

Full Length Research Paper

Antifungal and cytotoxic activity of purified biocomponents as carvone, menthone, menthofuran and pulegone from *Mentha* spp.

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Medicinal plants are attractive sources in the search of bioactive compounds in the treatment of infectious diseases. Considering that the infectious agent often develops resistance to existing treatments rapidly, the searches for such compounds are a never-ending process. One such infection, caused by *Candida* spp. is candidiasis, which is a public health problem. Additionally, many strains have resistance to traditional therapies. Therefore, we tested the antifungal activity of four compounds present in *Mentha* spp. with promising antifungal precedents. We measured inhibition of growth by microdilution, disruption of biofilm viewed by electron microscopy, inhibition of germ tube formation by optical microscopy and toxicity on HaCaT cells. Tests showed that the compounds tested had antifungal activity with a minimum inhibitory concentration of 0.5 mg/mL, at least, 50% of biofilm inhibition in the 0.5 mg/mL concentration, an inhibition of polymorphism to 86% and the changes in the cell envelope of yeast (SEM) and cell viability above 50% among the *Candida* strains tested. Therefore, the compounds exhibit promising antifungal properties and provide a reasonable therapeutic window to be used in association with other traditional antimicrobial.

Key words: Carvone, menthone, menthofuran, pulegone, *Candida* spp., biofilm, cytotoxicity.

INTRODUCTION

The emergence and propagation of microorganisms resistant to traditional chemotherapies has become an important public health problem (Sprenger and Fukuda, 2016). *Candida* species also has developed strategies to evade antifungal treatment (Acker et al., 2014). Recently,

researchers have elucidated that *Candida* species have a series of mechanisms involving virulence factors that contribute to their infection, proliferation, adaptation, and long-term survival in the human body. Two factors important in virulence are (1) polymorphism, a

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morphological transition where the yeast cell forms filaments that aids in infection of the host (Jacobsen et al., 2012); and (2) biofilm formation, which reduces the susceptibility of microorganisms to various immunological and chemotherapeutic modes of action, contributing to persistent infection (Chandra and Mukherje, 2015).

In order, to combat the increase of microorganisms resistant, it becomes necessary to continually develop new classes of antifungal compounds. Plant extracts provide a promising source of molecules, due to the long history of mutual tolerance and co-regulation between plants and fungi (Saharkhiz et al., 2012). We chose to focus our attention on the essential oil of *Mentha* spp. because it has been shown to contain a number of bioactive compounds, including those that may have antimicrobial activity (Schelz et al., 2006; Mkaddem et al., 2009; Zore et al., 2011).

We evaluated carvone, menthone, menthofuran and pulegone present in *Mentha* spp. against yeasts of the genus *Candida* and how they affect growth and survival, germ tube formation and biofilm formation and maintenance. In addition, we evaluated the toxicity of these compounds on HaCaT cells.

MATERIALS AND METHODS

Planktonic anti-*Candida* assay, minimum fungicidal concentration (MFC) and inhibition of germ tube formation were performed with three independent experiments; biofilm and cytotoxicity assay were performed with two independent experiments in triplicate. All tests were performed include positive control (without compounds) and negative control (without cell suspension).

Reagent preparation

Carvone, menthone, menthofuran and pulegone (acquired from Sigma- Aldrich; the purity was adjusted to 100%) were diluted in aqueous Tween 80 (0.05%). The commercial antifungal fluconazole was diluted in dimethyl sulfoxide (DMSO).

Candida strains

Candida species standard: *Candida rugosa* (IZ 12), *Candida lusitanae* (IZ 06), *Candida glabrata* (IZ 07), *Candida utilis* (CBS 5609), *Candida krusei* (CBS 573), *Candida albicans* (ATCC 90028), *Candida albicans* (ATCC MYA-2876), *Candida guilliermondii* (CBS 566), *Candida tropicalis* (CBS 74), *C. albicans* (CBS 562), *Candida parapsilosis* (CBS 604) and *Candida dubliniensis* (CBS 7987). (CBS- Centraal bureau voor Schimmel cultures; IZ: Zimotécnico Institute-ESALQ-USP, Brazil; ATCC: American Type Culture Collection- The Global Bioresource Center. Clinical isolates obtained from oral cavity and oral prosthesis were provided by Prof. Dr. Marcelo Fabiano Gomes Boriollo: *Candida albicans* (314), *Candida albicans* (330), *Candida albicans* (335), *Candida albicans* (338), *Candida albicans* (368), *Candida albicans* (378) and *Candida albicans* (462).

