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Formic Acid and Acetic Acid Induce a Programmed Cell Death in Pathogenic *Candida* Species

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Abstract Cutaneous fungal infections are common and widespread. Antifungal agents used for the treatment of these infections often have undesirable side effects. Furthermore, increased resistance of the microorganisms to the antifungal drugs becomes the growing problem. Accordingly, the search for natural antifungal compounds continues to receive attention. Apoptosis is highly regulated programmed cell death. During yeast cell apoptosis, amino acids and peptides are released and can stimulate regeneration of human epithelium cells. Thus, detection of chemical compounds inducing apoptosis in yeast and nontoxic for humans is of great medical relevance. The aim of this study was to detect chemical compound inducing apoptosis in pathogenic *Candida* species with the lowest toxicity to the mammalian cells. Five chemical compounds—acetic acid, sodium bicarbonate, potassium carbonate, lithium acetate, and formic acid—were tested for evaluation of antifungal activity on *C. albicans, C. guilliermondii*, and *C. lusitaniae*. The results showed that acetic acid and formic acid at the lowest concentrations induced yeast cells death. Apoptosis analysis revealed that cells death was accompanied by activation of caspase. Minimal inhibitory concentrations of potassium carbonate and sodium bicarbonate induced *Candida* cells necrosis. Toxicity test with mammalian cell cultures showed that formic acid has the lowest effect on the growth of Jurkat and NIH 3T3 cells. In conclusion, our results show that a low concentration of formic acid induces apoptosis-like programmed cell death in the *Candida* yeast and has a minimal effect on the survivability of mammalian cells, suggesting potential applications in the treatment of these infections.

Introduction

Cutaneous fungal and bacterial infections are common and widespread. The treatment with synthetic drugs is expensive, and many side effects are observed. There is a relevant need to reveal novel compounds of biological origin with antimicrobial property due to the growing problem of the acquisition of resistance by the pathogenic microorganisms observed in clinical settings. The efficient treatment of fungal infections is particularly difficult since fungal cell structure and metabolism are similar to those of eukaryotic hosts. Recently, the scientists have focused on the potential antimicrobial effects of certain natural products, which have the advantage of being readily available, environmentally friendly, and low in toxicity [16, 29].

Organic acids have been used for decades in feed preservation, preventing feed from bacterial and fungal deterioration. These acids are hypothesized to cross the cell membrane of bacteria where the release in protons (H+)

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causes the cells to die [4]. Acetic acid and formic acid are generally recognized as safe antimicrobial agents, and the low dilute solutions of organic acids are generally without side effects [10, 31].

Sodium bicarbonate is widely used in the food industry, to formulate toothpaste, cosmetic products, and is known for its acid-neutralizing properties. There are some reports that sodium bicarbonate possesses antimicrobial activity against oral microorganisms [5]. It has been reported that this agent is virucidal and inhibits the growth of several fungi, but the mechanism of action is unclear [1, 20].

In literature, there are numerous studies regarding searching for antimicrobial agents, but only a few of them investigate the action of chemical compounds on microorganisms and their induced cell death. Apoptosis is highly regulated cellular suicide program. Assays for apoptotic and/or necrotic cell death such as viability, DNA fragmentation, exposition of phosphatidylserine, or cell integrity have been established and are routinely used in the field of yeast programmed cell death [7, 19]. When a cell undergoes apoptosis, it continues to produce proteins and ATP. Damaged cells are homeostatically removed without an inflammatory reaction occurring. In contrast, necrotic cell death is characterized by the loss of metabolic functions and the integrity of the cell membrane. The released cellular content induces an inflammatory response in the effected tissue. Thus, the finding of chemical compounds inducing apoptosis in yeast and nontoxic for humans is of great medical relevance and might provide a basis for future therapies.

Keeping the above facts in view, the present study was carried out to investigate the antifungal activity and induced cell death of five natural compounds against *Candida albicans*, *C. guilliermondii*, and *C. lusitaniae*. *C. albicans* we used as a model microorganism as it is a medically important commensal pathogen. *C. guilliermondii* and *C. lusitaniae* are infrequent cause of infections, but they are unique among *Candida* species due to their drug resistance [11, 12].

