# Supplementary material for manuscript *Antifungal Activity of commercial Hydrastis canadensis Extract and Its Alkaloid Fractions against Candida albicans: Roles of Oxidative Stress and Lipid Peroxidation*

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## *I. Supplementary material for HPLC-DAD analysis of goldenseal extract*

The analysis of goldenseal extract components by HPLC-DAD method using alkaloids' standards was presented in Figures S1-4. As indicated in Materials and Methods section 4.3. Goldenseal extract was separated on C18 column (3 μm; 150 × 4.6 mm; dr. Maisch) using 0.1% $H_3PO_4$/MetOH gradient as follows: 1 minute of 90% of 0.1% H3PO4 and 10% of MetOH; 60 minutes of 40% of 0.1% $H_3PO_4$ and 60% of MetOH and 90 minutes of 90% of 0.1% $H_3PO_4$ and 10% of MetOH. Other conditions: flow of 1 mL per minute, column temperature 23 °C, analyzed wavelength $\lambda$ = 215 and 270 nm. The standard curve was performed for each alkaloids' standards (BBR, Figure S1; PMT, Figure S2; CND, Figure S3; HYD and HDN, Figure S4) based on this the amount of certain alkaloid in goldenseal extract was determined.

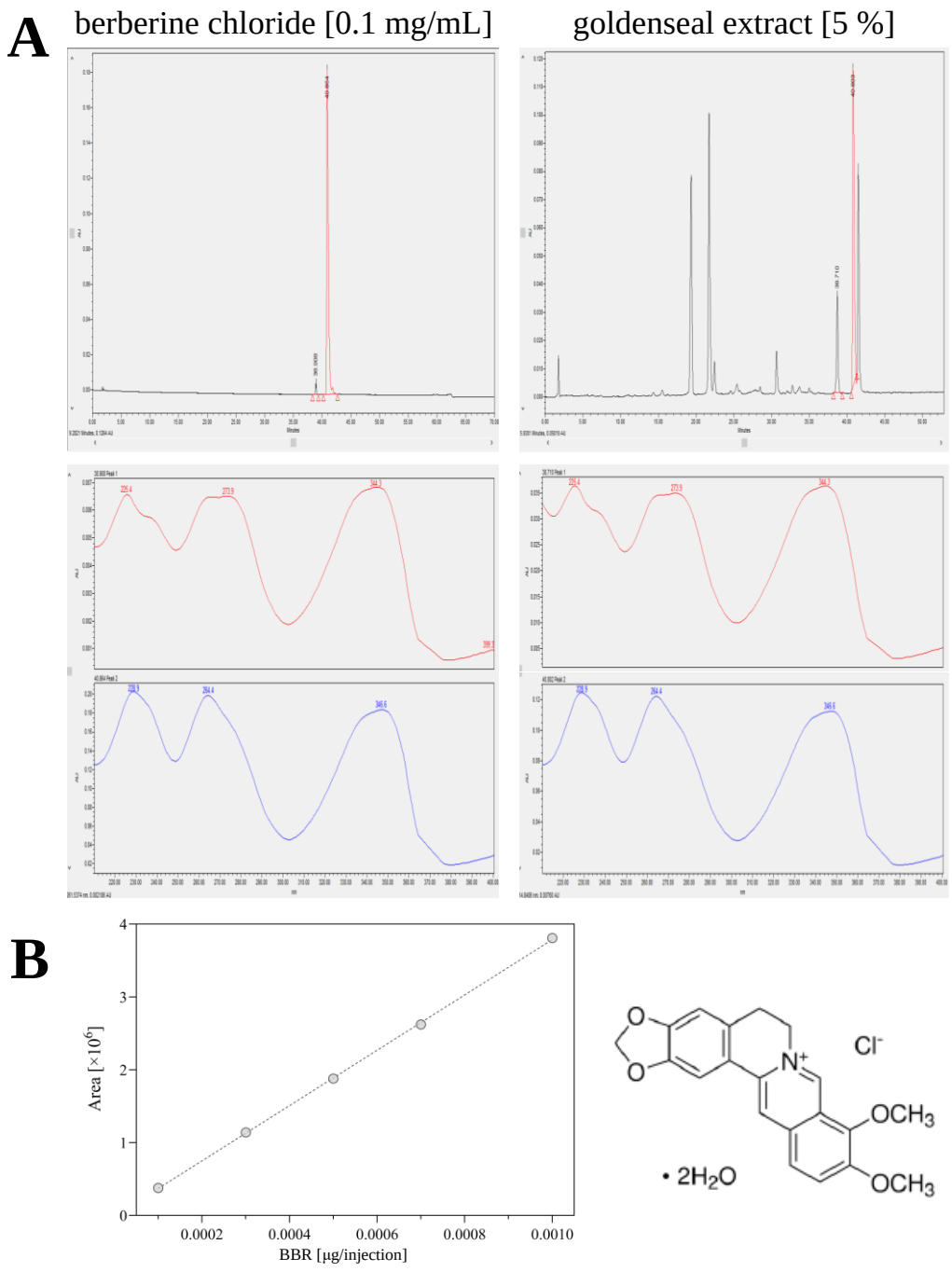



**Figure S1.** HPLC determination of berberine concentration in goldenseal extract based on berberine chloride standard (0.1 mg/mL). The chromatograms and absorption spectrum for berberine chloride and identification of berberine in goldenseal extract (red arrows) are presented on left and right panel of Figure **S1A**, respectively. The standard curve for berberine chloride was presented in Figure **S1B** along with the chemical structure of the standard.

