Oxidant and genotoxic-mediated strong antifungal activity of the essential oils from *Cupressus arizonica* var. *arizonica* and var. *glabra*

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Abstract

The composition and the evaluation of the antifungal activity and the mechanisms of action of the essential oils (EO) of *Cupressus arizonica* leaves of two varieties, *glabra* and *arizonica,* were studied. EOs were extracted by hydrodistillation and the chemical composition was determined by gas chromatography/mass spectrometry (GC-MS). Both var. *arizonica* and var. *glabra* EOs, displayed high contents of α-pinene (29.76% and 26.53%, respectively) and umbellulone (11.86% and 15.05%, respectively). The antifungal activity of the EOs of both varieties against pathogenic yeasts of the genus *Candida* was investigated and showed that very low concentrations of var. *glabra* EO, such as 5.10^{-2} µl/ml, were sufficient to inhibit growth of most of the species, while, all species, except *C. albicans* (MIC = 5.10^{-2}µl/ml), were inhibited for growth with only 10^{-2}µl/ml when the EO of var. *arizonica* was used.

The cytotoxicity of the EOs was assessed in *Saccharomyces cerevisiae* (used as a yeast experimental model) wild type and mutants affected in oxidative stress response and DNA repair pathways. Oxidative stress imposed by the EOs was determined by flow cytometry and the genotoxicity was assessed by yeast comet assay. A higher loss of yeast viability was observed with incubation of the EO from var. *arizonica* ($5x10^{-2}$ µl/ml, 60% viability loss) compared to var. *glabra* (5.10^{-2}µl/ml, 30% viability loss). DNA damage was observed as long comet tails when cells were exposed to the EO of var. *arizonica* and of var. *glabra*, (17 and 13 µm, respectively), compared to the negative control (5 µm). Intracellular oxidation increased in cells treated with the EOs, the var. *arizonica* being more active in the oxidant activity. The results obtained with the wild type yeast strain suggest that the EOs cause toxicity via an oxidative mechanism. To investigate the mechanism of oxidation, mutants affected in the oxidative stress response (*yap1*) and base excision repair DNA pathway (*apn1*) were investigated. The results show that the *yap1* and *apn1* yeast mutant strains are more sensitive to EOs than the wild type. For mutants affected in nucleotide excision repair (*rad4*), a pathway not involved in the repair of oxidative DNA damage, the results were similar to those obtained with the wild type.

Keywords: *Cupressus arizonica*; essential oil; yeasts; genotoxicity; oxidative stress

1. Introduction

The genus *Cupressus,* common name cypress, are native from warm temperate locations of the northern hemisphere. This genus, represented by 30 perennial species around the world, presents a large variety of forms, sizes and colors and some are extensively cultivated (Eckenwalder and James, 2009). A large number of species are known to possess different pharmacologic properties, namely due to their essential oils (EO) contents (Koukos et al., 2001). Some *Cupressus* species have been used in folk medicine. *Cupressus sempervirens*, a cypress native from the Eastern Mediterranean region, has been widely cultivated as an ornamental tree and used for medicinal purposes; the EO obtained from cones and young branches has anthelmintic, antipyretic, antirheumatic, antiseptic, astringent, balsamic and vasoconstrictive properties (Moerman, 1991). Additionally, taken internally, the EO is used in the treatment of whooping cough, the spitting up of blood, spasmodic coughs, colds, flu and sore throats (Lawless, 1995). Applied externally as a lotion or in a diluted way (e.g. using an oil such as almond), it astringes varicose veins and hemorrhoids, tightening up the blood vessels (Lawless, 1995). A resin is also obtained from the tree by making incisions in the trunk, which has a vulnerary action on slow-healing wounds vessel. *Cupressus macrocarpa* is also used for its medicinal value; a decoction of the foliage has been used in the treatment of rheumatism (Monteuus and Bailly, 1985).

Cupressus arizonica, the Arizona cypress, is a species native to the southwest of America. There are five varieties identified by botanists: var. *arizonica* (*Carz*), var. *glabra* (*Cglb*), var. *nevadensis*, var. *montana* and var. *stephensonii*. *Carz* and *Cglb* are the varieties most frequently found in the world because of their use in gardens and as source of timber (Askew and Schoenike, 1982). Both varieties, *Carz* and *Cglb*, have been introduced in Tunisia in arboretums since 1960 (Bouroulet, 1994). The Arizona cypress is widely cultivated as an ornamental tree, used for windbreaks in desert areas and as a timber source. Recently, the EO of this species has been reported to have important biological activities, namely larvicidal activity (Sedaghat et al., 2011), antimicrobial activity (Chéraif et al., 2007), and antifungal activity against *Aspergillus flavus* (Ali et al., 2013) and the anthracnose-causing fungal plant pathogens *Colletotrichum acutatum*, *C. fragariae* and *C. gloeosporioides* (Adams et al., 1997).

During the last few decades, fungal infections have been considered as serious health and life-threatening diseases, particularly among immune-compromised patients. As the number of these patients grows gradually, the incidence of opportunistic fungal infections has been increasing (Chamegriha et al., 1997). In addition, many pathogenic fungi are also responsible for a wide range of superficial infections affecting human health (Pierre-Leandri et al., 2003). The increasing impact of these infections, incidence of drug-resistant pathogens, and the toxicity of the available antifungal drugs, are important factors that lead to heightened interest in the study of alternative natural products such as EOs (Cavaleiro et al., 2006). The objective of the present work is to evaluate the EOs of *Carz* and *Cglb* as potential products against fungal infections by characterizing their chemical composition and investigating the degree and mechanisms of cytotoxicity.

1. Material and Methods

1.1. Plant material and extraction of the essential oils

The aerial parts of *Carz* and *Cglb* were collected from the El Kriieb arboretum (North West of Tunisia). The leaves were separated, dried at room temperature, and used for the extraction of the EOs. The EOs were extracted by hydrodistillation of dried plant material (150 g of each sample in 500 mL of distilled water) using a Clevenger-type apparatus for 3 h. The oils were stored in sealed glass vials at 4-5 °C prior to analysis and bioactivity experiments.

1.2. Analysis of the essential oils

Chemical analysis of the EOs was done by GC/MS in a Hewlett-Packard 5972 MSD System. An HP-5 MS capillary column (30 m x 0.25 mm ID, film thickness of 0.25 µm) was used for separation of compounds and directly coupled to the mass spectrometer. The carrier gas was helium, with a flow rate of 1.2 ml/min. The oven temperature was programmed at 50 °C for 1 min, then 50-240 °C at 5 °C/min, and subsequently held isothermal for 4 min. Injector port: 250 °C, detector: 280 °C, split ratio: 1:50. Volume injected: 0.1 µl of EO 1% solution (diluted in hexane); mass spectrometer: HP5972 recording at 70 eV; scan time: 1.5 s; mass range: 40-300 amu. The software used to handle mass spectra and chromatograms was ChemStation. The identification of the compounds was based on mass

spectra (compared with Wiley 275.L, 6th edition mass spectral library). Further confirmation was done from Kovats retention index data generated from a series of alkanes retention indices.

