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BIOMEDICAL SCIENCES

Nuclear and mitochondrial genome instability induced by fractions of ethanolic extract from *Hovenia dulcis* Thunberg in *Saccharomyces cerevisiae* strains

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Abstract: *Hovenia dulcis* is a plant commonly used as a pharmaceutical supplement, having displayed important pharmacological properties such antigiardic, antineoplastic and hepatoprotective. The purpose of this work was investigate the cytotoxic, genotoxic and mutagenic potential from fractions of *Hovenia dulcis* ethanolic extract on *Saccharomyces cerevisiae* strains FF18733 (wild type) and CD138 (ogg1). Ethanolic extract from *Hovenia dulcis* leaves was fractioned using organic solvents according to increasing polarity: Hexane (1:1), dichlorometane (1:1), ethyl acetate (1:1) and butanol (1:1). Three experimental assays were performed, such as (i) inactivation of cultures; (ii) mutagenesis (canavanine resistance system) and (iii) loss of mitochondrial function (petites colonies). The findings shown a decrease in cell viability in FF18733 and CD138 strains; all fractions of the extract were mutagenic in CD138 strain; only ethyl acetate and butanol fractions increased the rate of petites colonies for CD138 strains. Ethyl acetate and n-butanol fractions induces mutagenicity, at the evaluated concentrations, in mitochondrial and genomic DNA in CD138 strain, mediated by oxidative lesions. In conclusion, it is possible to infer that the lesions caused by the extract fractions could be mediated by reactive oxygen species and might reach multiple molecular targets to cause cellular damage.

Key words: Citotoxicity, medicinal plant, mutagenicity, Rhamnaceae.

INTRODUCTION

Hovenia dulcis Thunb. is a plant commonly used as a pharmaceutical supplement, having recently been considered as bearing nutraceutical properties (Yang et al. 2013, Lim et al. 2015, De Biaggi et al. 2020). A member of the Rhamnaceae family, this hardy tree usually grows in Asia, Eastern China and Korea. Its roots, seeds, branches, leaves and fruits have exhibited pharmacological effects, such as being antifatigue, antidiabetic, neuroprotective, antigiardia, antineoplastic and hepatoprotective (Na et al. 2013, Yang et al. 2019, Li et al. 2005, Gadelha et al. 2005, Castro et al. 2002, Dong et al. 2018).

The compounds isolated from this species includes triterpenes, alkaloids, flavonoids, and triterpenes glycosides, isoled from the roots, barks, leaves, fruits, and seeds of *H. dulcis* (Kang et al. 2017, Xu et al. 2003, Park et al. 2016a, Ding et al. 1997, Yoshikawa et al. 1996, 1997).

Triterpenes isolated from *H. dulcis* roots (27-O-protocatechuoyl-3-dehydroxyisoceanothanolic acid, 27-O-protocatechuoyl-3-dehydroxycolubrinic acid,

27-O-protocatechuoyl-3-dehydroxyepicolubrinic acid, 27-O-syringoylbetulinic acid) induced antiproliferative activity in HSC-T6 hepatic stellate cells (Kang et al. 2017). Hovenitin I isolated from the methanolic fraction from a *Hoveniae Semen Seu Fructus,* showed protective activity on liver injury induced by D-galactosamine/lipopolysaccharide or carbon tetrachloride in mice (Yoshikawa et al. 1997).

At the molecular level, ethanolic extract of branches from *H. dulcis* exhibit antiangiogenic activisuppressing both in VEGFR2 signaling and HIF-1α expression in hepatocarcinoma cell line Hep G2 (Han et al. 2017). Ethanolic extract of fruits from *H. dulcis* suppressed lipopolysaccharide (LPS)-stimulated inflammatory responses through nuclear factor-κB (NF-κB) pathway in Raw 264.7 cells (Park et al. 2016b). Aqueous *H. dulcis* seeds extracts reduced lipid accumulation in oleic acid-induced steatosis of HepG2 cells via activation of the AMPK and PPARα/CPT-1 pathway (Kim et al. 2016). In addition, in a comparative study on *H. dulcis* antioxidant activity by investigating *in vivo* and *in vitro* materials, higher antioxidant activity was observed in calli extracts when compared to the leaf extract (Ribeiro et al. 2015).

