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Original Article

Efficacy of *Pluchea dioscoridis* leaf extract against pathogenic *Candida albicans*

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Abstract

Introduction: This study aimed to evaluate the efficacy of *Pluchea dioscoridis* leaf extract on growth, survival, morphogenesis, and virulence gene expression of *Candida albicans.*

Methodology: Anticandidal activity was studied using the hole-plate method against four pathogenic *C. albicans* strains from clinical isolates. The effect of the extract on the growth profile of the yeast was also examined via a time-kill assay. Microscopic observations using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were made to determine the major alterations in the microstructure of *C. albicans*. Quantitative changes in phospholipase, hemolysin, and secreted aspartyl proteinase (SAP1 and SAP10) genes expression as virulence factors were analyzed using real-time polymerase chain reaction (RT-PCR).

Results: The extract exhibited high anticandidal activity, with the zones of inhibition between 0.5 and 6 cm, and recorded a minimum inhibitory concentration (MIC) value of 30 mg/mL. The time-kill study suggested that the extract possessed yeasticidal properties at higher concentrations and eradicated the growth of yeast cells. The SEM and TEM micrographs exhibited major abnormalities that occurred on the yeast cells after being exposed to the extract, resulting in complete alterations in their morphology and collapse of the cells beyond repair. At MIC concentration, phospholipase, proteinase, and hemolysin gene expression was reduced to 90%, 70%, 90% for SAP1, and 40% for SAP10,respectively, compared to that obtained from untreated *C. albicans*, as demonstrated by quantitative reverse transcription (RT)-PCR analysis.

Conclusions: The *P. dioscoridis* leaf extract may be an effective anticandidal agent to treat pathogenic yeast infections.

Key words: *C. albicans*; *P. dioscoridis*; Virulence factors; Realtime PCR, Biocontrol.

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Introduction

Candidal infection represents one of the most rapidly increasing healthcare infections, resulting in a significant mortality rate in hospitalized patients [1]. *Candida* spp. are now recognized as major agents of hospital-acquired infections [2]. These species are the most common fungal pathogens of humans, and they are causative agents of skin, oral, and vaginal candidiasis, giving rise to severe morbidity in millions of individuals worldwide [3,4]. *Candida* is a common commensal of the gastrointestinal and urogenital tracts of humans [5]. In fact, it is one of the leading causes of opportunistic fungal infections in immune-compromised individuals, including AIDS patients, transplant recipients, and cancer patients [6,7]. The increasing resistance of *C. albicans* to antifungal compounds and the reduced number of available drugs have led to the search for new therapeutic alternatives from plants. In addition, the remedial uses of commercially available antifungal drugs have induced varieties of toxic side effects [8,9].

The exploration of new and effective natural products showing antifungal activity against *C. albicans* is likely to play a significant role in overcoming drug resistance. Medicinal plants represent an interesting source of novel antimicrobial compounds. They are used medicinally in different countries and are a source of many potent and powerful drugs [10]. Raw drugs are extracted from a wide range of medicinal plant parts, and plants contain other various drugs that have not yet been discovered [11]. Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, phenolics, and flavonoids, which have been found *in vitro* to have antimicrobial properties [12].

Extracellular hydrolytic enzymes seem to play an important role in candidal overgrowth [13], as these enzymes facilitate adherence and tissue penetration, and hence the invasion of the host. Among the most

important hydrolytic enzymes produced by *C. albicans* are phospholipases and secreted aspartyl proteinases (SAPs), which are encoded by 10 SAP genes that seem to play different roles in *C. albicans* infection. Furthermore, the ability of *C. albicans* to acquire elemental iron through hemolysin production is pivotal in its survival and ability to establish infections within humans [14].

The application of genome-wide expression profiling to determine how drugs achieve their therapeutic effect presents the pharmaceutical industry with an exciting new tool for drug mode of action studies. The use of polymerase chain reaction (PCR) for the amplification of specific mRNA after reverse transcription enables the study of gene expression [15,16]. It is a sensitive method for the detection of mRNA expression levels, as mRNA tells us about the response of microorganisms to the surrounding environment. Backer *et al*. [17] used mRNA and reverse transcription (RT)-PCR to study the effect of itraconazole on *C. albicans* by analyzing the level of gene expression in several genes from the *C. albicans* genome before and after treatment of *C. albicans* with this compound. The present study attempted to study the potential anticandidal activity of *P. dioscoridis* and its mode of action on *Candida* virulence factors using specific RT-PCR.

