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Olive leaf extract activity against *Candida albicans* and *C. dubliniensis* – the *in vitro* viability study

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Olive leaf extract is characterized by a high content of polyphenols (oleuropein, hydroxytyrosol and their derivatives), which is associated with its therapeutic properties. The objective of the present research was to evaluate the antifungal activity of olive leaf extract against *Candida albicans* ATCC 10231 and *C. dubliniensis* CBS 7987 strains. Minimum inhibitory concentrations (*MIC*) of the extract were determined by several *in vitro* assays. The extract showed a concentration depended effect on the viability of *C. albicans* with *MIC* value of 46.875 mg mL^{-1} and *C. dubliniensis* with *MIC* value 62.5 mg mL^{-1}. Most sensitive methods for testing the antifungal effect of the extracts were the trypan blue exclusion method and fluorescent dye exclusion method while *MIC* could not be determined by the method according to the EUCAST recommendation suggesting that herbal preparations contain compounds that may interfere with this susceptibility testing. The fluorescent dye exclusion method was also used for the assessment of morphological changes in the nuclei of treated cells. According to the obtained results, olive leaf extract is less effective against the tested strains than hydroxytyrosol, an olive plant constituent tested in our previous study.

Keywords: antifungal activity, *Candida albicans*, *Candida dubliniensis*, olive leaf extract

Olive leaf extract is well known for its broad health benefits. Its therapeutic activities have been associated with its high content of biophenols (oleuropein, hydroxytyrosol and their derivatives) (1–7). Published reports on olive leaf revealed antioxidative activity as well as cardioprotective and chemopreventive effects (8). It has been demonstrated that olive leaf extract with 80 % (*m*/*m*) ethanol, purified and dissolved in water before administration has blood pressure lowering activity in rats (9). Antihypertensive activity of olive leaf extract was confirmed by a similar effect at the dose of 500 mg given twice daily in a

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flat-dose fashion in subjects with stage-1 hypertension in comparison with captopril given at a dose 12.5–25 mg twice daily (10). In 2008, Fabiani *et al.* (11) provided results suggesting that the complex mixture of phenols extracted from virgin olive and olive-mill wastewater reduced the DNA damage at concentrations of 1 μmol L^{-1} when coincubated in the medium with H_2O_2 (40 μmol L^{-1}). Another study (12) reported a dose-dependent cytotoxic effect of olive leaf extract, obtained with an 80:20 ethanol/water mixture, on HL60 human promyelocytic leukemia cells with IC_{50} of 10 μL mL^{-1}, indicating, by cell staining with acridine orange and ethidium bromide, the apoptotic pathway as the probable mechanism for the cytotoxic effect.

The plant extract from olive leaves was reported to have anticancer, antioxidative and antinflammatory properties (12, 13). In addition, the extract has demonstrated antimicrobial activity against pathogens (14), including the bacteria *Salmonella typhi*, *Vibrio parahaemoliticus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeuroginosa*, *Bacillus subtilis* (15–17).

Also, olive leaf extract has shown wide-ranging antimicrobial activity against *Campylobacter jejuni*, *Helicobacter pylori* and *Staphylococcus aureus* (including methicillin-resistant *S. aureus*) at minimum inhibitory concentrations (*MIC*s) as low as 0.31–0.78 % (*V*/*V*) (8). The study was made by Lee and Lee (18) to assess antimicrobial activity of individual phenolics (oleuropein, rutin, vanillin, caffeic acid) in comparison with combined phenolics in olive leaf extract. The results indicated higher antimicrobial effect of the combination of olive leaf extract phenolics than individual phenolics against *Bacillus cereus* and *Salmonella enteritidis*, suggesting synergistic effects when phenolic compounds were present in a mixture. The objective of the present study was to assess the activity of commercial olive leaf extract on the viability of yeasts *C. albicans* and *C. dubliniensis* using different susceptibility tests. Our recent study reported the effects of the biophenol compound hydroxytyrosol (HT) found in olive tree products against the yeasts of *Candida* spp. and dermatophytes (19). It demonstrated that HT has considerable *in vitro* antifungal potential. Nevertheless, further studies are required to elucidate the effects of other individual constituents of olive leaves and olive oil as well as olive leaf extract.

EXPERIMENTAL

Chemicals

Olive leaf material in a dry powder form was obtained from Magdis, Zagreb, Croatia**.** All chemicals and reagents, unless otherwise specified, were purchased from Sigma Chemical Co., USA.