Planktonic anti-*Candida* assay

The assay planktonic anti-*Candida* (Table 1) was made according

to the CLSI, 2008- M27-A3 reference method. In summary, yeast was inoculated in Sabouraud Dextrose Agar (SDA) culture medium and incubated aerobically at 37° C for 24 h. After that, was diluted to 2.5×10^3 CFU/mL in RPMI culture medium and measured at A_{530} . In microtiter plates, were made dilution series with compounds in RPMI (concentration range from 8 to 0.125 mg/mL) and added the cell. Fluconazole was tested (concentration range 64 to 0.5 µg/mL). The plates were incubated for 48 h at 37°C in aerobic conditions, followed by visual reading to evaluate cell growth. The concentrations that no showed cell growth was considered minimum inhibitory concentration (MIC).

Minimum fungicidal concentration (MFC)

The fungal viability after incubation with test compounds (concentration range 16 to 0.125 mg/mL) was performed by plating an aliquot of the MIC plate well contents to SDA plates and incubating at 37°C for 48 h. MFC was determined as the lowest concentration of a given compound that did not allow the growth of any fungal colony on SDA after the incubation period. The read was made visually (Gullo et al., 2012).

Inhibition of germ tube formation

The compounds were tested at sub-MIC concentrations (Table 2). *Candida albicans* MYA-2876 cells were grown on SDA for 24 h at 37°C under aerobic conditions and adjusted to 2.5×10^7 CFU/mL in PBS (1x) and Neubauer chamber under optical microscope (magnification 400x). The cell suspension was added a 1:1 mixture of RPMI culture medium (with diluted compounds) and fetal bovine serum. The cells count that forming germ tubes were conducted using an optical microscope at 400 x magnifications at intervals of 2, 4 and 6 h of aerobic incubation at 37°C and compared to positive control. Values were calculated as percentage (Consolaro et al., 2005).

Effect of compounds on biofilm

Inoculum adjustment

C. albicans MYA-2876 were incubated in yeast extract peptone dextrose (YPD) liquid medium for 24 h in orbital shaker (30 rpm) at 30°C. An aliquot of 7 mL of the inoculum was centrifuged at 10.8 g for 4 minutes. This process was performed twice with a phosphate buffered saline (PBS) (1x) wash step. The pellet was resuspended in 7 mL of RPMI. Cell concentration was determined using a Neubauer chamber under optical microscope (magnification 400x); the suspension was diluted to 1.0×10^6 CFU/mL in RPMI (Pierce et al., 2008).

Biofilm formation

The cell suspension (100 µL) was added to a sterile microtiter plate with U-shaped wells. The cell suspension were incubated for 2 h under agitation (100 rpm) at 37°C. After incubation, the plate was washed 3 times with saline (0.9%). Solutions of the compounds (concentration range 16 to 0.125 mg/mL) were added to the wells. The cells were incubated for 24 h at 37°C under aerobic conditions. After incubation period, were compared to the positive control (Da Silva et al., 2010).

Mature biofilm

The cell suspension (100 µL) was added to a sterile microtiter plate

Table 1. MIC and MFC of the compounds tested against *Candida* spp.