Methodology

Microorganisms and cultural maintenance

Three pathogenic *C. albicans* strains obtained from El Borg Research Laboratory, Tanta, Egypt, were used throughout the study. The *C. albicans* strains were designated as 1, 2, 3, and were isolated from patients with oral candidiasis, vaginitis, and yeast onychomycosis, respectively.

All strains were inoculated onto fresh Sabouraud dextrose agar (SDA) (Pharmacopoeia, New York, USA) plates and incubated at 37°C for 48 hours. Next, isolates were re-identified by the production of chlamydoconidia on cornmeal agar (Sigma, St. Louis, USA) and green color colonies on CHROMagar *Candida* (CHROMagar *Candida*, Oxoid, Hampshire, UK). From this selective and differential medium, the isolates were identified by classical methods [18] and compared to *C. albicans* standard strain (ATCC66027).

Plant material and plant identification

Fresh leaves of *P. dioscoridis* (*Conyza*) were collected from different habitats identified in the Nile Delta based on a work by Ahmed [19]. The plant identification was carried out in Tanta Herbarium, Tanta University (TANE), based on authentic materials. The leaves were washed thoroughly two to three times with running water and once with sterile distilled water, and then the leaves were air-dried under shade [20].

Preparation of crude extract and its antifungal activity

The extract was prepared in ethanol at room temperature by a simple extraction method described by Deshpande *et al*. [21]. The antifungal activities of ethanol extract of *P. dioscoridis*, with ketoconazole as a positive control and ethanol as a negative control, were assayed by the hole-plate method [22]. Freshly prepared suspension of each *C. albicans* (0.5 mL of 1×10^4 cells/mL) was mixed with 9.5 mL of sterile SDA (Pharmacopoeia, New York, USA) at 45°C, poured onto sterile Perti dishes, and left to solidify at room temperature. Regular wells were made in the inoculated agar plates by a sterile corn borer with 0.8 cm diameter. Each well was aseptically filled up with 0.2 mL from *P. dioscoridis* leaf extract and ketoconazole (30 mg/mL) separately. After incubation, antifungal activities were assayed by measuring the inhibition zone diameter. Three replicas were made for each tested suspension and all plates were incubated at 37°C for 24 hours.

Minimum inhibitory concentration

Different concentrations (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 mg/mL) were separately prepared from *P. dioscoridis* leaf extract and ketoconazole in 70% ethanol. Suspension of *C. albicans* (0.5 mL of 1×10^4 cells/mL) was mixed with 9.5 mL of each concentration in a sterile test tube and incubated for 24 hours at 25°C. Then, 0.2 mL of each mixture was spread on the surface of previously sterile SDA plates for two days at 37°C. Colony-forming units were counted, represented graphically, and MIC was recorded for both antifungal materials [23]. Three replicas were made for each tested suspension and compared to all plates.

Time-kill study of C. albicans in the presence of ethanolic leaf extract of P. dioscoridis

The extract was added to 25 mL of yeast extract-peptone-dextrose broth in a 50 mL Erlenmeyer flask to achieve concentrations of 0 (control), 15 (0.5 MIC), 30 (MIC) and 60 (2 MIC) mg/mL after addition of the inoculum (0.5 mL of 1×10^4 cells/mL). The experiments were conducted in triplicate, and all the flasks were incubated in a shaking incubator at 37°C with agitation at 100 rpm. One milliliter of the mixture within each flask was withdrawn at every 4 hour intervals starting from 0 until 48 hrs of cultivation, and

the cell growth was monitored by measuring optical density at 540 nm [24].

Determination of phospholipase activity

All *C. albicans* isolates were screened for extracellular phospholipase activity by measuring the size of the zone of precipitation after growth on egg yolk agar (Sigma, St. Louis, USA) [25]. The egg yolk medium consisted of 13.0 g Sabouraud glucose agar (SGA), 11.7 g NaCl, 0.11 g $CaCl_2$, and 10% sterile egg yolk (all in 184 mL distilled water). First, the components without the egg yolk were mixed and sterilized, then the egg yolk was centrifuged at 500 g for 10 minutes at room temperature, and 20 mL of the supernatant was added to the sterilized medium. Standard inoculum of *Candida* isolates (0.5 mL of 1×10^4 cells/mL) were deposited onto the egg yolk agar medium and left to dry at room temperature. A further 0.5 mL of saline, but without yeast cells, was overlaid with the plate and left to dry at room temperature. Each culture was then incubated at 37°C for 48 hours, after which the diameter of the precipitation zone around the colony (an indicator of phospholipase activity) was determined. Phospholipase activity (P_z value) was expressed as the ratio of the diameter of the colony to the diameter of the colony plus the precipitation zone (in mm) [26]. The assay was performed in duplicate.