1.3. Yeast strains, culture and sample preparation

Wild type and some mutants of *Saccharomyces cerevisiae* and several *Candida* species were used throughout this work (Table 1). *Saccharomyces cerevisiae* mutants include *yap1*, an oxidative stress-sensitive mutant affected in the gene *YAP1*, encoding the basic leucine zipper transcription factor involved in the transcription of a set of genes of the oxidative stress response (Coleman et al., 1999; Schnell and Entian, 1991). Other mutants include the *apn1*, affected in *APN1*, encoding the major apurinic/apyrimidinic endonuclease of the base excision repair (BER) pathway involved in the removal of oxidized nitrogenous bases and the mutant *rad4*, affected in *RAD4*, encoding a subunit of the nuclear excision repair factor 2 of the nucleotide excision repair (NER) pathway involved in the recognition of bulky, non-oxidative DNA damage.

All yeast strains were maintained on YPDA (yeast peptone dextrose agar) medium, containing yeast extract (1%), peptone (2%), glucose (2%) and agar (2%). For the preparation of liquid cultures, 5–10 ml YPD (YPDA lacking agar) was inoculated with a single colony of yeast and incubated overnight at 30 ºC, 200 rpm, and diluted with fresh medium to a density of 1.2×10^7 cells/ml. The cells were harvested after two generations by centrifugation (2 min at 14000 × *g*, 4 ºC), washed twice with the same volume of ice-cold deionized water and diluted back to the same concentration with ice-cold deionized water or ice-cold S buffer (1M sorbitol, 25 mM KH_2PO_4, pH 6.5).

1.4. Viability assay

Yeast cells from exponentially growing cultures were harvested by centrifugation at 14,000 x *g*, 2 min, at 4 ºC, washed twice with the same volume of sterilized deionized H_2O at 4 ºC and suspended in the same volume of S buffer. Aliquots of the suspension were incubated at 30 ºC, 200 rpm, in the presence of the EO of *Carz* or *Cglb* at different concentrations (0.1 to $1x10^{-3}$ µl/ml) or α-pinene ($3x10^{-3}$ to $6x10^{-5}$ µl/ml) for 90 min, harvested by centrifugation (2 min at 5000 × *g*, 4 ºC), washed twice with sterilized deionized H_2O at 4 ºC and suspended in the same volume of sterilized deionized H_2O. One hundred

microliters of the suspensions were serially diluted to 10^{-4} in deionized sterilized H_2O and spread on YPDA Petri dishes. After incubation at 30 °C for 48 h, the colonies were counted and the viability was calculated as percentage of colony-forming units (CFU), taking 100% viability for the sample of cells treated without EO (but containing the same amount of ethanol).

1.5. Comet assay

The yeast comet assay was performed as described previously (Azevedo et al., 2011). Briefly, from the S buffer cell suspension, approximately 10^6 cells were harvested by centrifugation (2 min, 14,000 × *g*, 4 °C), re-suspended in zymolyase buffer (2 mg/ml zymolyase, 20,000 U/g, ImmunO^{TM} 20T, in S buffer and 50 mM ß-mercaptoethanol) and incubated at 30 °C for 30 min, 200 rpm in order to digest the cell walls. Cell wall-devoid cells (spheroplasts) were washed twice by centrifugation (2 min, 14,000 × *g*, 4 °C) with ice-cold S buffer, incubated at 30 ^{0}C, 200 rpm, with the EO from *Carz* or *Cglb*, (10^{-3} to 5.10^{-5}µl/ml) for 90 min and washed twice with ice-cold S buffer as before. For the negative and positive controls, the EO was replaced by, respectively, the same amount of ethanol or H_2O_2 (10 mM in S buffer). The spheroplasts were washed twice as before, re-suspended in 1.5% (w/v in S buffer) low melting point agarose at 30 ºC and, immediately, 60 µl of the suspension was spread on a microscopy glass slide with a base layer of 0.5% (w/v in deionized water) normal melting point agarose. The suspension was covered with a coverslip and the glass slide was placed on ice for 5 min until agarose becomes solidified. The coverslip was gently removed and the glass slide was incubated in lysis buffer (30 mM NaOH, 1 M NaCl, 0.05% w/v laurylsarcosine, 50 mM EDTA, 10 mM Tris–HCl, pH 10) for 20 min to denature proteins and unwind genomic DNA. Subsequently, the slides were incubated twice in electrophoresis buffer (30 mM NaOH, 10 mM EDTA, 10 mM Tris–HCl, pH 10) for 20 min and the samples were then submitted to electrophoresis in the same buffer for 10 min at 0.7 V/cm, 4 ºC. After electrophoresis, the samples were neutralized by incubation in neutralization buffer (10 mM Tris–HCl, pH 7.4) for 10 min at 4 ºC, and fixed by two consecutive 10 min incubations in 76% (v/v) and 95% (v/v) ethanol. The slides were then air-dried and visualized in a fluorescence microscope upon staining with GelRed (10 µg/ ml; Biotium). The representative images of each slide, containing at least 50 comets, were acquired at a magnification of ×400 using a Leica Microsystems

DM fluorescence microscope. The tail length of the comets was analyzed with the free edition of CometScore™ software and the analytic parameter tail length (in µm) was chosen as a measure of the DNA damage.

1.6. Flow cytometry

Yeast cells from exponentially growing cultures were harvested as above, washed twice with the same volume of ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4), diluted to 0.02 optical density at 600 nm and 500 µl were used for the measurement of the auto fluorescence. The cells were loaded with 50 µM dichlorofluorescein diacetate (H_2DCFDA) by incubation at 30 °C, 200 rpm, during 1 h in the dark, after which they were washed twice by centrifugation as described above with the same volume of ice-cold PBS. Aliquots of 1 mL were mixed with the EO of each variety at different concentrations, or with the same volume of ethanol for the negative control, and incubated for 90 min, at 30 °C, 200 rpm, in the dark. Approximately twenty thousand cells of each sample were analyzed by flow cytometry in an Epics® XLTM cytometer (Beckman Coulter) equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. The green fluorescence was collected through a 488 nm blocking filter, a 550 nm long-pass dichroic and a 225 nm band-pass filter. The data were analyzed and histograms were made with the Flowing Software.

1.7. Statistical analyses

Each experiment was done at least in three independent experiments, in triplicate, and the results are presented as the mean value ± the standard deviation (SD). Comet assay results are the mean ± SD of three independent samples in which at least 50 comets were analyzed. GraphPad prism version 5 was used for statistical analyses. Statistical analyses of the data were performed using ANOVA One-Way, and the means were compared using Tukey's multiple comparison test. *P*-values less than 0.05 were considered to be significant.