Despite therapeutic advantages, some plants are potentially toxic, carcinogenic and teratogenic (Déciga-Campos et al. 2007, Akinboro & Bakare 2007). *In vitro* studies have shown that many plant species used in traditional medicine have toxic or mutagenic/carcinogenic properties (Cardoso et al. 2006, Demma et al. 2009, Mohd Fuat et al. 2007). In line with this notion, it is important to screen medicinal plants for their mutagenic and/or toxic potentiality, their properties and efficacy. This assessment can provide for an appropriate development of potential chemotherapeutic drugs, as well as safe use of plant-derived medicines and as a measure of safety for the continued long-term use of these plants (Cavalcanti et. al. 2006, Verschaeve et al. 2004, Verschaeve & Staden 2008).

Saccharomyces cerevisiae yeast strains feature many technical advantages over other systems, such as a short-time generation, facilitated genetic manipulations, inexpensive growth media, simple sterile technique requirement, easy laboratory maintenance, possibility of long-term storage, about one fifth of yeast genes are members from orthologous gene families associated with human diseases (Tenreiro et al. 2013). Thus, yeast has become an important tool and has been used by researchers as a eukaryotic model to study many cellular mechanisms, such as cell division, replication, metabolism, intracellular transport. *S. cerevisiae* has been used to detect toxic and/or mutagenic activities, to study mitochondrial functions, aging processes, and various pathologies associated with mitochondria, DNA repair and response to oxidative stress (Breitenbach et al. 2013, Tenreiro et al. 2013, Boiteux & Robertson 2013, Cankorur-Cetinkaya et al. 2013, Silva et al. 2014, 2016, Melo et al. 2011).

Living cells are exposed to different ways of stress during their life cycle including oxidative stress through reactive oxygen species (ROS) formation. Normally, ROS levels are controlled by cellular antioxidant systems, but increase in ROS levels leads to oxidative stress (Wallace 2014, Boiteux & Robertson 2013). It has been known, for several decades, that oxidative DNA damage accumulates in cells over time, this accumulation being linked to neurodegenerative disorders and cancer (Wallace 2014). The major oxidative DNA damages, as type 7, 8-dihydro-8-oxoguanine (8-oxoG) lesions, are removed by the base excision repair (BER) pathway (Wallace 2014, Boiteux & Robertson 2013). These lesions, if not removed, can lead to change-producing mutations in cellular functions, as well as inactivate tumor

suppressor genes and/or activate oncogenesis (Friedberg et al. 2006, Wallace 2014, Boiteux & Robertson 2013, Chalissery et al. 2017).

Therefore, the purpose of this study was investigate *S. cerevisiae* strains exposed to ethanolic extract fractions from *H. dulcis* in relation to their citotoxic and genotoxic potential, the induction of cell death as well as mutagenic potential (nDNA and mtDNA).

MATERIALS AND METHODS

Plant material

Hovenia dulcis leaves were collected in Petrópolis, Rio de Janeiro State, Brazil, in October 2015. A voucher specimen is kept at the Herbário da Universidade do Estado do Rio de Janeiro, HRJ-12818.

Chemical agents and reagents

Agar, Yeast Nitrogen Base without amino acids (YNBD), Bacto peptone and Yeast extract were purchased from *Difco™, Sweden*. Glucose, Ethanol P.A. Triphenyltetrazoliumchloride (TTC) and Dimethyl sulfoxide (DMSO) were purchased from *Merck, Brazil*. 4-Nitroquinoline-1-oxide (4-NQO) and canavanine (Can) were purchased from *Sigma, USA*. Phosphate-buffered saline (PBS) powder was purchased from *Laborclin, Brazil*. 4-NQO powder was dissolved in 10% ethanol solution, being used for experimental positive control. PBS powder was dissolved in distilled H_2O, sterilized by autoclaving (20 min, 121°C), stored at room temperature (PBS solution). It was used to wash cells and experimental negative control. 1% DMSO solution (1% DMSO) was 1 % in distilled H_2O.