Determination of hemolysin activity

Hemolysin activity was evaluated with a blood plate assay [27]. Media were prepared by adding 7 mL of fresh sheep's blood to 100 mL SGA supplemented with glucose at a final concentration of 3% (w/v). The final pH of the medium was 5.6. A standard inoculum of *Candida* isolates (0.5 mL of 1×10^4 cells/mL) was deposited onto the medium. A further 0.5 mL of saline but without yeast cells was overlaid with the same plate. The plate was then incubated at 37°C for 48 hours. After incubation, colony and halo diameters were measured with a ruler, and hemolytic activity was expressed by the ratio of the diameter of the colony to the outer diameter of the zone of degradation.

Determination of proteinase activity

Extracellular proteinase activity of all *C. albicans* isolates were analyzed regarding Bovine serum albumin (BSA) degradation based on the technique described by Staib [28]. Suspension of 0.5 mL 1×10^4 cells/mL was inoculated onto a 1% BSA plate. The plate was incubated for 5 days at 37°C, flooded with 1.25% naphthalene black solution for 15 minutes, and washed with 90% (v/v) methanol/water destaining solution, followed by 36 hours of decolorization with several changes of destaining solution. The ratio of the diameter of the colony to that of the transparent zone of proteolysis (in mm) was used as an index (Pr_z value) to represent the extent of proteinase activity by *C. albicans*. The assay was conducted in duplicate.

Effect of P. dioscoridis extract on virulence factors

After determination of virulence factors, *C. albicans* strain 1 seemed to be the most virulent isolate. A suspension of *C. albicans* strain 1 (0.5 mL 1×10^4 cells/mL) was mixed with 9.5 mL of *P. dioscoridis* extract (MIC 30 mg/mL) and ketoconazole (MIC 60 mg/mL) separately in sterile test tubes and incubated for 24 hours at 37°C. Then, these cells were used to determine phospholipase, hemolysin, and proteinase activities, as described above, to compare between the effect of *P. dioscoridis* extract and ketoconazole on these virulence factors.

Scanning and transmission electron microscope observations

Scanning electron microscope observations were carried out on *C. albicans* strain 1 cells. One milliliter of the *C. albicans* cell suspension at a concentration of 1×10^4 cells per milliliter was inoculated on SDA plates, which were then incubated at 37°C for 6 hours. The extract (2 mL), at a concentration of 30 mg/mL, was then dropped onto the inoculated agar and was further incubated for another 36 hours at the same incubation temperature. The untreated culture was used as a control. A small block of yeast containing agar withdrawn from the inoculated plates was fixed for scanning electron microscopy. Photos were taken with a scanning electron microscope, (JEOL, JSM–5200 LV, Tokyo, Japan) in the Faculty of Medicine, Tanta University, Tanta, Egypt. The TEM analyses were performed on samples harvested at the same hours and fixed in McDowell-Trump fixative prepared in 0.1 M phosphate solution (pH 7.2), rinsed in buffer three times, and post-fixed in 1% osmium tetroxide in buffer for 2 hours at 4°C. Then the sample was serially dehydrated with 50%, 75%, 95%, and 100% ethanol concentrations, respectively, and embedded in Epon-Araldite resin (Sigma, St. Louis, USA) to make the blocks of the cells. The resin with the embedded *C. albicans* cells was cut into ultra-thin sections in the ultramicrotomy process. Finally, the ultra-thin sections of the cells were stained with 2% uranyl acetate and lead citrate and observed under a transmission electron microscope (JEOL, JEM – 100 SX, Tokyo, Japan) [29].