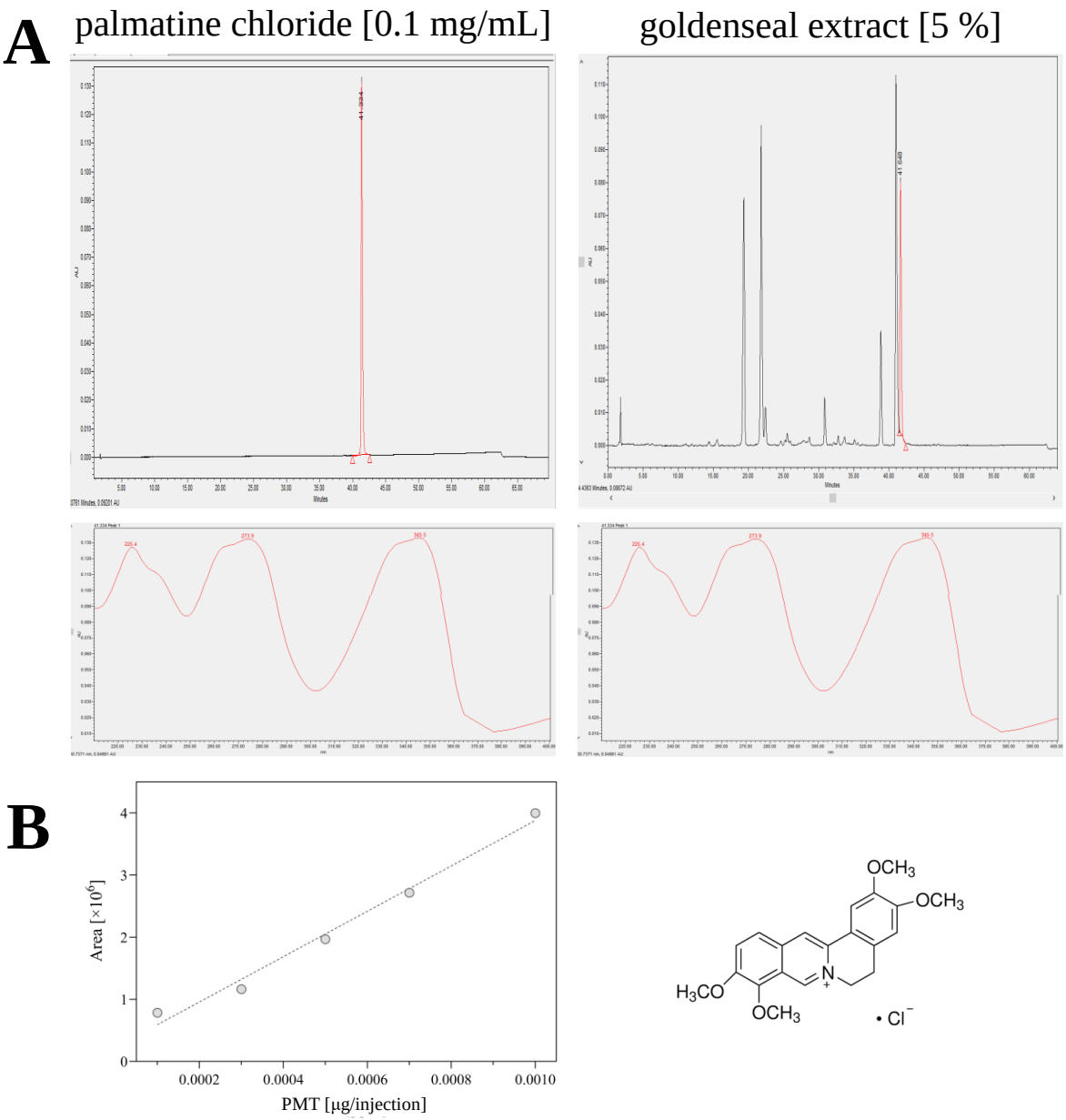



**Figure S2.** HPLC determination of palmatine concentration in goldenseal extract based on palmatine chloride standard (0.1 mg/mL). The chromatograms and absorption spectrum for palmatine chloride and identification of palmatine in goldenseal extract (red arrows) are presented on left and right panel of Figure **S2A**, respectively. The standard curve for palmatine chloride was presented in Figure **S2B** along with the chemical structure of the standard.

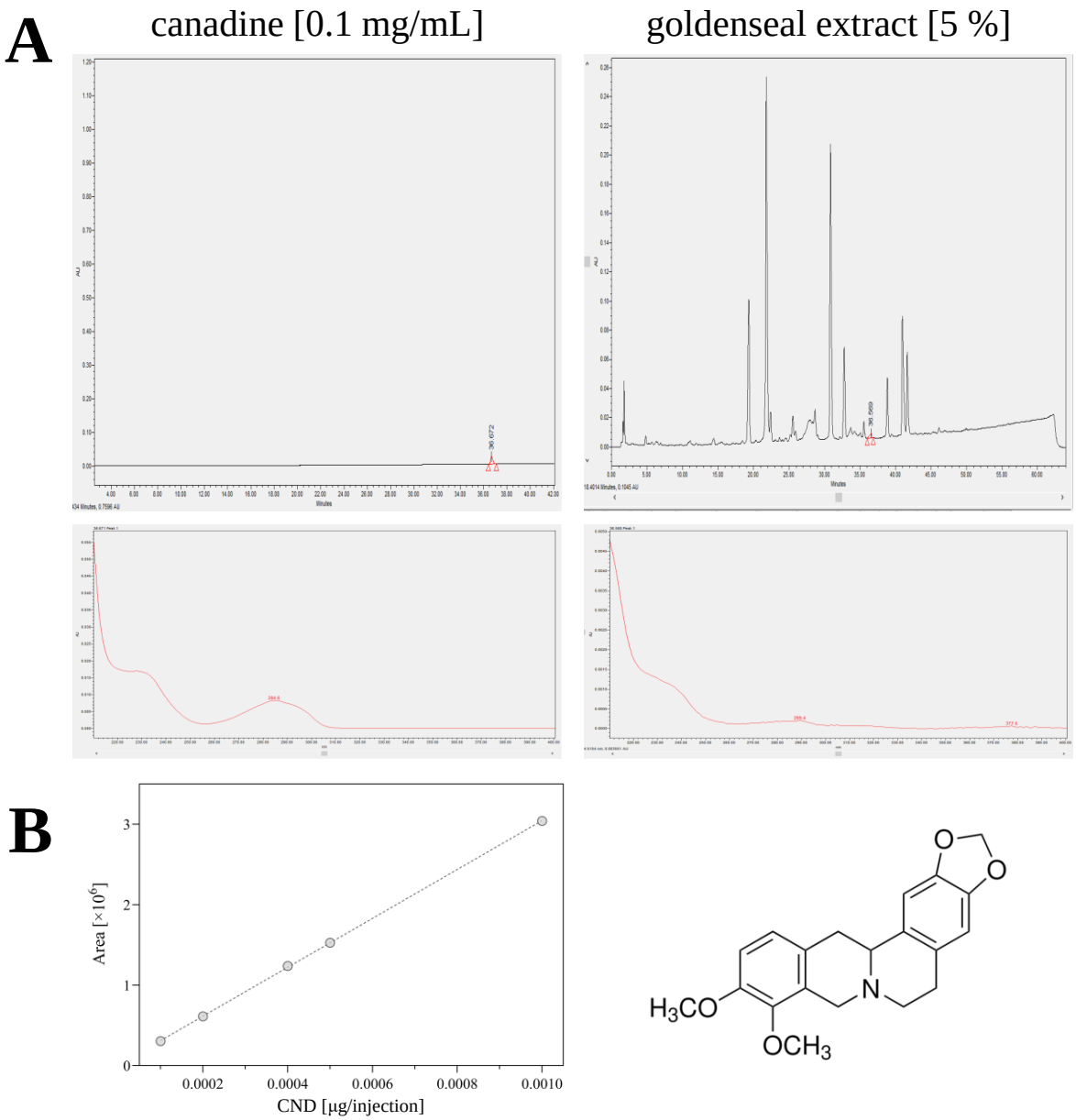



**Figure S3.** HPLC determination of canadine concentration in goldenseal extract based on canadine standard (0.1 mg/mL). The chromatograms and absorption spectrum for canadine and identification of canadine in goldenseal extract (red arrows) are presented on left and right panel of Figure **S3A**, respectively. The standard curve for canadine was presented in Figure **S3B** along with the chemical structure of the standard.