2- Results and Discussion

2.1- Chemical analysis of the essential oils

The chemical composition of the EOs from *Carz* and *Cglb* was analyzed by GC/MS, and results are shown in Table 2. A total of 55 compounds were identified comprising 96.62% and 90.42% of the EO content for *Carz* and for *Cglb*, respectively. The major constituents, in both varieties, were α-pinene (29.76% and 26.53%, respectively), umbellulone (11.86% and 15.05%), terpinen-4-ol (5.72% and 4.08%), limonene (4.09% and 4.12%), β-sesquiphellandrene (3.11% and 2.01%), δ-terpinene (2.86% and 2.06%) and camphor (2.68% and 1.83%). Therefore, the EO from the leaves of both varieties of *C. arizonica* can be considered α-pinene and umbellulone-rich oils. This is in accordance with previous studies of EOs from leaves of *C. arizonica* EO cultivated in Tunisia (Cheraif et al., 2007). However, EOs from leaves of specimens from Italy, USA (Texas) and Algeria contained only 7.8%, 7.6% and 10.5% α-pinene, respectively (Adam et al., 1997; Chanegriha et al., 1997; Flamini et al., 2003). Moreover, umbellulone is more abundant in the EO from *C. arizonica* cultivated in Italy (45.1%) and Algeria (37.3%) than in Tunisia, which reached only 15.05% in *Cglb* and 11.86% in *Carz* (Table 2). The comparison of EOs from both varieties revealed significant differences in the chemical composition (Table 2), namely camphene hydrate and α-cedrene, two constituents present in *Carz* with a proportion of 3.82% and 4.12% respectively, and β-cubebene, calmanene and 14-norcadin-5-en-4-one present only in *Cglb*. Interestingly, cis-muurola-4(14),5-diene is a compound found in significant amounts in EO of cultivated specimens from Iran (10%) and north Tunisia (9.4%) (Afsharypuor and Tavakoli, 2005; Cherail et al., 2007). In our samples, this compound was not detected and this result is similar to EOs from Italian, Texan, Algerian and French *C. arizonica* (Adam et al., 1997; Chanegriha et al., 1997; Pierre-Leandi et al., 2003). The differences found between the main constituents of the EO obtained from *C. arizonica* cultivated in Tunisia and those cultivated in other countries can be related to the climate and soils differences (Chéraif et al., 2007). Our data support the observation that the composition of *Cupressus* EO is significantly dependent on the geographic origin of the plants, since the relative amounts of the most abundant components can be considerably different in EOs from plants cultivated in Texas (Adams et al., 1997), Argentina (Malizia et al., 2000) and France (Pierre-Leandri et al., 2003); umbellulone can be found in much higher amounts than α-pinene in

Algerian plants (Chanegriha et al., 1997); or the main constituents do not include umbellulone in plants cultivated in Iran (Afsharypuor and Tavakoli, 2005).

In EOs of both varieties (Table 2), there is a marked predominance of monoterpene hydrocarbons (47.22% and 41.24%, respectively), oxygen containing monoterpenes (33.03% and 27.8%) and sesquiterpene hydrocarbons (11.23% and 19.06%). Monoterpene content is higher in *Carz* (80.25%) than in *Cglb* (69.04%), while sesquiterpenes are more abundant in *Cglb* (20.32%) than *Carz* (14.75%).

2.2- Antifungal activity of the essential oils of *C. arizonica* var. *arizonica* and var. *glabra*

In this study we tested antifungal activity of *C. arizonica* EO of varieties *Carz* and *Cglb*, against the yeast model organism *S. cerevisiae* and a group of pathogenic *Candida*, including the most virulent ones: *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. dubliniensis* and *C. bracarensis* (Table 1). The *Candida* species were selected by their relevance as human fungal pathogens and frequency of clinical cases (Moran et al. 2012; Clark et al. 2002; Dar-Odeth et al. 2003; Pfaller et al. 2003; Pereira et al., 2008). *Candida albicans*, a species most frequently isolated in patients, is responsible for about 50% of candidemia, whereas *C. glabrata* represents 10-20% of candidemia (Eggimann et al. 2003). *Candida tropicalis* is one of the more common *Candida* causing human diseases in tropical countries, and is considered the most prevalent pathogenic yeast species of the *Candida*-non-*albicans* group (Rajendra et al. 2010). *Saccharomyces cerevisiae* is a model organism that can allow us to study the mechanisms of EOs antifungal activity.

We tested several concentrations of *Carz* and *Cglb* EOs and determined their minimum inhibitory concentrations (MICs) that inhibited the growth of selected yeasts (Table 3). The growth of *S. cerevisiae* and *Candida* species was very sensitive to EOs, especially *C. tropicalis* (with a MIC of 10^{-3} and 10^{-2}, for *Cglb* and *Carz* EOs, respectively).

Saccharomyces cerevisiae cells were exposed to the EO of varieties *Carz* and *Cglb* and several aliquots were harvested, diluted, and spread on YPDA plates in order to count colonies and estimate viability as CFUs. *Saccharomyces cerevisiae* viability significantly decreased in a dose-dependent manner from 10^{-3} µl/ml up to 10^{-1}µl/ml EOs concentrations where nearly all cells lost viability (Fig. 1). As depicted in Fig. 1, when cells were incubated with 10^{-2}µl/ml and $5x10^{-2}$µl/ml EO of *Carz*, the

viability was lower (approximately 65% and 40%, respectively; Fig. 1B) when compared to the EO from *Cglb* (approximately 90% and 70%, respectively; Fig. 1A). These results suggest that the EO from *Carz* is more cytotoxic than the EO obtained from *Cglb*, a trend that was already observed for MIC values of *C. tropicalis* (Table 3).

Antifungal activity of EOs from different plants has been reported extensively in the literature (Bakkali et al., 2008). Previous publications reported the antimicrobial activity of the EO from *C. arizonica* against several bacteria (Cheraif et al., 2007), *Aspergillus flavus* (Karbin et al., 2009) and the strawberry anthracnose causing fungal plant pathogens *Colletotrichum acutatum*, *C. fragariae* and *C. gloeosporioides* (Ali et al., 2013).

2.3- Mechanisms of action of the essential oils of *C. arizonica* var. *arizonica* and var. *glabra*

2.3.1- The essential oils of *C. arizonica* var. *arizonica* and var. *glabra* are more cytotoxic to yeast mutants affected in the oxidative stress response

In order to clarify the mechanism of action of these EOs, the cytotoxicity was investigated in *S. cerevisiae* mutant strains affected in the oxidative stress response and in DNA repair pathways. As depicted in Fig. 1, a remarkable decrease in viability was observed in *S. cerevisiae* mutant strains affected in the oxidative stress response (*yap1* and *apn1*), as compared with the wild type strain. Viability of *yap1* mutant strain was significantly affected (circa 20% less, Fig. 1C-D) when compared with the wild type viability, for as low as 10^{-3} µl/ml of EO. Increased sensitivity was also observed with the *apn1* mutant strain (Fig. 1E-F). Interestingly, *rad4* was the mutant strain less affected by both EOs (Fig. 1G-H), viability being comparable to that of the wild type strain (Fig. 1A-B). These results strongly suggest that the toxicity of the EOs is mediated by an oxidative stress-inducing mechanism since the *yap1* mutant is unable to activate the cellular response against oxidative stress and the *apn1* mutant is affected in the repair of oxidative DNA damage. On the other hand, the *rad4* mutant strain, which displays an EO resistance similar to the wild type, is fully able to repair oxidative DNA damage as it is only affected in the NER pathway (De Laat et *al*., 1999; Kamileri et al., 2012), which is not

involved in oxidative DNA damage. The cytotoxic effects of some EOs mediated by oxidative stress or prooxidant mechanisms and/or mitochondria damage have been reported before (Bakkali et al., 2008).

The presence of a significant amount of α-pinene in both EOs, as shown in Table 2, suggests that this compound might be a major cytotoxic agent since it is known to induce oxidative stress (Singh et al., 2006; Iwamoto et al., 2012; Pinto et al., 2013), with different targets, including the DNA. Therefore, we decided to investigate if this compound promotes similar effects in the viability of all *S. cerevisiae* strains tested. We used equivalent amounts of α-pinene present in the quantities of EOs (based in the composition data, Table 1) used in the viability tests (Fig. 1). In fact, for the concentrations of α-pinene tested we observed a similar effect in viability (Fig. 2), *S. cerevisiae* wild type and *rad4* mutant strains being less sensitive than *yap1* and *apn1* mutants. Therefore, these results corroborate α-pinene citoxicity and since this compound is the most abundant in the EOs *Carz* and *Cglb* (Table 2), it is likely that it might be also the main cytotoxic agent of these oils.