RNA isolation and cDNA synthesis

Total RNAs were isolated from the 36-hour *C. albicans* strain 1, harvested by centrifugation, washed twice with sterile distilled water, and resuspended in sterile distilled water (one sample treated with 30 mg of *P. dioscoridis* leaf extract and one control sample untreated) in liquid SDA medium as mentioned previously, using TRIzol (Oxoid, Hampshire, UK) according to the manufacturer's protocol. The integrity of RNA samples was assessed by agarose gel electrophoresis. First strand cDNA was synthesized using an Advantage RT-for-PCR Kit (Clontech, Alto, USA). The first strand cDNA synthesis was performed in a total volume of 25 µL. Primers are listed in Table 1 using Primer Express software (PE Applied Biosystems, Austin, USA) for phospholipase, hemolysin, and proteinase (SAP1 and SAP2) according to Naglik *et al*. [30], Bennett *et al*. [31] and Nergi *et al*. [32]. The reaction mixture contained 2.5 µL of 5 9 buffer with MgCl2, 2.5 µL of 2.5 mM dNTPs, 4 µL from the oligo (dT) primer (20 pol/µL), 2 µg RNA, and 200 U reverse transcriptase enzyme (Fermentas, Boston, USA). RT-PCR amplification was performed in a thermal cycler (Eppendorf, Berlin, Germany) programmed at 42°C for 1 hour and 72°C for 10 minutes. cDNA was then stored at -20°C until use.

Real-time PCR amplification conditions

The cDNA of the two samples (control and treated) were subjected to semi-quantitive PCR using the primers listed in Table 1. The real-time PCR reaction consisted of 12.5 µL of 2 × Quantitech SYBR Green RT Mix (Sigma, St. Louis, USA), 1 µL of 25 pm/µL forward primer, 1 µL of 25 pm/µL reverse primer,1 µL of the cDNA (50 ng), and 9.25 µL of RNase-free water for a total of 25 µL. Samples were spun before being loaded in the rotor wells. The real-time PCR program was as follows: initial denaturation at 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. Data was taken during the extension step. This reaction was performed using a Rotor-Gene 6000 system (Qiagen, New York, USA). The 18S rRNA gene was used as housekeeping gene (reference gene) in this test. Threshold cycle values represent the PCR cycle in which an increase in fluorescence, over a defined threshold, first occurred for each amplification plot.

Real-time Q-PCR data analysis

The relative expression ratio was accurately quantified and calculated according to Rasmussen [33]. Accordingly, for each biological sample, the difference (Δ) in quantification cycle value (Ct) between the target (Ct [target]) averaged from three technical repeats) and the reference (Ct [reference]), a fixed Ct value was used for all samples) was first transformed into relative quantities (RQ) using the exponential function with the efficiency (E) of the PCR reaction.

The CT (threshold of cycle) value of each detected gene was determined by automated threshold analysis on an ABI System (Thermo Fisher Scientific, Carlsbad, United States) The CT value of each target gene was normalized to CT (reference) to obtain ΔCT (target), where

ΔCT (target) = (CT [target] – CT [reference]), ΔCT (control) = (CT [control] – CT [reference]). The relative expression quantity of the target gene was indicated to

ΔΔCT = (ΔCT [target] – ΔCT [control]) according to 2-ΔΔCt algorithm. Values higher than 1 in a parameter express an increase (up-regulation), while values lower than 1 express a decrease (down-regulation).

Statistical analysis

Statistical analysis was carried out on the data measuring *P. dioscoridis* leaf extract and ketoconazole effects on growth of *C. albicans* through analysis of variance (ANOVA) one-way tests by SPSS, version 17 (IBM, Armonk, USA).

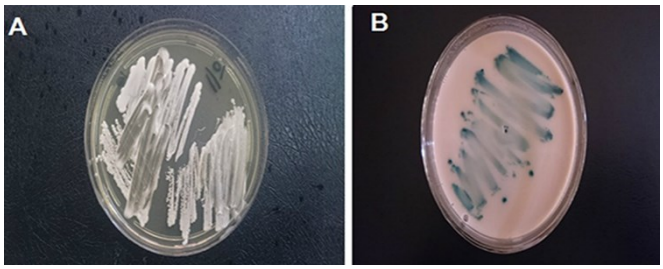
Table 1. Primer list for the targets.