Strains	Carvone		Menthone		Menthofuran		Pulegone		Fluconazole
	MIC (mg/mL)	MFC (mg/mL)	MIC (mg/mL)	MFC (mg/mL)	MIC (mg/mL)	MFC (mg/mL)	MIC (mg/mL)	MFC (mg/mL)	MIC (µg/mL)
Cr 12	4	8	8	8	8	8	1	2	-
Cl 06	2	4	4	4	2	2	1	2	0,5
Cg 07	4	8	8	-	8	8	2	2	32
Ck 573	2	2	8	8	4	4	1	1	16
Ca 90028	2	4	8	8	4	4	2	4	-
Ca 2876	2	4	8	8	4	4	2	2	-
Cg 566	1	1	4	8	4	4	1	1	1
Ct 94	2	4	-	-	8	8	2	2	-
Ca 562	4	8	8	8	8	8	2	4	-
Cp 604	1	2	8	-	4	8	1	2	4
Cd 7987	2	4	8	8	4	8	1	2	1
Ca 314	1	2	8	8	4	8	1	1	-
Ca 330	1	2	8	8	8	8	1	1	-
Ca 335	1	2	8	8	4	8	1	1	-
Ca 338	0,5	1	8	8	4	8	1	1	-
Ca 368	0,5	2	4	8	4	8	1	1	-
Ca 378	0,5	2	8	8	4	8	1	1	-
Ca 462	1	4	8	8	4	8	1	1	-

-, Without activity; Cr 12, *C. rugosa* IZ; Cl 06, *C. lusitanae* IZ; Cg 07, *C. glabrata* IZ; Ck 573, *C. krusei* CBS; Ca 90028, *C. albicans* ATCC; Ca 2876, *C. albicans* ATCC; Cg 566: *C. guilliermondii* CBS; Ct 94, *C. tropicalis* CBS; Ca 562, *C. albicans* CBS; Cp 604: *C. parapsilosis* CBS; Cd 7987: *C. dubliniensis* CBS. *C. albicans* clinical isolates: Ca 314; Ca 330; Ca 335; Ca 338; Ca 368; Ca 378; Ca 462.

Table 2. Inhibition of germ tube formation expressed in percentage.

Incubation period (hours)	Inhibition percentage			
	Carvone (1 mg/mL)	Menthone (4 mg/mL)	Menthofuran (2 mg/mL)	Pulegone (1 mg/mL)
2	72	65	65	79
4	79	64	69	85
6	79	66	70	86

with U-shaped wells. The cells were incubated aerobically at 37°C for 24 h. Solutions of the compounds (concentration range 16 to 0.125 mg/mL) were added to the wells. The suspension cell with compounds was incubated for 24 h at 37°C under aerobic conditions. After incubation period, were compared to the positive control (Pierce et al., 2008).

Biofilm quantification

After incubation period, the plates were washed 3x with saline (0.9%) for remove planktonic cells. The quantification of the fungal cell viability was calculate using a colorimetric XTT [2,3-bis(2-metoxi-4-nitro-5-sulfo-fenil) -2 H -tetrazolium-5-carboxanilida] reduction assay, in which were add in the plates 80 µl solution XTT and measured in reader microplate spectrophotometer at A₄₉₀.

Scanning electron microscopy (SEM) of the biofilm

The biofilm formation and mature biofilm samples (in 0.5 mg/mL

concentration) were grown in culture slides (BD Falcon). After the incubation period, the supernatant was discarded and biofilm fixed with glutaraldehyde (2.0%) for 30 min followed by drying ambient temperature. Specimens were dehydrated with increasing concentrations of ethanol (50, 70, 90 and 100%) for 10 min each. After that, specimens were dried, metallized with gold and observed by SEM (JEOL JSM 5600LV) to view the biofilm structure in the compounds presence. The images were selected randomly.