2.3.2- The essential oils of *C. arizonica* var. *arizonica* and var. *glabra* increase intracellular oxidation in yeast cells

Higher sensitivity of the mutant strains affected in oxidative stress response, *yap1* and *apn1* to *Carz* and *Cglb* EOs strongly suggests an oxidative activity in their cytotoxic effects (Figs. 1 and 2). Hence, we hypothesized that the EOs would have an intracellular effect in terms of redox state. To investigate whether the oxidant effect of the EOs is mediated by altered intracellular redox state in yeast, cells incubated with both oils were analyzed by flow cytometry with a redox-sensitive probe, H_2DCFDA, which is fluorescent in the oxidized form. This lipophilic compound permeates the cells where it is deacetylated to dichlorofluorescein by intracellular esterases. The deacetylated form is hydrophilic and becomes trapped inside the cells.

Intracellular fluorescence of cells has shifted in a dose-dependent manner to higher values when cells were incubated with the EOs (Fig. 3A-H). This suggests that cells undergone intracellular oxidation as a result of the activity of EOs of both varieties. Once again, *Carz* EO was more active, displaying more pronounced shifts of fluorescence towards higher values, which correlates with results of

viability (Fig. 1), and higher α-pinene content compared to *Cglb* EO (Table 2). All samples were monitored under the fluorescence microscope, which confirmed that fluorescence was exclusively intracellular (Fig. 3I-J).

The oxidative activity of both EOs would be more pronounced in cells with impaired oxidative stress response pathways. Therefore, we investigated the intracellular oxidation level in the mutants tested previously using 10^{-3} µl/ml EO, a concentration that did not promote marked effects in loss of viability (Fig. 1A and 1B) and intracellular oxidation (Fig. 3G and 3H) in wild type cells. As depicted in Fig. 4, *yap1* and *apn1* mutant strains displayed increased intracellular oxidation upon treatment with both EOs (Fig. 4A-4H), while the *rad4* mutant was not affected (Fig. 4I-4L). These results are in accordance with the previous observations on viability, where impairment in the oxidative stress response (in *yap1* and *apn1* mutants) rendered strains more sensitive. As above, the intracellular origin of the fluorescence was confirmed by fluorescence microscopy as depicted in a representative sample (Fig. 4M-N).

The *yap1* mutant affected in the transcription factor Yap1 that regulates transcription of genes of the oxidative stress response is a clear example of a high sensitive yeast strain when challenged with oxidative toxicants (Kuge and Jones, 1994). In this work, the higher sensitivity of this strain in the presence of *Carz* and *Cglb* EOs, when compared with the wild type strain (Figs. 1-4), is a strong indication that these oils are oxidative toxicants. Additionally, the *apn1* mutant, affected in the pathway involved in the repair of DNA oxidative damage, was more susceptible to the EOs (Figs. 1-4) than the *rad4* mutant involved in the removal of bulky, non-oxidative DNA damage, which was as sensitive as the wild type (Figs. 1 and 4). It is interesting to note that we observed the same behavior when we used α-pinene instead of EOs, suggesting that this is a major antifungal active compound of *Cupressus* EOs (Fig. 2).

2.3.3- The essential oils of *C. arizonica* var. *arizonica* and *glabra* are genotoxic to yeast cells

One of the cellular targets of oxidative stress is DNA. Therefore it is conceivable that the *Carz* and *Cglb* EOs have a genotoxic effect in yeast cells, as occurred with other EOs (Bakkali et al. 2008). To assess genotoxicity we analysed the DNA damage provoked by the EOs with the yeast comet assay.

Yeast spheroplasts were treated with 10^{-3} µl/ml, 5.10^{-4} µl/ml, 10^{-4} µl/ml or 5.10^{-5} µl/ml EO from *Carz* and *Cglb* and the DNA damage was subsequently analyzed. As expected, cells exposed to the EOs of both varieties displayed increased DNA damage, assessed as a comet tail length, in a dose-dependent manner (Fig. 5A and 5B). At the higher EO concentration tested, 10^{-3}µl/ml, in which yeast viability is unaffected (Fig. 1), DNA damage was significantly higher than in the negative control. Interestingly, the EO from *Carz* was more active (Fig. 5A and 5B), which correlates with the higher cytotoxic activity of this oil in the parental and mutant strains. These results suggest that the oxidant activity of both EOs target the genome of yeast cells, the EO from *Carz* being more active than the one from *Cglb*.

Oxidative stress-mediated genotoxicity similar to *Carz* and *Cglb* EOs was previously reported for EOs from *Piper gaudichaudianum* (Sperotto et al., 2013), *Cymbopogon* species (palmarosa, citronella and lemongrass) and *Chrysopogon zizanioides* (vetiver) (Sinha et al., 2014). However, the fact that some of these EOs are also reported as having the opposite effect, especially at low concentrations (Sinha et al., 2011; Sinha et al., 2014), together with the activities observed at very low concentrations in *Carz* and *Cglb*, suggests that these EOs have considerably high antifungal activity with high potential for human applications.

3- Conclusion

In this work we showed that the EOs from *Carz* and *Cglb* have powerful antifungal activity, namely against several relevant pathogenic yeasts. We provide compelling evidences based on approaches using the availability of *S. cerevisiae* mutant strains affected in specific cellular processes in order to identify putative EO cytotoxic activities. Antifungal activity of *C. arizonica* EO is mediated by an oxidative process leading to increased intracellular oxidation and DNA damage. Moreover, α-pinene is a major compound responsible for the biological effects induced by EO from *Carz* and *Cglb*. The high antifungal activity of these EOs makes them good candidates for antifungal agents against pathogenic yeasts.

4- Acknowledgements

This work is supported by national funds by FCT - Portuguese Foundation for Science and Technology, under the projects UID/AGR/04033/2013, PTDC/AGR-ALI/105169/2008 and PEst-OE/AGR/UI4033/2014. The authors would like Célia Pais for providing *C. glabrata*, *C. dubliniensis*, *C. parapsilosis* and *C. bracarensis* species for this work and to Cristina Ribeiro for all the support in flow cytometry analyses.

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469 Table 1

470 Yeast strains used in this work.

Strain name	Genotype	Reference or origin
Saccharomyces cerevisiae BY4741	*MAT*a*; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0*	Brachmann et al., 1998
Saccharomyces cerevisiae rad4	*MAT*a*; his3D1; leu2D0; met15D0; ura3D0; YER162c::kanMX4*	Euroscarf, Germany
Saccharomyces cerevisiae yap1	*MAT*a*; his3D1; leu2D0; met15D0; ura3D0; YML007w::kanMX4*	Euroscarf, Germany
Saccharomyces cerevisiae apn1	*MAT*a*; his3D1; leu2D0; met15D0; ura3D0; YKL114c::kanMX4*	Euroscarf, Germany
Candida albicans ATCC 18804	Wild type, clinical isolate	ATCC, USA
Candida glabrata 8D	Wild type, clinical isolate	Department of Biology, University of Minho, Portugal
Candida dubliniensis CIPO 82	Wild type, clinical isolate	Correia et al., 2004
Candida parapsilosis 28 B	Wild type, clinical isolate	Correia et al., 2004
Candida tropicalis IGC 3097	Wild type, clinical isolate	Instituto Gulbenkian de Ciência, Portugal
Candida bracarensis NCYC 3133	Wild type, clinical isolate	Correia et al., 2006

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Table 2

Essential oils composition (% w/w) from leaves of *C. arizonica* var. *arizonica* and var. *glabra* cultivated in Tunisia. Phytochemical analysis was done using gas chromatography/mass spectrometry (GC-MS) and data are the mean (±SE) of three independent analyses for each variety (%EO values are significantly different in the same line at: * $p< 0.05$, ** $p< 0.01$, and *** $p< 0.001$). RI: retention index, %EO: percentage of EO.