Key		Forward	Reverse
		***Candida albicans* phospholipase gene**	
1	ACT	CGCTTCTTCTCAATCTTCTGCC	CGAATGGATGGACCAGATTCGTCG
		***Candida albicans* proteinase gene**	
2	SAP1	TTTCATCGCTCTTGCTATTGCTT	TGACATCAAAGTCTAAAGTGACAAAACC
3	SAP10	CCCGGTATCCAATAGAATCGAA	TCAGTGAATGTGACGAATTTGAAGA
		***Candida albicans* hemolysin gene**	
4	HLP	GTGCTGGTGACACTGCTGCT	TCCGATTCATCCACTATTTC
		Housekeeping gene (reference gene)	
18S rRNA		GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

Results

Figure 1 showed *C. albicans* on SDA and its green colonies on chromagar. Anticandidal activity of the ethanolic leaf extract of *P. dioscoridis* is shown in Table 2. The extract inhibited all the tested isolates in this study, the diameters of inhibition zones ranging from 5.5 to 6 cm, better than ketoconazole as a standard drug. Ethanol did not show any inhibition against tested strains. There were significant differences in the anticandidal activities of different concentrations of extract compared with ketoconazole and the rate of untreated fungal growth (Table 3). Subsequent experiments were conducted to determine the MIC values of the extract and ketoconazole against the *C. albicans* strains. Table 3 also showed that the MIC of extract was 30 mg/mL, while the MIC of ketoconazole was 55 mg/mL. This high concentration may be due to the resistance of tested isolates. A time-kill study was conducted over a period of 48 hours with the aim of assessing the anticandidal activity with 0.5 MIC (15 mg/mL), MIC (30 mg/mL), and 2 MIC (60 mg/mL) values, and the results are shown in Figure 2. At 0.5 MIC value, there were drastic drops in OD after 12 hours of cultivation, which leads to the stationary phase of yeast growth compared to control (untreated cell). However, at MIC and 2 MIC values, the extract eradicated the cell numbers. The results showed the potency of the extract as an anticandidal agent against *C. albicans*.

Figure 1. Growth of *C. albicans* after 48 hours. (A) White to cream colonies on SDA (B) Green colonies on CHROM agar.



Using the agar plates containing substrates for particular enzymes, such as BSA, egg yolk, or sheep's blood, all strains of *C. albicans* tested in the present study differed in terms of enzymes produced. This study focused on *C. albicans* strain 1 because it showed the largest inhibition zone and it was found to be the most pathogenic, as it was the highest for the production of extracellular phospholipase, hemolysin, and proteinase ($P_z = 0.4$; $H_z = 0.2$; $Pr_z = 0.1$, respectively) (Table 4). Significantly different enzymatic and hemolytic activity could be detected between control strain 1 and those pretreated for 24 hours with *P.*

Table 2. Anticandidal activitiy of the *P. dioscoridis* extract against different pathogenic strains of *C. albicans* based on their zones of inhibition.

***C. albicans* strains**	**Zones of inhibition (cm)**	
	***P. dioscoridis* extract (30 mg/mL)**	**Ketoconazole (30 mg/mL)**
Candida albicans strain 1	6	3
Candida albicans strain 2	6	2.5
Candida albicans strain 3	5.8	2.5

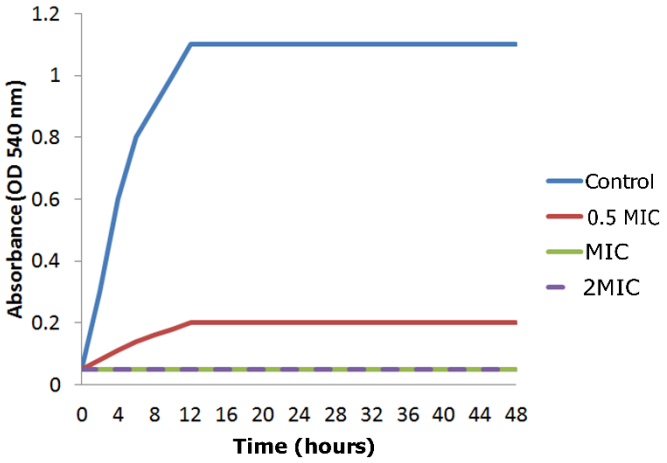
C. albicans 1: isolated from oral candidiasis; *C. albicans* 2: isolated from vaginitis; *C. albicans* 3: isolated from yeast onychomycosis

Table 3. Effect of *P. dioscoridis* on growth of *C. albicans* and its minimum inhibitory concentration.

