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Precise Demonstration of Salt-Stress Induced Antifungal Activity of *Origanum onites* Essential Oil and *Taraxacum officinale* Extract Against Drug Resistant Isolates of *Candida albicans* and *Aspergillus fumigatus* Using Micro-Colony Method

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Research Article

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ABSTRACT

The rise of drug-resistant fungal pathogens has intensified the need for novel antifungal agents. Plants are a significant source, although effective concentrations in extracts are often low. Accurate *in vitro* assays are essential for validating these compounds. This study uses the micro-colony method, measuring hyphal growth and cell diameter under a microscope with digital imaging, to assess antifungal activity quickly and precisely. We evaluated *Origanum onites* essential oil and *Taraxacum officinale* methanol extract against drug-resistant *Candida albicans* and *Aspergillus fumigatus* strains. Yeast cell pigmentation was also assessed using image processing tools. To enhance compound penetration, mono and divalent salts (100mM KCl, NaCl, $CaCl_2$) were added to the media. In salt-free media, *Origanum onites* essential oil inhibited *Candida albicans* (MIC: 0.3 µl/ml; MFC: 0.03 µl/ml) and *Aspergillus fumigatus* (MIC: 0.15 µl/ml; MFC: 0.03 µl/ml), while *Taraxacum officinale* extract was ineffective. Salt stress increased *Origanum onites* activity against *Aspergillus fumigatus* (MIC: 0.075 µl/ml) but had minimal impact on *Candida albicans*. Salt stress enabled *Taraxacum officinale* extract to inhibit *Candida albicans* (EC50: 12.71 µg/ml) and reduced its pigmentation dose-dependently without affecting toxicity against *Aspergillus fumigatus*. These results demonstrate that the micro-colony assay effectively evaluates plant-derived antifungal compounds and detects subtle dose-response variations in pathogenic fungi.

Keywords: Antifungal drug resistance, Micro-colony method, Plant metabolites, Salt stress

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Introduction

Certain mold (e.g., *Aspergillus fumigatus* Fresenius) and yeast fungi (e.g., *Candida albicans* (C.P. Robin) Berkhout) infect human tissues, causing fungal diseases (mycoses) such as aspergillosis and candidiasis, which can be fatal in some cases [1]. Synthetic antifungal drugs (azoles, echinocandins, and polyenes) are the main treatment options for mycoses [1-3]. However, fungal pathogens develop resistance to antifungal drugs through various mechanisms, including efflux proteins (ATP-binding cassette transporters and major facilitators), reduced drug penetration (selective permeability and excessive pigmentation), and modification of the drug target (point mutations hindering fungicide binding and increased transcription of the target site) [2,3].

Plants accumulate a plethora of antifungal compounds, such as alkaloids, flavonoids, and isoprenoids, with different cellular targets, including cell wall formation and protein biosynthesis [4,5]. Therefore, plants, especially endemic species, are invaluable reservoirs of novel antifungal drugs to combat resistant fungal pathogens. The European Pharmacopoeia and National Committee for Clinical Laboratory Standards (NCCLS) guidelines dictate the use of sensitive reference microorganism strains to validate prospective antimicrobial agents [6]. Given the rise of resistance, screening potent antifungal compounds on resistant isolates is a wise strategy.

Appropriate and precise *in vitro* assays are essential for validating effective antifungal compounds. Diffusion and turbimetric methods are two main microbiological assays of antibiotics outlined by the European Pharmacopoeia [6]. These methods have been utilized under various terminologies, such as disc diffusion halo, colony radial growth, paper disc diffusion assays, and microdilution techniques like the minimum inhibitory concentration assay outlined by NCCLS (2004) [7-9].

Previously, the micro-colony assay, which involves measuring the hyphal growth of the germinated conidium during the early incubation period using microscopy and a digital microscope camera, was developed to screen resistance to azole fungicides and dose responses to alternative oxidase inhibitors in slow-growing plant pathogens, such as the filamentous fungi *Fusicladium effusum* G. Winter and *Cercospora arachidicola* Hori [10-13]. The micro-colony method requires shorter incubation periods to validate antifungal compounds compared to other assays. Additionally, it is compatible with definitive data analysis methods, such as normal distribution tests, regression analysis, and analysis of variance tests. Data perception is based on the average growth of the

population at each concentration, which is not possible with other methods [10-12].

Hypothetically, high osmotic pressure could accelerate the influx of low molecular mass substances into fungal cells [14]. For instance, salt stress in the fungal organism *Zygosaccharomyces rouxii* (Boutroux) Yarrow induced several physiological alterations and increased nystatin toxicity [14]. In the gram-positive bacterium *Corynebacterium glutamicum*, 1.5 M NaCl-mediated osmotic pressure induced the uptake of the osmoprotectant glycine betaine, but choline uptake remained unchanged [15]. In *Escherichia coli* (Migula) Castellani and Chalmers bacteria, 0.3 M NaCl increased the cellular uptake of organic compounds containing humic substances [16]. In the oomycete *Pythium porphyrae* Takahashi and Sasaki, KCl and $MgCl_2$ increased the membrane permeability of an anti-Pythium protein [17].

This study aimed to: i) validate the micro-colony assay to screen the antifungal potential of *Origanum vulgare* L. (Lamiaceae) essential oil and the endemic species *Taraxacum officinale* methanol extract against a fluconazole-resistant strain of *Candida albicans* ATCC 10231 and a drug-resistant clinical isolate of *Aspergillus fumigatus* [18]; ii) evaluate the role of salt stress during *in vitro* testing, as low molecular mass antifungal compounds might be underscored due to lack of internal penetration, and salt-mediated osmotic pressure could be used to increase the cellular penetration of substances in the plant extract; and iii) assess the pigmentation of *Candida albicans* yeast cells with images captured for the micro-colony assay.

Material and Methods

Plant Material

Oregano plants were purchased from a local market in Izmir, Turkey. In Western Anatolia, a common type of oregano plant is Origanum onites L., also known as Cretan oregano or Turkish oregano. This variety is well-known for its potent essential oils and is widely cultivated and used in the region.

Preparation of Origanum onites Essential Oil

Steam distillation was performed according to the "Determination of Essential Oils in Herbal Drugs" protocol in the European Pharmacopoeia (EDQM, 2010). A total of 68.0 g of *Origanum onites* dried flowers and leaves were placed in a 2000 mL round-bottom flask with 800 mL of distilled water (EDQM, 2010). The distillation was conducted without xylene for 6 hours using a Clevenger apparatus (Ildam, NS 7/5, Ankara, Turkey) and a single electric stove at 1500 W maximum power (Arçelik, Bolu, Turkey) (EDQM, 2010). The water-free *Origanum onites* essential oil was recovered using anhydrous sodium sulphate (Merck, Darmstadt, Germany) and stored at 4.0 °C in a glass container.

Preparation of Methanolic Extracts

Taraxacum officinale plant material (leaves and stems) was collected in the Spring of 2024 from the rural areas surrounding the Sivas Province, Anatolia, Turkey. The collected plant material was dried at room temperature for two weeks. The dried material was then ground into a fine powder using a mechanical grinder. For the extraction process, 50 g of the powdered plant material was soaked in 500 mL of methanol (analytical grade) in a conical flask. The mixture was left to macerate at room temperature for 72 hours with occasional shaking to ensure thorough extraction of the phytochemicals. After the maceration period, the mixture was filtered using Whatman No. 1 filter paper to remove the plant residues. The filtrate was then concentrated under reduced pressure at 40°C using a rotary evaporator (Heidolph Instruments, Schwabach, Germany) to remove the methanol, yielding a semi-solid crude methanolic extract. The extract was further dried in a desiccator over anhydrous calcium chloride to obtain a constant weight. The resulting methanolic extract was stored in an airtight container at 4°C until further use.