No	compound	RI	%EO *glabra*	%EO *arizonica*	*P*
1	tricyclene	925	0,28 ± 0,14	0,2 ± 0,1	
2	α-thujene	930	0,75 ± 0,03	0,91 ± 0,09	
3	α-pinene	939	26,53 ± 0,73	29,76 ± 0,3	*
4	camphene	954	0,61 ± 0,03	0,59 ± 0	
5	Sabinene	975	1,7 ± 0,1	2,51 ± 0,01	***
6	β-pinene	979	0,68 ± 0,05	0,71 ± 0,01	
7	β-myrcene	990	0,85 ± 0,04	0,75 ±	
8	δ-4-carene	1002	0	0,44 ± 0	***
9	α-phellandrene	1005	0,32 ± 0,16	0	***
10	δ-3-carene	1011	1,02 ± 1,02	1,72 ± 0	***
11	α-terpinene	1017	0,83 ±	1,07±	*
12	*p*-cymene	1024	1,47 ± 0,18	1,56 ± 0	
13	*p*-cymen-8-ol	1026	0,35 ± 0,03	0,65 ± 0	***
14	Limonene	1029	4,12 ± 0,18	4,09 ± 0,01	
15	δ-terpinene	1059	2,06 ± 0	2,86 ± 0,01	***
16	α-terpinolene	1088	0,85 ± 0,08	1,03 ± 0,01	
17	Linalool	1096	0,4 ± 0,05	0,72 ± 0	**
18	β-fenchol	1121	1,48 ± 0,01	1,38 ± 0	**
19	α-campholenal	1125	0,18 ± 0,09	0,38 ± 0	*
20	(*Z*)-pinocarveol	1139	0,9 ± 0	0,59 ± 0	***
21	p-menthe-2-en-1-ol	1140	0	0,38 ± 0,01	***
22	camphor	1146	1,83 ± 0,11	2,68 ± 0,01	***
23	camphene hydrate	1149	0,23 ± 0,11	3,82 ± 0,03	***
24	Pinocarvone	1164	0	0,98 ± 0,01	***
25	Borneol	1169	0	0,53 ± 0	***
26	Umbellulone	1171	15,05 ± 0,26	11,86 ± 0,04	***
27	terpinen-4-ol	1177	4,08 ± 0,02	5,72 ± 0	***
28	myrtenal	1195	0	0,32 ± 0	***
29	(*E*)-carveol	1217	0	0,49 ± 0,01	***
30	β-citronellol	1225	0,6 ± 0,01	0,56 ± 0,01	
31	thymol methylether	1235	0,36 ± 0,03	0,42 ± 0	***
32	piperitone	1252	0	0,33 ± 0,01	***
33	Bornyl acetate	1285	0,54 ± 0,03	0,89 ± 0,01	***
34	Thymol	1290	0,49 ± 0,01	0,33 ± 0,02	*
35	α-terpinyl acetate	1350	0,89 ± 0,01	0	***
36	ionole	1377	1,26 ± 0,02	0,83 ± 0,02	***
37	β-cubebene	1388	6,71 ± 0,02	0,32 ± 0	***

38	α-cedrene	1411	0,66 ± 0,08	4,12 ± 0,03	***
39	Aromadendrene	1441	1,65 ± 0	0,89 ± 0,06	***
40	δ-murrolene	1479	0,69 ± 0,02	0,31 ± 0,03	***
41	δ-curcumene	1480	0,55 ±	0	
42	germacrene D	1485	0	0,27 ± 0,13	**
43	β-sesquiphellandrene	1522	2,01 ± 0	3,11 ± 0,02	***
44	delta-cadinene	1524	0	1,04 ± 0,01	***
45	Calmanene	1529	4,5 ± 0,07	0,17 ± 0,02	***
46	(*Z*)-Cadina-1,4-diene	1534	0,36 ± 0	0	
47	α-calacorene	1545	0,53 ± 0	0	
48	(*E*)-nerolidol	1563	0,23 ± 0,11	0	
49	β-caryophylleneepoxide	1583	0	0,32 ± 0,03	***
50	Cedrol	1600	1,36 ± 0,08	1,01 ± 0,08	***
51	α-cadinol	1654	0,77 ± 0	0,57 ± 0,01	***
52	Cadalene	1674	0,4 ± 0,03	0	***
53	14-norcadin-5-en-4-one	1697	2,78 ± 0	0,79 ± 0,03	***
54	*epi*-manoyloxide	1987	0,36 ± 0	0,07 ± 0,07	*
55	labd-(13E)-8,15-diol	2428	0,7 ± 0,03	1,55 ± 0	***
	Monoterpene hydrocarbons		41,24	47,22	***
	Oxygenated monoterpenes		27,8	33,03	***
	Total monoterpenes		69,04	80,25	***
	Sesquiterpene hydrocarbons		19,06	11,23	***
	Oxygenated sesquiterpenes		1,26	3,52	**
	Total sesquiterpenes		20,32	14,75	***
	Oxygenated diterpenes		1,06	1,62	
	Total		**90,42**	**96,62**	***

479

480

481 Table 3

482 Minimum inhibitory concentrations (µl/ml) of the EOs of *C. arizonica* var. *arizonica* and var. *glabra*
483 in several pathogenic *Candida* species and *S. cerevisae*.

Essential oils	Yeast species						
	C. albicans	*C. bracarensis*	*C. dubliniensis*	*C. glabrata*	*C. parapsilosis*	*C. tropicalis*	*S. cerevisae*
C. arizonica var. *glabra*	$5x10^{-2}$	$5x10^{-2}$	$1x10^{-2}$	$5x10^{-2}$	$5x10^{-2}$	$1x10^{-3}$	$1x10^{-1}$
C. arizonica var. *arizonica*	$5x10^{-2}$	$1x10^{-2}$	$1x10^{-2}$	$1x10^{-2}$	$1x10^{-2}$	$1x10^{-2}$	$1x10^{-1}$

484

Figure captions

Fig. 1. Viability of *S. cerevisiae* and mutant strains in the presence of the EO of *C. arizonica*. *S. cerevisae* wild type (A and B) and mutant yeast cells *yap1* (C and D), *apn1* (E and F) and *rad4* (G and H) were incubated with the EO, from *C. arizonica* var. *glabra* (A, C, E and G) and var. *arizonica* (B, D, F and H), at different concentrations (10^{-3}, 10^{-2}, $5x10^{-2}$ or 10^{-1} µl/ml) for 90 min at 30 °C. Aliquots of each suspension were harvested, serially diluted and spread on YPDA plates. Colonies were counted after 48 h incubation and viability was calculated as percentage of control (absence of EO corresponding to 100% viability). Data are the mean±SD of three independent experiments (significantly different in relation to control group at: * $p< 0.05$, ** $p< 0.01$, and *** $p< 0.001$).