Concentration (mg/mL)		**Diameter of inhibition zone (cm)**	
		Pluchea dioscoridis	**Ketoconazole**
0		0.0 ± 0.0^{a12}	0.0 ± 0.0^{a}
10		0.5 ± 0.1^{b}	0.2 ± 0.0^{b}
15		2.5 ± 0.4^{c}	0.5 ± 0.1^{c}
20		3.0 ± 0.3^{d}	1.5 ± 0.2^{d}
25		4.5 ± 0.2^{e}	2.0 ± 0.1^{e}
30		6.0 ± 0.0^{f}	2.8 ± 0.3^{f}
35		6.0 ± 0.0^{f}	3.2 ± 0.1^{g}
40		6.0 ± 0.0^{f}	3.8 ± 0.1^{h}
45		6.0 ± 0.0^{f}	4.5 ± 0.2^{i}
50		6.0 ± 0.0^{f}	5.5 ± 0.1^{j}
55		6.0 ± 0.0^{f}	6.0 ± 0.0^{j}
60		6.0 ± 0.0^{f}	6.0 ± 0.0^{j}
ANOVA	**F value**	**1,900.23**	**799.87**
	P value	**1.7602×10^{-32}**	**5.55×10^{-28}**

[1] Values are the mean of three replicates ± SD; [2] Values with the same letter in the same column are insignificant ($p \leq 0.01$).

Figure 2. Growth profiles of *C. albicans* strain 1 after being treated with 0.5 MIC (15 mg/mL), MIC (30 mg/mL) and 2 MIC (60 mg/mL) of *P. dioscoridis* extract.

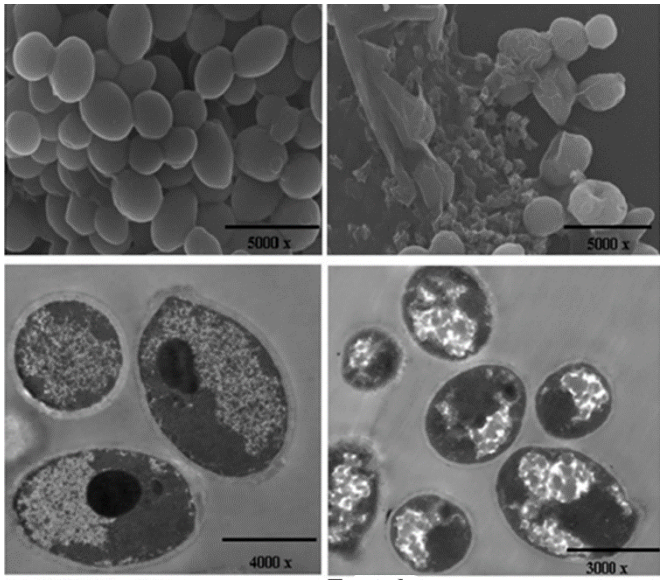


dioscordis at MICs that caused a statistically significant reduction in activity (Table 5).

A clearer view of the effect of the *P. dioscoridis* extract against *C. albicans* cells is shown on SEM micrographs in Figure 3. Many cells, oval and smooth in appearance are shown, as well as some at a budding stage as controls. After 24 hours of extract exposure, treated cells appeared as shrunken cells, and completely collapsed cells were eventually observed. At this stage, the damage of the cells was beyond repair and the cells lost their metabolic functions completely.

Observation on SEM micrographs suggested the cells had undergone distinct morphological and cytological alterations. Further evidence of these changes can be clearly observed from the TEM

Figure 3. SEM and TEM micrographs of the untreated and *Pluchea dioscoridis*-treated *C. albicans* strain 1 cells.



micrographs, which reaffirm some disorganization of the yeast cells and also the destruction of their cytoplasms and organelles (Figure 3). Control cells showed a typical structure of *C. albicans* cells with nucleolus and organelles. The cytoplasm contained elements of the cell membrane system and was enveloped by a typical cell wall of yeast cells. After extract exposure, the treated cells exhibited notable alterations in the cell membrane and cell wall. The cytoplasmic volume decreased, and there was deformed or stunted budding formation with disorganization;

Table 4. Phospholipase, hemolysin, and proteinase activities of *C. albicans* strains.