Micro-colony Assay

The experiments were performed in triplicate, and more than three doses were used for each substance to achieve a geometric dose-response progression (EDQM, 2010). *Origanum vulgare* essential oil was serially diluted in DMSO to reach concentrations of 0.3, 0.15, 0.1, 0.075, and 0.03 µl/ml in the media. *Taraxacum officinale* extract was serially diluted in DMSO to reach concentrations of 3.0, 1.5, 1.0, and 0.3 µg/ml in the media. *Origanum onites* essential oil is soluble in 1% DMSO, which is adequate for preparing serial dilutions for biological assays. A concentrated stock solution was prepared by dissolving 10 µL of the essential oil in 990 µL of 1% DMSO, resulting in a 1% (v/v) stock solution. To achieve serial dilutions, 500 µL of the 1% stock solution was added to 500 µL of 1% DMSO to make a 0.5% solution. This process was repeated to achieve concentrations of 0.25%, 0.125%, 0.0625%, and 0.03125%. The final working concentrations in the media were obtained by adding the appropriate volume of each diluted solution to the growth medium to reach the desired concentrations of 0.3, 0.15, 0.1, 0.075, and 0.03 µL/mL. Each concentration of the essential oil solution was thoroughly mixed with the growth medium using a magnetic stirrer for approximately 30 seconds to ensure even distribution of the essential oil in the medium. By using 1% DMSO as the solvent, the solubility of the essential oil was maintained, and the serial dilution process ensured accurate and reproducible concentrations for the antifungal assays. In a previous experiment, the azole fungicide fluconazole failed to inhibit resistant strains of *Aspergillus fumigatus* and *Candida albicans* [19]. Azole fungicides bind to key enzymes lanosterol 14α-demethylase and C5-sterol desaturase, leading to the inhibition of ergosterol biosynthesis [3]. Therefore, another standard fungicide, thiabendazole, with a different mode of action, was used as a reference inhibitor. The microtubule inhibitor thiabendazole (Sigma-Aldrich, Taufkirchen, Germany) was

serially diluted in acetone to reach concentrations of 100, 10, 1.0, and 0.1 µg/ml in the medium [20].

The growth medium for the *Aspergillus fumigatus* testing was Potato Dextrose Agar (PDA) at 40 g/L (Merck, Darmstadt, Germany). The growth medium for the *Candida albicans* testing was Sabouraud Dextrose Agar (SDA) at 40 g/L (Merck, Darmstadt, Germany). The control plates were amended only with DMSO or acetone. Additionally, another group of control plates was prepared without the addition of DMSO to screen for the toxic effects of the solvent. The content of DMSO was less than 1% in all concentrations [21].

For each concentration and control, salt-amended "medium b" was prepared. In *Saccharomyces cerevisiae* Meyen ex E.C. Hansen, 100mM KCl, NaCl, and $CaCl_2$ in the medium reduced resting membrane potential and altered amiodarone toxicity [22]. To achieve osmotic pressure, 100mM concentrations of KCl, NaCl, and $CaCl_2$ were added to the media before sterilization. The autoclaved media were kept in a water bath set to 60°C throughout the experiment to prevent solidification and maintain consistent viscosity. For each concentration of *Origanum onites* essential oil, *Taraxacum officinale* extract, and thiabendazole fungicide, a separate flask with a magnetic stirrer bar was used to ensure precision in substance concentration. After serial dilution, the substances were added to the flasks and mixed with a magnetic stirrer for approximately 30 seconds to achieve thorough dispersal in the media. After mixing, 4 ml of the media was immediately introduced onto Petri plates (90x17 mm, glass) using a 5 ml capacity pipette. The plates were stored on a shelf until the medium solidified.

Candida albicans cells and *Aspergillus fumigatus* conidia were suspended and washed with sterile distilled water. The number of conidia and yeast cells to be inoculated on each plate was estimated using a haemocytometer (Bürker-Türk, Wertheim, Germany) to obtain final inoculums of 3 x 10^6 *Aspergillus fumigatus* conidia and 1 x 10^5 *Candida albicans* yeast cells. The *Aspergillus fumigatus* conidia and *Candida albicans* cells were introduced and spread on Petri plates with a glass rod and incubated at 27°C and 37°C for 16 and 12 hours, respectively. After the incubation period, the plates were kept at 4°C in a cold storage room. Plates were measured as separate stocks to prevent activation and further growth during the measurement process.

Images of the micro-colonies at 40X magnification were captured with Olympus Soft Imaging Solutions software under the compound microscope Olympus CX31 using model Lc 20 Micro digital microscope camera (Olympus Corp., Tokyo, Japan). The micro-colonies were measured with the Motic Images Plus 2.0 software (Motic Inc., Xiamen, China). If the fungal colonies' growth exceeded the range of the focus area, half of the diameter was measured and multiplied by two, as seen in the control plate of Figure 10.

Relative inhibition in radial growth was obtained using the equation for each concentration using the formula; ϕ=micro-colony measurement on plant extract, essential oil or fungicide, ω = micro-colony measurement on control, ε = relative inhibition in fungal growth;

$$\varepsilon = 1 - \left(\frac{\sum_{i=1}^{n}(log\varphi i)/n\varphi}{\sum_{i=1}^{n}(log\omega i)/n\omega}\right)$$

Pigmentation Assay

The pigmentation of the Candida albicans micro-colonies was analyzed using Microsoft Paint Version 6.0 (Build 6002, Service Pack 2.0). Images captured for the micro-colony assay were opened in the Paint program. A single location on a micro-colony was selected using the "Pick Color" tool. Subsequently, the "Red, Green, and Blue" values were identified using the "Edit Colors" and "Define Custom Colors" tools.

Relative inhibition in pigmentation was obtained using the equation for each concentration using the formula; β = pigmentation value on plant extract, essential oil or fungicide, α = pigmentation value on control, and σ = relative inhibition in pigmentation;

$$\sigma = \left[\left(\frac{\sum_{i=1}^{n}(\log(red+blue+green)\beta i)/n\beta}{\sum_{i=1}^{n}(log(red+blue+green)\alpha i)/n\alpha}\right) * 100\right] - 100$$

Data Analysis

For each concentration, fungal development was validated based on the measurement of approximately 10 distinct micro-colonies. Instead of arithmetic values, log10-transformed values of each micro-colony were used to increase the precision of growth estimation. These measurements were estimated as the growth values of the sub-population at each concentration. The normality of the population at each testing concentration was verified using the Shapiro-Wilk validity test at a significance level of P = 0.05. Additionally, differences in fungal development and pigmentation based on dose-response among the different replicates and treatments were verified with a one-way analysis of variance (ANOVA) test at a significance level of P = 0.05. However, for regression analysis and dose-response curves, the data set from one replicate was used to represent the growth or pigmentation value at each concentration. To demonstrate the influence of salt stress on radial growth and pigmentation, data sets from one replicate of salt-free and salt-amended media were compared and verified using a one-way ANOVA test at a significance level of P = 0.05. The range in growth values, minimum inhibitory concentration (MIC), i.e., the concentration that inhibits fungal development, and minimum fungicidal concentration (MFC), i.e., the concentration that reduces fungal development, were estimated (NCCLS). The fit of the dose-response curve was evaluated with linear regression analysis (critical value = 0.80). The effective concentration (EC50) that inhibits 50% of the fungal growth was estimated with the linear regression equation. Data analysis was conducted using the PAST version 2.12 software [23].

Results

Evaluation of Antifungal Activity of Origanum onites Essential Oil on Candida albicans Using Micro-Colony Method

In salt-free media, *Origanum onites* essential oil was toxic to *Candida albicans* in a dose-dependent manner (Figure 1). The MIC value was evaluated as 0.3 µl/ml, and the MFC was evaluated as 0.03 µl/ml (Table 1 and Figure 2). The population growth estimations in all concentrations, except for the observation at a concentration of 0.75 µl/ml, showed a log-normal

distribution (Table 1). The growth estimations of three replicates had similar mean values, except for the observation at 0.1 µl/ml (Table 1). Salt stress altered the estimated growth of the population at all testing concentrations (Table 1 and Figure 2). In salt-amended medium (medium b), the population growth estimations at all concentrations showed a log-normal distribution (Table 1). The growth estimations of three replicates in salt-amended medium had different mean values (Table 1). However, based on relative inhibition profiling, salt stress did not alter the MIC value, which remained at 0.3 µl/ml. Based on linear regression validation, the relative inhibition versus concentration values fit the linear regression models for both salt-free medium (R^2 = 0.9175) and medium b (R^2 = 0.8556) (Figure 3). The EC50 values estimated for the salt-free medium, using the regression model equation (y = 371.36x - 20.026), and for medium b (y = 344.33x - 13.82) were 0.188566 µl/ml and 0.185345 µl/ml, respectively (Figure 3).