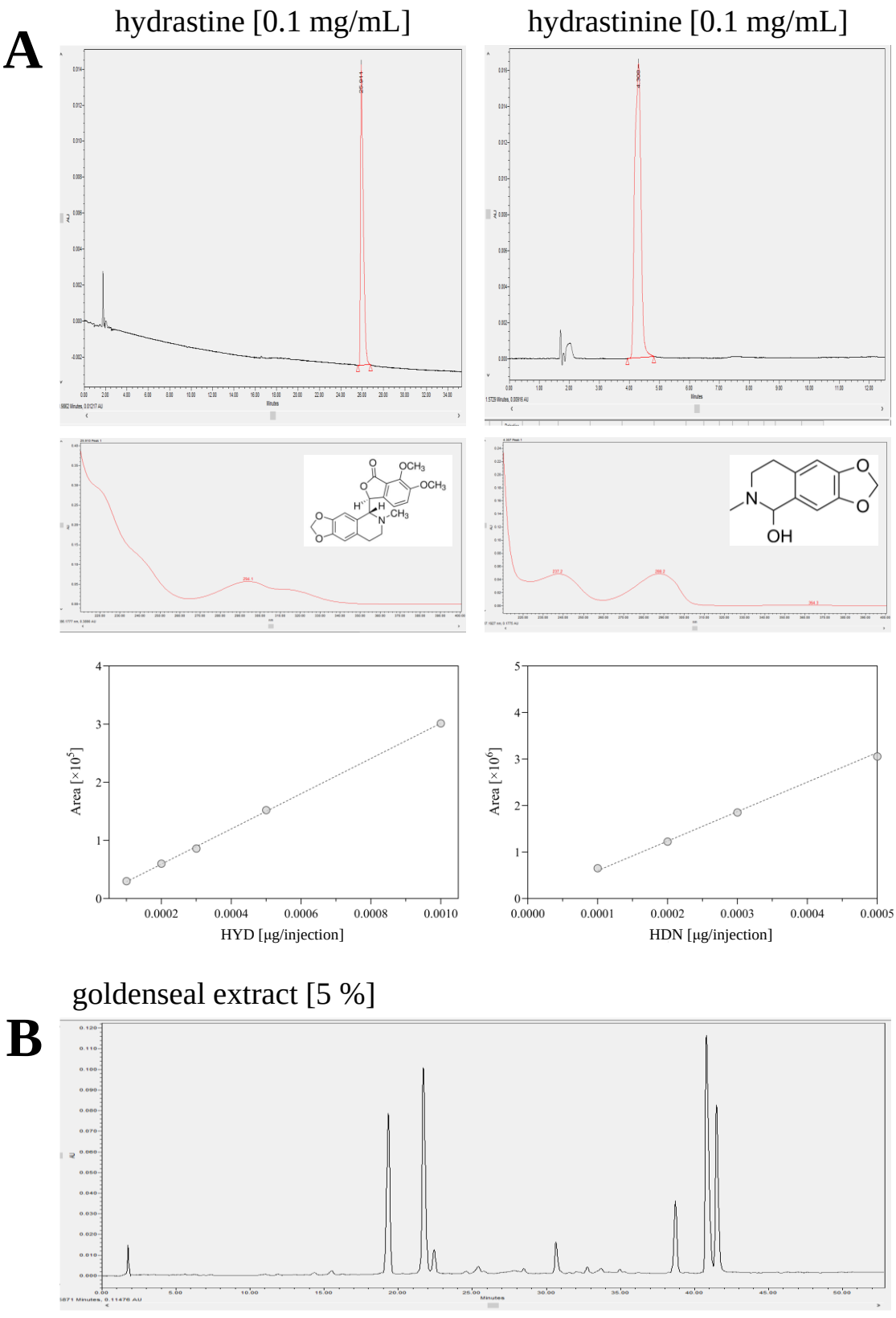



**Figure S4.** HPLC determination of hydrastine and hydrastinine presence in goldenseal extract based on hydrastine and hydrastinine standards (0.1 mg/mL). The chromatograms and absorption spectrum for hydrastine and hydrastinine along with the chemical structure of the standards are presented on left and right panel of Figure **S4A**, respectively. The chromatogram for goldenseal extract indicating no matching peaks for hydrastine and hydrastinine was presented in Figure **S4B**.

***II. Supplementary material for identification of major alkaloids present in fraction 2 of goldenseal extract***

This section provides detailed information for single ion chromatograms of alkaloids identified by $[M]^+$ or $[M+H]^+$ ions (Figure S5) and identified by $[M+2H]^{2+}$ and $[M+H]^+$ ions (Figure S7) for fraction 2 (F2) of goldenseal extract. Detailed description of analysis conditions was provided in section 4.4. of Material and Methods in manuscript.