Cytotoxicity of the compounds

HaCaT cells (epithelial cells from normal human keratinocytes immortalized but not transformed) were grown in RPMI/FBS and adjusted to 6.5x10⁴ CFU/mL in Neubauer chamber under optical microscope (magnification 400x). Posteriorly, 100 µL of the cells was added to microtiter plate and incubated for 24 h at 37°C and 5% CO₂. Subsequently, the compounds (concentration range 16 to 0.125 mg/mL) were added in the cells and incubated for 24 h under the same conditions. The cells were fixed with trichloroacetic acid (TCA) 10% and incubated for 1 h at 4°C. The plates were washed

with distilled water (3x), dried at ambient temperature and stained with sulforhodamine B (SRB, 0.4) dissolved in 1% aqueous acetic acid, washed 4 times with distilled water, and then treated with SRB in 10 μ M TRIZMA pH 8.0. The cell suspension were measured at A₅₃₀ in microplate reader to evaluate cell viability after treatment with the compounds (Endo et al., 2010) and reported as IC₅₀%, the concentration of the compound that induces 50% lysis or cell death (Liu et al., 2016).

Statistical analysis

The biofilm and cytotoxicity tests were analyzed by the Dunnett statistical test (ANOVA) using the software Bioestat 5.0; a p<0.05 was considered statistically significant.

RESULTS

Planktonic anti-*Candida* assay

The compounds tested showed inhibitory activity against nearly all *Candida* spp. for the concentration range tested, with the exception of menthone for *C. tropicalis* CBS 94 (Table 1). Carvone and pulegone showed inhibitory activity at lower concentrations compared to menthone and menthofuran. A commercially available antifungal agent tested was Fluconazole, to compare the results of inhibition obtained by isolated compounds of essential oils obtained from *Mentha* spp. Fluconazole showed inhibitory activity only in some strains tested.

Minimum fungicidal concentration (MFC)

Carvone and pulegone expressed MFC for the strains tested in lower concentrations compared to the other compounds. The concentration of 2 mg/mL of carvone and 1 mg/ml of pulegone showed most fungicidal activity inhibiting most species tested (Table 1).

Inhibition of germ tube formation

Pulegone showed the greatest efficiency inhibiting until 86% germ tube formation compared to other compounds and positive control as shown in Table 2.

Action of compounds on biofilm

The compounds tested prevented at least 50% adhesion, inhibited progress of biofilm formation, and damaged of mature biofilms at low concentrations: 0.250 mg/mL for carvone and pulegone; 1 mg/ml for menthone and 0.5 mg/mL for menthofuran compared to the positive control (Figure 1).

SEM assay was made to view the action of the compounds on biofilm. The images obtained showed changes in cells as pore formation, roughness and

presence of leakage of cellular contents after treatment with the compounds. The results of biofilm formation are shown in Figure 2 and mature biofilm in Figure 3.

Cytotoxicity of the purified compounds

Cytotoxicity evaluation of the compounds in HaCaT cells using menthone and menthofuran showed cell viability above 50% at concentrations 4 to 0.125 mg/mL; carvone and pulegone showed cell viability above 50% at concentrations of 8 to 0.125 mg/mL compared to positive control (Figure 4).

DISCUSSION

In planktonic anti-*Candida* assays, all compounds showed antifungal activity, however, carvone and pulegone inhibited the growth of most strains in less concentrations compared to other compounds. These results indicate a greater antifungal potential of carvone and pulegone against these species. The results obtained with the commercial antifungal Fluconazole did not exhibited antifungal activity against some species tested, including clinical isolates. These results corroborate with Ramesh et al. (2010) demonstrating that clinical isolates strains of *Candida* spp. were resistant to Fluconazole. In this sense, the results obtained was satisfactory because the compounds showed antifungal activity in species that Fluconazole did not showed, suggesting that such compounds may be a possible source for new drugs, especially as adjuvant. Minimum fungicidal concentration was performed to confirm cell death in MIC through observation of no colony growth in solid medium and classify fungistatic or fungicidal activity by compounds (Gullo et al., 2012). Carvone and pulegone expressed MFC for the strains tested in lower concentrations demonstrating most fungicidal character compared to the other compounds.

We also observe that the compounds tested acted in virulence factors of *Candida* spp. such as hyphae and biofilm. The germ tube formation is the beginning of the growth of hyphae (Ellepola and Saramayake, 2001). We observed that compounds reduced germ tube formation. These results are important because this form is related to the invasiveness of the fungus to the host tissue (Jacobsen et al., 2012; Mayer et al., 2013). In biofilm tests, the compounds reduced biofilm formation and deconstructed the mature biofilm at low concentrations tested. Considering that biofilms are associated with approximately 80 of infections caused by *Candida* spp. (Tsang et al., 2012), such compounds can be promise as therapeutic agents against this virulence factor.