Materials and Methods

Yeast Strains and Cultivation Conditions

The study was performed on *C. guilliermondii* and *C. lusitaniae* strains isolated from the skin of patients with clinical diagnosis of atopic dermatitis and identified applying assimilation test api ID32C (bioMerieux sa, France) [32]. Physiological identification of these isolates was confirmed by 26S rDNA sequences analysis. Briefly, the D1/D2 region of the large subunit of the 26S rDNA was PCR-amplified from the genomic DNA using the universal primer pair F63/LR3. The amplified fragments were purified using GeneJET^{TM} PCR Purification kit (Thermo Fisher Scientific) and sequenced using the Sanger dideoxy chain-termination method. A similarity search of the D1/D2 26S rDNA sequences against database entries was performed using the Basic Local Alignment Search Tool (BLAST) program provided by the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST). *C. albicans* ATCC 10231 strain was also included in this study. Yeasts were grown on Sabouraud agar medium (SA) (2 % glucose, 1 % peptone, 1.5 % agar) for further research.

Antifungal Screening

Antifungal testing was adapted from the CLSI guideline M27-A3 [8]. Five chemical compounds—acetic acid, sodium bicarbonate, potassium carbonate, lithium acetate, and formic acid—were tested for evaluation of antifungal activity. Acetic acid, a known inducer of apoptosis in yeast, was used as a control. Minimum inhibitory concentrations (MICs) were determined by a microdilution method in 96-well microdilution plates using Sabouraud broth (SB) (2 % glucose, 1 % peptone). Analyzed yeast strains were subcultured on SA at 35 °C for 24 h. 100 µl of prepared yeast suspension in SB medium was added to 100 µl of SB medium supplemented with known concentrations of tested antifungal compounds (final inoculum size 7×10^2–3×10^3 CFU/ml). 100 µl of each yeast strain suspension was added to 100 µl of SB medium for the positive control. Medium alone was used for the sterility control. Each strain was tested in duplicate on each media. Microwell plates were incubated at 35 °C for 48 h aerobically. The first two wells without visible growth as compared to the positive control were defined as the MIC and subcultured on the SA medium for 5 days at 35 °C.

Time-kill Assay

The time-kill assay was performed by the broth macrodilution method. In brief, a starting inoculum of 10^5–10^6CFU/ml and a final concentration of the antifungal compound equal to the MIC were used in the assay. Flasks containing 50 ml of SB medium with the appropriate antifungal agent were mixed with 50 ml of SB medium containing the test organism in logarithmic growth phase. The flasks were incubated with shaking at 35 °C. Aliquots were removed, and OD_{560} was measured at 0-, 2-, 4-, 6-, 8-, 10-, 20-, 22-, 24-, 26-, 28-, 30-, 32-, and 48-hour time point. Time-killing curves were constructed by plotting the OD_{560} versus time over 48 h. Medium without antifungal agents was used as a control. The assay was repeated three times. Results were interpreted by the antifungal effect between the tested different compounds.

Apoptosis Analysis

All strains were grown over night aerobically on a rotary shaker at 35 °C. Cells were harvested and incubated for 2, 4, and 24 h in SB medium supplemented with the minimal inhibitory concentrations of tested compounds. Nucleus morphology analysis was performed by staining yeast cells with 4 µl DAPI for 10 min at room temperature. Samples were analyzed by fluorescent microscopy using 330–380 nm wavelength.