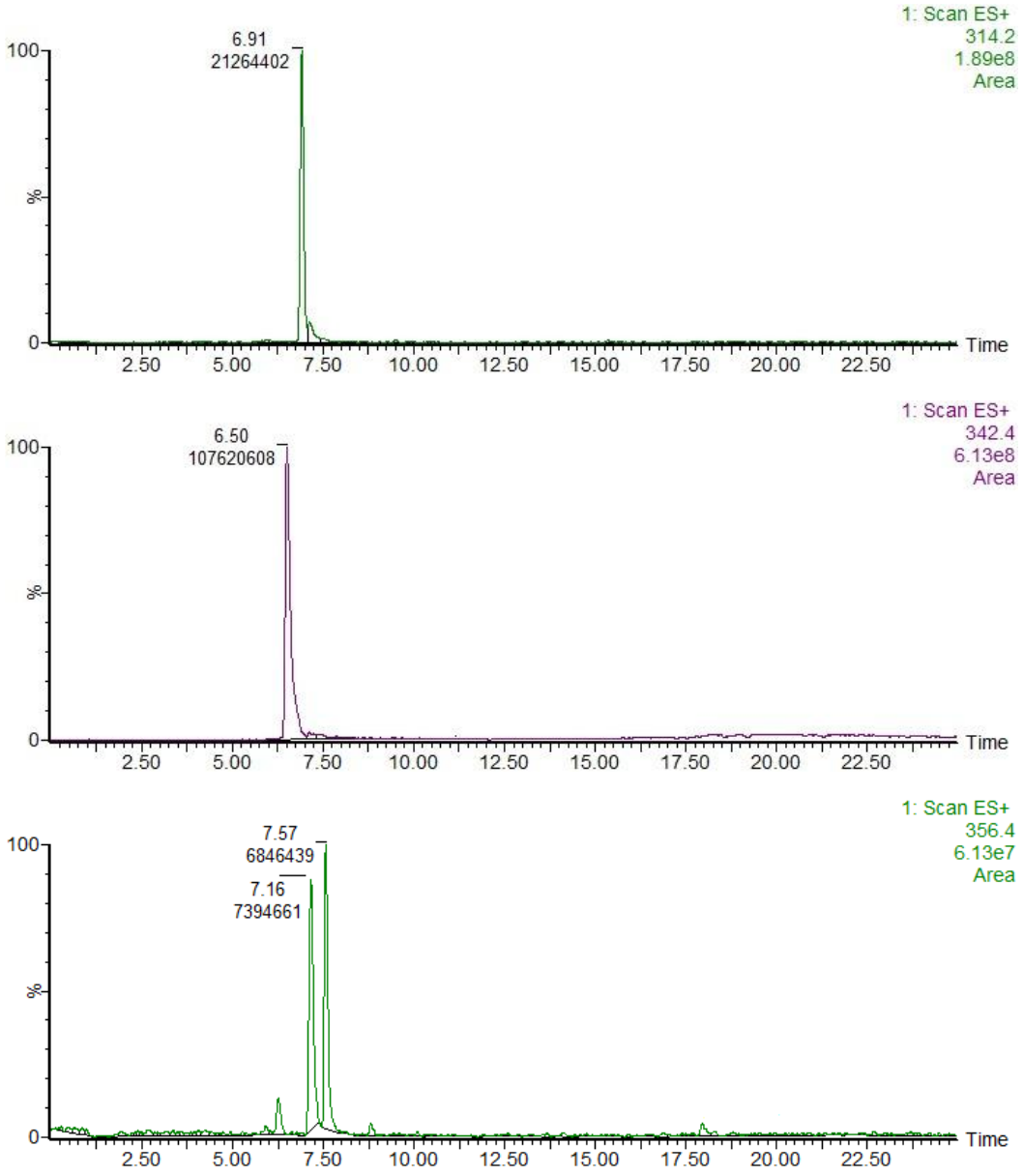



**Figure S5.** Single ion chromatograms of alkaloids identified by $[M]^+$ or $[M+H]^+$ ions. The peaks are signed by retention time (rt, min) and peak areas (arbitrary units); *m/z* 314, 342, 356 (rt = 7.16) - $[M]^+$ of magnocurarine, magnoflorine and xanthoplanine, respectively; *m/z* 356 (rt = 7.57) - $[M+H]^+$ of tetrahydropalmatine.

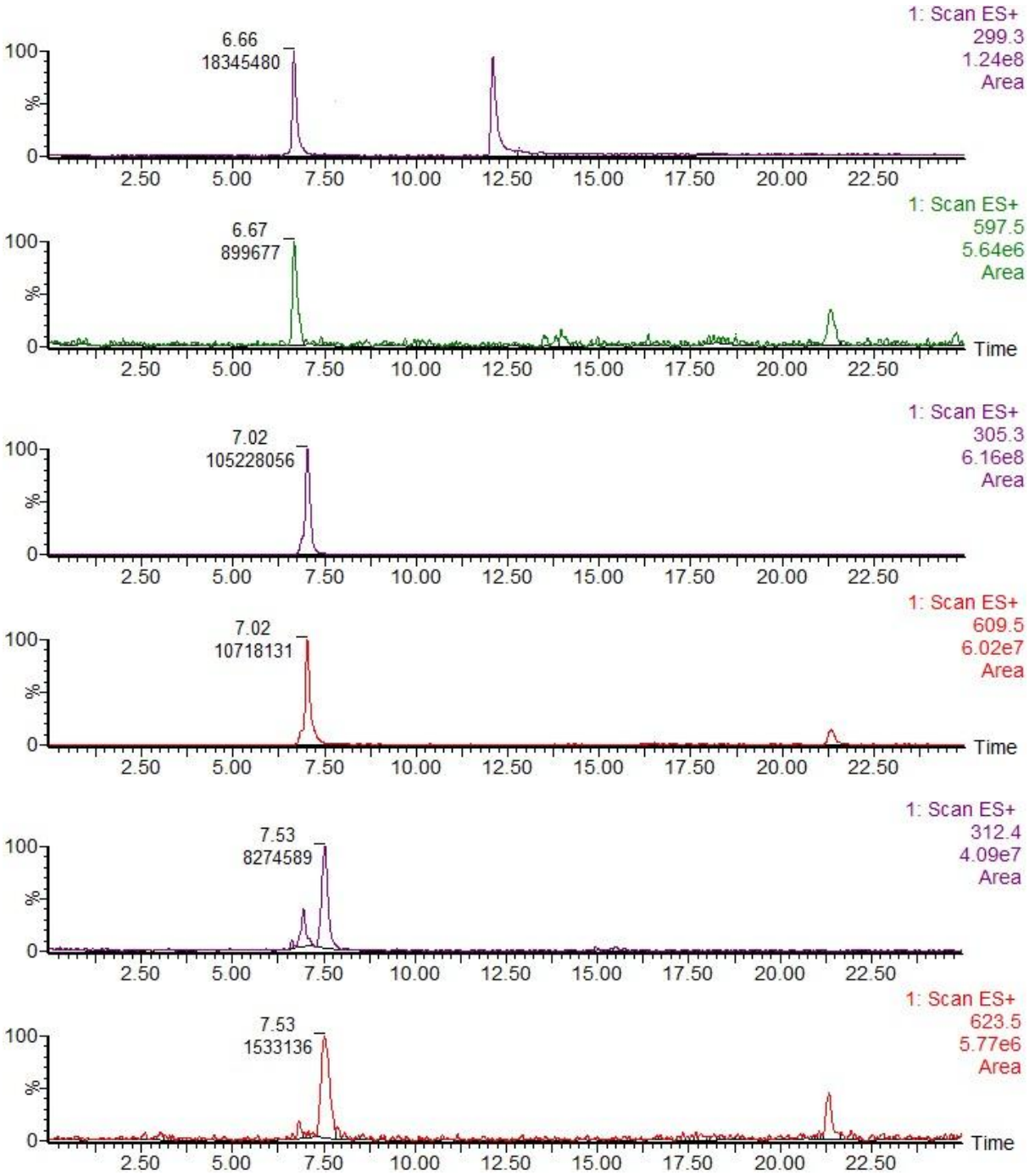



**Figure S6.** Single ion chromatograms of alkaloids identified by $[M+2H]^{2+}$ and $[M+H]^{+}$ ions. The peaks are signed by retention time (rt, min) and peak areas (arbitrary units); *m*/*z* 299, 305, 312 - $[M+2H]^{2+}$ of guattegaumerine, berbamine and tetrandrine, respectively; *m*/*z* 597, 609 and 623 - $[M+H]^{+}$ of guattegaumerine, berbamine and tetrandrine, respectively.

Figure S7 provides the product ion spectra of the identified alkaloids. The summarized data for accurate *m*/*z* measurements and diagnostic product ions obtained for the identified alkaloids were presented in Table S1.