Olive leaf extract was prepared by water extraction procedure. Phosphate buffer pH 7.4 was added to yield a stock solution of the extract in 500 mg mL^{-1} concentration. Amphotericin B stock solution in 1 mg mL^{-1} concentration was prepared in dimethylsulphoxide (DMSO). Both stocks were maintained at –20 °C and used within several days.

Microorganisms

Strains of *C. albicans* ATCC 10231 and *C. dubliniensis* CBS 7987 were obtained from the stock cultures of microorganisms (Department of Microbiology, Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia).

Determination of polyphenols by HPLC

Reverse-phase HPLC with diode array detection was used to determine polyphenolic composition of olive leaf extract. Commercial extract was dissolved in a methanol/water solution (80/20, *V*/*V*) and filtered throught a 0.45 μm polyvinylidene difluoride (PVDF) filter (Macherey-Nagel, Germany).

HPLC analysis was carried out according to the method of the International Olive Council (20), using a Varian ProStar System. Separation of the polyphenolic compound was conducted on an RP C18 column (Luna 250 × 4.6 mm, 5 μm, Phenomenax, USA) at room temperature. Relative amounts of 0.2 % phosphoric acid (eluent A), methanol (eluent B) and acetonitrile (eluent C) were varied throughout the analysis at a flow rate 1 mL min^{-1} to achieve separation. The gradient was as follows: at 0 min, 96 % A, 2 % B and 2 % C; from 0 to 40 min, percentages changed from their initial values to 50 % A, 25 % B and 25 % C; from 40 to 45 min, percentages changed to 40 % A, 30 % B and 30 % C; from 45 to 60 min, percentages changed to 50 % B and 50 % C and remained there until 70 min; from 70 to 72 min, the percentages returned to their initial values and remained there for another 10 min. Phenolic compounds were detected at 280 nm and identified by comparing their retention times and UV spectra (from 200 to 400 nm) with those of the following standards: 4-hydroxybenzoic acid, apigenin, caffeic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid, galic acid, hydroxytyrosol, luteolin, oleuropein, pinoresinol, protocatechuic acid, sinapic acid, syringaldehyde, syringic acid, *trans*-cinnamic acid, vanillic acid, vanillin and tyrosol. Quantification of polyphenolic compounds was performed using the calibration curve constructed by injecting standard solutions (1–25 μg mL^{-1}) of tyrosol. Results are given in g of tyrosol per 100 g of dry extract and are presented as the average of three determinations.

Antifungal susceptibility testing

Inoculum suspensions for yeasts were prepared from fresh cultures of yeast strains with physiological saline adjusted to the cell density of 0.5 McFarland units using a nephelometer Densimat (Biomérieux, France). The test was performed with working suspensions prepared as 1 in 10 dilution in physiological saline containing 5×10^5 CFU mL^{-1}.

MIC was determined by the serial twofold microdilution method with RPMI 1640 broth containing 2 % (*m*/*V*) glucose (RPMI 1640 2 % G, Sigma-Aldrich, USA) in 96-well microtiter plates with flat bottoms, as recommended in the EUCAST Definitive Document EDef 7.2 (21). Microdilution plates were incubated at 37 °C for 24 h aerobically and the absorbance was measured with a microdilution plate reader Labsystems iEMS Reader MF (Labsystems Oy, Finland) at 405 nm.

In addition, the subcultivation method was used by inoculating 10 μL of the sample from each dilution on the surface of Sabouraud 2 % (*m*/*V*) glucose agar and incubation was carried out at 35 °C for 24 h. The positive control for yeasts was performed with amphotericin B, and the negative one was an extract-free control. *MIC* was defined as the lowest concentration of olive leaf extract that allows no more than 20 % growth of microbes in comparison with the extract-free control.