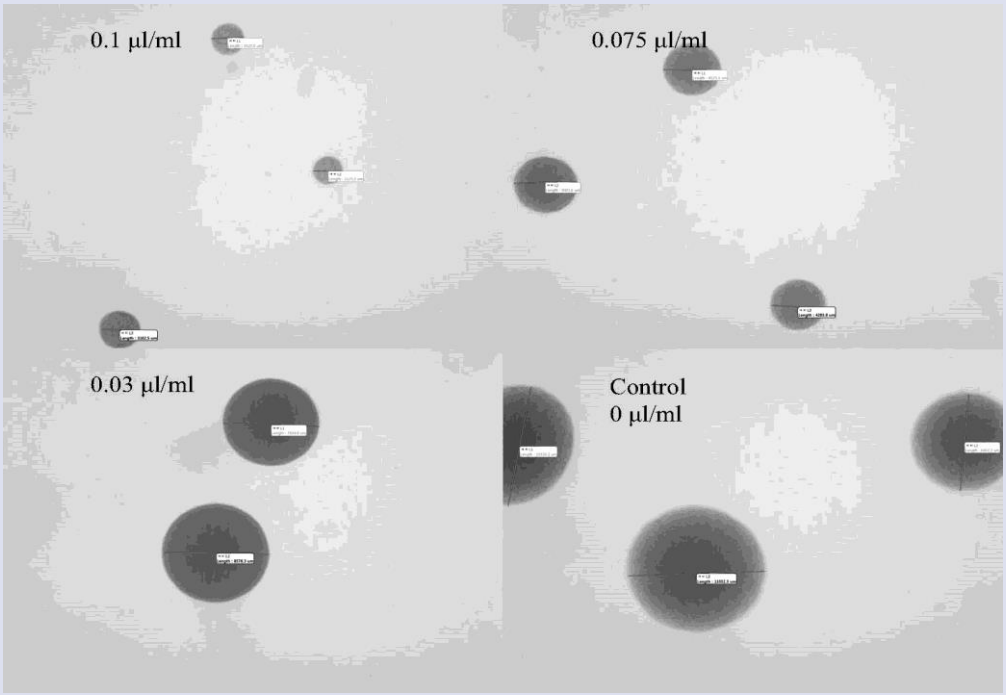

Figure 1. After 12 hours of incubation, in salt-free medium *Origanum onites* essential oil is reducing the diameter of *Candida albicans* micro-colonies in a dose-depended, measured at 40X magnification.

Table 1. Influence of *Origanum onites* essential oil on the fungal development of *Candida albicans* using micro-colony method

Concentration (µl/ml)	Mean [c]	Range [e]	Shapiro-Wilk W [f]	F-value [g]	F-value [h]
0.3	0	0	N/A	N/A	N/A
0.15	2.38776	2.580241- 2.114611	$0.9313_{(n=13)}$	$2.127_{(2,23)}$	$5.255_{(1,23)}$ [a,d]
0.1	2.52171	2.646011-2.427486	$0.9429_{(n=8)}$	$13.69_{(2,23)}$ [a,d]	$72.26_{(1,23)}$ [a,d]
0.075	2.635704	2.711807-2.521792	0.8258 [a] $_{(n=10)}$	$1.786_{(2,31)}$	$11.58_{(1,18)}$ [a,d]
0.03	2.836544	3.031408-2.547159	$0.9287_{(n=8)}$	$0.5238_{(2,27)}$	$15.87_{(1,24)}$ [a,d]
0	2.908963	3.214287-2.672375	$0.9709_{(n=10)}$	$0.2291_{(2,25)}$	$0.05205_{(1,19)}$
0.3[b]	0	0	N/A	N/A	
0.15[b]	2.498476	2.635986-2.371068	$0.9625_{(n=12)}$	$6.689_{(2,24)}$ [a,d]	
0.1[b]	2.267903	2.37328-2.070407	$0.8503_{(n=4)}$	$5.433_{(1,9)}$ [a,d]	
0.075[b]	2.454632	2.559428-2.410777	$0.834_{(n=8)}$	$14.9_{(2,17)}$ [a,d]	
0.03[b]	2.643603	2.899054-2.435526	$0.9399_{(n=11)}$	$6.955_{(2,25)}$ [a,d]	
0[b]	2.871262	3.077622-2.602277	$0.9349_{(n=11)}$	$0.06331_{(2,25)}$	

[a] *p* value less than 0.05 significant difference

[b] Medium b contains 100mM of KCl, NaCl, and $CaCl_2$

[c] Mean of the Log_{10} transformed growth values for the first replicate

[d] F value bigger than F value at the 0.05 significance level of the degrees of the freedom for one-way ANOVA test results

[e] Mean of the Log_{10} transformed growth values for the first replicate

[f] Validation of the log normal distribution of the population with the Shapiro-Wilk W assay for the first

[g] Validation of the similarity of the log_{10} transformed growth values of three replicates using one-way ANOVA test

[h] Validation of the similarity of the log_{10} transformed growth values on salt amended and salt free medium using one-way ANOVA test

N/A; not available

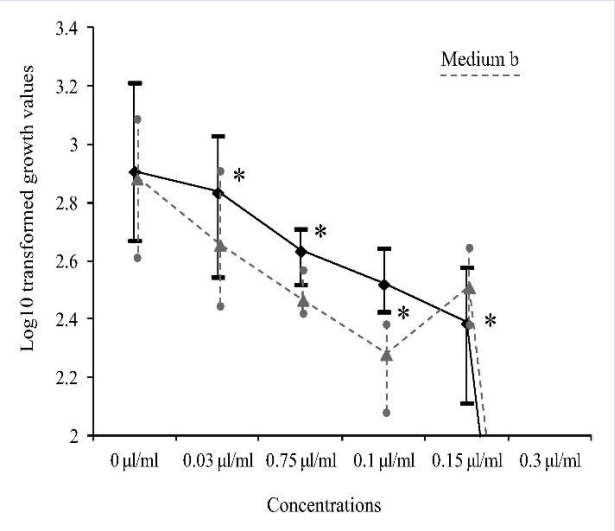

Figure 2. Dose response curve of *Candida albicans* based on the log10 transformed growth values versus the different concentrations of *Origanum onites* essential oil in salt-free (medium a) and salt-amended (medium b) media. * Sign represents the significant differences between two treatments at the corresponding concentration using one-way ANOVA.

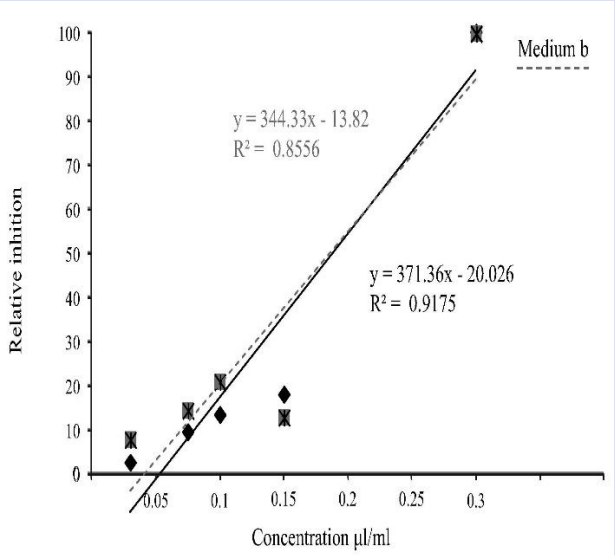

Figure 3. The linear regression models of *Candida albicans* based on the relative inhibition versus the different concentrations of *Origanum onites* essential oil in salt-free (medium a) and salt-amended (medium b) media.

