Evaluation of antimicrobial, cytotoxic and chemopreventive activities of carvone and its derivatives

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Considering the reported activity of carvone in the literature, this study aimed to evaluate the antimicrobial, cytotoxic and chemopreventive activities of (+)- and (-)-carvone, (+)- and (-)- hydroxydihydrocarvone and α,β-epoxycarvone. (+)-Hydroxydihydrocarvone (HC+), (-)-hydroxydihydrocarvone (HC-) and α,β-epoxycarvone (EP) were obtained by synthesis using (+)-carvone (C+) or (-)-carvone (C-) as precursors. The antifungal activity (MIC and MFC) were evaluated against *Candida parapsilosis*, *C. tropicalis*, *C. krusei* and *C. albicans* and the antibacterial activity (MIC and MBC) against *Escherichia coli* and *Staphylococcus aureus*. The cytotoxicity assays were performed with human cancer cell lines HepG-2 and SiHa and the normal strain MRC-5 through sulphorrodamine B assay. Chemoprevention was evaluated through quinone reductase assay. Our results showed no cytotoxicity on tumor and normal cell lines and no induction of the quinone reductase enzyme. C- and HC- presented activity against *E. coli*. All compounds presented weak antifungal activity against *C. tropicalis* and *C. parapsilosis.* EP and C+ showed moderate activity against *C. krusei*. Results suggest the potential use of carvones and its derivatives as antifungal agents against *Candida* yeasts. The absence of cytotoxicity in cell lines indicates safety in the use of these compounds.

Keywords: Carvone/antimicrobial activity. Carvone/antifungal activity. Carvone /cytotoxicity. Carvone/ chemoprevention.

INTRODUCTION

Natural products diversity is an important source in the search for bioactive compounds. Plants have a wide range of secondary metabolites that are important for the discovery of drugs for various diseases such as cancer and infections by microorganisms. Essential oils are generally a mixture of secondary metabolites including terpenes and phenolic compounds, mainly monoterpenes, sesquiterpenes, phenylpropanoids and other low molecular weight volatile compounds. Essential oils are responsible for the characteristic odor of aromatic plants which is important for attracting pollinators and seed dispersers, moreover, it acts as a defense mechanism against predators and diseases. Many of them present pharmacological activity and may be used therapeutically (De Sousa, 2015; Bakkali *et al.*, 2008; Wagner, Elmadfa, 2003). Carvone and related metabolites are monoterpene constituents of essential oils that presented interesting activities including antibacterial, antifungal, anticonvulsivant and cytotoxic to cancer cell lines (Carvalho, Fonseca, 2006; Chen *et al.*, 2006; Freire; Costa, 2006; Stammati *et al.*, 1999).

The enantiomers (4*S*)-(+)-carvone and (4*R*)-(-)-carvone (Figures 1a and 1b) are monoterpenes found in essential oils from various plant species, commonly used in food and pharmaceutical products, such as *Carum carvi* L., *Anethum graveolens* L., *Mentha spicata* L. and *Mentha viridis* (L.) L. The carvone isomers or essential oils rich in carvone were effective against a wide spectrum of human

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pathogenic fungi (eg. *Candida albicans*) and bacteria (eg. *Campylobacter jejuni* and *Listeria monocytogenes*) (Carvalho, Fonseca, 2006; Mcgeady, Wansley, Logan, 2002; Rault *et al.*, 2013). Zheng, Kenney and Lam (1992) demonstrated that carvone induced the detoxifying phase II enzyme glutathione *S*-transferase in several mouse target tissues, which shows the potential cancer chemopreventive action of the compound. Moreover, carvone inhibited viability and proliferation of Hep-2 cells (human carcinoma epithelial cells) probably inducing apoptosis without causing DNA damage (Stammati *et al.*, 1999). De Sousa *et al.* (2007a) also demonstrated its depressant effects on central nervous system and the anticonvulsivant action of (+)-carvone. The monoterpene α,β-epoxycarvone (Figure 1c) is an essential oils found in *Carum carvi* L. (Iacobillis *et al.*, 2005), *Catasetum maculatum* Kunth. (Lindquist *et al.*, 1985), *Kaempferia galanga* L. (Jirovetz *et al.*, 2001) and other aromatic plants (Kaiser, 1997). This monoterpene presented antimicrobial activity against *Candida albicans* and *Staphylococcus aureus* (Arruda *et al.*, 2006). Additionally, it showed anticonvulsant, antinociceptive, anti-inflammatory, antiulcerogenic and antioxidant activities (De Sousa *et al.*, 2007b; De Almeida *et al*, 2008; Siqueira *et al.*, 2012; Salgado *et al.,* 2015). The semisynthetic compounds (+)-hydroxydihydrocarvone and (-)-hydroxydihydrocarvone (Figure 1d and 1e) were obtained from hydration of (+)-carvone or (-)-carvone. The (-)-Hydroxydihydrocarvone demonstrated antinociceptive and anti-inflammatory activities along with depressant effects on central nervous system (De Sousa, Oliveira, Almeida, 2006; De Sousa *et al.*, 2010; Oliveira *et al.*, 2007; Oliveira *et al.*, 2009). Regarding toxicological studies, data from literature indicates low toxicity of all these compounds (De Sousa *et al.*, 2007a; De Sousa *et al.*, 2007b; Oliveira *et al.*, 2009); only (+)-hydroxydihydrocarvone have not been evaluated regarding its toxicological parameters.