The antifungal action of the compounds can be attributed to their chemical class. Other members of the terpenoids are known to destabilize cell membranes and increase cellular permeability which permit the disruption

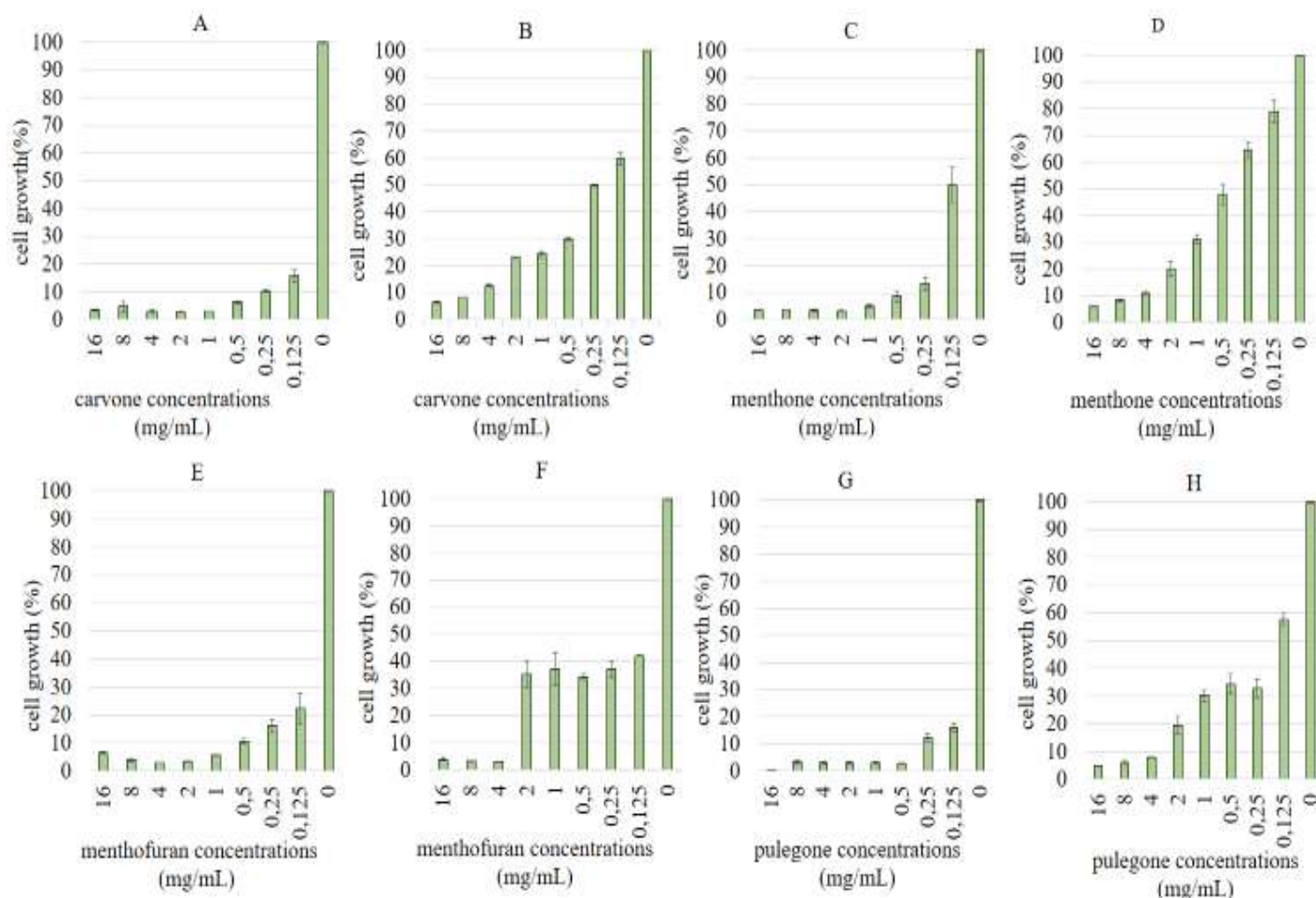


Figure 1. Graphs representing growth (%) of *Candida albicans* ATCC-2876 biofilm in the compounds presence (concentration range 16.0 mg/ml to 0.125 mg/ml). All results showed statistical significance (Dunnett test, ANOVA). 0 mg/ml indicates positive control. Biofilm formation in the presence of (A) carvone; (c) menthone; (E) pulegone. Mature biofilm exposed to (B) carvone; (D) menthone; (f) menthofuran; (H) pulegone.

and death of the microorganisms (Zore et al., 2011). The SEM assay was performed to evaluate the action of the compounds in *C. albicans* MYA-2876 biofilms. The images obtained showed changes in cells as the presence of pores and ridges in the cell envelope and extravasation of cellular contents confirming this hypothesis.

With the purpose to evaluate the therapeutic applicability of the compounds, we performed cytotoxicity assays on HaCaT epithelial cells. The compounds showed results with cell viability above 50% in the most part concentrations tested although high concentrations of some neared the 50% viability limit. The low cytotoxicity of compounds isolated from *Mentha* spp. has also been demonstrated by Amaral et al. (2015) who evaluated cytotoxicity of rotundifolone, a major constituent of the essential oil of *Mentha x villosa* against tumor cell lines HCT-116 and SF-295. The data obtained in the initial cytotoxicity assays indicate a potential

therapeutic window for these compounds isolated to be used as antifungal agents.

Conclusion

The compounds tested modify the pathogenic *Candida* spp. fungus in many ways, including inhibition of growth, formation and maintenance of biofilms, as well as germ tube formation. Although each compound and strain has their unique profile of action or resistance, the low cytotoxicity and therapeutic effect of these compounds make them worthwhile candidates for complementing antifungal clinical protocols.

Conflicts of Interests

The authors have not declared any conflict of interests.