Evaluation of Antifungal Activity of Taraxacum officinale Extract on Candida albicans Using Micro-Colony Method

In salt-free medium, *Taraxacum officinale* methanol extract was not toxic to *Candida albicans* in a dose-dependent manner (Figure 4). The lethal concentration of e *Taraxacum officinale* extract was not within the testing concentration range (Table 2); therefore, the MIC value was not determined. The population growth estimations in all concentrations showed a log-normal distribution (Table 2). The growth estimations of three replicates had different mean values at all concentrations (Table 2).

In salt-amended medium, *Taraxacum officinale* extract showed toxicity at the high concentrations of 3 µg/ml and 1.5 µg/ml (Figures 4, 5, and Table 2). Salt stress altered the estimated growth of the population at these high concentrations. In salt-amended medium, the population growth estimations at all concentrations showed a log-normal distribution (Table 2). The growth estimations of three replicates in salt-amended medium had similar mean values at all concentrations (Table 2). Therefore, salt stress increased the toxicity of *Taraxacum officinale* extract and significantly reduced the variability in the response of the *Candida albicans* subpopulation (Table 2). Based on linear regression validation, in salt-free medium, the relative inhibition versus concentration values did not fit the linear regression model (R^2 = 0.0765) (Figure 6). Conversely, in salt-amended medium, the relative inhibition versus concentration values fit the linear regression model (R^2 = 0.939) (Figure 6). The EC50 value estimated with the regression model equation (y = 3.828x + 1.3298) was 12.71426 µg/ml.

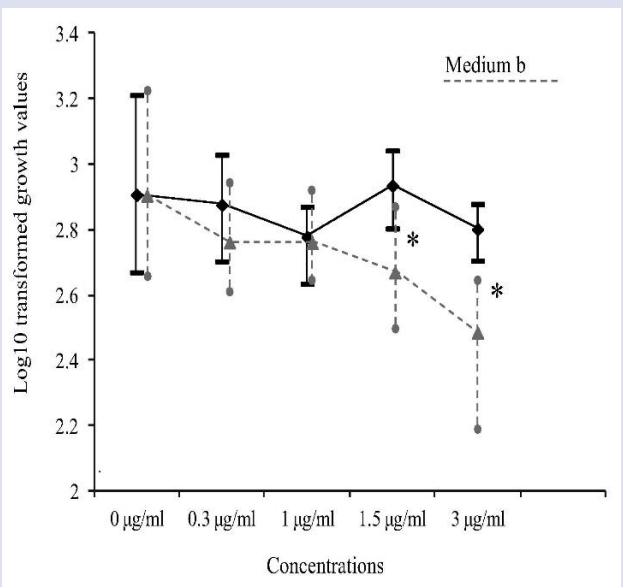

Figure 4. Dose response curve of *Candida albicans* based on the log10 transformed growth values versus the different concentrations of *Taraxacum officinale* extract in salt-free (medium a) and salt-amended (medium b) media. * Sign represents the significant differences between two treatments at the corresponding concentration using one-way ANOVA

Table 2. Influence of *Taraxacum officinale* extract on the fungal development of *Candida albicans* using micro-colony method

Concentration (µg/ml)	Mean [c]	Range [e]	Shapiro-Wilk W [f]	F-value [g]	F-value [h]
3.0	2.803592	2.880814-2.70774	$0.8311_{(n=8)}$	$6.408_{(2,18)}$ [a,d]	$38.5_{(1,16)}$ [a,d]
1.5	2.936823	3.044344-2.806248	$0.969_{(n=8)}$	$3.78_{(2,23)}$ [a,d]	$67.71_{(1,17)}$ [a,d]
1.0	2.779769	2.872215-2.636789	$0.904_{(n=11)}$	$3.492_{(2,25)}$ [a,d]	$3,661_{(1,17)}$
0.3	2.877884	3.031408-2.705864	$0.9241_{(n=7)}$	$5.77_{(2,19)}$ [a,d]	$0.007084_{(1,19)}$
0	2.908963	3.214287-2.672375	$0.9709_{(n=10)}$	$0.2291_{(2,25)}$	$0.05205_{(1,19)}$
3.0[b]	2.489879	2.631139-2.200577	$0.8856_{(n=10)}$	$0.003273_{(2,19)}$	
1.5[b]	2.674407	2.854367-2.511883	$0.9519_{(n=10)}$	$2.635_{(2,20)}$	
1.0[b]	2.764484	2.908485-2.658202	$0.966_{(n=7)}$	$2.784_{(2,18)}$	
0.3[b]	2.76606	2.930898-2.620656	$0.9431_{(n=12)}$	$0.3313_{(2,25)}$	
0[b]	2.871262	3.077622-2.602277	$0.9349_{(n=11)}$	$0.06331_{(2,25)}$	

[a] *p* value less than 0.05 significant difference

[b] Medium b contains 100mM of KCl, NaCl, and $CaCl_2$

[c] Mean of the Log_{10} transformed growth values for the first replicate

[d] F value bigger than F value at the 0.05 significance level of the degrees of the freedom for one-way ANOVA test results

[e] Mean of the Log_{10} transformed growth values for the first replicate

[f] Validation of the log normal distribution of the population with the Shapiro-Wilk W assay for the first

[g] Validation of the similarity of the log_{10} transformed growth values of three replicates using one-way ANOVA test

[h] Validation of the similarity of the log_{10} transformed growth values on salt amended and salt free medium using one-way ANOVA test

N/A; not available

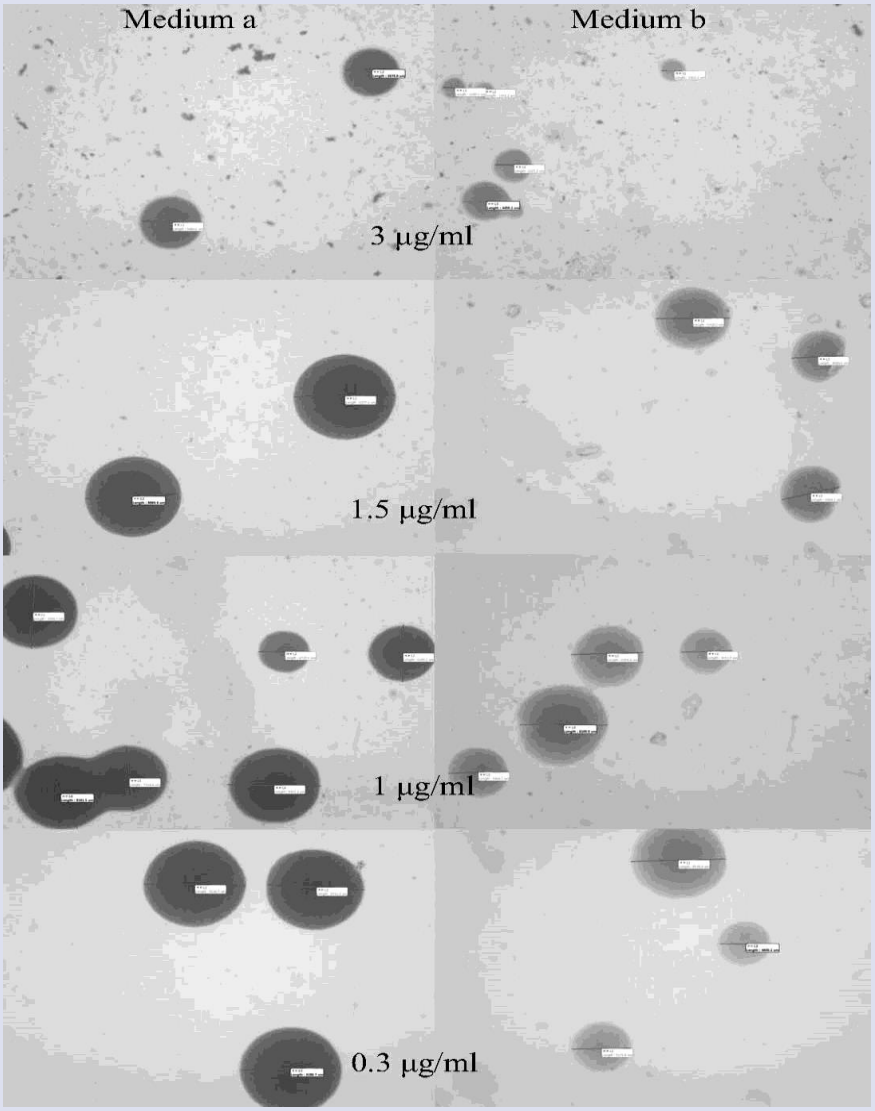

Figure 5. After 12 hours of incubation, in salt-amended medium high concentrations (3 µg/ml and 1.5 µg/ml) *Taraxacum officinale* extract is reducing the diameter and the pigmentation of *Candida albicans* micro-colonies measured at 40X magnification