Considering the reported activities of carvone in the literature, this study aimed to evaluate the antimicrobial, cytotoxic and chemopreventive activities of (+)- and (-)-carvone, (+)- and (-)-hydroxydihydrocarvone and α,β-epoxycarvone. Although enantiomers have similar physicochemical properties, they have different behavior in chiral environments, such as endogenous drug receptors, membrane proteins and enzymes. The antifungal activity was evaluated against *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei* and *Candida albicans* and the antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*. The cytotoxicity assays were performed with human cancer cell lines HepG-2 (hepatocellular carcinoma) and SiHa (cervical tumor cell)
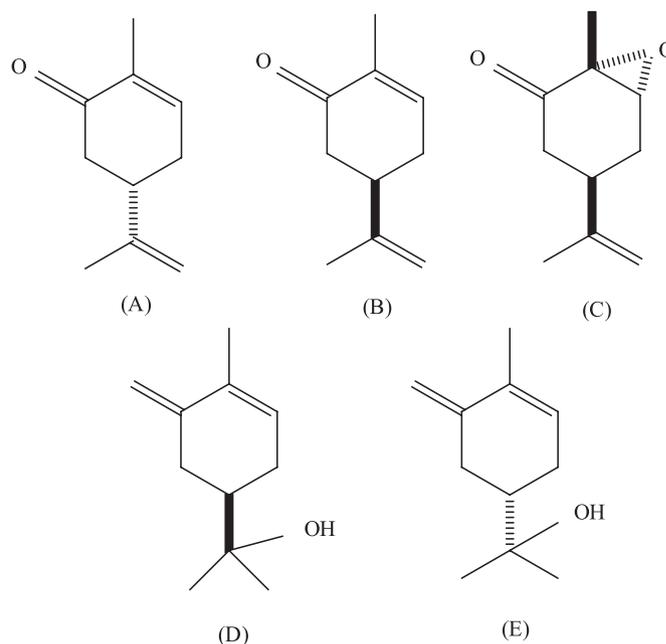

FIGURE 1 - Chemical structures of **(a)** (4*S*)-(+)-carvone, **(b)** (4*R*)-(-)-carvone, **(c)** α,β-epoxycarvone, **(d)** (-)-hydroxydihydrocarvone and **(e)** (+)-hydroxydihydrocarvone.

and normal strain MRC-5 (normal lung fibroblast) through sulphor rodamine B assay.

Regarding potential chemopreventive action, the compounds were also evaluated in the induction of quinone reductase assay. Cancer quimioprevention involves the use of natural or synthetic agents to prevent, reverse or retard the carcinogenesis process.

MATERIAL AND METHODS

Compounds

(4*S*)-(+)-carvone and (4*R*)-(-)-carvone (C- and C+) were purchased from Sigma-Aldrich®. Derivatives (+)-hydroxydihydrocarvone and (-)-hydroxydihydro-carvone (HC+ and HC-) were obtained from hydration of C+ and C- according to the method described by Bucho e Wuest (1979). The hydration was performed by reaction with 50% sulfuric acid, agitating in a magnetic stirrer for 40 h at room temperature. α,β-Epoxy-carvone (EP) was prepared from C- according to Klein and Ohloff (1963).