Externalization of phosphatidylserine at the outer layer of plasma membrane was assayed by staining cells with FITC/AnnexinV (Roche) and PI [19]. 1 x 10^6 yeast cells were harvested and washed with digestion buffer (1.2 M sorbitol, 0.5 mM $MgCl_2$, 35 mM K_2HPO_4, pH 6.8). Cells wall was digested with 15 U/ml lyticase and 5.5 % glusulase for 2 h at 30 °C for generation of protoplasts. Protoplasts were harvested by centrifugation and washed twice with 200 µl AnnexinVbinding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) containing 1.2 M sorbitol. To the 38 µl of cell suspension, 2 µl FITC/AnnexinV and 2 µl PI were added. Cells were incubated at the room temperature for 30 min. After staining, yeast cells were harvested by centrifugation and applied onto polylysine coated slides. Samples were analyzed by fluorescent microscopy using 400–500 nm and >560 nm wavelength.

Fluorescein FragEL™ DNA Fragmentation Detection Kit was used for the detection of DNA strand breaks. Analysis was performed by following manufacturer's instruction. Briefly, 1 x 10^6yeast cells were harvested and fixed for 10 min at room temperature in 4 % PBS buffered formaldehyde. Formaldehyde was removed by washing yeast cells with TBS. 100 µl of proteinase K (20 µg/ml) was used for permeabilisation of the specimen. Proteinase K was removed by centrifugation at 1,000 rpm for 5 min at 4 °C. Cells were harvested, stained with Fluorescein FragEL™ reaction mixture, and analyzed by fluorescent microscopy using 450–500 nm wavelength. Yeast cells were additionally treated with DNaseI for generation of positive control.

Active caspase were detected by staining cells with FITC-VAD-FMK (CaspACE™In Situ Marker Kit, Promega) kit. 1 x 10^6 cells were harvested and washed with PBS buffer. Cells were incubated with 10 µM CaspACE™In Situ Marker for 20 min at 30 °C in the dark. After staining cells were harvested, washed with PBS, and applied onto polylysine coated slides. Samples were analyzed by fluorescent microscopy using wavelength of 490–525 nm.

Toxicity Test with Mammalian Cell Cultures

Jurkat and NIH 3T3 cells were cultured in RPMI medium with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 % fetal calf serum. All cell culture reagents were from HyClon (ThermoFisher Scientific). Jurkat cells were seeded 0.25 x 10^6 cells per well in six-well plate. The cell concentration was 0.25 x 10^5/ml. NIH 3T3 cells were seeded in 24-well plate 3 x 10^4 cells per well. The untreated and antifungal agents treated cells were incubated at 37 °C, 5 % of CO_2 up to 3 days. The cells were analyzed after 1, 2, and 3 days. Apoptotic cells were stained with 7AAD (Molecular probes) and analyzed by FACS Calibur (BD Biosciences, USA). For the analysis of cell proliferation, the cells prior the culturing were labeled with CFSE (Molecular probes). For this purpose, Jurkat and NIH 3T3 cells were resuspended in RPMI medium with 10 % FCS at a concentration 0.3–1 x 10^7/ml. CFSE was added to the cells at a final concentration of 1 µM and incubated for 5 min at 37 °C. The not bound CFSE were washed out from the cells with RPMI medium. The labeled cells were analyzed by FACS Calibur.

Results

The sequence analysis of the ribosomal gene confirmed previous physiological identification of clinical *C. guilliermondii* (accession No. KJ396345) and *C. lusitaniae* (accession No. KJ396344) isolates.

MICs of tested compounds are presented in Table 1. The results showed that acetic acid and formic acid exhibited the most intense antifungal activity on all tested *Candida* strains.

The time-kill assay was used to study time-dependent antifungal activities of antimicrobial agents. The results showed that potassium carbonate possessed the fastest antifungal activity (Fig. 1). Cells were completely eliminated already after 2 h of incubation with MIC of potassium carbonate. No colony forming units were obtained after 6 h of treatment of *Candida* cells with acetic acid. Formic acid eliminated the cells after 20 h of incubation,

Table 1 Minimum inhibitory concentrations of tested antifungal agents

	$NaHCO_3$	K_2CO_3	CH_3COOH	HCOOH	CH_3COOLi
C. albicans	5 %	2.5 %	0.25 %	0.1 %	1 %
C. guilliermondii	5 %	2.5 %	0.3 %	0.2 %	1 %
C. lusitaniae	5 %	2.5 %	0.25 %	0.1 %	1 %