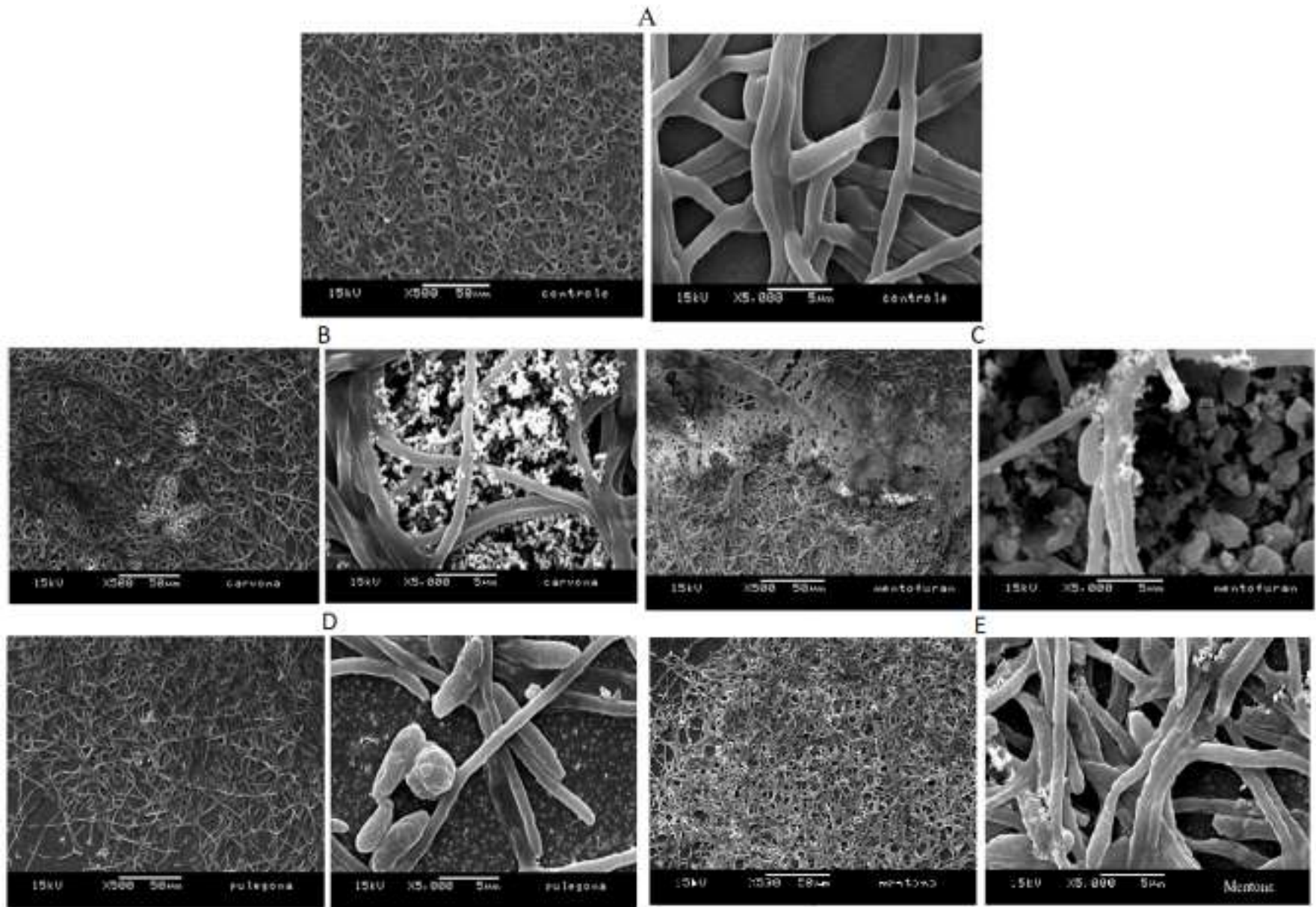


Figure 2. Effects of compounds on mature MYA-2876 cell viewed 500x and 5000x magnification. (A) Control without compounds. (B) Biofilm exposed to carvone; (D) pulegone; (E) menthone.