Preparation of test samples

For antimicrobial testing the substances C+, C-, H+, H- and EP were solubilized (10.0 mg/mL) in Tween 80 (1 % in PBS). Carvones were tested at concentrations of 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039 and 0.019

mg/mL. In the cytotoxicity and quinone reductase assays the concentrations of carvones were 50.0, 16.6, 5.5, 1.8 and 0.6 µg/mL.

Microorganisms

Strains of bacteria and yeast used in this study were *Bacillus subtilis* (ATCC 9362), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 10536), *Candida albican*s (ATCC 64548), *Candida parapsilosis* (ATCC 22019), *Candida tropicalis* (ATCC 750), and *Candida krusei* (ATCC 6258).   A bacterial colony of each strain was inoculated in brain heart infusion (BHI) broth and incubated at 37 °C for 24 h. The final concentration of cells in suspension was adjusted to 0.5 McFarland standard (approximately 1.5×10^8 cells/mL). Yeast colonies were incubated for 48 h at 35 °C and suspended in 5 mL of 0.9% sterile saline solution. Yeast cells were counted in Neubauer chamber and adjusted to a final concentration of $2.5 – 5.0 \times 10^3$ cells/mL.

Positive controls

Ampicillin was used as positive control in antibacterial assays with stock solution concentration of 50 mg/mL (deionized water). Amphotericin B was used as a positive control for *Candida* strains with stock solution concentration of 32 mg/mL (dimethylsulfoxide: RPMI-1640 medium 1:5; v/v).

Determination of Minimum Inhibitory Concentration (MIC) by microplate dilution Method and Minimum Fungicidal Concentration (MFC)

MIC was determined using microplate dilution assays according to reference document M27-A3 of the CLSI with modifications (CLSI, 2008). RPMI-1640 containing glutamine and phenol red was used in *Candida* strains maintenance, the medium, without sodium bicarbonate, was supplemented with 3-(*N*-morphin)-propanesulfonic acid (MOPS) at 0.165 M, pH 7.0. In each well of a microplate, 100 µL of sterilized RPMI-1640 medium was added. Then, 100 µL of carvone samples were added to the first well of each column (columns 1-8). In the column 9, 100 µL of amphotericin B was added to the first well. Serial dilution was performed by transferring 100 µL from the first to the next until the end of the line. Finally, 100 µL of yeast was added to each well to a final concentration of 2.5×10^3 cells/mL. Controls: yeast growth control (medium and yeast), solvent control (100 µL of 1%

Tween 80 with and without yeast), sterility control (only medium), compound control (compound and medium) and positive control (amphotericin B). The microplates were incubated for 48 h at 35 °C under agitation. Fungal growth inhibition was assessed by the addition of 20 µL of 2% aqueous solution 2,3,5-triphenyltetrazolium chloride (TTC) that was read after incubation at 35 °C for 1 h. The lowest concentration which no visible growth was observed was considered the minimum inhibitory concentration (MIC). Tests were performed in triplicate. The minimal fungicidal concentration (MFC) was determined by plating the contents of each sample from MIC test into Petri dish containing Saboraund agar. The plates were incubated for 48 h at 35 °C. MFC was evaluated by absence or presence of yeast growth.

Determination of Minimum Inhibitory Concentration (MIC) by microplate dilution method and Minimum Bactericidal Concentration (MBC)

MIC was determined using dilution microplate method, according to reference document M7-A9 of the CLSI with modifications (CLSI, 2012). For that, 100 µL of BHI (brain heart infusion) was added to the wells. Then, 100 µL of carvone (or derivatives) sample was added to the first well of each column (columns 1-8). In the column 9, 100 µL of ampicillin was added in the first well. Serial dilution was performed transferring 100 µL from the first well to the subsequent until the end of each line. Finally, 100 µL of bacterial suspension (1:200; BHI) was added to each well. The final concentration of bacteria in each well was 2.5×10^5 cells/mL. Controls: solvent control (Tween 80 1% with and without bacteria), growth control (bacteria and medium), sterility control (only medium), compound control (compound and medium) and positive control (ampicillin). Microplates were incubated for 24 h at 37 °C. The growth inhibition was detected by adding 20 µL of a 0.01% aqueous solution of resazurin after incubation at 37 °C for 1 h. Tests were performed in triplicate. The minimal bactericidal concentration (MBC) was determined by plating each sample of the MIC test into Petri dishes containing agar Mueller-Hinton. Petri dishes were incubated for 24 h at 37 °C. MBC was evaluated by absence or presence of bacterial growth.