Determination of viability by the trypan blue dye exclusion method

To determine the viability of *Candida* blastopores, the trypan blue dye exclusion method was used. *C. albicans* ATCC 10231 and *C. dubliniensis* CBS 7987 were grown in Sabouraud 2 %

(*m*/*V*) glucose broth medium at 37 °C for 24 h aerobically on an Orbital Shaker-Incubator ES–20 Grant-bio (Grant Instruments Ltd., UK). The cells were washed twice with physiological saline and cell density was adjusted to 5 McFarland units (5×10^7 CFU mL^{-1}) using Densimat nephelometer. A hundred and fifty μL of *C. albicans* inoculum suspension was inoculated in RPMI 1640 with 2 % (*m*/*V*) glucose containing olive leaf extract in concentrations of 46.875, 15.625, 1.5625 and 0.390625 mg mL^{-1}. Positive control was performed with 1 μg mL^{-1} of amphotericin B and 10 % (*V*/*V*) of H_2O_2. A hundred and fifty μL of *C. dubliniensis* inoculum suspension was inoculated in RPMI 1640 with 2 % (*m*/*V*) glucose, containing olive leaf extract in concentrations of 187.5, 62.5, 6.25, 1.563, 0.625 and 0.098 mg mL^{-1} and positive control was performed with 2 μg mL^{-1} of amphotericin B and 10 % (*V*/*V*) of H_2O_2. After 18 h of aerobic incubation, cells were harvested by centrifugation at 2 rpm for 2 minutes. Cell viability was determined using the Countess™ (Invitrogen, USA) automated cell counter software. Cells were stained with 0.4 % trypan blue solution in 0.81 % NaCl and 0.06 % K_2HPO_4 and immediately counted using a pre-calibrated counter. Twenty μL of cell suspension was mixed with 20 μL of 0.4 % trypan blue solution. Percentage of viable blastospores and $\log_{10}$ CFU mL^{-1} were determined after treatment with different concentrations of olive leaf extract *vs.* untreated control; results were graphically presented.

Assessment of morphological changes by the fluorescent dye exclusion method

Assessment of morphological changes in the nuclei was performed with *C. albicans* ATCC 10231 and *C. dubliniensis* CBS 7987 as test strains. A hundred and fifty μL of *C. albicans* inoculum suspension in physiological saline of 5 McFarland units (5×10^7 CFU mL^{-1}) was added to 900 μL of RPMI 1640 2 % G broth containing 46.875, 15.625, 1.563 and 0.391 mg mL^{-1} of olive leaf extract. Positive control was performed with 1 μg mL^{-1} of amphotericin B and 10 % (*V*/*V*) of H_2O_2. A hundred and fifty μL of *C. dubliniensis* inoculum suspension in physiological saline of 5 McFarland units (5×10^7 CFU mL^{-1}) was added to 900 μL of RPMI 1640 2 % G broth containing 187.5, 62.5, 6.25, 1.563, 0.625 and 0.098 mg mL^{-1} of olive leaf extract and control was performed with 2 μg mL^{-1} of amphotericin B and 10 % (*V*/*V*) of H_20_2. The samples were incubated at 35 °C for 1 h; additional measurement was carried out with samples incubated at 35 °C for 18 h. After incubation, ethidium bromide and acridine orange were added to the samples in a final concentration of 100 μg mL^{-1} (1:1; *V*/*V*). The samples were analyzed by determining viable, apoptotic and necrotic cells.

Statistical analysis

One-way ANOVA with Dunnett's multiple comparison post-test was used to compare the viability of cells under treatment with untreated cells (NC) for the trypan blue exclusion method (GraphPad Prism v5 for Windows, GraphPad Prism Software Inc.). Statistical analysis of the data obtained by the fluorescent dye-exclusion method was performed using a chi-square test.

RESULTS AND DISCUSSION

Determination of polyphenols by HPLC analysis in the olive leaf water extract identified the following compounds: oleuropein, hydroxytyrosol, tyrosol, pinoresinol, protocatechuic acid and apigenin (Table I). Oleuropein was found to be the most abundant com-

Table I. Phenolic compounds of Olea europaea *by HPLC*

Compound	Retention time (min)	Concentration of phenolic compounds (g of tyrosol/100 g of dry extract)
Hydroxytyrosol	13.9	0.252 ± 0.033
Protocatechuic acid	14.4	0.036 ± 0.015
Tyrosol	17.2	0.144 ± 0.030
Oleuropein	35.8	9.281 ± 0.185
Pinoresinol	40.9	0.088 ± 0.039
Apigenin	46.1	0.024 ± 0.013
n.i.[a]		9.807 ± 0.362

[a] Sum of all non-identified phenolic compounds

pound in the analyzed extract (9.281 ± 0.185 g of tyrosol/100 g of dry extract). The HPLC chromatogram of the phenolic compounds of olive leaf extract with standard phenolics is shown in Fig 1.