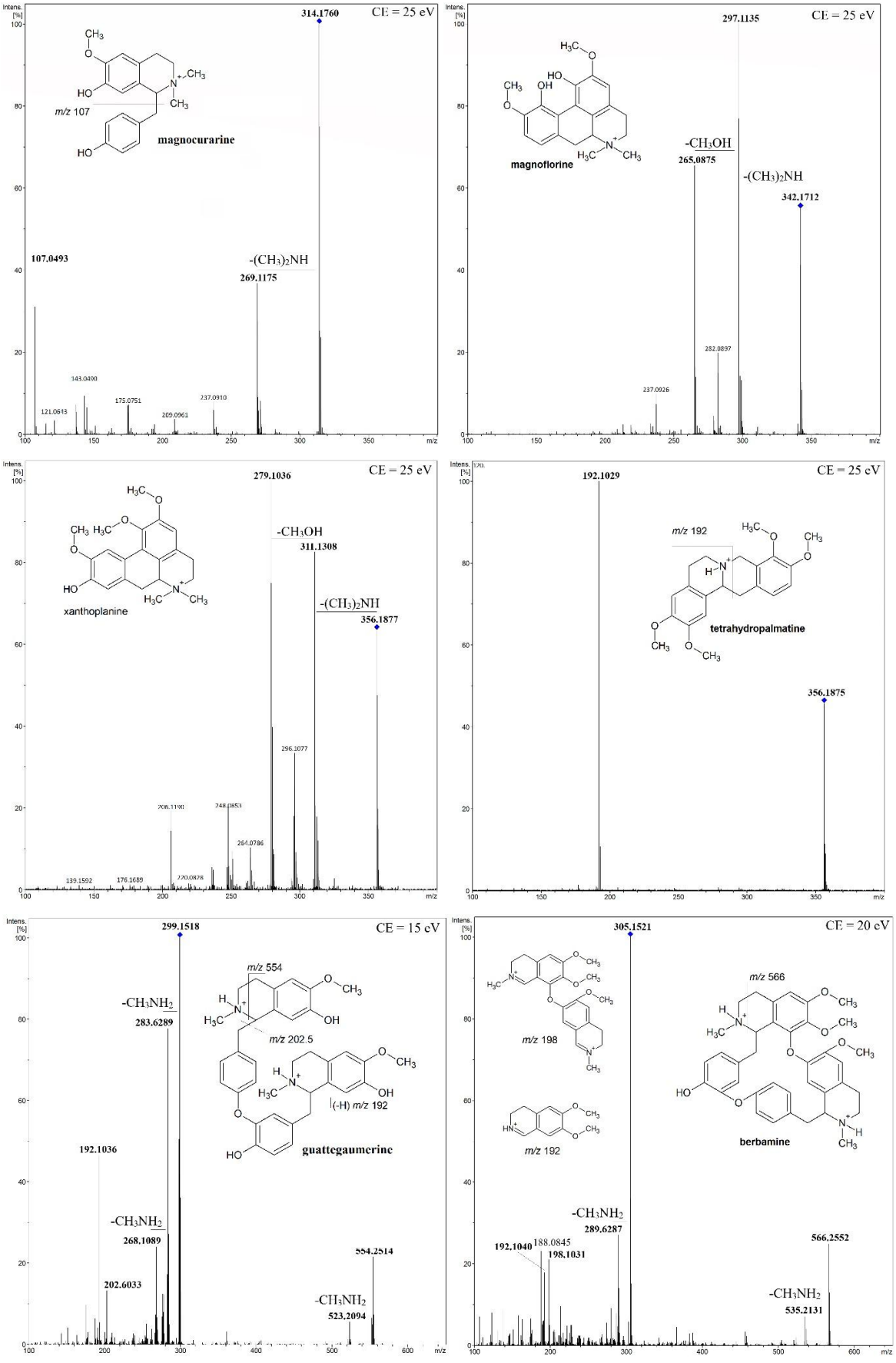



**Figure S7.** The product ion spectra of the identified alkaloids.

**Table S1.** Accurate *m/z* measurements and diagnostic product ions obtained for the identified alkaloids.

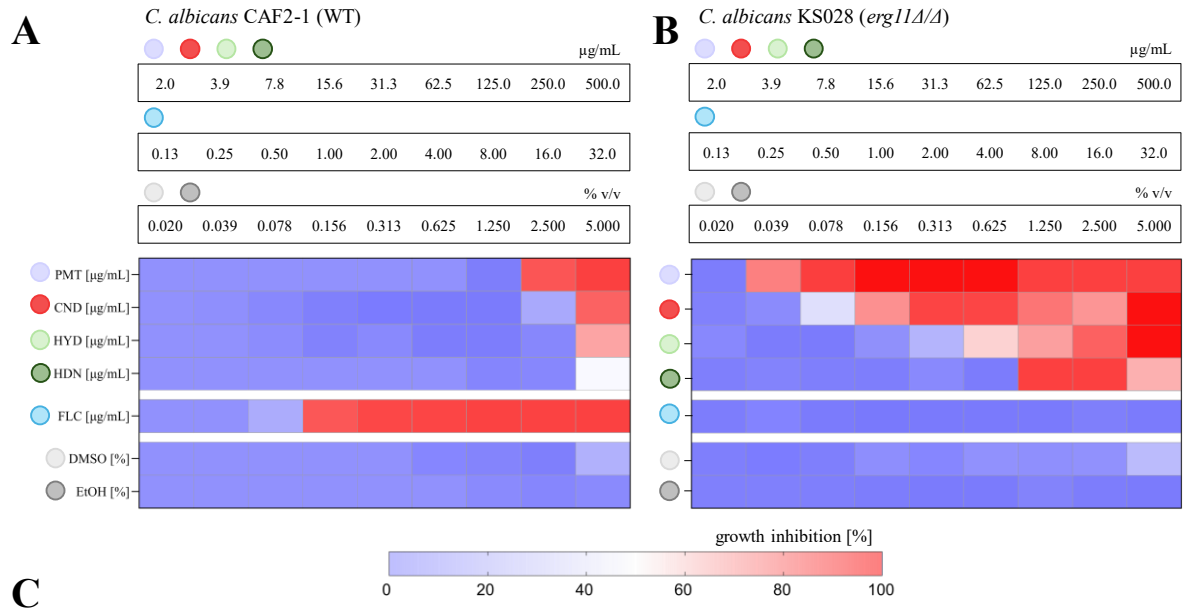
| Compound | Retention time (min) | Precursor ion measured *m/z* | Exact *m/z* | Error (ppm) | Diagnostic product ion m/z (reference)* |
|---|---|---|---|---|---|
| magnoflorine | 10.7 | 342.1712 | 342.1700 | 3.5 | 297, 265 [13, 14] |
| magnocurarine | 10.8 | 314.1760 | 314.1751 | 2.8 | 269, 107 [12, 13] |
| guattegaumerine | 11.2 | 299.1518 | 299.1519 | 0.3 | 554, 192 [18, 19] |
| xanthoplanine | 11.3 | 356.1877 | 356.1857 | 5.6 | 311, 297 [15] |
| berbamine | 11.6 | 305.1521 | 305.1516 | 1.6 | 566, 188 [20, 21] |
| tetrahydropalmatine | 11.7 | 356.1875 | 356.1857 | 5.1 | 192 [16, 17] |
| tetrandrine | 12.0 | 312.1598 | 312.1597 | 0.3 | 580, loss of $CH_3NH_2$ [22] |

** References have been cited in the main document.*

### *III. Supplementary material for determination of antifungal activity of goldenseal extract, fractions and alkaloids*

Data presented in Figure S8 are supplement for results presented in Figure 5 in manuscript. Figure S8 includes growth inhibition (%; Figure S8A and Figure S8B) and MIC50 values for palmatine (PMT) and canadine (CND) performed in higher range of concentrations (500.0 – 2.0 μg/mL) since the range of concentration for these alkaloids presented in manuscript did not cause the growth inhibition of *C. albicans* CAF2-1 (WT) and KS028 (*erg11Δ/Δ*) strains (range of concentration for these alkaloids presented in manuscript reflects concentration of those compounds in goldenseal extract). Since we did not detect the hydrastine (HYD) or hydrastinine (HDN) in analyzed goldenseal extract (Figure S4, supplementary material) the determination of antifungal activity of HYD and HDN was also provided in supplementary material in Figure S8.

We also provided positive drug control for this study by using fluconazole (FLC). This result emphasizes the *C. albicans* KS028 (*erg11Δ/Δ*) as FLC-resistant strain. Additionally, in Figure S8 supplies information on impact of alkaloids, FLC (solvent: DMSO) and goldenseal extract (ethanol, EtOH) solvents on *C. albicans* CAF2-1 (WT) and KS028 (*erg11Δ/Δ*) growth.




