**Supplementary material**

**TITLE**

# Antimycotic activity of ozonized oil in liposome eye drops against *Candida* spp.


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**Footnote**

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**MEMBRANE DEPOLARIZATION IN *C. albicans***

**Table S1.** Intensity of fluorescence $D_0$, background; $D_s$, released fluorescence at several concentrations of Ozodrop®; $D_{max}$, maximum of depolarization of *C. albicans* plasma membrane in presence of saponin 0.05%. Fluorescence intensity was measured with $\lambda_{ex}$ = 559 nm and $\lambda_{em}$ = 575 nm by Perkin Elmer Luminescence Spectrometer LS50B (Perkin Elmer, Italy) Comparisons between multiple groups were performed by ANOVA One-way repeated measure test on raw data, followed by Dunnett test with respect to $D_0$. Error is expressed as ± standard error (SEM) of the mean of three independent experiments.

| | $D_0$ | | $D_s$ | | | $D_{max}$ | | |
|---|---|---|---|---|---|---|---|---|
| **Ozodrop® %(v/v)** | **Mean** | **±SE of mean** | **Mean** | **±SE of mean** | ***p-value*** | **Mean** | **±SE of mean** | ***p-value*** |
| 0.05 | 50.73 | 0.63 | 48.77 | 0.90 | 0.80097 | 358.04 | 4.87 | 1.56634E-7 |
| 0.10 | 52.80 | 2.37 | 56.12 | 1.82 | 0.84611 | 372.64 | 10.31 | 2.04369E-6 |
| 0.20 | 52.58 | 1.26 | 63.78 | 1.31 | 0.05863 | 358.10 | 5.23 | 1.93309E-7 |
| 0.39 | 54.65 | 1.27 | 82.86 | 0.91 | 4.38938E-4 | 360.32 | 3.75 | 3.33129E-8 |
| 0.78 | 50.56 | 1.07 | 84.19 | 5.05 | 0.02962 | 327.67 | 11.14 | 9.62392E-6 |
| 1.56 | 54.58 | 1.10 | 119.83 | 5.12 | 8.31708E-4 | 353.48 | 9.64 | 2.0071E-6 |
| 3.125 | 59.85 | 2.02 | 162.64 | 8.38 | 2.57142E-4 | 380.87 | 11.79 | 2.78997E-6 |