The effect of olive leaf extract against the yeast strains *C. albicans* ATCC 10231 and *C. dubliniensis* CBS 7987 was evaluated by *in vitro* susceptibility assays (*MIC*s are given in Table II).

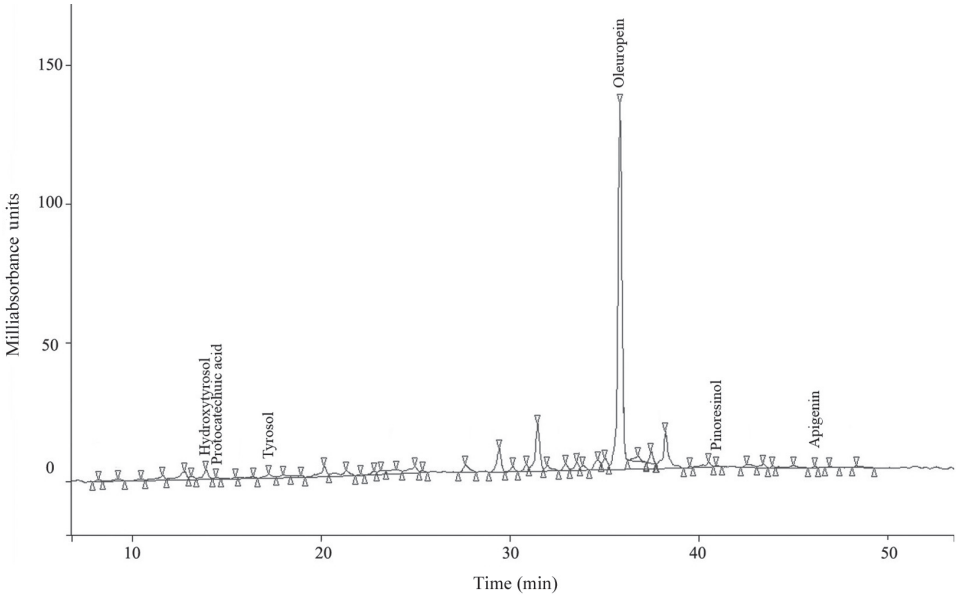

Fig. 1. HPLC chromatogram of phenolic compounds of olive leaf extract.

Table II. Comparison of MIC *values obtained for* C. albicans *and* C. dubliniensis

Test strain	*MIC* (mg mL^{-1})				
	Subcultivation method	EUCAST method[a]	Trypan blue exclusion method	Fluorescent dye exclusion method	
				1 h	18 h
C. albicans ATCC 10231	NA	NA	46.875	> 46.875	46.875
C. dubliniensis CBS 7987	NA	NA	62.5	62.5	62.5

[a] EUCAST recommendation EDef 7.2 (20).
MIC – concentration yielding 80 % growth inhibition compared to the growth in extract-free medium.
NA – no activity observed.