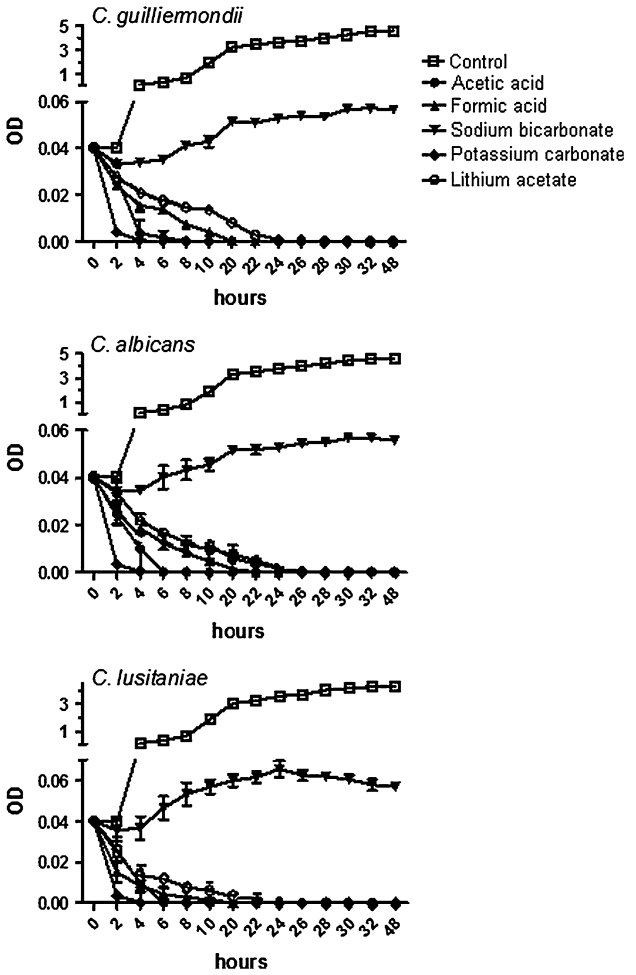

Fig. 1 Viability of *Candida* strains in SB medium supplemented with MICs of analyzed compounds

while sodium carbonate exhibited delayed antifungal activity. Comparison between the different *Candida* species showed that *C. guilliermondii* had the highest resistance to all tested compounds as compared to *C. lusitaniae* and *C. albicans*.

For the apoptosis induction analysis, four chemical compounds (potassium carbonate, sodium bicarbonate, formic acid, and acetic acid) were selected. Lithium acetate showed good antifungal activity, but it is commonly used for the permeabilisation of the cell membrane. It leads to inability to test effect of lithium acetate for the externalization of phosphatidylserine in yeasts. Therefore, lithium acetate was excluded from the further analysis. Yeast cells were incubated with MICs of tested compounds for four hours.

TUNEL staining is commonly used for the detection of apoptotic cells, but DNA fragmentation can simultaneously occur during yeast cell death by the necrotic pathway resulting in the high percentage of TUNEL positive cells in the population (Table 2). TUNEL staining alone is not suitable to count the real number of cells dying in apoptotic manner, and more precise methods have to be applied to confirm the results. Therefore, for the apoptosis analysis, we performed TUNEL staining, phosphatidylserine staining, and active caspase staining analysis.

Double staining of yeast cells with AnnexinV and propidium iodide allowed the discrimination between early apoptotic cells exhibiting phosphatidylserine externalization (AnnexinV+/PI−), late apoptotic/secondary necrotic cells, which showed both PS exposure and membrane permeability (AnnexinV+/PI+), and cells with ruptured membrane indicative of primary necrosis (AnnexinV−/PI+) (Supplementary data Fig. 1). The highest percentage of the cells in all analyzed strains displaying markers of early and late apoptosis was detected after the treatment of yeasts with formic and acetic acids (Fig. 2). Phosphatidylserine externalization was observed in more than 80 % cells of all analyzed strains after the 4 h of treatment with MIC of formic acid. Incubation of analyzed strains with acetic acid induced apoptosis in more than 82 % of cells.