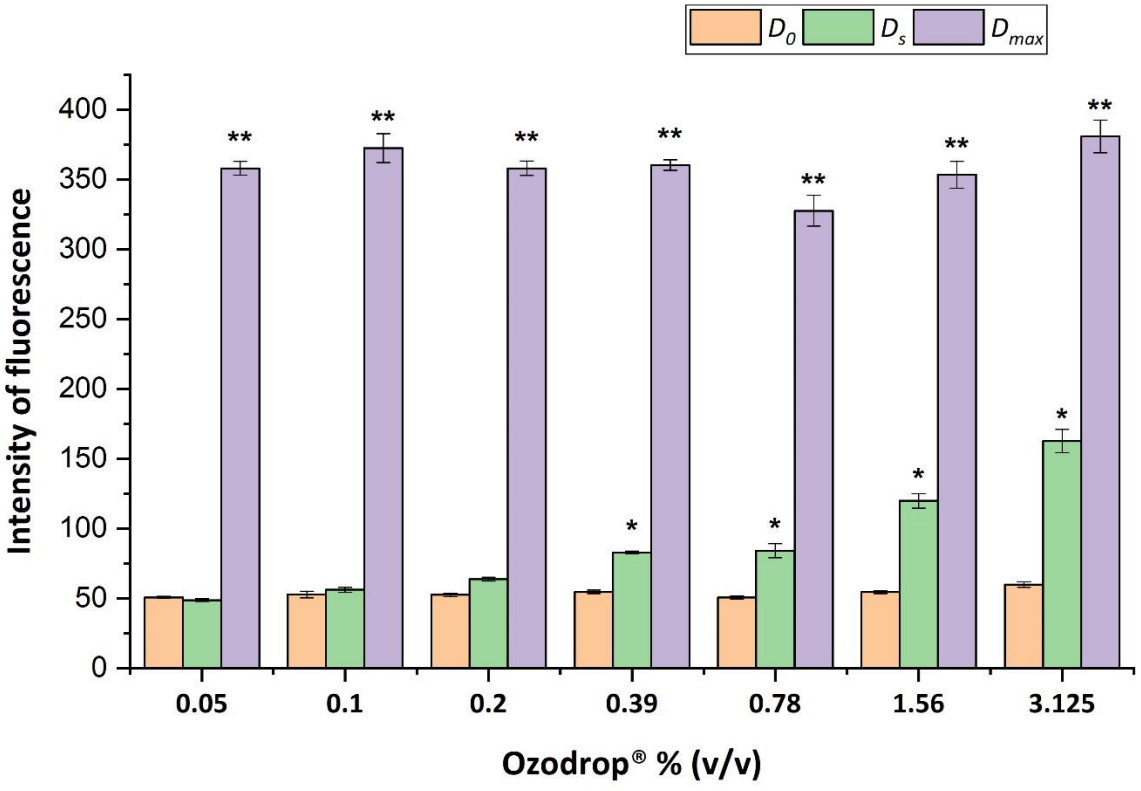


**Figure S1.** Intensity of released fluorescence in presence of several Ozodrop® dilutions. *, $p$<0.05; **$p$<0.0001. More detailed information are reported above in Table S2.

**CELL VIABILITY ASSAY**

**Table S2**. Cell viability was evaluated using the MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide. Data obtained from viability assay were reported as percentage of killed cells with respect to untreated cells. Comparisons between multiple groups were performed by ANOVA One-way repeated measure test on raw data, followed by Dunnett test with respect to untreated cells. Error is expressed as ± standard error (SEM) of the mean of three independent experiments.

| | Raw data (O.D. at 570 nm) | | | % of killed cells | |
|---|---|---|---|---|---|
| **Ozodrop® %(v/v)** | **Mean** | **±SE of mean** | ***p-value*** | **Mean** | **±SE of mean** |
| 0 | 0.84 | 0.02 | | 0.00 | 0.00 |
| 0.78 | 0.72 | 0.03 | 0.0454 | 13.93 | 3.60 |
| 1.56 | 0.60 | 0.04 | 4.72E-04 | 27.66 | 5.75 |
| 3.125 | 0.60 | 0.01 | 4.64E-04 | 27.79 | 2.23 |
| 6.25 | 0.46 | 0.01 | 6.55E-06 | 44.59 | 1.07 |
| 12.5 | 0.25 | 0.03 | 4.40E-11 | 70.53 | 3.10 |