Cell lines and cell culture

The cell lines used in cytotoxicity assays was HepG2 (human hepatocellular liver carcinoma, ATCC®HB-8065™), courtesy of Dra. Dayse Maria

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Favero Salvadori (Department of Patology, UNESP), SiHa (human cancer of cervix, ATCC®HB-35™), courtesy of Dra. Luisa Lina Villa (Laboratory of Virology, Ludwig Institute) and MRC-5 (human lung fibroblast, ATCC®CCL-171™). In the quinone-reductase assay, the cell line used was Hepa c1c7 (murine hepatocellular carcinoma, ATCC®CRC-2026™), courtesy of Dr. John M. Pezzuto of College of Pharmacy, University of Hawaii. The medium used for *in vitro* culture of HepG2, SiHa and MRC-5 was Dulbecco´s Modified Eagle Medium (DMEM) and for Hepa c1c7 was Alfa Modification of Eagle Medium (α-MEM). DMEM and α-MEM were supplemented with fetal bovine serum for cells maintenance. The cells were cultured in bottles at 5% CO_2 atmosphere and 37 °C until the cell monolayer reached confluence (80%). After, cells were washed with Hanks and trypsin-EDTA solutions to detach cells and counting.

Sulforhodamine B (SRB) assay

SRB is used to measure the protein content in adherent cell cultures using 96 wells microplate. The plates were pre-incubated with culture medium (DMEM supplemented with 10% fetal bovine serum, 100 μL/well) for 24 h at 37 °C in incubator with 5% CO_2. Wells without cells were used as controls, doxorrubicine as positive control (36.79 μM) and dimethyl sulfoxide (DMSO 1%) as vehicle control. After 24 h of incubation cells adhesion and growth were observed. Then, the medium was discarded and treatments, controls and DMEM without fetal bovine serum were added in each well. After 24 h of incubation, the cells were fixed with trichloroacetic acid (100 μL/well) and placed under refrigeration for 1 h at 4 °C. TCA was removed and plates were washed in low-flow of water three times and dried. Following, plates were stained for 20 min at room temperature with SRB 0.4% (50 μL/well). In mildly acidic conditions the colorant binds to basic amino acid residues in fixed cells. This reaction provides an estimate of the total protein mass. The plates were washed with 1% acetic acid solution and dried at room temperature. The unbound dye was removed after washing, the dyestuff bound to the protein was solubilized in a basic medium (Tris base, 10 mM, pH 10.5) to determine the optical density in a plate reader at 570 nm. The colorimetric evaluation gives an estimate of total protein mass. The assay was conducted as described in Skehan *et al*. (1990) with modifications (Voigt, 2005). Cellular concentration used was 1.4×10^4 cells/mL.

We calculated the average absorbance of the negative control (MAbsNC) and vehicle (MabsVC), for each concentration of the tested substances (MabsT) and positive control (MabsPC). The percentage of living cells was given by:

$$\% \text{ survival} = [(\text{MAbsT} - \text{MAbsNC})/(\text{MAbsVC} - \text{MAbsNC})] \times 100$$