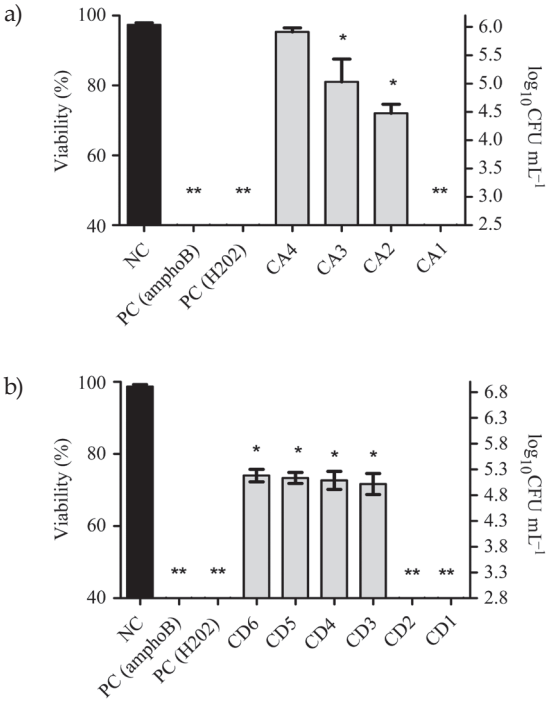

Fig. 2. a) Viability of *C. albicans* ATCC 10231 under treatment with olive leaf extract applied at four concentrations (46.875 (CA1), 15.625 (CA2), 1.563 (CA3) and 0.391 (CA4) mg mL^{-1}) using trypan blue exclusion method (PC – positive control: amphotericin B 1 μg mL^{-1} and H_2O_2 10 % (*V*/*V*); NC – negative control, untreated cells, **p* < 0.05 or ***p* < 0.01 in comparison to NC); b) Viability of *C. dubliniensis* CBS 7987 under treatment with olive leaf extract applied at six concentrations: 187.5 (CD1), 62.5 (CD2), 6.25 (CD3), 1.563 (CD4), 0.625 (CD5) and 0.098 mg mL^{-1} (CD6) using trypan blue exclusion method (PC – positive control: amphotericin B 2 μg mL^{-1} and H_2O_2 10 % (*V*/*V*); NC – negative control, untreated cells, **p* < 0.05 or ***p* < 0.01 in comparison to NC). Results represent the mean of three experiments (± SD).

According to the results gained by Klančnik *et al.* (22), broth microdilution testing was the most accurate method for assessing the antimicrobial effect of plant extracts. Our findings indicated that this method was not always suitable for determining the antimicrobial activity of plant extracts, since colored samples can interfere with measurement of optical density. Optimally, evaluation of viable cells with a microdilution plate reader should utilize a colorless sample to minimize the interference (23). Trypan blue exclusion method and the fluorescent dye exclusion method indicated lower *MIC* concentrations for yeast strains in comparison with *MIC* determination according to the EUCAST recommendation and the subcultivation method, therefore suggesting these methods as more sensitive for the screening of antifungal activity. However, the trypan blue exclusion method has restrictions since it is not suitable for measurements of samples containing less than 10^4 CFU. The colony formation assay (subcultivation method) is used only to provide information about cell proliferation, while trypan blue and fluorescent dye exclusion assays can additionally provide an insight into the morphological changes and membrane integrity of the treated cells as the dyes cross the compromised cell membrane and stain cellular targets and structures in dead cells. Fig. 2 shows the viability of *C. albicans* and *C. dubliniensis* under treatment with olive leaf extract using the trypan blue exclusion method.

The trypan blue exclusion assay results indicated that reduction of viable blastospores following 18 h of incubation was concentration dependent; however, as previously stated, the samples that contained less than 10^4 CFU mL^{-1} could not be measured.

Olive leaf extract treatment against *C. albicans* in concentrations of 46.875, 15.625 and 1.5625 mg mL^{-1} caused a statistically significant decrease of cell viability compared to the negative control (46.875 mg mL^{-1}, $p < 0.01$; 15.625 mg mL^{-1}, 1.5625 mg mL^{-1}, $p < 0.05$; one-way ANOVA with Dunnett's multiple comparison post-test). Decrease of viability of *C. dubliniensis* under treatment with olive leaf extract was found to be statistically significant compared to the control at six tested concentrations (187.5 and 62.5 mg mL^{-1}, $p < 0.01$; 6.25, 1.563, 0.625 and 0.098 mg mL^{-1}, $p < 0.05$, one-way ANOVA with Dunnett's multiple comparison post-test).

We have previously reported high susceptibility of medically important yeasts and dermatophyte strains to hydroxytyrosol treatment, which is a phenolic constituent in olive fruit and leaves of the olive (1). Considerable effects of hydroxytyrosol were observed against *C. albicans* ATCC 10231 and *C. dubliniensis* with *MIC* values of 6.25 mg mL^{-1} for both test strains. Hydroxytyrosol has demonstrated much more inhibitory activity than aqueous extract of olive leaf. Karygianni *et al.* (24) reported that olive leaf extract extracted by mechanical stirring for 12 h with acetone and dissolved in DMSO showed the *MIC* value determined by the microdilution assay against *C. albicans* of 10 mg mL^{-1}. Also, ethanolic olive leaf extract showed *MIC* of 15 mg mL^{-1} against a *C. albicans* isolate in the study by Halawi *et al.* (25). The difference between the antifungal activities of the extract could be attributed to the sample origin, different extraction procedure and different chemical profile of the extract (14). In addition, use of a combination of susceptibility tests is desirable since the results obtained for antimicrobial activity from different susceptibility tests do not always correlate with each other.

Results of the quantitative fluorescent assay for simultaneous identification of apoptotic and necrotic cells in cultures incubated with olive leaf extract for 1 and 18 h are shown in Fig. 3.

