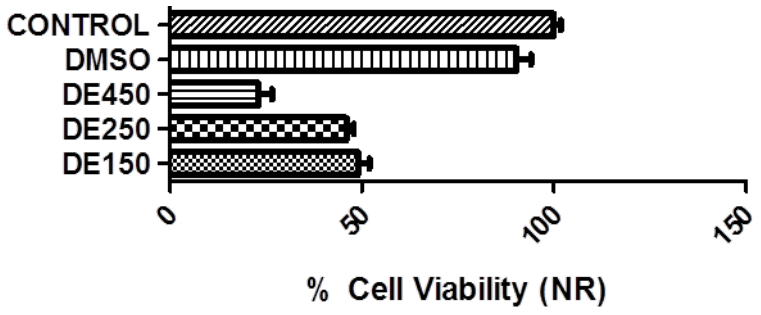

The production of alkaline phosphatase was assessed using the BCIP-NBT test **(FIGURE 4)**. This assay showing that, in the presence of essential oil, the cells remained viable and functional. For the dichloromethane and hexane extracts, there was significant reduction in cell viability, by both MTT **(FIGURE 2)** and NR **(FIGURE 3)**, at concentrations above 250µg/mL. However, data from the alkaline phosphatase assay showed that the dichloromethane extract was more compatible with cell viability than the extract prepared with hexane **(FIGURE 4)**.

**FIGURE 4:** Percentage of production of alkaline phosphatase in the presence of different concentrations (µg/mL) of *S. terebinthifolius* essential oil (EO), hexane (HE) and dichloromethane (DE) extracts.




The antitumor activity of the dichloromethane extract in Caco-2 cells was assessed using the NR assay **(FIGURE 5)**. The antiproliferative activity of the dichloromethane extract was dose-dependent. However, the extract inhibited cell growth by 50% even at the lowest concentration tested (150µg/mL). Obtained similar results with dichloromethane extract of *S. terebinthifolius* leaves with human colon carcinoma (HCT-8), melanoma (MDA-MB-435) and leukemia (HL-60) cell lines [21]. At 150µg/mL, the extract showed cytotoxicity on osteoblasts (MTT and NR assays) and did not affect cell function (alkaline phosphatase). These data indicate the non-existence of a selectivity of the cytotoxic effect shown by the dichloromethane extract, since the extract did reduce cell viability in osteoblast cultures at the same dose at which cell death was observed in the Caco-2 cells.

**FIGURE 5:** Percentage of cellular viability of Caco-2 cells in the presence of different concentrations (µg/mL) of *S. terebinthifolius* dichloromethane extract (DE) evaluated by Neutral Red (NR) method.




## Conclusions

From the analysis of the results, it can be concluded that extracts of *Schinus terebinthifolius* Raddi fruits in dichloromethane and hexane, complexed with hydroxypropyl-β-cyclodextrin (HP-β-CD), present antifungal

activity against the fungus *Malassezia furfur*. All samples inhibited their growth, and when complexed with HP-β-CD, the inhibitory concentration was reduced by 50%.

These data indicate the not existence of a selectivity of the cytotoxic effect shown by the dichloromethane extract, since the extract did reduce cell viability in osteoblast cultures at the same dose at which cell death was observed in the Caco-2 cells.

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