Induction of quinone reductase assay

To evaluate the carvones as inducers of quinone reductase, 10^4 cells/mL of mouse Hepac1c7 cells were seeded in two sterile 96 wells microplates. In one plate (Plate 1), the cytotoxicity assay was carried out using crystal violet dye and in a second plate (Plate 2), the quinone reductase assay (NQO1) was performed. After 24 h of pre-incubation, the medium was replaced by 190 μL of fresh culture medium and 10 μL of test compounds, 4´-bromoflavone (0.01 μM) or DMSO (vehicle control). NQO1 activity was assessed after 48 h of cell exposure using 3-(4,5-dime-tylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction to blue formazan based on NADPH-dependent menadiol system. Plate 1: after 48 h, the medium was removed and 100 μL of 0.2% crystal violet solution in 2 % ethanol was added. Subsequently, 200 μL of SDS 0.5% in 50% ethanol were added. The absorbance was determined in a microplate reader at 590 nm (Power Wave 200 Microplate Scanning, Bio-Tek Instrument®). Plate 2: medium was removed and 50 μL of 0.8% solution of digitonin in 2 mM EDTA (pH 7.8) was added. The plate was incubated at 37° C for 10 min (mild agitation). Finally, 200 μL of the following reactional mixture was added to the wells: water – 28 mL; Tris-HCl (0.5 M, pH 7.4) – 1.5 mL; tween 20 (1,5 %) – 0.2 mL; FAD (7.5 mM) – 20 μL; glucose-6-phosphate (150 mM) – 0.2 mL; NADP (50 mM) – 18.0 μL; albumin bovine serum – 20 mg; MTT – 9.0 mg; glucose-6-phosphate dehydrogenase – 60 U; menadione (50 mM) – 30 μL. The microplate was stirred until the blue color began to appear. The plates were placed in spectrophotometer and measured at 590 nm (Scanning Microplate Power Wave 200, Bio-Tek Instrument®) (Prochaska *et al*., 1988; Fahey *et al*., 2004).

NQO1 induction ratio (IR) was represented by specific enzyme activity of cells treated compared to control (DMSO 0.5%). Compounds were considered active if IR > 2.0. 4'-bromoflavone, a potent NQO1 inducer, was used as positive control for the assay.

Statistical analysis

One-way analysis of variance (ANOVA) with Tukey´s post-test was employed for the statistical analysis for cytotoxicity and chemoprevention results. Data were expressed as the mean ± SD.

RESULTS AND DISCUSSION

Antimicrobial assays

Compounds C- and HC- only presented antibacterial activity against *S. aureus* at the highest concentration (MIC/MBC: 2.5 mg/mL) (Table 1). C+, HC+ and EP did not show antibacterial activity. Data from literature showed activity of EP against *S. aureus* using a diffusion method in plates with solid medium (Arruda *et al.*, 2006). Also from literature, C- and C+ inhibited the growth of *E. coli* at concentrations of 9.6 mg/mL (Naigre *et al.*, 1996) and 1.5 mg/mL (MIC) (Helander *et al.*, 1998), respectively. All these literature data differ from results found in our study.

Table I shows the results of MIC for antifungal activity and MFC. Carvones presented weak activity against *C. tropicalis* with values of MIC/MFC of 2.5 mg/mL and low inhibitory action against *C. parapsilosis* was also observed (MFC: 1.25 mg/mL). C+ presented intermediate activity to *C. Krusei* (0.625 mg/mL). Carvones presented activity against *C. albicans* in this study, C+ and C- were the most active compounds with MIC/MFC values of 0.312 and 0.625 mg/mL, respectively. Scientific literature reports that essential oils rich in C+ inhibited the growth of this fungus (Jirovetz *et al.*, 2003) and that carvone inhibited the transformation of *C. albicans* to its pathogenic form (Mcgeady, Wansley, Logan, 2002). Similarly to carvone and its derivatives, several monoterpenes also showed activity against *C. albicans*, including linalool, citral, citronellol, thymol, eugenol and carvacrol (Zore *et al.*, 2001; Alvarez *et al.*, 2012). All samples inhibited *C. krusei* and EP and C+ were the most active compounds against this yeast (MIC/MFC: 0.625 mg/mL).

Cytotoxicity assay

All samples presented low cytotoxicity in the cell lines tested at the concentrations of 0.62 to 50.00 μg/mL and did not reach IC_{50} or IC_{20} values (Figure 2). *In vitro* assays have been used to evaluate toxicity, determine activity and mechanism of action of natural antitumor products (Nagle *et al.*, 2004). Despite carvone and its derivatives did not show cytotoxicity in this study, previous studies showed cytotoxic activity against prostate and breast cancer cell lines, P-815 (murine mastocytoma), K-562 (human chronic myeloid leukemia), CEM (acute lymphoblastoid leukemia T) and MCF-7 (human breast adenocarcinoma) (Stammati *et al.*, 1999; Chen *et al.*, 2006; Jaafari *et al.*, 2012). Natural carvones are present in low concentrations in food and have applications in industry and agriculture, increasing the human exposure to these compounds (Carvalho, Fonseca, 2006). The low toxicity observed in this study suggests safety in the use of carvones.