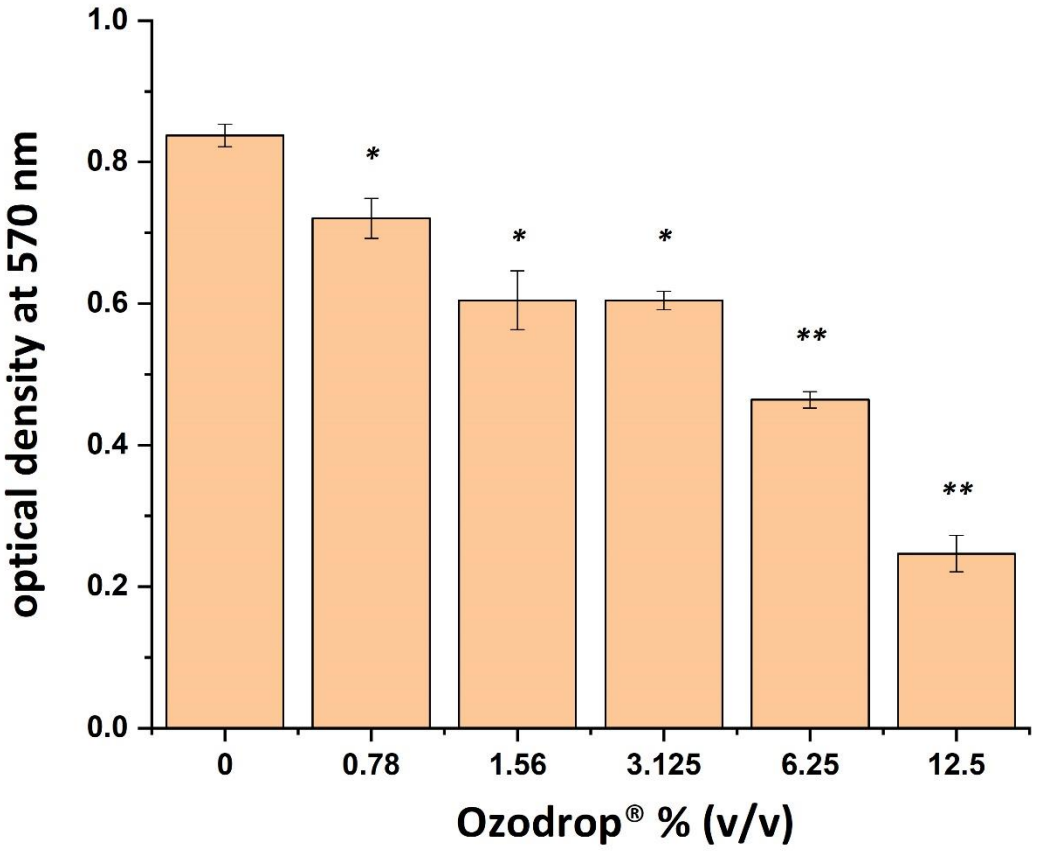


**Figure S2**. Raw data from optical density at 570 nm. *, $p<0.05$; **$p<0.0001$. More detailed information are reported above in Table S3.

**DETERMINATION OF LIPID PEROXIDATION**

Whole-cell lipid membrane peroxidation in *C. albicans* cells was colorimetrically evaluated by lipid peroxidation assay kit (MAK085, Sigma-Aldrich, St. Louis, MO, USA) following manufacturer's protocol. Absorbance was measured at 530 nm ($A_{530}$) by microplate reader iMark, BioRad. The amount of the malondialdehyde (MDA) in the samples was determined using a standard curve as indicated by the manufacturer. Calibration curve was obtained by measuring the absorbance at 530 nm of standard solutions of malondialdehyde in a range from 0 to 20 nmol.

**Table S3.** Standard solutions of malondialdehyde used for the determination of the calibration curve.

| Malondialdehyde (nmol) | Replicates (ABS at 530 nm) | | | Mean (ABS at 530 nm) | ±SE of mean |
|---|---|---|---|---|---|
| 0.00 | 0.04 | 0.053 | 0.07 | 0.05 | 0.00953 |
| 4.00 | 0.25 | 0.256 | 0.26 | 0.26 | 0.00463 |
| 8.00 | 0.44 | 0.462 | 0.46 | 0.45 | 0.00689 |
| 12.00 | 0.64 | 0.637 | 0.64 | 0.64 | 0.00203 |
| 16.00 | 0.82 | 0.845 | 0.85 | 0.84 | 0.0104 |
| 20.00 | 1.13 | 1.110 | 1.110 | 1.115 | 0.00709 |