Fig. 2. Viability of *S. cerevisiae* in the presence of α-pinene. *S. cerevisiae* wild type strain (A) and mutants *yap1* (B), *apn1* (C) and *rad4* (D) were incubated with α-pinene at different concentrations ($6x10^{-5}$, 10^{-4}, $3x10^{-4}$ and $3x10^{-3}$ µl/ml) for 90 min at 30 °C. Aliquots of each suspension were harvested, serially diluted and spread on YPDA plates. Colonies were counted after 48 h incubation at 30 ºC and viability was calculated as percentage, taking 0 µl/ml concentration as reference (100% viability). Data are the mean ±SD of three independent experiments (significantly different in relation to control group at: * $p< 0.05$, ** $p< 0.01$, and *** $p< 0.001$).

Fig. 3. Intracellular oxidation of *S. cerevisiae* cells exposed to *C. arizonica* EO from var. *arizonica* (A, C, E and G) and var. *glabra* (B, D, F and H). *S. cerevisae* wild type cells were loaded with H_2DCFDA and then exposed to EO at different concentrations (10^{-2}, $5x10^{-3}$ and 10^{-3} µl/ml) or the same volume of ethanol (A and B) for 90 min and analyzed for fluorescence by flow cytometry. Data are from a representative experiment from three independent replicas. Representative cell of a sample loaded with H_2DCFDA and photographed by fluorescence microscopy (Leica DM5000) after treatment with EO (I and J). Zoom 100X (I) and 400X (J).

Fig. 4. Intracellular oxidation of *yap1* (A-D), *apn1* (E-H) and *rad4* (I-L) yeast mutant strains exposed to *C. arizonica* EO from var. *arizonica* (A, C, E, G, I and K) and var. *glabra* (B, D, F, H, J and L). Mutant yeast cells were loaded with H_2DCFDA and then exposed to 10^{-3} µl/ml EO for 90 min and analyzed for fluorescence by flow cytometry. Data are from a representative experiment from three independent replicas. Representative cells

of a sample loaded with H_2DCFDA and photographed by fluorescence microscopy (Leica DM5000) after treatment with EO (M and N). Zoom 100X (M) and 400X (N).

Fig. 5. Genotoxicity of *C. arizonica* EO from var. *glabra* (A) and var. *arizonica* (B) in *S. cerevisiae* cells. *S. cerevisae* wild type spheroplasts were treated with EOs at different concentrations ($5x10^{-5}$, 10^{-4}, $5x10^{-4}$ or 10^{-3} µl/ml) for 90 min at 30 °C. DNA damage was analyzed with the yeast comet assay (see Materials and Methods). Controls included cells treated with the EO diluting solvent (ethanol; C-) or cells treated with 10 mM H_2O_2 (C+). Mean±SD values are from three independent experiments (significantly different in relation to control group at: * $p< 0.05$, ** $p< 0.01$, and *** $p< 0.001$).

Figure 1

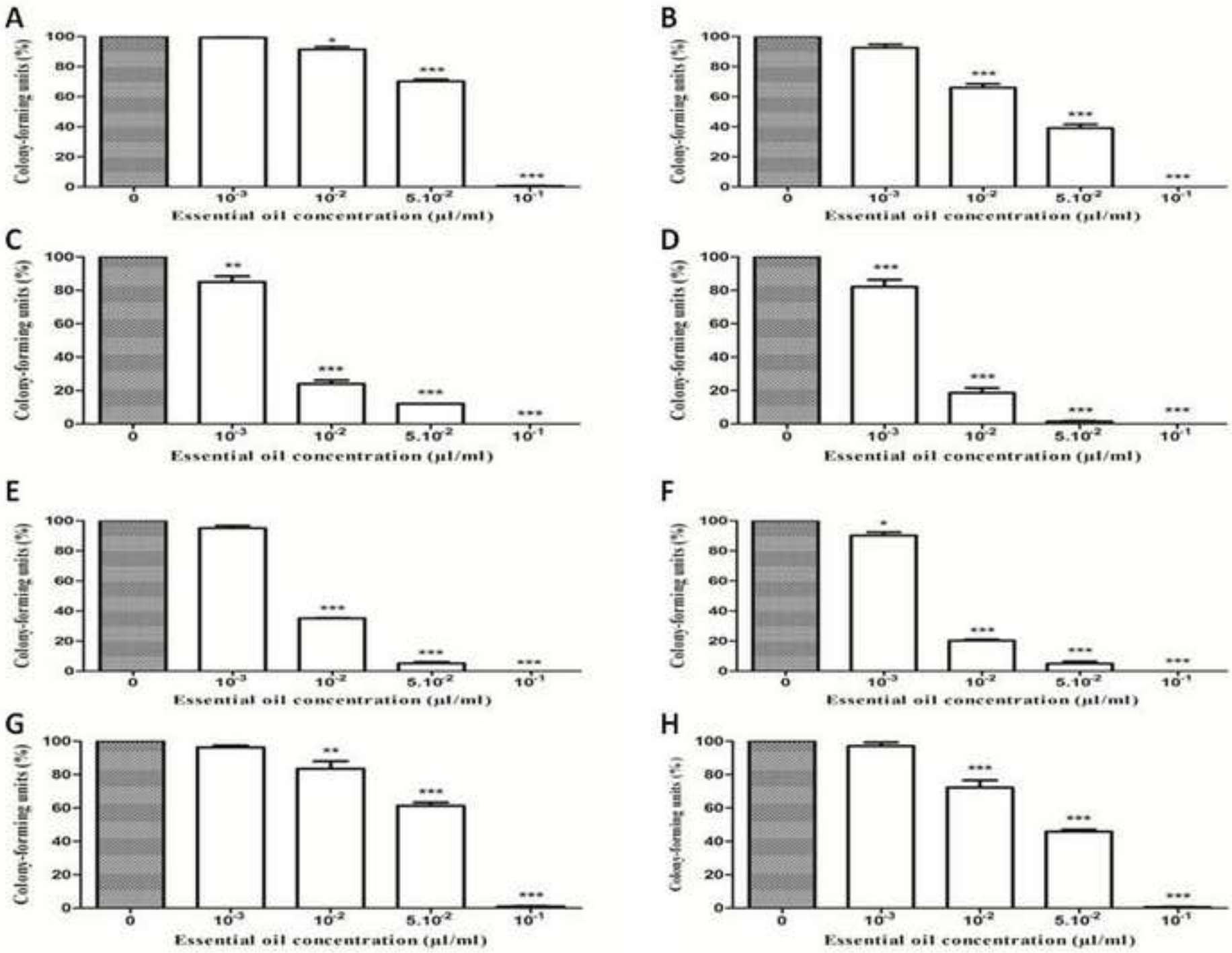

A
B
C
D
E
F
G
H
Colony-forming units (%)
Essential oil concentration (µl/ml)
0
10-3
10-2
5.10-2
10-1