Quinone reductase assay

Three independent experiments were performed to evaluate the carvone and derivatives ability to induce quinone reductase enzyme. The cell strain used was Hepa c1c7 and 4'-bromoflavone was used as positive control. Carvones did not show induction of quinone reductase at tested concentrations (Figure 3). The percentage of living cells by crystal violet assay also demonstrated that these compounds did not present cytotoxic activity.

CONCLUSIONS

Plants' secondary metabolites are an important source to development of new drugs. This study evaluated

TABLE I - Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of carvones against S. aureus and E. coli

Compounds	*S. aureus* MIC/MBC (mg/mL)	*E. coli* MIC/MBC (mg/mL)	*C. parapsilosis* MIC	MFC	*C. tropicalis* MIC	MFC	*C. krusei* MIC	MFC	*C. albicans* MIC	MFC
C+	> 2.500	>2.500	1.250	1.250	2.500	2.500	0.625	0.625	0.312	0.312
C-	>2.500	2.500	-	-	2.500	2.500	1.250	1.250	0.625	0.625
HC+	>2.500	>2.500	1.250	1.250	2.500	2.500	1.250	1.250	1.250	1.250
HC-	>2.500	2.500	1.250	1.250	2.500	2.500	2.500	2.500	1.250	1.250
EP	>2.500	>2.500	0.625	1.250	2.500	2.500	0.625	0.625	1.250	1.250
Ampicillin	3,91±1,56	31,25								
Amphotericin B			0.500	2.000	0.100	4.000	1.000	4.000	0.500	2.000