Incubation of the *C. lusitaniae, C. guilliermondii, and C. albicans* in media supplemented with MICs of sodium bicarbonate and potassium carbonate led to the necrosis induction (Fig. 3).

Early and late apoptotic phenotypes detected after the treatment with carbonates were less than 20 % in all three strains. After the treatment with sodium bicarbonate, over 60 % of the cells displayed AnnexinV−/PI− phenotype. Incubation of the analyzed strains with potassium carbonate led to the necrotic pathway of cell death (Fig. 3). Over 82 % of the cells of all the analyzed strains displayed necrotic markers, and markers of early and late apoptosis were registered in less than 10 % of the cells.

Table 2 DNA fragmentation and caspase activation (%) in yeast cells after treatment with MICs of antifungal agents

	C. albicans		*C. guilliermondii*		*C. lusitaniae*	
	TUNEL+	CASPASE+	TUNEL+	CASPASE+	TUNEL+	CASPASE+
$NaHCO_3$	36.47 ± 4.587	25.78 ± 2.598	35.23 ± 5.267	23.56 ± 3.578	37.56 ± 4.869	27.15 ± 2.561
K_2CO_3	15.34 ± 3.578	12.47 ± 1.578	13.83 ± 2.159	11.59 ± 1.442	16.86 ± 2.567	13.85 ± 1.896
CH_3COOH	76.89 ± 5.743	85.71 ± 5.12	74.51 ± 4.897	85.53 ± 5.731	79.2 ± 6.012	88.79 ± 3.823
HCOOH	72.23 ± 4.963	87.58 ± 5.465	67.86 ± 5.364	82.15 ± 6.589	75.18 ± 4.125	86.75 ± 4.127

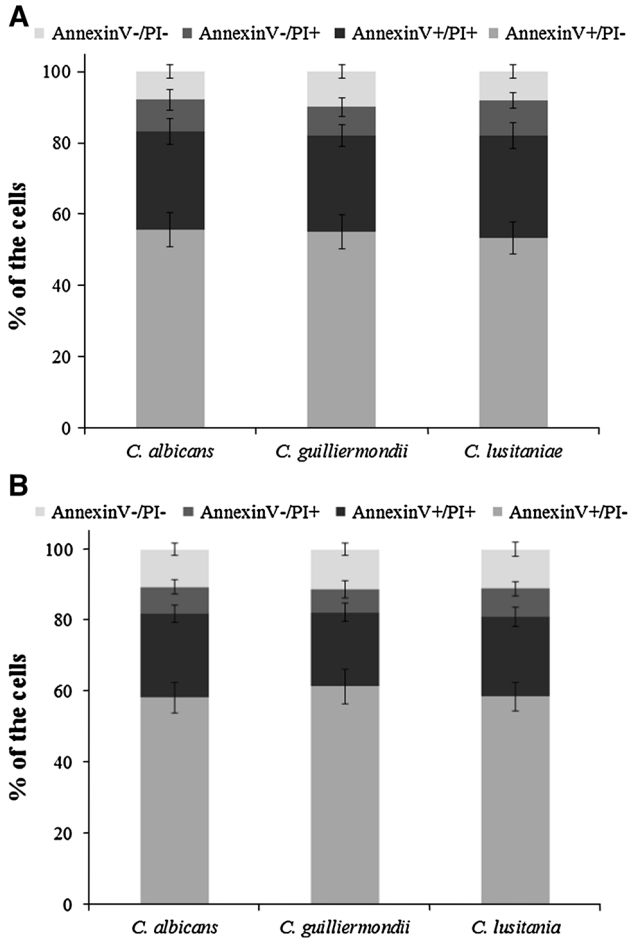

Fig. 2 Externalization of the phosphatidylserine in the outer layer of yeast cells plasma membrane after the treatment with MIC of acetic acid (**a**) and formic acid (**b**). Early apoptotic cells—AnnexinV+/PI−, late apoptotic/secondary necrotic cells—AnnexinV+/PI+, necrotic cells—AnnexinV−/PI+, viable cells—AnnexinV−/PI−