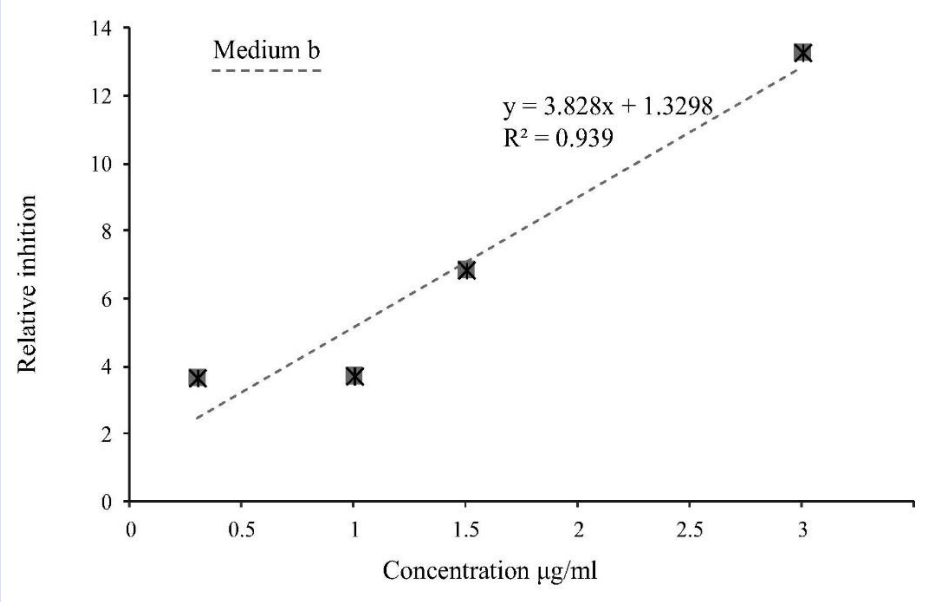

Figure 6. The linear regression model of *Candida albicans* based on the relative inhibition of the fungal growth versus the different concentrations on *Origanum onites* essential oil on salt-amended medium (medium b)

Evaluation of Pigmentation in Candida albicans in Response to Origanum onites Essential Oil

In salt-free medium, *Origanum onites* essential oil reduced the pigmentation of *Candida albicans* cells (Figure 7). The estimated pigmentation values at all testing concentrations showed a log-normal distribution (Table 3). The pigmentation of three replicates had similar mean values except at concentrations of 0.1 µl/ml and 0.075 µl/ml (Table 3). Salt stress altered the estimated pigmentation of the population at all testing concentrations except 0.075 µl/ml (Figure 7 and Table 3). In salt-amended medium, the pigmentation of three replicates had similar mean values except at 0.15 µl/ml (Table 3). The relative inhibition in pigmentation versus concentration values fit the linear regression models in both salt-free (R^2 = 0.8616) and salt-amended (R^2 = 0.8274) media (Figure 8). The EC50 values estimated with the regression model equation for the salt-free (y = 393.05x - 30.121) and salt-amended (y = 357.95x - 20.047) media were 0.203844295 µl/ml and 0.195689342 µl/ml, respectively (Figure 8).

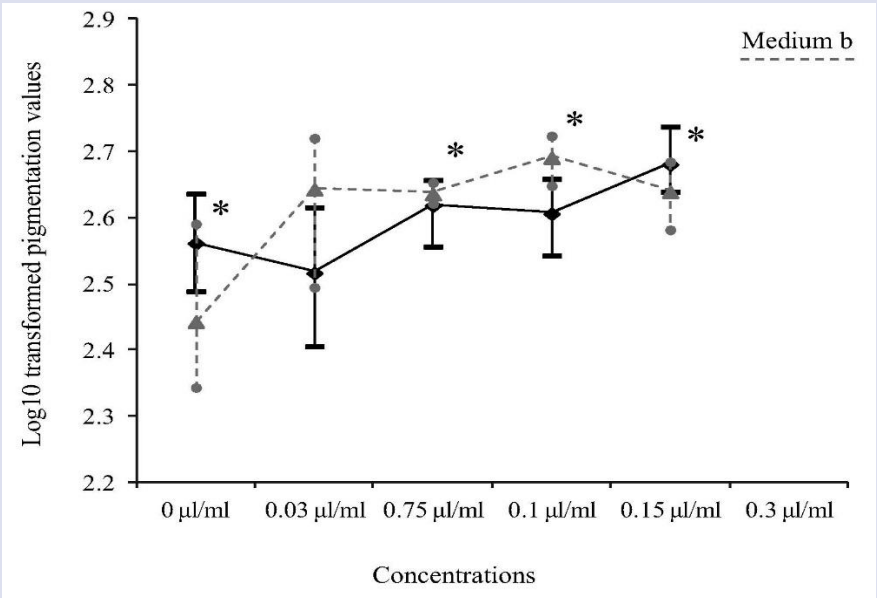

Figure 7. Dose response curve of *Candida albicans* based on the log10 transformed pigmentation values versus the different concentrations of *Origanum onites* essential oil in salt-free (medium a) and salt-amended (medium b) media. * Sign represents the significant differences between two treatments at the corresponding concentration using one-way ANOVA

Table 3. Influence of *Origanum onites* essential oil on the pigmentation of *Candida albicans* using image processing

Concentration (µl/ml)	Mean [c]	Range [e]	Shapiro-Wilk W [f]	F-value [g]	F-value [h]
0.3	0	0	N/A	N/A	N/A
0.15	2.681604	2.738781-2.640481	$0.9234_{(n=13)}$	$0.6505_{(2,24)}$	$7.828_{(1,23)}$ [a,d]
0.1	2.60703	2.659916-2.544068	$0.9824_{(n=8)}$	$6.535_{(2,23)}$ [a,d]	$29.51_{(1,13)}$ [a,d]
0.075	2.619661	2.658011-2.557507	$0.9145_{(n=10)}$	$7.822_{(2,30)}$ [a,d]	2.791
0.03	2.517378	2.617000-2.40654	$0.9589_{(n=8)}$	$0.07002_{(1,13)}$	$16,65_{(1,17)}$ [a,d]
0	2.562559	2.63749-2.489958	$0.9638_{(n=11)}$	$57.59_{(2,25)}$ [a,d]	$16.43_{(1,19)}$ [a,d]
0.3[b]	0	0	N/A	N/A	
0.15[b]	2.64394	2.689309-2.586587	$0.9486_{(n=12)}$	$4.801_{(2,24)}$ [a,d]	
0.1[b]	2.695653	2.728354-2.653213	$0.9551_{(n=7)}$	$0.4435_{(1,9)}$	
0.075[b]	2.64086	2.658011-2.62634	$0.9534_{(n=6)}$	$2.208_{(1,17)}$	
0.03[b]	2.64771	2.725095-2.499687	$0.8801_{(n=11)}$	$2.816_{(2,25)}$	
0[b]	2.447628	2.595496-2.348305	$0.9557_{(n=11)}$	$8.069_{(2,25)}$ [a,d]	

[a] *p* value less than 0.05 significant difference
[b] Medium b contains 100mM of KCl, NaCl, and $CaCl_2$
[c] Mean of the Log_{10} transformed pigmentation values for the first replicate
[d] F value bigger than F value at the 0.05 significance level of the degrees of the freedom for one-way ANOVA test results
[e] Mean of the Log_{10} transformed pigmentation values for the first replicate
[f] Validation of the log normal distribution of the population with the Shapiro-Wilk W assay for the first
[g] Validation of the similarity of the log_{10} transformed pigmentation values of three replicates using one-way ANOVA test
[h] Validation of the similarity of the log_{10} transformed pigmentation values on salt amended and salt free medium using one-way ANOVA test
N/A; not available