Yeast strain, media, growth conditions and reagents

S. cerevisiae parental strains FF18733 (Mata, *his*7, *leu*2, *lys*1, *ura*3, trp1) (Heude & Fabre 1993) and their derivative CD138 (*ogg*1::TRP1) (Thomas et al. 1997, Pádula et al. 2004) were grown at 28 °C in YPD medium (1% yeast extract, 1% bacto-peptone, 2% glucose, with 2% agar for plates) or YNBD medium (2% glucose, 0.7% yeast nitrogen base without amino acids with 2% agar for plates) supplemented with appropriate amino acids and bases. Supplemented YNBD (YNBD) medium, lacking arginine, but containing canavanine (Sigma), at 60 mg/l was used for selective growth of canavanine-resistant (Can^R) mutants. Yeast cultures were grown to a cell density of ~1 $\times 10^8$ cells/ml (stationary phase) in YPD medium, at 28°C, under shaking. Cells were harvested, washed twice with PBS solution, and resuspended in the same solution.

Preparation of the *H. dulcis* ethanolic extract

Fresh leaves from *H. dulcis* (fresh weight = 1826 g) were washed with water and dried in an oven (45 °C) for 24 hours. After drying in the oven, this resulted in a dry weight (DW) of 532 g. The dried material was then macerated and immersed in ethyl alcohol 95% for 2 days and filtered on Whatman paper (no 1). The filtrate was separated and stored, and more ethyl alcohol 95% was added to the plant material. This process was performed for 2 weeks. Subsequently, all filtrate was subjected to rotary evaporation at 40 °C, resulting in a 26 g crude ethanolic extract residue.

The yield percentage of crude ethanolic extract was 4.8% based on the weight of dry plant material, determined through the following equation: Yield % = m_r / DW x 100. In which: m_r = crude extract residue mass (g), DW = dry weight plant material (g).

Fractions Preparation

The crude ethanolic extract residue (5.5 g) was dissolved in distilled water. The solution was placed in a separating funnel, n-hexane 1:1 (v

/ v) was added, following vigorous stirring and separation of the two phases, and hexane fraction was isolated. This procedure was repeated three times. Subsequently, the residual extract was fractioned using organic increasing polarity solvents - dichloromethane (1:1), ethyl acetate (1:1) and butanol (1:1) - in a manner similar to that described for hexane. Subsequently, all fractions were subjected to rotary evaporation at 40 °C, resulting in a residue of 1.30g (hexane fraction), 1.18g (dichloromethane fraction), 0.29g (ethyl acetate fraction), 0.87g (butanol fraction).

Fraction yield percentages were 0.24% (hexane fraction), 0.22% (dichloromethane fraction), 0.05 % (ethyl acetate fraction) and 0.16% (butanol fraction) based on the DW dry plant material (g) weight, determined through the following equation: Yield % = m_f / DW x 100. In which, m_f = fraction residue (g) mass, DW = dry weight plant material (g).

The extract fractions were stored at 4 °C and subsequently diluted in 1% DMSO for use in the pharmacological tests.

Evaluation of cytotoxic potential of *H. dulcis* ethanolic fractions

Cultures thus obtained were treated with different ethanolic extract fractions concentrations from *H. dulcis* (3.125, 6.25, 12.5, 25, 50, 75, 100, 125, 150, 175, 200 µg/mL) and incubated (28 °C) under agitation, for 60 min. For the control, the cultures were treated with 4-Nitroquinoline-1-oxide (4-NQO) (1 µg/mL) powerful chemical mutagen and carcinogen (Arima et al. 2006, Kanojia & Vaidya 2006), 1% DMSO solution (solvent used to dilute the extract fractions) or PBS solution (buffer solution used to dilute *S. cerevisiae* strains). Subsequently, appropriate treated cell dillutions were performed and plating (YPD-agar plates). Colonies were counted after 2 days at 28 °C (Silva et al. 2016). Values represent the mean of three isolated experiments. The concentration of the sample which inhibits 50% of the observed effect (IC_{50}) was determined by using the linear equation (y = ax + b) using Excel® software package Office 97. The program then created a trend line and selected the items to display the equation on the graph and present the R-square value on the graph (correlation coefficient). Then by using a y = 0.5 value we calculated the value of x (dose corresponding to IC_{50}).