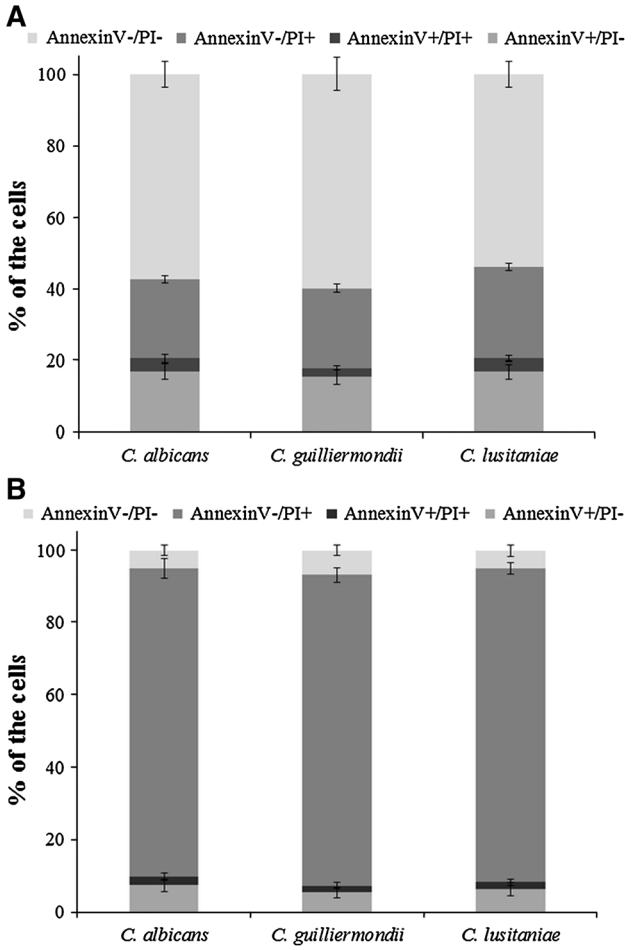

Fig. 3 Externalization of the phosphatidylserine in the outer layer of yeast cells plasma membrane after the treatment with MIC of sodium bicarbonate (**a**) and potassium carbonate (**b**). Early apoptotic cells—AnnexinV+/PI−, late apoptotic/secondary necrotic cells—AnnexinV+/PI+, necrotic cells—AnnexinV−/PI+, viable cells—AnnexinV−/PI−

TUNEL and active caspase analysis results are presented in Table 2 (Supplementary data Figs. 2, 3). The highest percentage of the cells with fragmented DNA and active caspase was detected after treatment of cells with formic and acetic acids. The percentage of the apoptotic cells observed after treatment with formic acid statistically did not differ ($p > 0.05$) from the percentage of apoptotic cells observed after treatment with acetic acid, which is a known inducer of apoptosis in yeast cells. Meanwhile, potassium carbonate induced apoptosis in less than 20 % of the cells. After treatment of yeasts with sodium carbonate, apoptosis related phenotypes were detected in less than 40 % of the total cell population.

Apoptosis analysis results were confirmed by CFU count that showed that over 40 % of the *C. lusitaniae*, *C. guilliermondii*, and *C. albicans* cells did not produced the colonies after the treatment of the cells with MIC of sodium bicarbonate (Fig. 4). Therefore, less than 28 % of