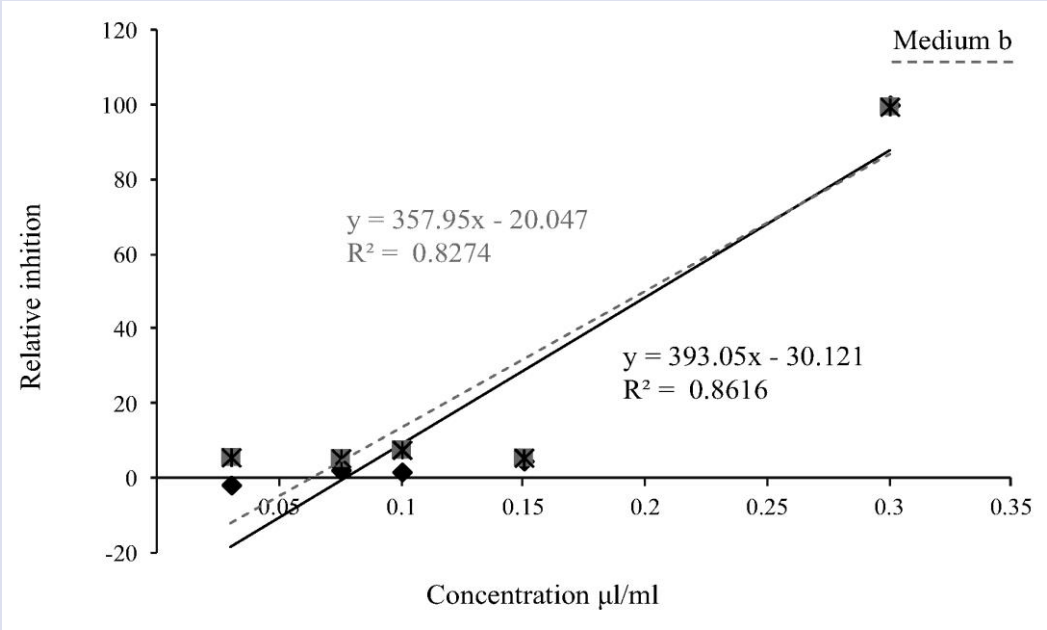

Figure 8. The linear regression model of *Candida albicans* based on the relative inhibition of the fungal growth versus the different concentrations on *Origanum onites* essential oil on salt-free (medium a) and salt-amended (medium b) media

Evaluation of Pigmentation in *Candida albicans* in Response to *Taraxacum officinale* Extract

In salt-free medium, only the highest tested concentration of *Taraxacum officinale* extract, 3.0 µg/ml, reduced the relative pigmentation of *Candida albicans* cells (Figure 5). The estimated pigmentation values at all testing concentrations showed a log-normal distribution (Table 4). The pigmentation of three replicates had similar mean values except at concentrations of 1.5 µg/ml and 1.0 µg/ml (Table 4). Salt stress altered the estimated pigmentation of the population at all testing concentrations (Table 4). In salt-amended medium, the pigmentation of three replicates had similar mean values (Table 4). However, the relative inhibition in pigmentation versus concentration values did not fit linear regression models in salt-free (R^2 = 0.1597) and salt-amended (R^2 = 0.0782) media.

Table 4. Influence of *Taraxacum officinale* extract on the pigmentation of *Candida albicans* using image processing

Concentration (µg/ml)	Mean [c]	Range [e]	Shapiro-Wilk W [f]	F-value [g]	F-value [h]
3.0	2.595216	2.641474-2.563481	$0.9169_{(n=8)}$	$1.515_{(2,18)}$	$19.4_{(1,16)}$ [a,d]
1.5	2.474952	2.545307-2.428135	$0.9421_{(n=9)}$	$7.466_{(2,23)}$ [a,d]	$84.54_{(1,17)}$ [a,d]
1.0	2.560618	2.627366-2.465383	$0.9488_{(n=11)}$	$5.785_{(2,25)}$ [a,d]	$16.64_{(1,17)}$ [a,d]
0.3	2.543203	2.607455-2.481443	$0.9948_{(n=9)}$	$2.937_{(2,20)}$	$81.92_{(1,19)}$ [a,d]
0	2.562559	2.63749-2.489958	$0.9638_{(n=11)}$	$57.59_{(2,25)}$ [a,d]	$16.43_{(1,19)}$ [a,d]
3.0[b]	2.658418	2.70757-2.60206	$0.9612_{(n=10)}$	$0.1574_{(1,17)}$	
1.5[b]	2.641549	2.710117-2.596597	$0.8936_{(n=10)}$	$3.262_{(2,20)}$	
1.0[b]	2.64116	2.700704-2.604226	$0.9248_{(n=8)}$	$2.683_{(2,18)}$	
0.3[b]	2.679605	2.720159-2.628389	$0.9488_{(n=12)}$	$13.18_{(2,25)}$	
0[b]	2.447628	2.595496-2.348305	$0.9557_{(n=11)}$	$8.069_{(2,25)}$ [a,d]	

[a] *p* value less than 0.05 significant difference
[b] Medium b contains 100mM of KCl, NaCl, and $CaCl_2$
[c] Mean of the Log_{10} transformed pigmentation values for the first replicate
[d] F value bigger than F value at the 0.05 significance level of the degrees of the freedom for one-way ANOVA test results
[e] Mean of the Log_{10} transformed pigmentation values for the first replicate
[f] Validation of the log normal distribution of the population with the Shapiro-Wilk W assay for the first
[g] Validation of the similarity of the log_{10} transformed pigmentation values of three replicates using one-way ANOVA test
[h] Validation of the similarity of the log_{10} transformed pigmentation values on salt amended and salt free medium using one-way ANOVA test
N/A; not available

Evaluation of Antifungal Activity of Origanum onites Essential Oil on Aspergillus fumigatus Using Micro-Colony Method

In salt-free media, *Origanum onites* essential oil was toxic to *Aspergillus fumigatus* in a dose-dependent manner (Figures 10 and 11). The MIC value was evaluated as 0.15 µl/ml, and the MFC was evaluated as 0.03 µl/ml. The population growth estimations in all testing concentrations showed a log-normal distribution (Table 5). The growth estimations of three replicates had different mean values except for the observation at 0.1 µl/ml (Table 5). Salt stress drastically altered the toxicity of *Origanum onites*, with the MIC value re-evaluated as 0.075 µl/ml (Table 5). Salt stress affected the estimated growth of the population in all testing concentrations. The MFC concentration remained 0.03 µl/ml, and based on a one-way ANOVA test, salt stress did not alter fungal growth at this concentration. Based on linear regression validation, in salt-free medium, the relative inhibition versus concentration values fit the linear regression model (R^2 = 0.9223) (Figure 12). The EC50 values estimated with the regression model equation (y = 781.24x - 26.688) in salt-free medium was 0.098161896 µl/ml (Figure 12).