Evaluation of the mutagenic potential of *H. dulcis* ethanolic extract fractions

Stationary phase cultures were diluted to 5 x 10^2 cells/ml in YPD medium. FF18733 strain were treated with ethanolic extract fractions from *H. dulcis* (IC_{50}), hexane (125 µg/mL), dichloromethane (100 µg / mL), ethyl acetate (50 µg/mL) and butanol (150 µg/mL). CD 138 strain were treated with ethanolic extract fractions from *H. dulcis* (IC_{50}), hexane (100 µg/mL), dichloromethane (50 µg/mL), ethyl acetate (12.5 µg/mL) and butanol (50 µg/mL). 4NQO (1 µg/mL), or 1% DMSO solution were used as positive and negative controls, respectively, for both strains. Yeast strains were treated for 48 hours, at 28 °C. under shaking. Canavanine-resistant mutants (Can^R) were determined after plating appropriate dilutions on YNBD-agar plates supplemented with canavanine and amino acids (Uracil 0.2%, Histidine 2%, Leucine 1%, Lysine 0.4% and Tryptophan 0.2%), and mutants were counted after 3 days at 28 °C (Silva et al. 2016, Thomas et al. 1997). Values are the mean of three isolated experiments.

Measurement of petite colonies induction (mitochondrial mutants)

Stationary phase FF18733 cultures were treated with ethanolic extract fractions from *H. dulcis* (IC_{50}), hexane (125 µg/mL), dichloromethane (100 µg/mL), ethyl acetate (50 µg/mL) and butanol (150 µg/mL). CD138 strain were treated with

ethanolic extract fractions from *H. dulcis* (IC_{50}), hexane (100 µg/mL), dichloromethane (50 µg/mL), ethyl acetate (12.5 µg/mL) and butanol (50 µg/mL). 4NQO (1 µg/mL), or 1% DMSO solution were used as positive and negative controls, respectively, for both strains. Yeast strains were treated for 24 hoursat 28 °C. under shaking. Treated cultures were spread out on YPD-agar plates and incubated at 28 °C for 4 days. The phosphate buffer with agar (1%) containing triphenyltetrazolium chloride (TTC) poured onto YPD-agar plates and mitochondrial mutants form white and small colonies (*petite colonies*), scored by TTC color assay (Ogur et al. 1957, Chen & Clark-Walker 2000, Silva et al. 2016). Values are the mean of three isolated experiments. At least 2500 colonies (total 7500) of each strain were scored to determine *petite* percentage.

Statistical analysis

The experiments were performed in triplicate. The data obtained in each experiment were submitted to variance analysis (ANOVA oneway) and post test of Tukey multiple comparisons using the GraphPad InStat 4.0 program adopting a confidence level of 95%. IC_{50} values obtained in the experiments were confirmed with those determined using Excel® software. Sample concentration which inhibits 50% of the observed effect (IC_{50}) was determined using the linear equation (y = ax + b) using the Excel® software package Office 97. Then the program created a trend line and selected the items to display equation on the graph and present the R-square value on the graph (correlation coefficient). Then, using a value of y = 0.5 we calculated the value of x (dose corresponding to IC_{50}).

RESULTS

Effect of *Hovenia dulcis* ethanolic extract fractions on *S. cerevisiae* strains survival

The results show a significant decrease (**p< 0.01 ***p<0.001) in the survival fraction of the extract fractions when compared to control (PBS and 1% DMSO). The IC_{50} was determined for FF18733 strain [hexane (125 µg/mL), dichloromethane (100 µg/mL), ethyl acetate (50 µg/mL) and butanol (150 µg/mL)] and CD138 strain [hexane (100 µg/mL), dichloromethane (50 µg/mL), ethyl acetate (12.5 µg/mL) and butanol (50 µg/mL)] (Figure 1a and 1b). The ethyl acetate fraction had greater cytotoxic potential, with IC_{50} values calculated from 58.050±0.057 µg/mL for the FF18733 and 19.405±0.039 µg/mL strain for the CD138 strain (Table I).

Mutagenesis evaluation

In order to evaluate possible mutagenic potentiality, *S. cerevisiae* strains were treated with IC_{50} fractions and analyzed by quantification of Can1 gene mutant frequency. Figure 2 shows data from spontaneous and induced mutagenesis by fractions from *H. dulcis* ethanolic extract (IC_{50}). There was a significant increase in the mutation rate induced by the fractions only in CD138 strain (*p< 0.05 **p< 0.01 ***p<0.001) in comparison with spontaneous mutation rate (1% DMSO solution or PBS solution) and 4NQO (positive control), confirming its strong mutagenic activity.