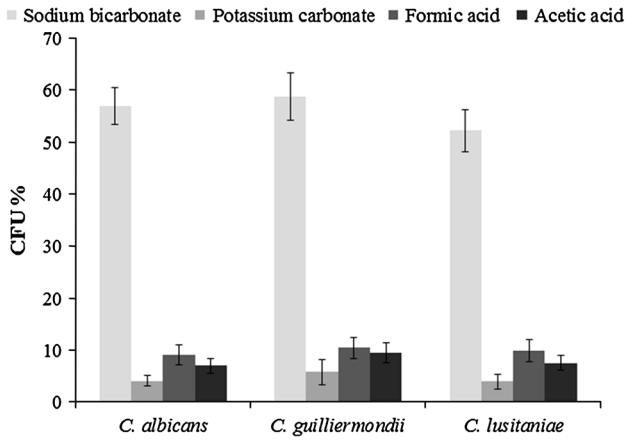

Fig. 4 Apoptosis analysis results were confirmed by CFU count. Yeast cells were incubated in the SB medium supplemented with MICs of tested compounds for 4 hours. After incubation, cells were plated on the SA plates and appeared colonies were counted after 48 hours of growth

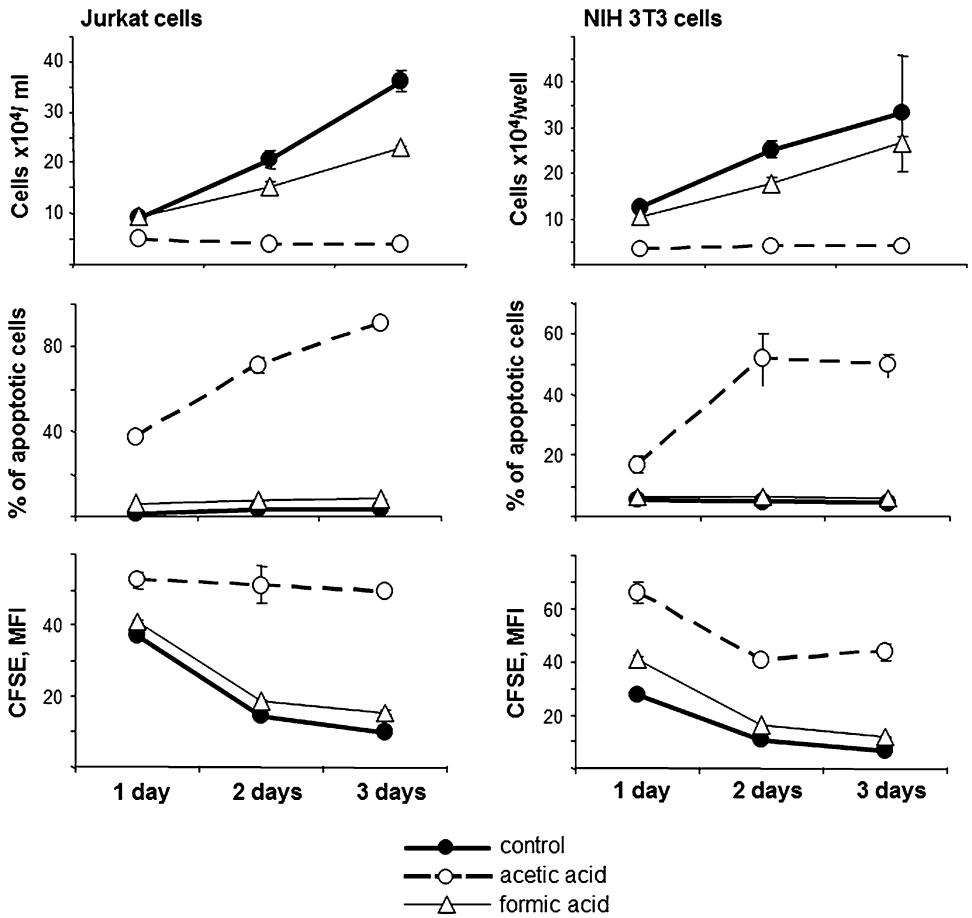

Fig. 5 The influence of acetic acid and formic acid on the growth of mammalian cell lines

the cells showed apoptotic phenotype indicating that remaining cells were committed to necrosis. Treatment of the cells with potassium carbonate caused necrosis induction in more than 78 % of the cells. Apoptosis was induced in more than 75 % of the cells after the incubation with acetic acid and formic acid.