Assessment of morphological changes in the cell nuclei by fluorescent dye exclusion method is based on the principle that viable cells exclude ethidium bromide and the appearance of their nuclei with an intact structure is bright green. Non-viable cells had orange to

red colored chromatin of organized structure, while apoptotic cells were bright green with highly condensed or fragmented nuclei (26).

Olive leaf extract treatment of *C. albicans* applied at four concentrations caused a statistically significant ($p < 0.0001$, Pearson chi-square test) decrease of cell viability compared to the negative control after 1 and 18 h of incubation. Cytotoxic effect of the olive leaf extract

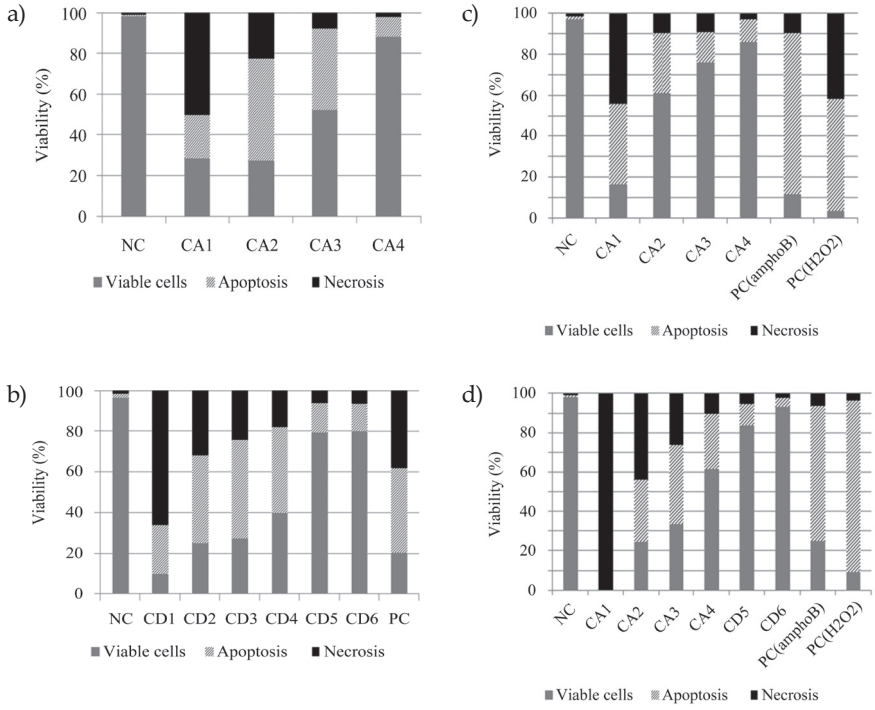

Fig. 3. a) Detection of viable, apoptotic and necrotic cells of *C. albicans* ATCC 10231 following 1 h *in vitro* exposure to olive leaf extract at four concentrations: 46.875 (CA1), 15.625 (CA2), 1.563 (CA3) and 0.391 mg mL^{-1} (CA4) (NC – negative control, untreated cells, viable *vs.* dead cells $p < 0.0001$; apoptosis *vs.* other cells $p < 0.0001$; necrosis *vs.* other cells $p < 0.0001$ in comparison to negative control); b) detection of viable, apoptotic and necrotic cells of *C. dubliniensis* CBS 7987 (CD) following 1 h *in vitro* exposure to olive leaf extract applied at six concentrations: 187.5 (CD1), 62.5 (CD2), 6.25 (CD3), 1.563 (CD4), 0.625 (CD5) and 0.098 mg mL^{-1} (CD6) (PC – positive control: amphotericin B 2 μg mL^{-1} and H_2O_2 10 % (*V*/*V*); NC – negative control, untreated cells, viable *vs.* dead cells $p < 0.0001$; apoptosis *vs.* other cells $p < 0.0001$; necrosis *vs.* other cells $p < 0.0001$ in comparison to negative control); c) detection of viable, apoptotic and necrotic cells of *C. albicans* ATCC 10231 following 18 h *in vitro* exposure to olive leaf extract applied at four cocentrations (PC – positive control: amphotericin B 1 μg mL^{-1} and H_2O_2 10 % (*V*/*V*); NC – negative control, untreated cells, viable *vs.* dead cells $p < 0.0001$; apoptosis *vs.* other cells $p < 0.0001$; necrosis *vs.* other cells except CA4 $p < 0.0001$ in comparison to negative control); d) detection of viable, apoptotic and necrotic cells of *C. dubliniensis* CBS 7987 following 18 h *in vitro* exposure to olive leaf extract (PC – positive control: amphotericin B 2 μg mL^{-1} and H_2O_2 10 % (*V*/*V*); NC – negative control, untreated cells, viable *vs.* dead cells except CD6 $p < 0.0001$; apoptosis *vs.* other cells $p < 0.0001$; necrosis *vs.* other cells except CD6, PC(amphoB) and PC(H_2O_2) $p < 0.0001$ in comparison to negative control).