Mitochondrial function

Triphenyl tetrazolium chloride color assay was used to investigate the frequency of mitochondrial mutants (petites). Following treatment of *S. cerevisiae* with *H. dulcis* ethanolic extract fractions, alteration in respiratory metabolism was observed, only in CD138 yeast strain. These alterations were observed in CD138 cultures treated with ethyl acetate and butanol

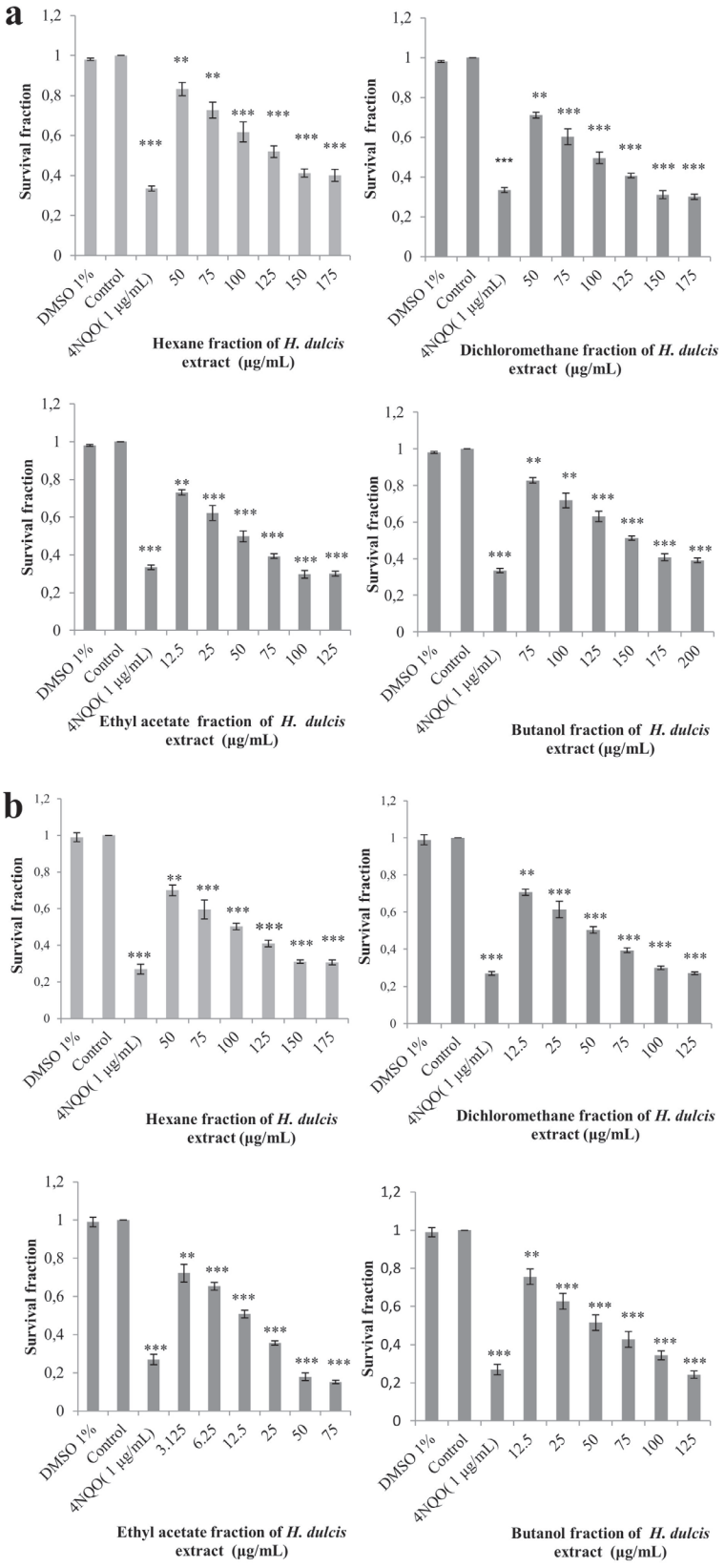

Figure 1(a). Survival fraction of *S. cerevisiae* strain FF18733 **(b)** Survival fraction of *S. cerevisiae* strain CD138 treated with different concentrations of the *H. dulcis* ethanolic extract. Evaluation after 60 min of treatment with *H. dulcis* extract fractions ** (*p* <0.01) *** (*p* <0.001) compared to control.

fractions (*p<0.05) (Figure 3). Each experiment was performed using, 4NQO as a positive control and PBS solution or and 1% DMSO solution as negative.