**C**

**minimal inhibitory concentration ($MIC_{50}$)**

| *C. albicans* strain | PMT [µg/mL] | CND [µg/mL] | HYD [µg/mL] | HDN [%] | FLC [µg/mL] | DMSO [%] | EtOH [%] |
|---|---|---|---|---|---|---|---|
| CAF2-1 | 250.0 | 500.0 | 500.0 | nd | 1.0 | nd | nd |
| KS028 | 3.9 | 15.6 | 62.5 | 125.0 | nd | nd | nd |

**Figure S8.** *C. albicans* CAF2-1 (WT, **A**) and KS028 (*erg11Δ/Δ*, **B**) growth inhibition (%) in presence of palmatine (PMT), canadine (CND), hydrastine (HYD), hydrastinine (HDN), fluconazole (FLC) as a control for *C. albicans* KS028 (*erg11Δ/Δ*) FLC-resistant strain and background DMSO and ethanol (EtOH). All agents analyzed were described with individual legend of colored circles. The $MIC_{50}$ value (which indicates minimal inhibitory concentration of certain antifungal agent needed to decrease *C. albicans* growth at least in 50%) was summarized in Table in Figure **2C**. The 'nd' means 'not determined' according to $MIC_{50}$ value.

### *IV. Supplementary material for investigation of intracellular ROS level*

The consistent description of analysis method for intracellular ROS investigation using $H_2$DCFDA probe was described in section 4.7 of Materials and Methods in manuscript. The histograms for representative samples from ROS $H_2$DCFDA flow cytometry analysis representing green fluorescence of *C. albicans* CAF2-1 (WT) event in autofluorescence (AF) and $H_2$DCFDA stained cells in control conditions were provided in Figure S9. Cells were first gated on single-event and then gated on PI-negative events. Next 5,000 events were collected for each sample. The gating strategy for PI-negative events were presented in Figures S10 and S11. For each analyzed sample the mean fluorescence presented in Figure 6A in manuscript were obtained after background (AF) subtraction to provide the reliable data for fluorescence of $H_2$DCFDA probe.

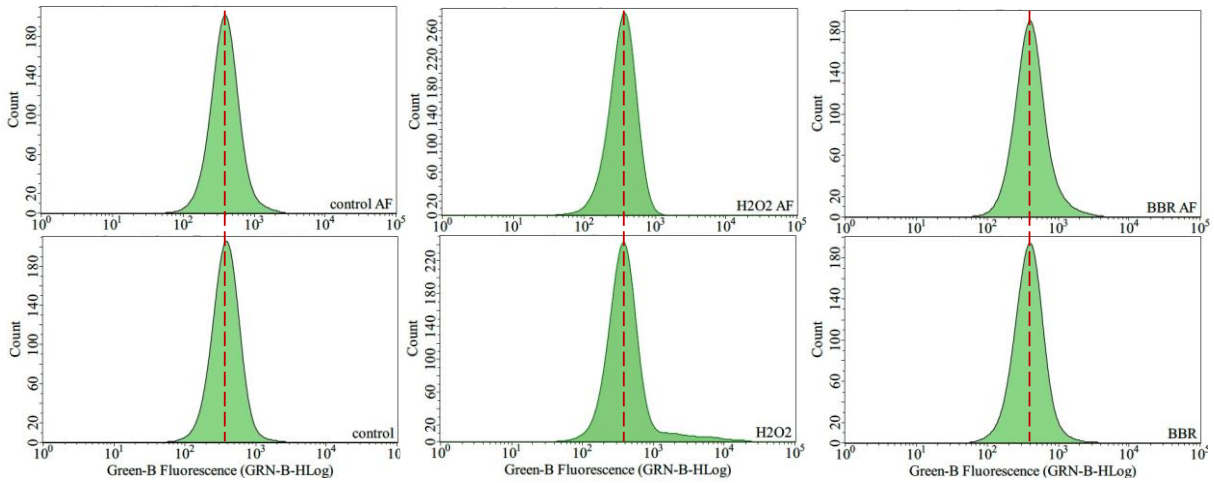



**Figure S9.** Histograms for representative samples from ROS $H_2$DCFDA flow cytometry analysis representing green fluorescence of *C. albicans* CAF2-1 (WT) event in autofluorescence (AF) and $H_2$DCFDA stained cells in control conditions, after treatment with $H_2O_2$ or BBR. The presented data were gated for single, PI-negative events.