was concentration dependent. Intergroup comparison using the Pearson chi-square test revealed statistically significant differences ($p < 0.0001$) between all of the four tested concentrations when the samples were incubated for 1 h. Also, when the samples were incubated for 18 h, highly statistically significant differences ($p < 0.0001$) between almost all of the four tested concentrations were revealed, while for samples 1.5625 and 0.3906 mg mL^{-1}, $p = 0.0018$.

Necrosis predominated over apoptosis only in the *Candida albicans* sample treated with olive leaf extract at the highest concentration (46.875 mg mL^{-1}). In other three samples, apoptosis was the predominant type of cell death for both incubation times.

It has to be mentioned that 46.875 mg mL^{-1} was highly cytotoxic to *C. albicans* after 18 h of incubation. The induction of apoptosis in that sample was comparable to positive controls (amphotericin B and H_2O_2), and the differences between these samples were not statistically significant. Furthermore, the induction of apoptosis in the 15.625 mg mL^{-1} sample was comparable to the positive control H_2O_2.

Longer treatment of *C. albicans* with olive leaf extract caused significant lowering of cell viability ($p = 0.0004$) only at 46.875 mg mL^{-1}, which was accompanied with a significant increase in the number of apoptotic cells ($p < 0.0001$). At the same time, the frequency of necrotic cells increased as well, but not significantly.

Compared to the negative control, treatment of *C. dubliniensis* with olive leaf extract at six concentrations, showed a statistically significant ($p < 0.0001$, Pearson chi-square test) loss of cell viability after 1 h of incubation, while after 18 h of incubation only 187.5–0.625 mg mL^{-1} concentrations caused a statistically significant decrease ($p < 0.0001$, Pearson chi-square test).

Cytotoxic effect of the olive leaf extract was concentration dependent. When the samples were incubated for 1 h, intergroup comparison using the Pearson chi-square test revealed statistically significant differences ($p < 0.0001$) between almost all of the six concentrations tested. An exception was the difference between 0.625 and 0.098 mg mL^{-1} samples.

Necrosis predominated over apoptosis only in the sample of *C. dubliniensis* treated with olive leaf extract at the highest concentration (187.5 mg mL^{-1}) after 1 h of incubation and it predominated at the two highest concentrations (187.5 and 62.5 mg mL^{-1}) after 18 h of incubation. In other samples, apoptosis was the predominant type of cell death.

When the samples were incubated for 18 h, intergroup comparison using the Pearson chi-square test revealed highly statistically significant differences ($p < 0.0001$) between almost all of the six concentrations tested. Still, significant differences were observed for 62.5 mg mL^{-1} *vs.* 6.25 mg mL^{-1} ($p = 0.0150$) and 0.625 mg mL^{-1} *vs.* 0.098 mg mL^{-1} ($p = 0.0002$).

It has to be mentioned that 187.5 mg mL^{-1} concentration was highly cytotoxic to *C. dubliniensis*, while the induction of apoptosis in the sample treated with 62.5 mg mL^{-1} was comparable after 18 h of incubation to the positive control amphotericin B and the difference between these samples was not statistically significant. Comparison between two treatment times revealed that longer treatment of *C. dubliniensis* with olive leaf extract caused a significant lowering of cell viability ($p < 0.0001$) only at the 187.5 mg mL^{-1} concentration, which was accompanied with a significant increase of the incidence of necrotic cells ($p < 0.0001$).

CONCLUSIONS

The results of the present study have confirmed that olive leaf extract has a cytotoxic effect on the viability of tested yeast strains and that the effect is concentration dependent. A combination of susceptibility tests was successfully applied in order to provide a more

comprehensive profile of the antifungal activity of the extract. Based on our results, we suggest the use of trypan blue and fluorescent dye exclusion methods as more sensitive for evaluating the *MIC*s of complex samples such as the olive leaf extract.

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