DISCUSSION

In the present study, it was observed, in both evaluated strains, that the fractions from the *H. dulcis* ethanolic extract caused cell inactivation, in all concentrations tested, thereby evidencing their cytotoxicity. These results corroborate´s Park & Chang (2007), report, in which the chloroform fraction of the *H. dulcis* methanolic leaf extract inhibited cell growth of human hepatoma lineage (HEPG2) and human colon adenocarcinoma (HT29 lineage). In mutagenesis assay, an increase in the frequency of mutations in the CD138 strain was observed after treatment with *H. dulcis* ethanolic fractions. The CD138 strain is deficient in the OGG1 gene. The OGG1 gene encodes a DNA glycosylase which catalyses the removal of 8-OxoG from damaged DNA. Thus, Ogg1-deficient *S. cerevisiae* strains (CD 138) exhibit a spontaneous G-C to T-A mutator phenotype which results in cell death after 8-oxoG induction (Thomas et al. 1997, Tsuzuki et al. 2007, Menck & Sluys 2017). Our results indicate that one or more components of the extract produced lesions in the Can1 gene (type 8-OxoG). These lesions have been repaired in the wild strain (FF18733) but not repaired in the mutant strain (CD138), which justifies a higher level of mutagenesis in CD138 strain.

Mitochondrial genome damage by genotoxic and/ or oxidizing agents can be assessed by the mitochondrial mutagenesis assay, also known as petit assay. An increase was observed in the frequency of petite colonies only in CD138 strains, after treatment with ethyl acetate and n-butanol fractions (*p< 0.05). Silva et al. (2014) have already observed this same effect in CD138 strain treated with *Cassia augustifolia*. In fact, mitochondrial DNA accumulate mutations in an order of magnitude higher than that of nuclear DNA, consistent with the vulnerability of the mitochondria to ROS-induced DNA damage (Kaniak-Golik & Skoneczna 2015). Ogg1 inactivation leads to increased frequency of mitochondrial petite mutants due to deletions in the mtDNA (rho gen) (Singh et al. 2001, Liu & Butow 2006). Alterations or loss of mitochondrial activity are one of the factors that trigger the intrinsic pathway of cell death programmed by

Table I. Cytotoxicity of *Hovenia dulcis* ethanolic extract fractions on strains of *S. cerevisiae*.

Strain	Fractions	IC50 (µg/mL)	Slope	R^2	95% confidence interval IC_{50}
FF18733	Hexane	134.351±0.057	0.004	0.973	134.294 – 134.408
	Dichloromethane	104.029±0.043	0.003	0.968	103.986 – 104.073
	Ethyl acetate	58.05±0.057	0.004	0.930	57.993 – 58.107
	Butanol	158.94±0.024	0.004	0.977	158.934 – 158.958
CD138	Hexane	104.818±0.035	0.003	0.968	104.783 – 104.853
	Dichloromethane	56.128±0.035	0.004	0.966	56.093 – 56.163
	Ethyl acetate	19.405±0.039	0.008	0.868	19.366 – 19.444
	Butanol	61.905±0.080	0.004	0.974	61.824 – 61.985

IC_{50}: Concentration that reduces viability by 50%.
Slope: Angular coefficient.
R^2: Correlation coefficient.

apoptosis (Menck & Sluys 2017). Our findings suggest that there was an increase in mtDNA damage and, consequently, there was a change in the activity of this organelle. Thus, the discovery that one of the fractions produces mitochondrial alteration´s makes this fraction an interesting object of study for a chemotherapeutic agent.