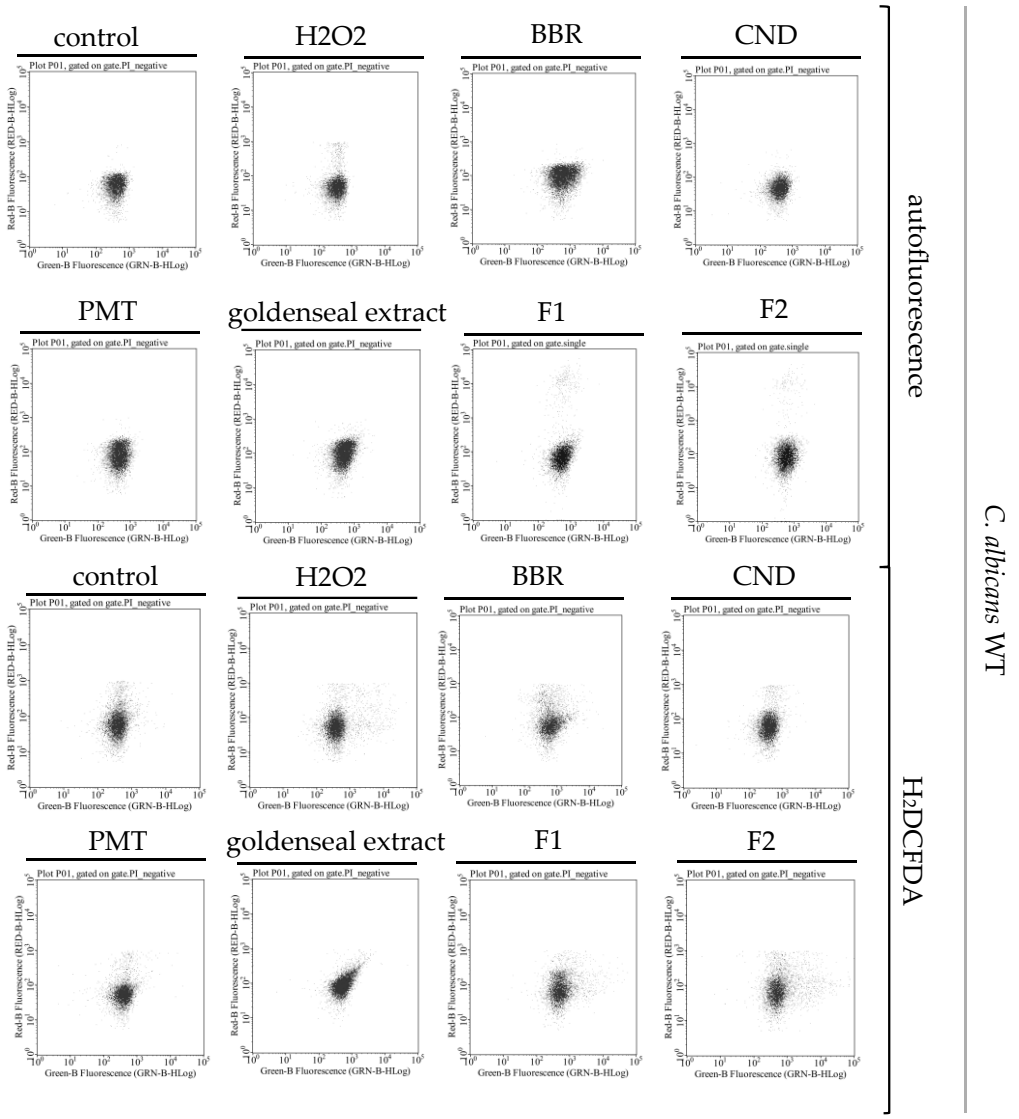



**Figure S10.** Dot plots representing gating strategy for $H_2$DCFDA flow cytometry analysis of intracellular ROS measurement for representative samples of *C. albicans* CAF2-1 (WT) cells in control conditions or treated with $H_2O_2$, BBR, PMT, CND, goldenseal extract, F1 and F2. Gating was performed firstly for single event and then population was gated on propidium iodide (PI) negative event (Red-B Fluorescence). Then such population was plotted and data were presented in Figure S10 as Red-B Fluorescence (PI-negative events) against Green-B Fluorescence ($H_2$DCFDA). Upper panel of the figure presents autofluorescence (AF) events (without addition of $H_2$DCFDA probe) and lower panel presents events for cells incubated with $H_2$DCFDA probe. All measurements were performed in three biological repetitions, and representative dot plots were presented in this supplementary material.

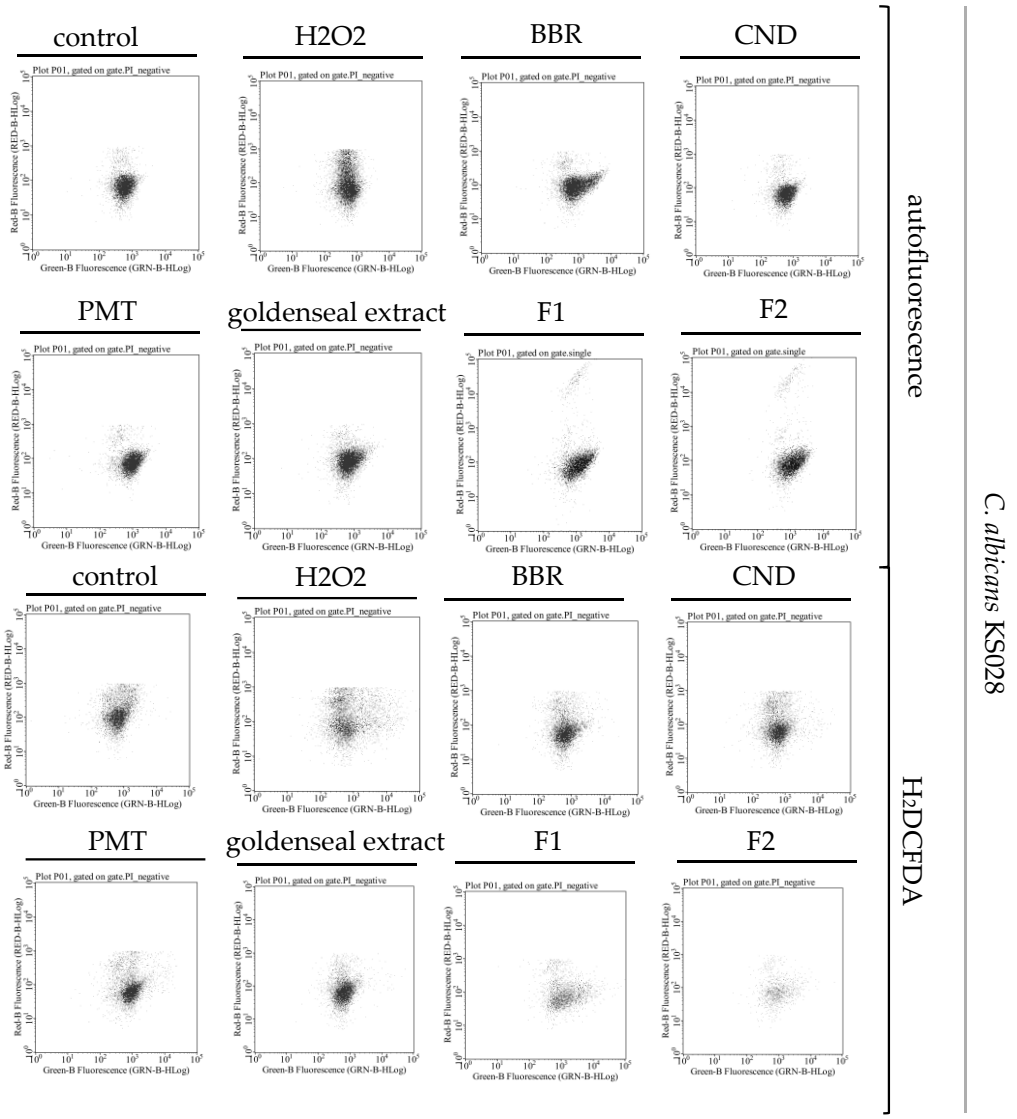



**Figure S11.** Dot plots representing gating strategy for $H_2$DCFDA flow cytometry analysis of intracellular ROS measurement for representative samples of *C. albicans* KS028 (*erg11Δ/Δ*) cells in control conditions or treated with $H_2O_2$, BBR, PMT, CND, goldenseal extract, F1 and F2. Gating was performed firstly for single event and then population was gated on propidium iodide (PI) negative event (Red-B Fluorescence). Then such population was plotted and data were presented in Figure S10 as Red-B Fluorescence (PI-negative events) against Green-B Fluorescence ($H_2$DCFDA). Upper panel of the figure presents autofluorescence (AF) events (without addition of $H_2$DCFDA probe) and lower panel presents events for cells incubated with $H_2$DCFDA probe. All measurements were performed in three biological repetitions, and representative dot plots were presented in this supplementary material.

Additional representative microphotographs of *C. albicans* CAF2-1 (WT) and KS028 (*erg11Δ/Δ*) cells treated with BBR, PMT, CND stained with $H_2$DCFDA probe were presented in Figure S12.