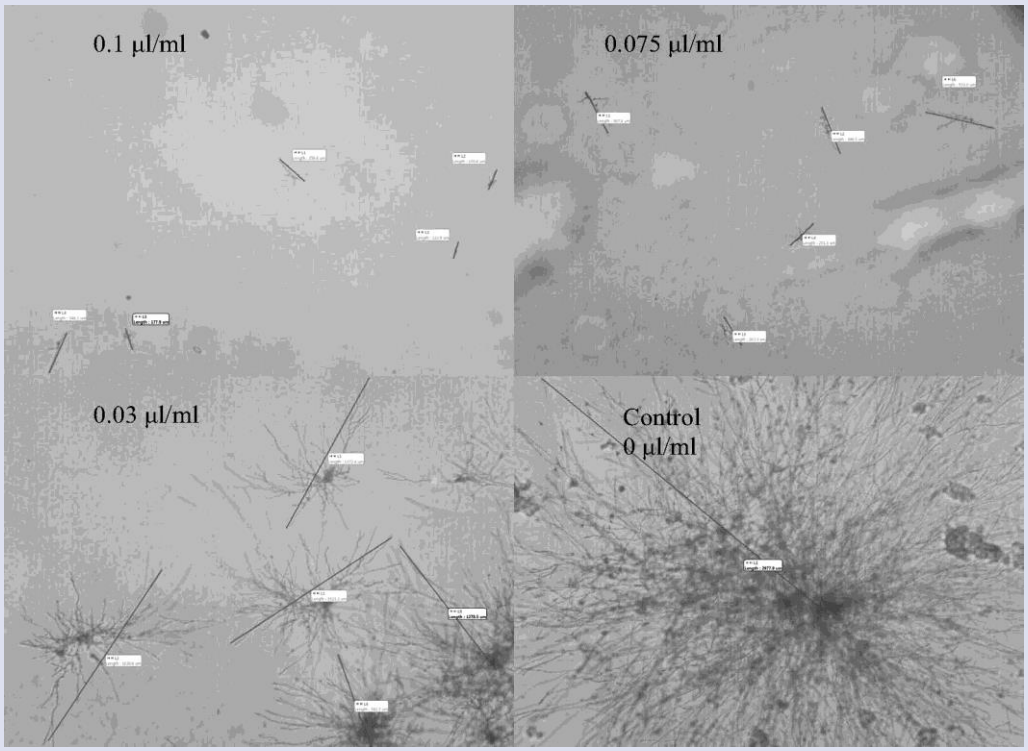

Figure 10. After 12 hours of incubation, in salt-free medium *Origanum onites* essential oil is reducing the diameter of *Aspergillus fumigatus* micro-colonies in a dose-depended, measured at 40X magnification

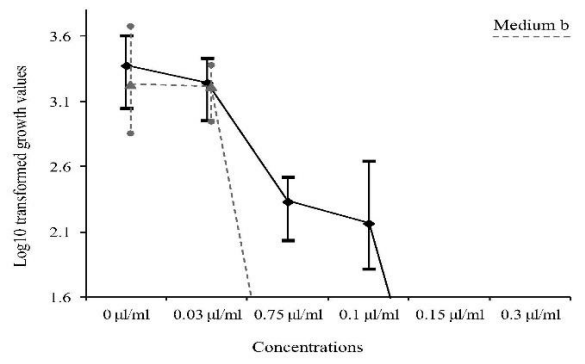

Figure 11. Dose response curve of *Aspergillus fumigatus* based on the log10 transformed growth values versus the different concentrations of *Origanum onites* essential oil in salt-free (medium a) and salt-amended (medium b) media. * Sign represents the significant differences between two treatments at the corresponding concentration using one-way ANOVA

Table 5. Influence of *Origanum onites* essential oil on the fungal development of *Aspergillus fumigatus* using micro-colony method

Concentration (µl/ml)	Mean [c]	Range [e]	Shapiro-Wilk W [f]	F-value [g]	F-value [h]
0.3	0	0	N/A	N/A	N/A
0.15	0	0	N/A	N/A	N/A
0.1	2.169116	2.648653-1.822168	$0.9397_{(n=19)}$	$2.414_{(1,27)}$	N/A
0.075	2.335844	2.525304-2.040602	$0.9118_{(n=16)}$	$29.63_{(2,31)}$ [a,d]	N/A
0.03	3.247178	3.437941-2.959995	$0.9586_{(n=16)}$	$5.502_{(2,39)}$ [a,d]	$0.02817_{(1,23)}$
0	3.379108	3.610788-3.053232	$0.8893_{(n=14)}$	$5.872_{(2,29)}$ [a,d]	$2.704_{(1,24)}$
0.3[b]	0	0	N/A	N/A	
0.15[b]	0	0	N/A	N/A	
0.1[b]	0	0	N/A	N/A	
0.075[b]	0	0	N/A	N/A	
0.03[b]	3.21874	3.386374-2.953711	$0.9362_{(n=9)}$	$1.148_{(2,\ 20)}$	
0[b]	3.234383	3.684432-2.863025	$0.9644_{(n=12)}$	$3.831_{(2,\ 25)}$ [a,d]	

[a] *p* value less than 0.05 significant difference
[b] Medium b contains 100mM of KCl, NaCl, and $CaCl_2$
[c] Mean of the Log_{10} transformed growth values for the first replicate
[d] F value bigger than F value at the 0.05 significance level of the degrees of the freedom for one-way ANOVA test results
[e] Mean of the Log_{10} transformed growth values for the first replicate
[f] Validation of the log normal distribution of the population with the Shapiro-Wilk W assay for the first
[g] Validation of the similarity of the log_{10} transformed growth values of three replicates using one-way ANOVA test
[h] Validation of the similarity of the log_{10} transformed growth values on salt amended and salt free medium using one-way ANOVA test
N/A; not available

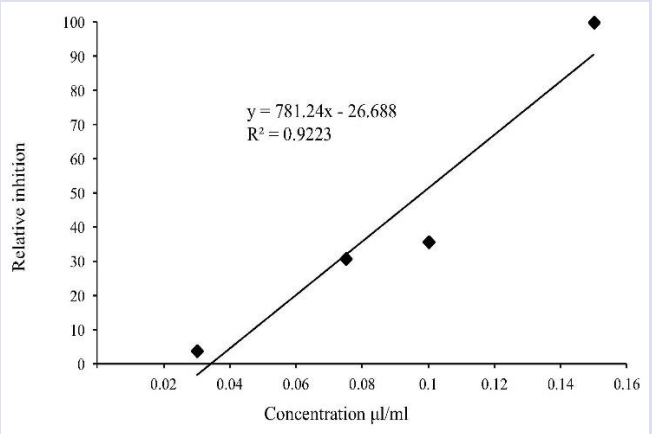

Figure 12. The linear regression model of *Aspergillus fumigatus* based on the relative inhibition of the fungal growth versus the different concentrations of *Origanum onites* essential oil on salt-free medium

Evaluation of Antifungal Activity of Taraxacum officinale Extract on Aspergillus fumigatus Using Micro-Colony Method

In salt-free media, *Taraxacum officinale* extract was toxic to *Aspergillus fumigatus* in a dose-dependent manner (Figure 13). The population growth estimations in all concentrations showed a log-normal distribution (Table 6). The growth estimations of three replicates had different mean values except for the observation at 0.3 µg/ml (non-toxic concentration) (Table 6). Salt stress did not alter the estimated growth of the population in all testing concentrations except 0.3 µg/ml (non-toxic concentration). In salt-amended medium, the population growth estimations in all concentrations showed a log-normal distribution except at 0.1 µg/ml (non-toxic concentration) (Table 6). The growth estimations of three replicates had different mean values except for the observation at 1.5 µg/ml (non-toxic concentration) (Table 6). Unlike *Candida albicans*, salt addition did not increase the toxicity of *Taraxacum officinale* extract. The lethal concentration of *Taraxacum officinale* extract was not within the testing concentration range; therefore, the MIC value was not determined. The MFC was estimated as 3.0 µg/ml in both salt-amended and salt-free media. Based on linear regression validation, the relative inhibition versus concentration values fit the linear regression models in both salt-free (R^2 = 0.9324) and salt-amended media (R^2 = 0.938) (Figure 14). The EC50 values estimated with the regression model equation for salt-free (y = 5.4926x - 6.5695) and salt-amended (y = 4.676x - 9.5868) media were 10.29922 µg/ml and 12.74311 µg/ml, respectively (Figure 14).