By joining all findings, it was possible to conclude that the ethyl acetate and butanol *H. dulcis* ethanolic extract fractions are cytotoxic and mutagenic, inducing oxidative lesions in genomic and mitochondrial DNA. Therefore, a precaution should be taken regarding the use of this medicinal plant by the general population, based on the mutagenic potential evidenced in this study. Further studies should be carried out to identify the substances responsible for these toxic and mutagenic activities, and to demonstrate the involvement of the extract in the induction of apoptosis via the intrinsic mitochondrial pathway.

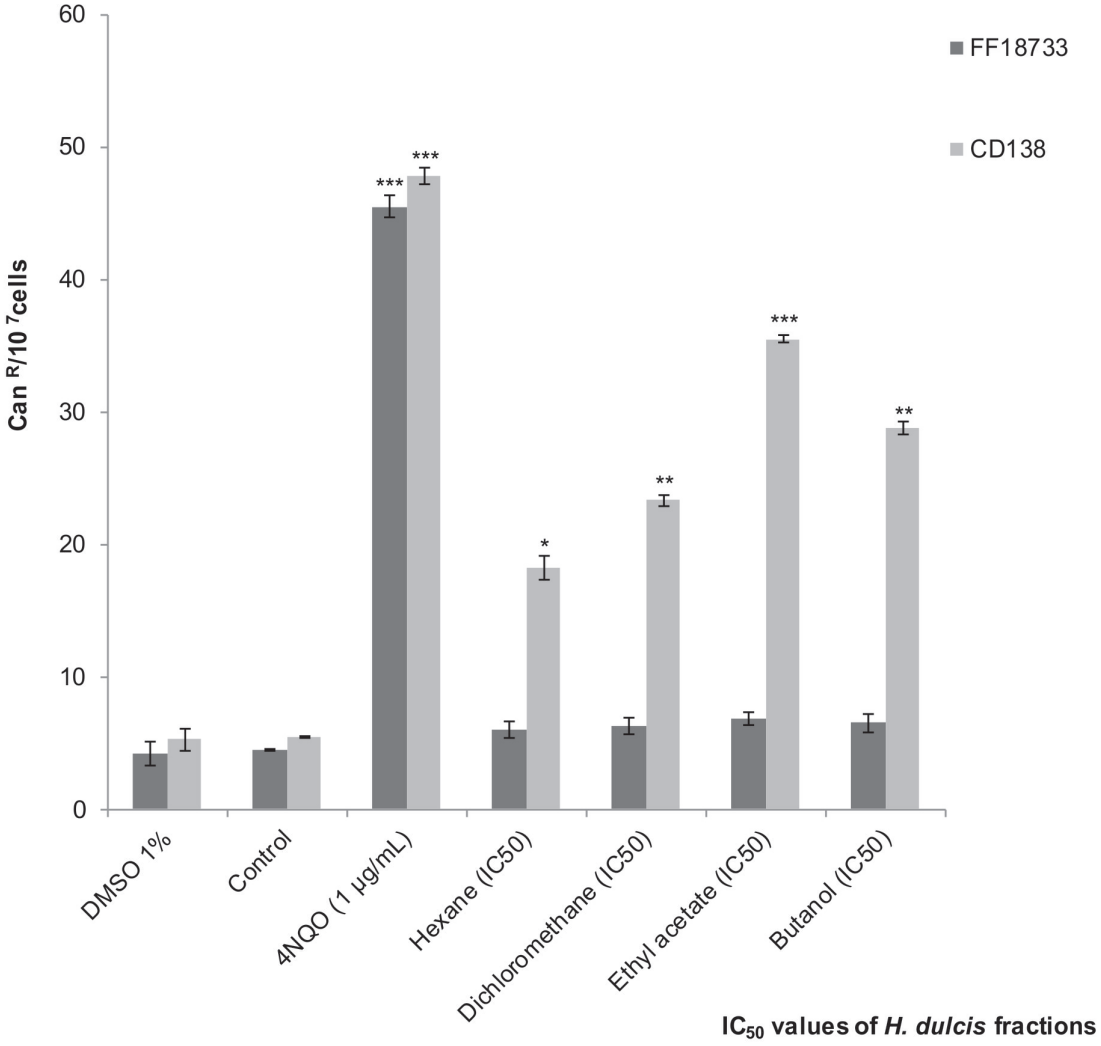

Figure 2. Spontaneous and exposure-induced mutagenesis frequency to fractions of *H. dulcis* ethanolic extract on *S. cerevisiae* strains, FF18733 and CD138. Evaluation after 48h of treatment with IC_{50} concentrations of *H. dulcis* ethanolic extract fractions *($p<0.05$) **($p<0.01$) * ($p<0.001$) compared to control.**