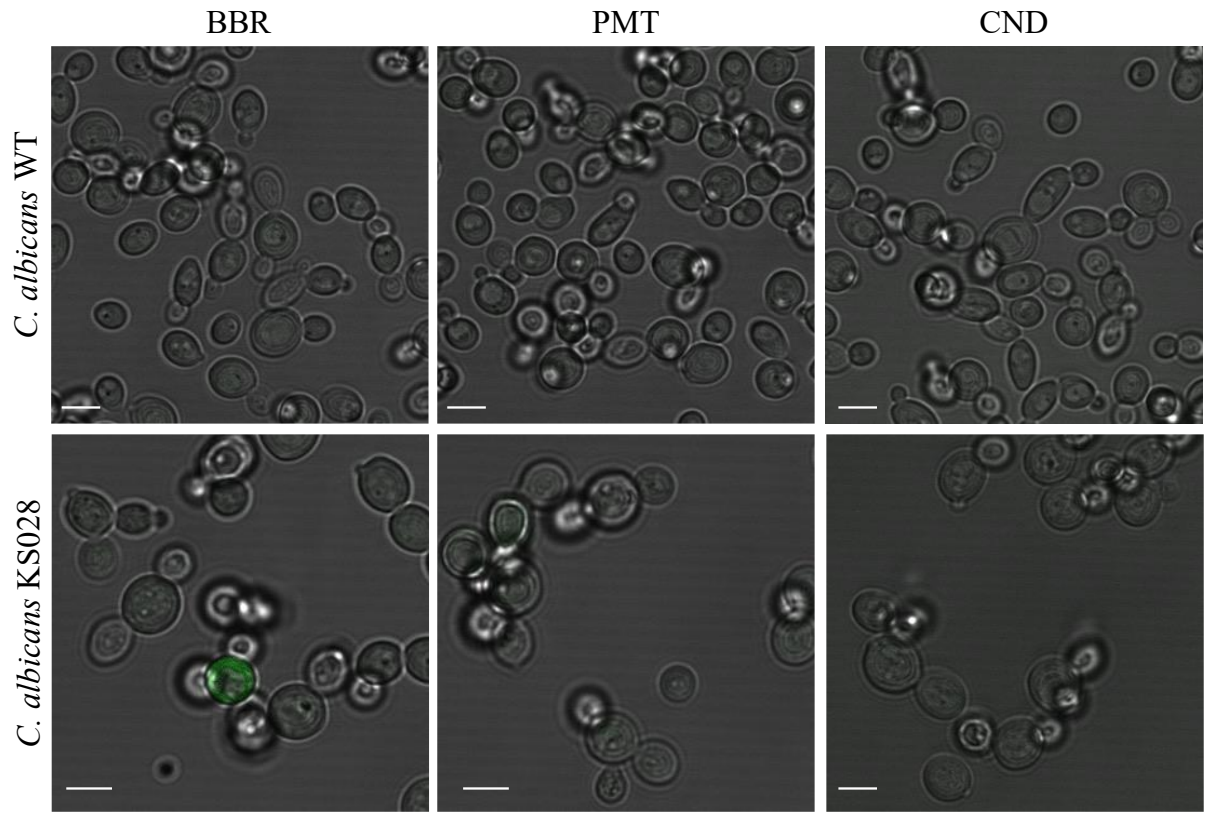



**Figure S12.** Reactive oxygen species (ROS) accumulation in *C. albicans* CAF2-1 (WT) and KS028 (*erg11Δ/Δ*) cells [**A**] after treatment with $MIC_{50}$ concentration of BBR, PMT, CND (according to its amount in goldenseal extract). The representative micrographs of *C. albicans* cells stained with $H_2$DCFDA probe were presented (scale bar = 5 μm).

### *V. Supplementary material for lipid peroxidation assay*

Figure S12 provides additional information about the gating strategy for lipid peroxidation assay. Events were first gated for single events and then gated according to population obtained for negative (YPD alone cultured cell) and positive control (cells cultured in presence of tBHP in concentration of 0.5 mM). Since *C. albicans* KS028 (*erg11Δ/Δ*) strain exhibit more heterogenous population in context of lipid peroxidation (due to the ergosterol deprivation from plasma membrane) the gating was corrected for those *C. albicans* CAF2-1 (WT) and KS028 (*erg11Δ/Δ*) individually with respect to population of negative and positive controls.

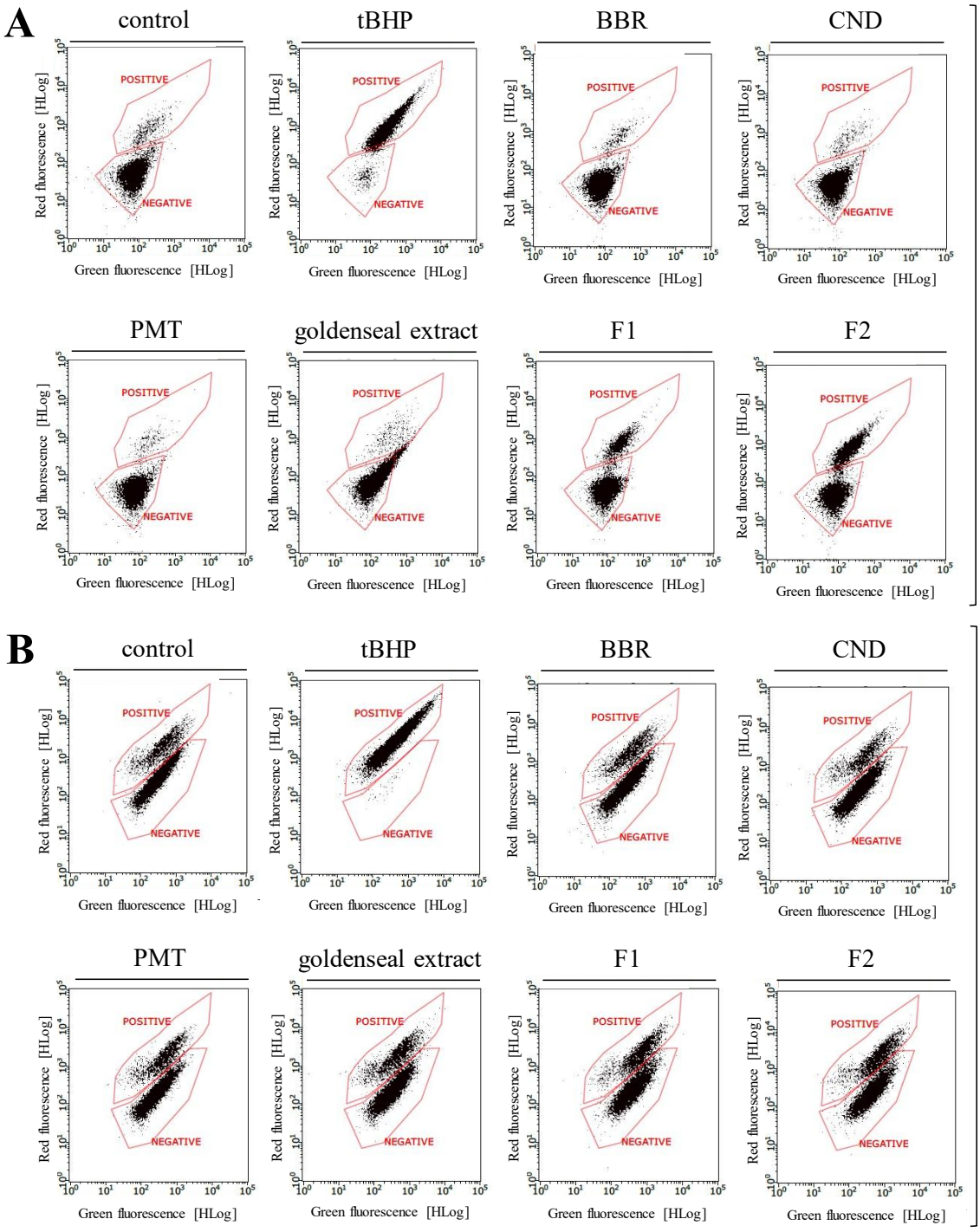



**Figure S13.** Dot plots representing gating strategy for BODIPY™ 581/591 C11 flow cytometry analysis for representative samples of *C. albicans* CAF2-1 and KS028 cells in control conditions or treated with tBHP, BBR, PMT, CND, goldenseal extract, F1 and F2. The presented data was gated for single events.