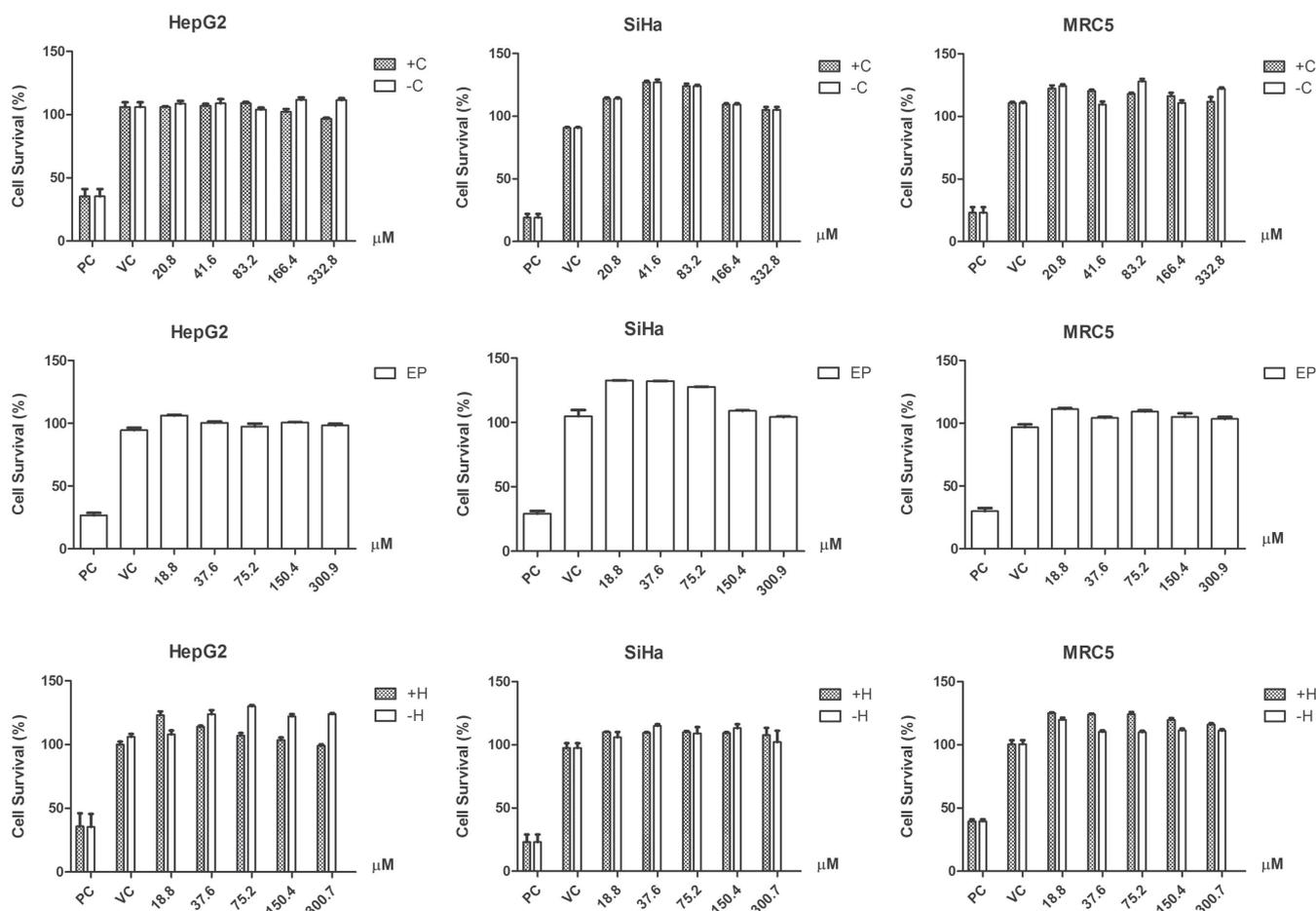Braz. J. Pharm. Sci. 2017;53(4):e00076

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FIGURE 2 - Determination of cell viability (%) in HepG2, SiHa and MRC-5 for (4S)-(+)-carvone and (4R)-(-)-carvone (C- and C+), (+)-hydroxydihydrocarvone and (-)-hydroxydihydrocarvone (HC+ and HC-) and α,β-epoxy-carvone (EP); three independent experiments (mean ± standard deviation). PC: positive control; VC: vehicle control. Statistical analysis: One-way ANOVA, post-test Tukey. P < 0.001.
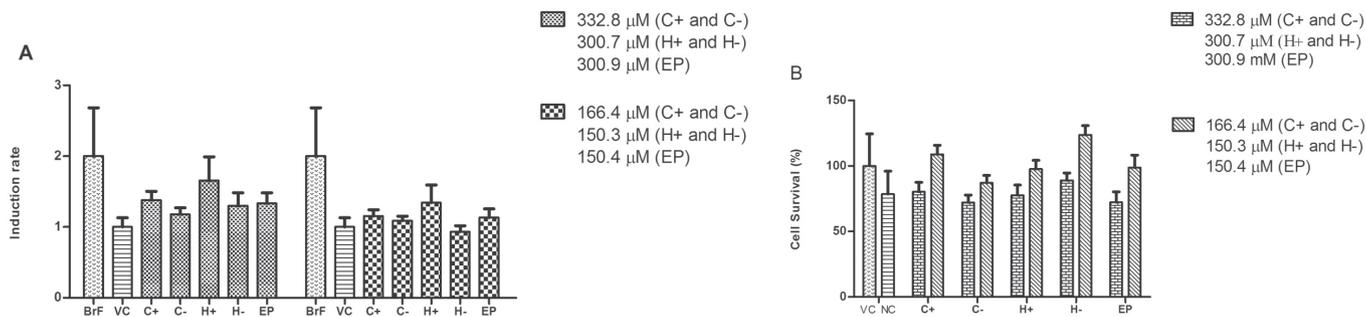

FIGURE 3 - (A)-induced enzyme QR (mean ±standard deviation) in Hepa c1c7. Positive control: BNF. (B)-cell viability (%) evaluated by crystal violet assay after treatment during 48 h. Statistical analysis: oneway ANOVA, post-test Tukey.

the activity of carvone and its derivatives against strains of bacteria and fungi, the cytotoxicity in human cells and the inhibition of quinone reductase in murine carcinoma cells. As conclusions, C- and HC- showed low activity against *E. coli*. However, EP, C+ and HC+ did not inhibit the growth of the bacteria strains tested. Compounds presented weak antifungal activity against *C. tropicalis* and *C. parapsilosis*. EP and C+ showed moderate activity against *C. krusei* similar to C+ and C- against *C. albicans*. All tested samples demonstrated fungistatic and fungicidal activity against

Candida yeasts and the most significant result was found with C+, C- and EP. These results suggest the potential use of carvones and its derivatives as antifungal agents against *Candida* yeasts. In citotoxicity SRB assay none of the compounds showed activity in HepG2, SiHa and MRC-5 cell lines and also did not inhibit the enzyme quinone reductase. The absence of cytotoxicity in both cancer and normal cell lines indicates the safe use of these compounds.

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