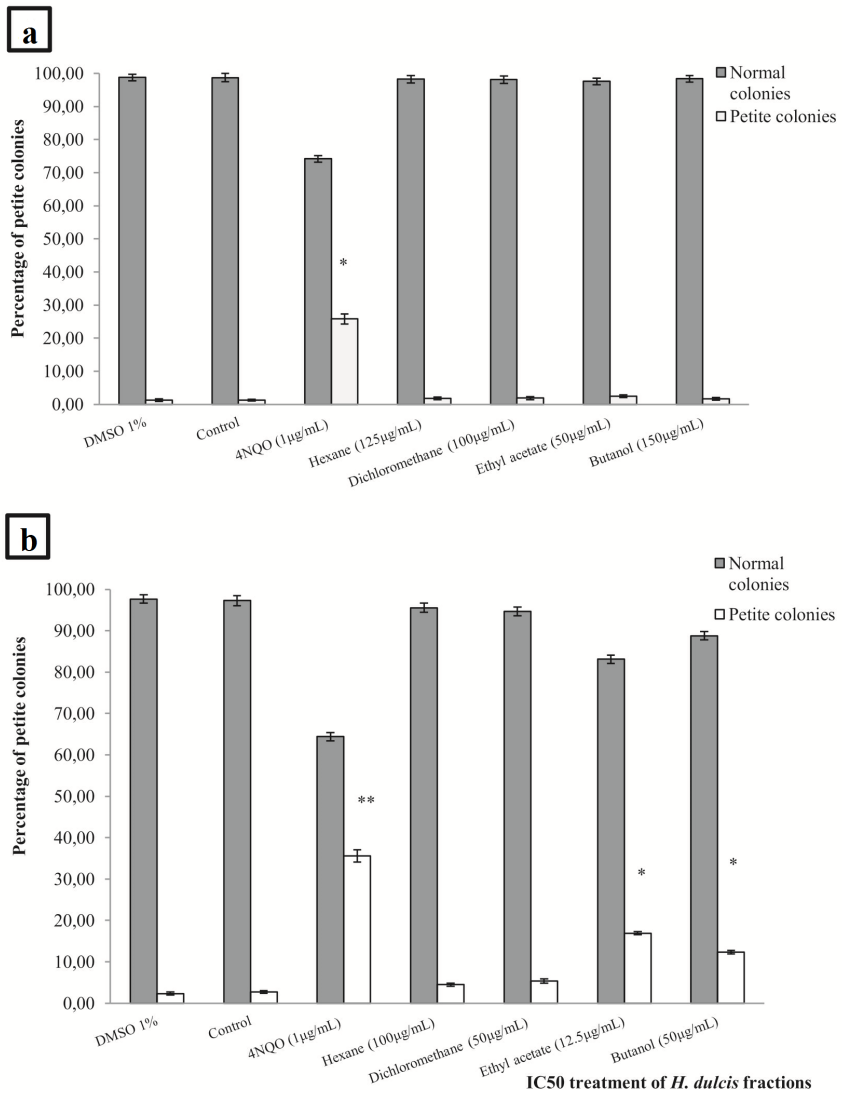

Figure 3. Petite colonies percentage after the treatment of *H. dulcis* extract fractions on *S. cerevisiae* strains, FF18733 (a) CD138 (b). Evaluation performed during 24 hours of incubation with fractions of *H. dulcis* ethanolic extract and controls *(p<0.05) ** (p <0.01) compared to control.

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**In memoriam*

Author contributions

FJSD and NA conceived the project, designed the experiments and contributed to manuscript writing and financial obtaining. LBNA, BBFC, BMN, LOC, CRS and TCC performed the experiments, analyzed the data, prepared the figures and wrote the manuscript. All then contributed equally to this work. ACL and MP analysed, interpreted the data and made critical revisions of the manuscript for intellectual content. ACL and MP contributed equally to this work. The authors declare that they have no conflict of interest.