Figure 2

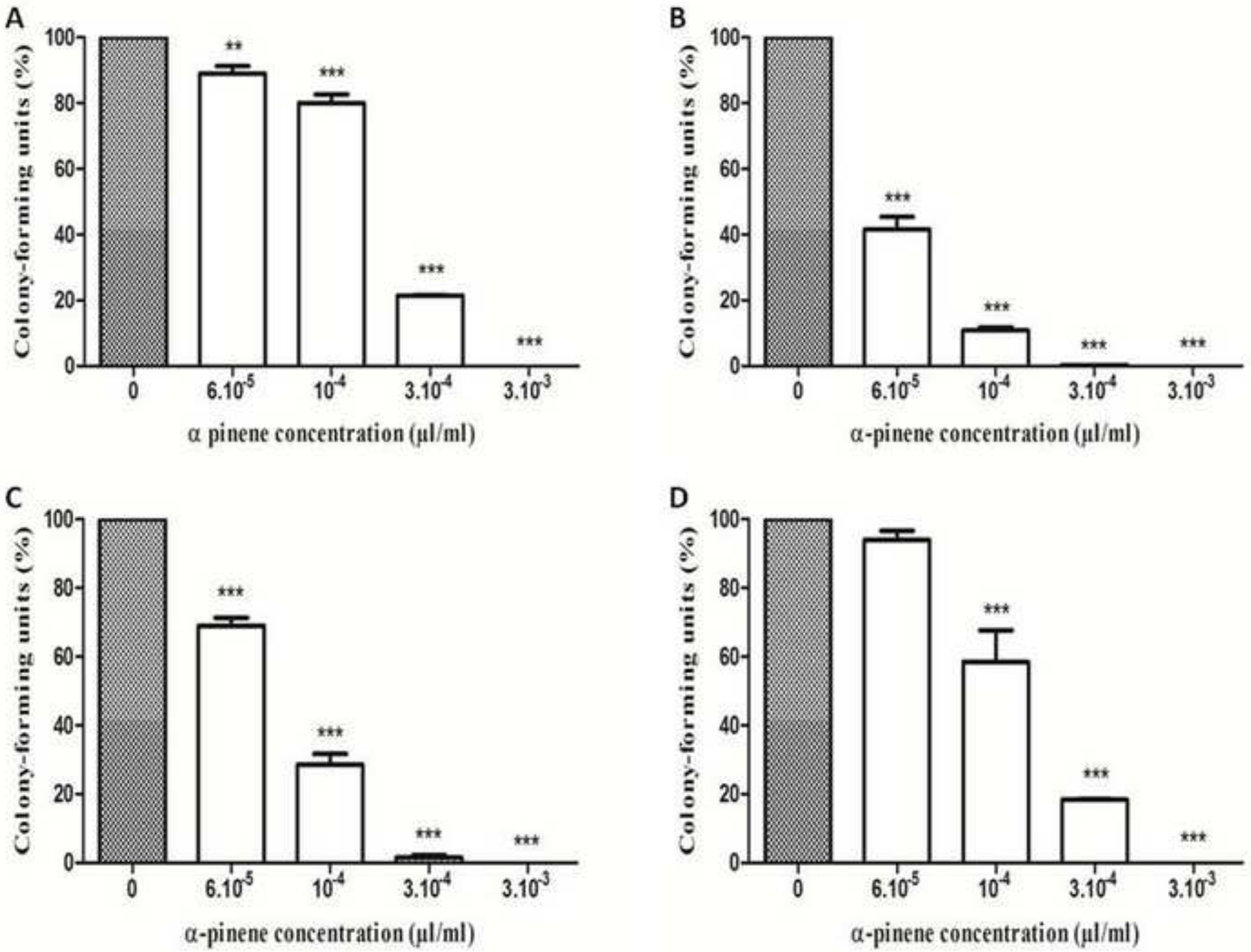

A
B
C
D
Colony-forming units (%)
100
80
60
40
20
0
**
***
0
6.10-5
10-4
3.10-4
3.10-3
α pinene concentration (µl/ml)
α-pinene concentration (µl/ml)