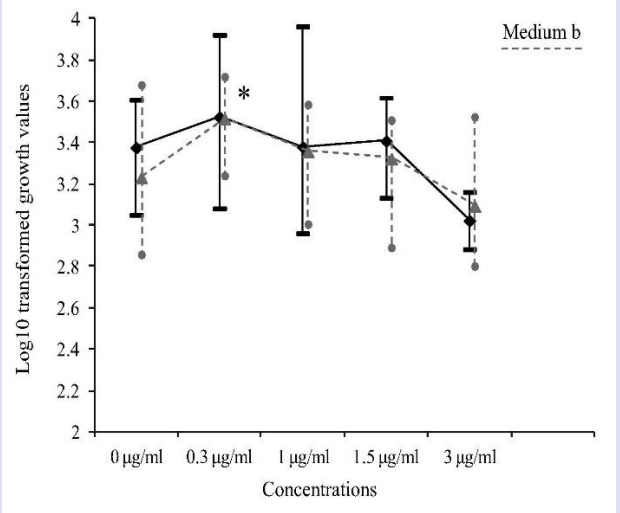

Figure 13. Dose response curve of *Aspergillus fumigatus* based on the log10 transformed growth values versus the different concentrations of *Taraxacum officinale* extract in salt-free (medium a) and salt-amended (medium b) media. * Sign represents the significant differences between two treatments at the corresponding concentration using one-way ANOVA

Table 6. Influence of *Taraxacum officinale* extract on the fungal development of *Aspergillus fumigatus* using micro-colony method

Concentration (µg/ml)	Mean [c]	Range [e]	Shapiro-Wilk W [f]	F-value [g]	F-value [h]
3.0	3.025688	3.164353-2.88773	$0.9772_{(n=11)}$	$9.53_{(2,28)}$ [a,d]	$0.7891_{(1,21)}$
1.5	3.410988	3.620864-3.135673	$0.9626_{(n=15)}$	$4.911_{(2,28)}$ [a,d]	$2.352_{(1,24)}$
1.0	3.381992	3.966264-2.96459	$0.9429_{(n=11)}$	$6.386_{(2,18)}$ [a,d]	$0.05602_{(1,22)}$
0.3	3.526891	3.925291-3.085219	$0.9774_{(n=10)}$	$2.887_{(2,24)}$	$34.29_{(1,18)}$ [a,d]
0	3.379108	3.610788-3.053232	$0.8893_{(n=14)}$	$5.872_{(2,29)}$ [a,d]	$2.704_{(1,24)}$
3.0[b]	3.095654	3.529456-2.807332	$0.8897_{(n=12)}$	$5.311_{(2,28)}$ [a,d]	
1.5[b]	3.326025	3.514056-2.897022	$0.8159_{(n=11)}$ [a]	$2.333_{(2,24)}$	
1.0[b]	3.359797	3.589279-3.01013	$0.8971_{(n=13)}$	$12.76_{(2,28)}$ [a,d]	
0.3[b]	3.519158	3.723685-3.245192	$0.9745_{(n=6)}$	$21.93_{(2,22)}$ [a,d]	
0[b]	3.234383	3.684432-2.863025	$0.9644_{(n=12)}$	$3.831_{(2,25)}$ [a,d]	

[a] *p* value less than 0.05 significant difference
[b] Medium b contains 100mM of KCl, NaCl, and $CaCl_2$
[c] Mean of the Log_{10} transformed growth values for the first replicate
[d] F value bigger than F value at the 0.05 significance level of the degrees of the freedom for one-way ANOVA test results
[e] Mean of the Log_{10} transformed growth values for the first replicate
[f] Validation of the log normal distribution of the population with the Shapiro-Wilk W assay for the first
[g] Validation of the similarity of the log_{10} transformed growth values of three replicates using one-way ANOVA test
[h] Validation of the similarity of the log_{10} transformed growth values on salt amended and salt free medium using one-way ANOVA test
N/A; not available

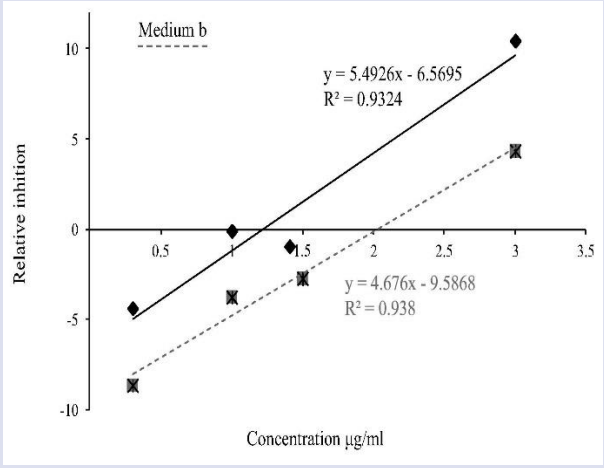

Figure 14. The linear regression models of *Aspergillus fumigatus* based on the relative inhibition versus the different concentrations of *Origanum onites* essential oil in salt-free (medium a) and salt-amended (medium b) media

Discussion

Based on our *in vitro* assays with *Candida albicans* and *Aspergillus fumigatus* at different concentrations of *Origanum onites* essential oil and *Taraxacum officinale* extract in salt-free (medium A) and salt-amended (medium B) treatments, the micro-colony method provides data on relative inhibition, log-normal distribution of growth values, significant similarities in growth values, linear regression models, EC50 values, and differences using mathematical and statistical models. Thus, the micro-colony assay meets the requirements (validity of mathematical model and verification of the dose response with a linear model) set for a microbiological assay by the European Pharmacopeia [6].

The main advantage of the micro-colony method is its unbiased and detailed perception of fungal development at the population level at each concentration using definitive data analysis methods. The micro-colony method has similarities with diffusion methods (e.g., disc diffusion halo assay, disc diffusion method, radial growth) which use a graduated ruler for measurement [6]. However, the micro-colony method is less time-consuming in preparation and requires a significantly shorter incubation period to obtain growth values, which is important, especially for mold fungi that must be incubated for 3-7 days [8]. The MIC assay in liquid medium is a reference and popular method which could be conducted without spectrometric instruments, and many scholars utilize visual observations and state as “sharp or obvious” decrease based on the decision of the observer [8,9,21]. Thus, in the MIC method, growth could be evaluated solely based on a two-level scheme, i.e., growth or inhibition at each concentration. Therefore, NCCLS has efforts to define “more reproducible reference testing methods” [21]. Conversely, the micro-colony method is more compatible with the fundamental concepts of instrumental analysis due to the measurement of growth using microscopic instruments.

The micro-colony method can demonstrate the impact of medium salinity causing slight alterations in the dose response. Additionally, the images of the colony growth could be stored for future experiments and shared with other research groups. However, the drawbacks of the method are the laborious and time-consuming process of micro-colony measurements and data analysis.

Our preliminary assays suggest that the yeast cell images captured for the micro-colony assays could be used to screen pigmentation in yeast cells. Pigmentation was significantly higher in the control of the salt-amended medium compared to the salt-free control. *Origanum onites* essential oil inhibited the pigmentation of the *Candida albicans* cells. Both *Origanum onites* essential oil and *Taraxacum officinale* extract, in combination with salt, reduced the relative pigmentation significantly in all testing concentrations. Melanin is the major pigment in fungi, which plays roles in pathogenicity and antioxidant activity [24]. Additionally, pigments incorporate with the fungal cell wall and hamper the penetration of toxins into the fungal cells. Therefore, compounds capable of inhibiting pigmentation are prospective reagents in antifungal drug co-formulations.

The toxicity of *Origanum onites* essential oil was proven with the micro-colony method on *Candida albicans* and *Aspergillus fumigatus*. The essential oil was more toxic to the filamentous fungus *Aspergillus fumigatus*. In several studies, an isopropyl cresol terpenoid molecule, carvacrol, was found to be the major component of *Origanum onites* essential oil, which could reach up to 86.9% of its overall content. The multi-site antifungal activity of the carvacrol molecule derives from its hydroxyl moiety and hydrophobicity [7,25]. In fungi, carvacrol is capable of inhibiting ergosterol biosynthesis, disrupting the cell membrane, altering its fatty acid composition, inducing Ca^{2+} burst, and triggering specific cellular events similar to rapamycin [7,25,26]. Thus, carvacrol and *Origanum onites* essential oil demonstrate broad-spectrum antimicrobial and toxic activity, capable of inhibiting mold fungi (*Penicillium roqueforti* Thom.), yeast fungi (*S. cerevisiae*), bacteria (*E. coli* and *Pseudomonas aeruginosa* (Schröter) Migula), and insect larvae (*Ochlerotatus caspius* Pallas) [26,27]. Based on the micro-colony assay, *Taraxacum officinale* extract did not exert strong antifungal activity. In our experiments, salt stress altered the response of *Candida albicans* to *Origanum onites* essential oil; however, the estimated relative inhibition was unchanged.

Conflicts of interest

There are no conflicts of interest in this work.

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