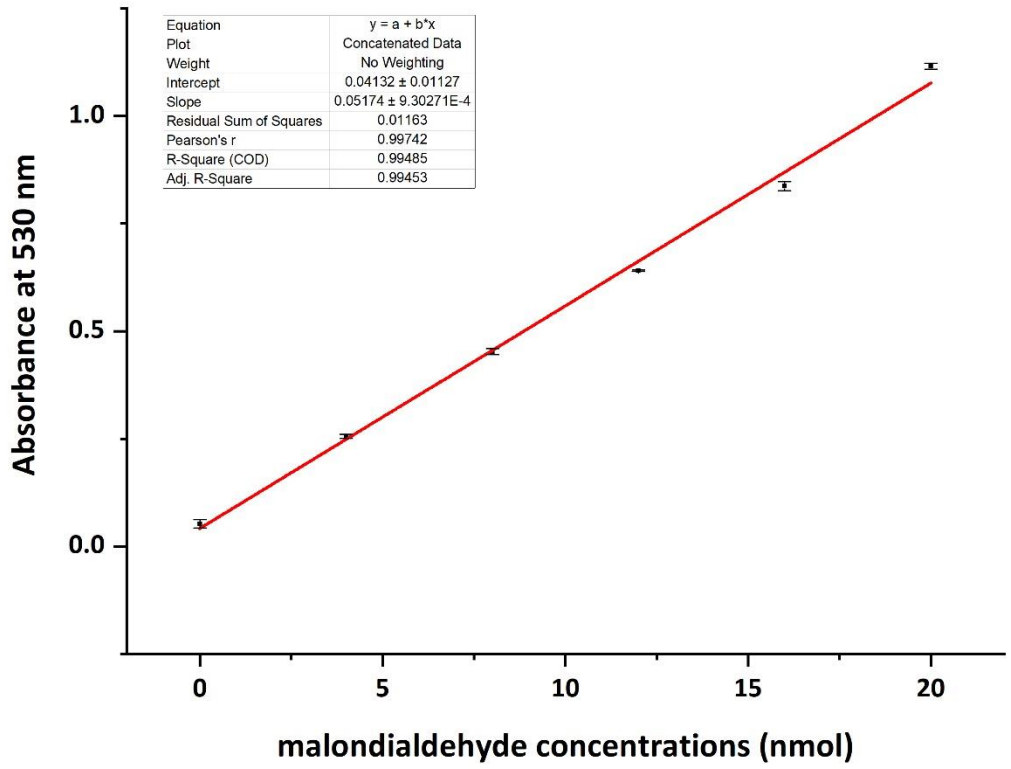


**Figure S3**. Calibration curve of malondialdehyde obtained by linear regression of concatenated data. Error is expressed as ± standard error (SEM) of the mean of three independent experiments.

**Table S4.** Whole-cell lipid membrane peroxidation in *C. albicans* cells was colorimetrically evaluated by lipid peroxidation assay kit (MAK085, Sigma-Aldrich, St. Louis, MO, USA) following manufacturer's protocol. Absorbance was measured at 530 nm ($A_{530}$) by microplate reader iMark, BioRad. Comparisons between multiple groups were performed by ANOVA One-way repeated measure test on raw data, followed by Dunnett test with respect to untreated cells. Error is expressed as ± standard error (SEM) of the mean of three independent experiments.

| **Ozodrop® %(v/v)** | **Mean** | **±SE of mean** | ***p-value*** |
|---|---|---|---|
| 0.00 | 0.10 | 0.01 | |
| 0.78 | 0.19 | 0.02 | 0.33201 |
| 1.56 | 0.30 | 0.04 | 0.01167 |
| 3.13 | 0.39 | 0.07 | 8.58E-04 |
| 6.25 | 0.78 | 0.04 | 1.20E-08 |
| 12.50 | 1.38 | 0.03 | 0 |

**OZODROP® INDUCES INTRACELLULAR ROS PRODUCTION**

**Table S5.** The fluorescent probe DCFH2-DA was used to assess ROS generation upon Ozodrop® treatment in a range from 1/4×MIC to 4×MIC (0.78% – 12.5% (v/v)). Statistically significant enhancement in fluorescence intensity, related to ROS levels generated by Ozodrop®, is observed. Hydrogen peroxide at 500 µM was used as positive control. Comparisons between multiple groups were performed by ANOVA One-way repeated measure test on raw data, followed by Dunnett test with respect to untreated cells. Error is expressed as ± standard error (SEM) of the mean of three independent experiments.

| **Ozodrop® %(v/v)** | **Mean** | **±SE of mean** | ***p-value*** |
|---|---|---|---|
| 0.00 | 32.52 | 3.01 | |
| 0.78 | 59.30 | 5.53 | 0.01531 |
| 1.56 | 57.52 | 5.82 | 0.02274 |
| 3.13 | 72.07 | 7.08 | 0.00107 |
| 6.25 | 84.10 | 9.50 | 1.14E-04 |
| 12.50 | 109.42 | 13.78 | 1.37E-06 |
| $H_2O_2$ 500 µM | 95.67 | 17.49 | |

**CYTOFLUORIMETRIC ANALYSIS OF MITOCHONDRIAL MEMBRANE POTENTIAL**

**Table S6**. Determination of $\Delta\Psi_m$ was carried out using the fluorescent probe JC-1. Samples were analysed by FACSCalibur flow cytometry equipped with Cell Quest software (Becton Dickinson, San Jose, CA, USA) for data acquisition. Data from 5,000 events per sample were collected and the fluorescence intensity shift from red to orange was measured and analysed in FL1 channel. A sample treated with 50 µM of Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; Cayman, Michigan, USA) was used to verify the maximum depolarization. Comparisons between multiple groups were performed by ANOVA One-way repeated measure test on raw data, followed by Dunnett test with respect to untreated cells. Error is expressed as ± standard error (SEM) of the mean of three independent experiments.

| **Ozodrop® %(v/v)** | **Mean** | **±SE of mean** | ***p-value*** |
|---|---|---|---|
| 0.00 | 15.77 | 0.15 | |
| 0.78 | 11.24 | 1.64 | 0.06991 |
| 1.56 | 7.23 | 0.39 | 1.55E-03 |
| 3.12 | 3.28 | 1.31 | 5.90E-05 |
| 6.25 | 2.58 | 1.34 | 2.97E-05 |
| 12.50 | 1.32 | 0.82 | 7.96E-06 |