Figure 3

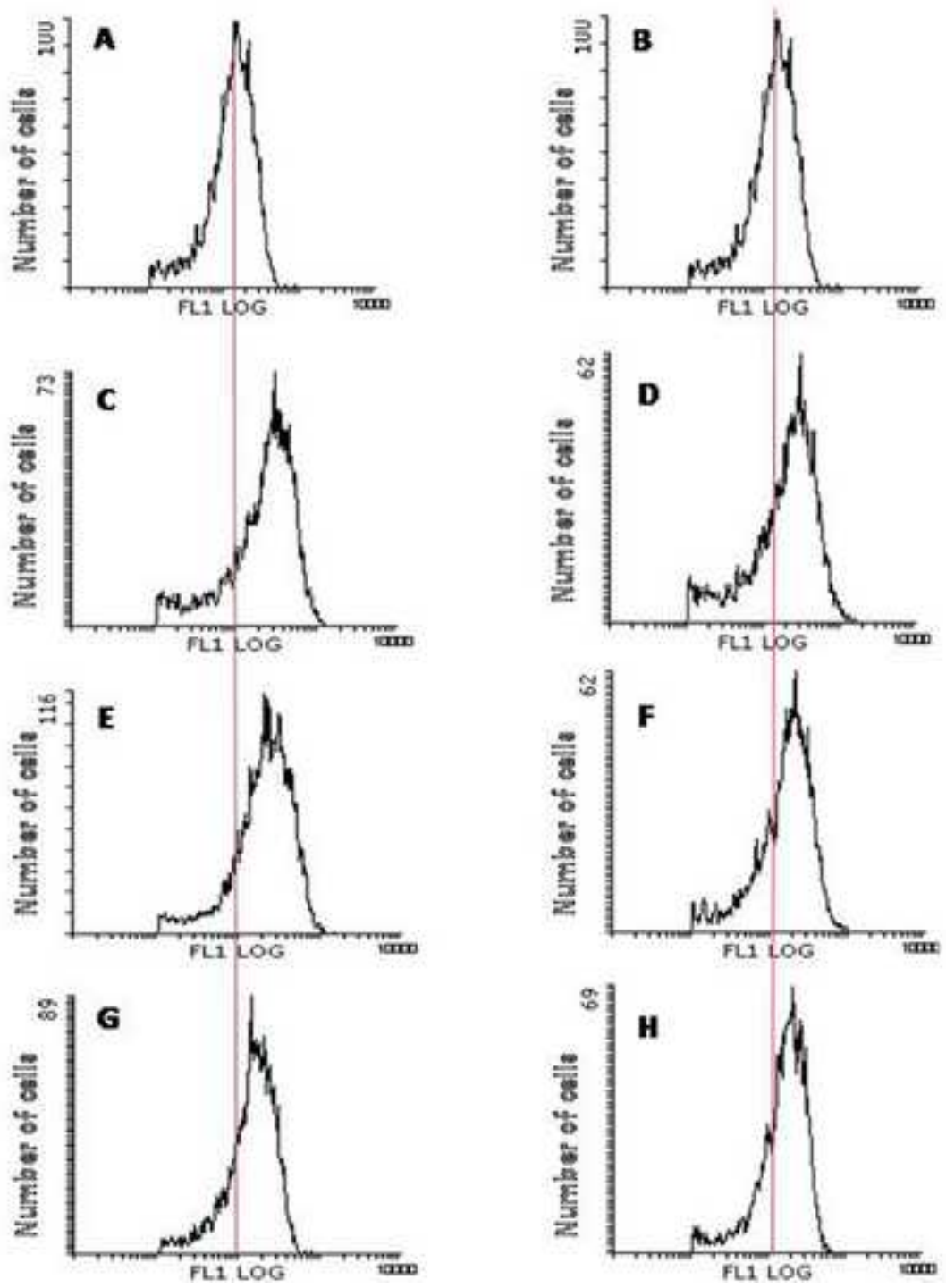


Figure 4

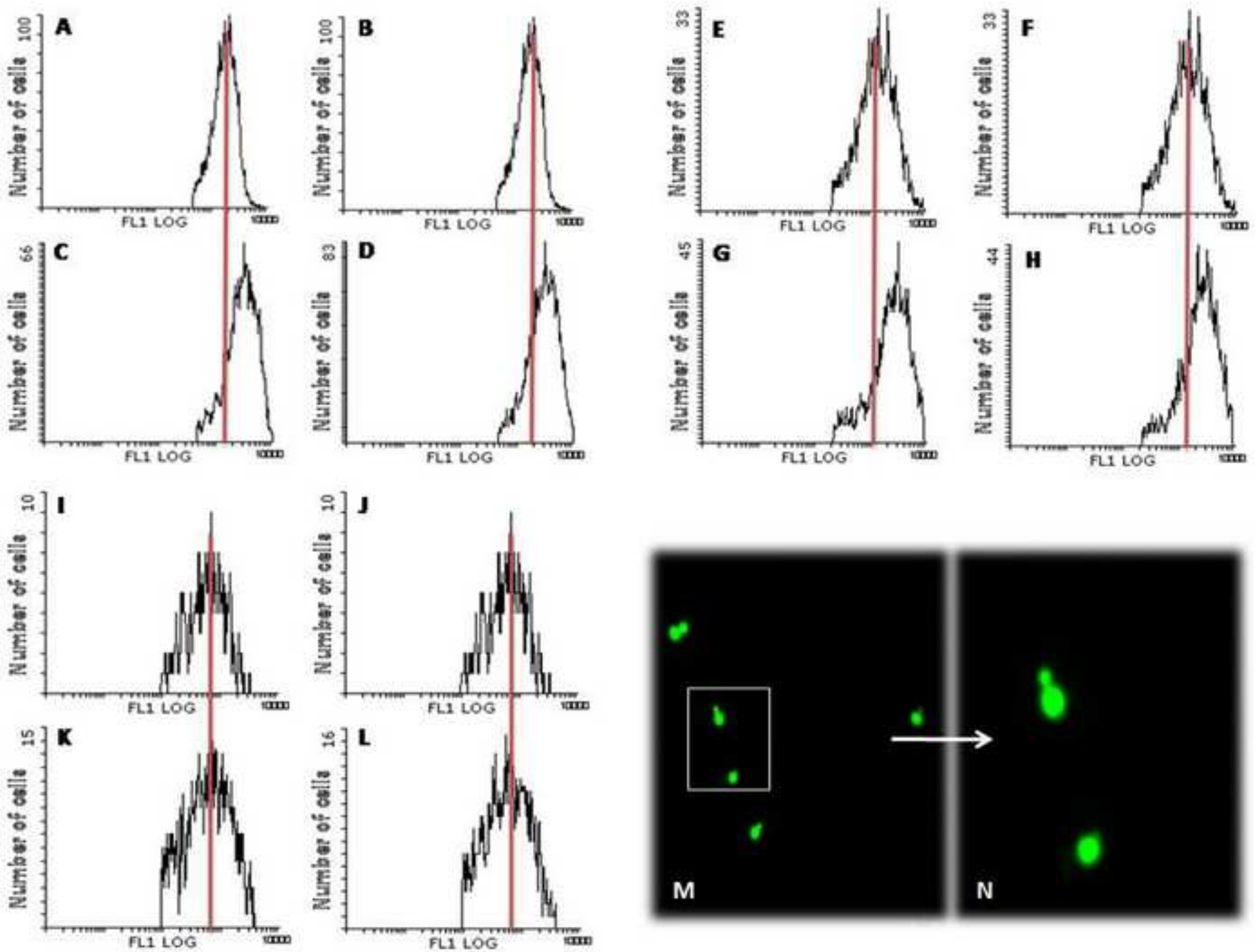
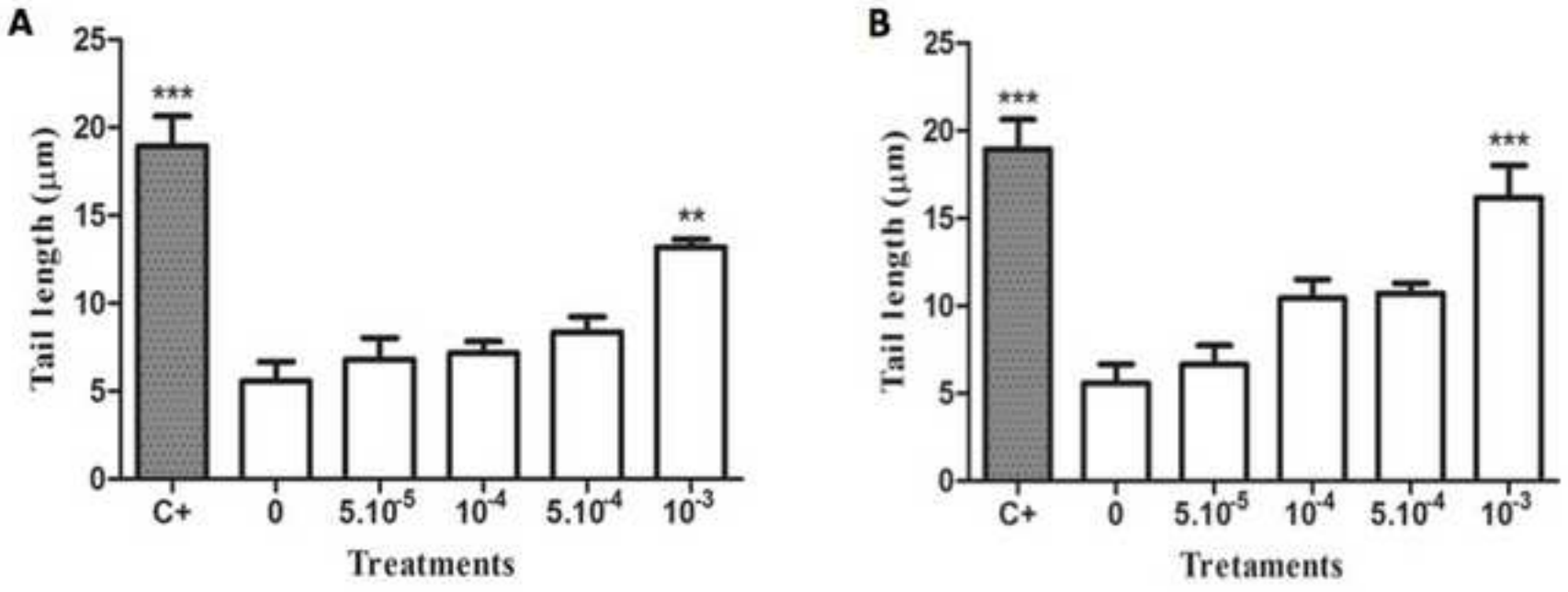


Figure 5


A
Tail length (µm)
25
20
15
10
5
0
***
**
C+
0
5.10-5
10-4
5.10-4
10-3
Treatments
B
Tail length (µm)
25
20
15
10
5
0
***
***
C+
0
5.10-5
10-4
5.10-4
10-3
Tretaments