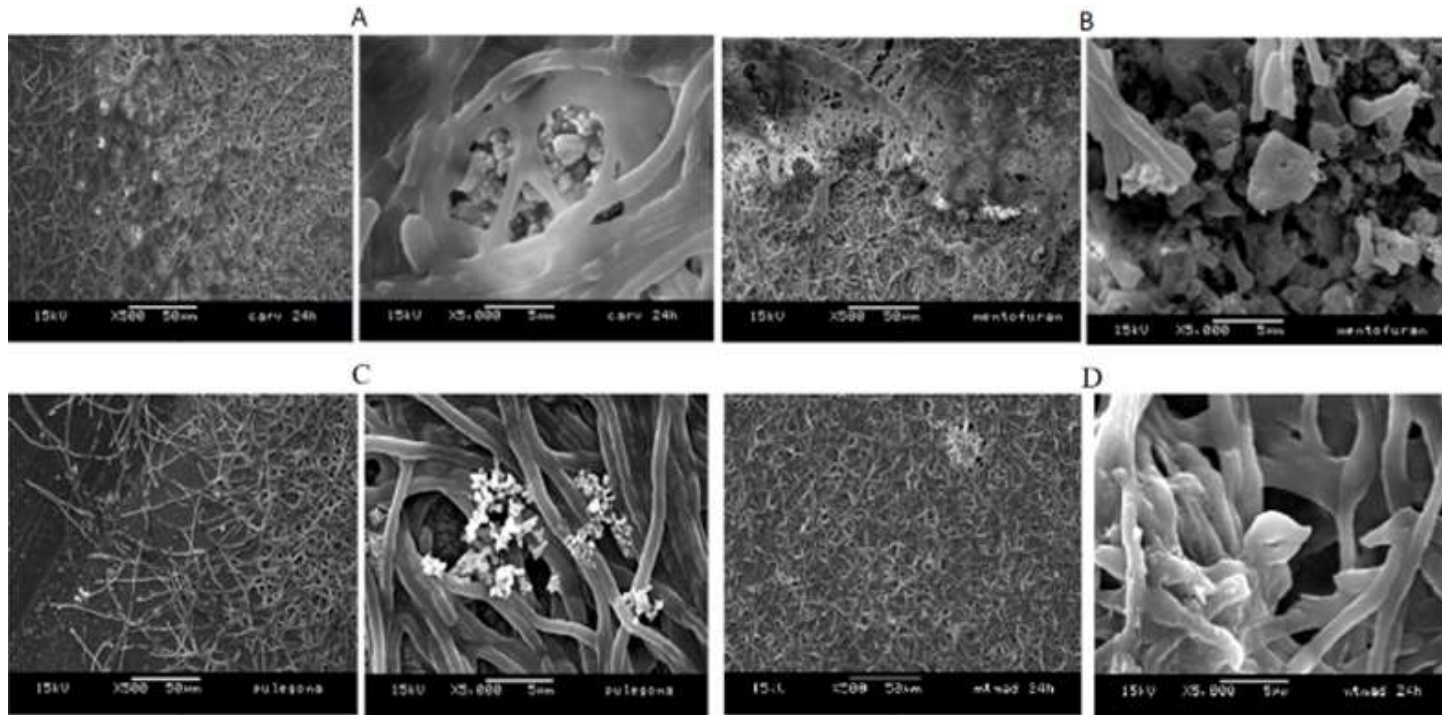


Figure 3. Effects of compounds on mature MYA-2876 cell viewed 500x and 5000x magnification. (A) Biodilm exposed to carvone; (B) pulegone; (D) menthone.

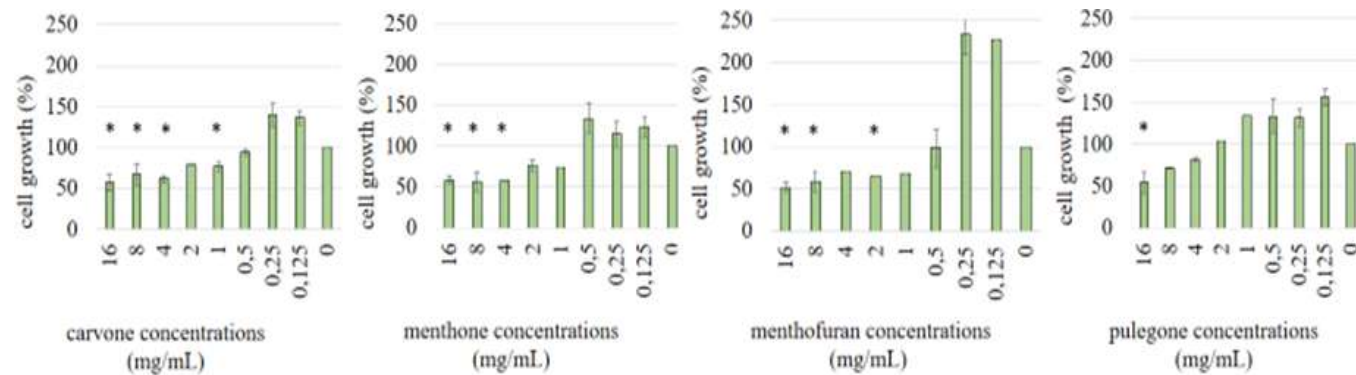


Figure 4. Graphs representing growth (%) of HaCat cells exposed to the compounds (concentration range 16 to 0.125 mg/ml). (A) HaCat cells exposed to carvone; (c) HaCat cells exposed to menthone; (C) HaCat cells exposed to menthofuran. (D) HaCat cells exposed to pulegone. *Statistically significant ($p < 0.05$). Dunnett, ANOVA.

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