Toxicity test with cell cultures was performed in order to appreciate the effects of chemicals on the capability of mammalian cells to replicate. The results showed that formic acid had the lowest effect on the survivability of Jurkat and NIH 3T3 cells (Fig. 5). Lithium acetate, sodium bicarbonate, and potassium carbonate caused massive wave of cell death in mammalian cell lines; therefore, formic acid had the lowest effect on the mammalian cell survival and proliferation.

Discussion

In this study, we investigated the antifungal activity of five compounds against three *Candida* species. Our results revealed that acetic acid, sodium bicarbonate, potassium carbonate, lithium acetate, and formic acid had almost equal antifungal activities on different *Candida* species. Moreover, the results showed that formic acid and acetic acid had highest effects against tested microorganisms.

In this research, we included acetic acid rather as control, because it is known that acetic acid possesses antibacterial and antifungal properties [14, 28]. It is a compound commonly used to induce yeast apoptosis. Acetic acid enters the cell by a process of membrane protein channel-facilitated diffusion, accumulates in a cell, and acidifies the intracellular environment. This process finally results in cell death by the apoptotic or necrotic pathway [18, 22].

Recently, it was shown that a low concentration of formic acid induces caspase independent cell death in *Saccharomyces cerevisiae*. Authors of this study hypothesized that formic acid caused cell death in yeast by inhibiting the activity of cytochrome oxidase by formate, resulting in the rapid depletion of intracellular ATP and sequential cell death [9]. In this study, we determined that treatment of *Candida* with formic acid follows a series of typical apoptotic morphological changes resembling that in mammalian cells. Unlike Lin Du et al. published results, the data obtained in the present study indicate that low concentration of formic acid induces caspase-dependent

cell death in yeast. These differences are probably related to yeast species used.

Antifungal activities of sodium bicarbonate and potassium carbonate have already been demonstrated [16, 23, 24, 27]. Several studies suggest that disturbance of internal homeostasis of potassium and sodium cations is responsible for a growth arrest of the cell [3, 15]. Our work demonstrated that treatment of the yeast cells with MICs concentrations of sodium bicarbonate and potassium carbonate resulted in cell death mostly by the necrotic pathway. The time-kill assay was used to evaluate time-dependent antifungal activities of tested agents. Results showed that potassium carbonate was faster-acting antifungal agent than sodium bicarbonate. Additionally, we observed that lower MICs concentrations of sodium bicarbonate and potassium carbonate stimulated yeast to hyphae switching (data not shown). There is opinion that the main functions of hypha formation are translocation between host environments and nutrient acquisition [6]. Phenotypic switching enables an organism to adapt easier to specific environments (partially due to nutrient availability) through selective gene expression. It was shown that this mechanism is involved in the infection process as loss of the ability to form hypha resulted in *C. albicans* avirulence [17]. It was shown that hyphal cells contain intracellular potassium at high concentrations in comparison with yeast cells [30]. Additionally, there is evidence that phenotypic switching participates in the fungal response to antifungal agents [21]. Keeping the above facts and our results in view, we suggest that applications of these compounds in the treatment of *Candida* infections are limited because an accidental use of an insufficient concentration can provoke hypha formation and exacerbation of a disease.

Formic (or methanoic) acid is very dangerous at high concentrations, but at low concentrations it is widely used in industry. Several studies have shown an antimicrobial activity of formic acid on bacteria and yeasts [2, 25, 26]. However, to our knowledge, there are no studies in literature evaluating its antimicrobial activity on medically important organisms.

Conclusion

In this study, we showed that a low concentration of formic acid induces apoptosis-like programmed cell death in the *Candida* yeast but has a minimal effect on the survivability of mammalian cells. Formic acid is an inexpensive material already used for medical purposes, and our results suggest that it could be used for the development of suitable candidate for treatment of fungal infections.

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