	***C. albicans* isolates**		
Activity (cm)[1]	**Strain 1**	**Strain 2**	**Strain 3**
Phospholipase (P_z)	0.4 ± 0.01^{a23}	0.5 ± 0.01^{b}	0.6 ± 0.08^{c}
Hemolysin (H_z)	0.2 ± 0.02^{a}	0.2 ± 0.0^{a}	0.3 ± 0.06^{b}
Proteinase (Pr_z)	0.1 ± 0.01^{a}	0.3 ± 0.05^{b}	0.2 ± 0.02^{c}

[1] Results are expressed as P_z, Pr_z, and H_z indexes calculated as the proportion of *C. albicans* colony diameter compared to the diameter of the precipitation or hemolysis zone. According to the applied scale, P_z, Pr_z, and H_z values equal to 1 indicate a negative reaction, while values of 0.7–0.99 indicate weak activity, 0.5–0.69 indicate medium activity, and values < 0.5 indicate strong secretory activity; [2] Values are the mean of three replicates ± SD; [3] Values with the same letter in the same column are insignificant ($p \leq 0.01$).

Table 5. Phospholipase, proteinase, and hemolysin activities of *C. albicans* strain 1 pretreated with *P. dioscoridis* at minimum inhibitory concentration.

	***C. albicans* strain 1**		
Activity (cm)[1]	**Control**	***Pluchea dioscoridis***	**Ketoconazole**
Phospholipase (P_z)	0.4 ± 0.01^{a23}	0.8 ± 0.08^{b}	0.4 ± 0.02^{a}
Hemolysin (H_z)	0.2 ± 0.02^{a}	0.8 ± 0.05^{b}	0.6 ± 0.07^{c}
Proteinase (Pr_z)	0.1 ± 0.01^{a}	0.9 ± 0.05^{b}	0.4 ± 0.03^{c}

[1] Results are expressed as Pz, Prz, and Hz indexes calculated as the proportion of *C. albicans* colony diameter compared to the diameter of the precipitation or hemolysis zone. According to the applied scale, P_z, Pr_z, and H_z values equal to 1 indicate a negative reaction, while values of 0.7–0.99 indicate weak activity, 0.5–0.69 indicate medium activity, and values < 0.5 indicate strong secretory activity. [2] Values are the mean of three replicates ± SD; [3] Values with the same letter in the same column are insignificant ($p \leq 0.01$).

also, some cells underwent severe disorganization within the cells, which led to collapsed and lysed cells.

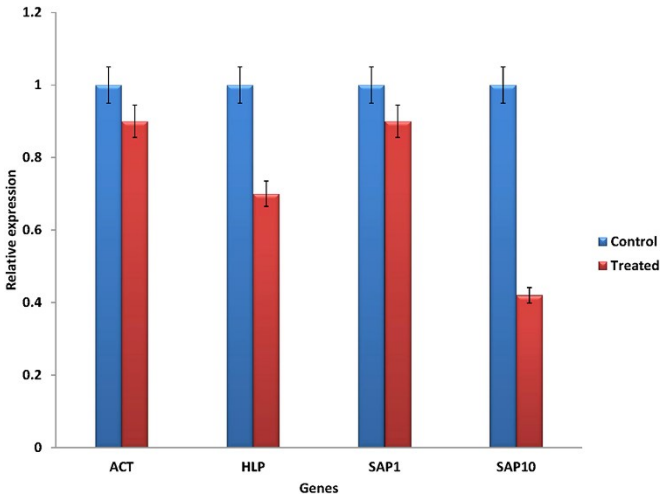
P. dioscoridis leaf extract had an effect on the ratio of phospholipase, hemolysin, and proteinase (SAP1 and SAP10) gene expression quantified by real-time PCR. Figure 4 shows significant reduction of those expressed genes in treated *C. albicans* up to 90%, 70%, 90% SAP1 and 40% SAP10 in respect to untreated one (100%). These results confirm the inhibitory effect of *P. dioscoridis* leaf extract on *C. albicans* virulence genes *in vitro.*

Discussion

C. albicans is a harmless commensal dimorphic yeast-like fungus in healthy humans, which can cause superficial as well as life-threatening systemic infections in immunocompromised patients [34]. *C. albicans* is the most virulent among the *Candida* species, and can cause several forms of candidiasis in debilitated humans. *C. albicans* can infect virtually all body sites because of its high adaptability to different host niches by the activation of appropriate sets of genes in response to complex environmental signals [35]. In this study, we concentrated on *C. albicans* because the management of *Candida* infections faces a number of problems, including a limited number of effective antifungal agents, the toxicity of the available antifungal agents, the resistance of *Candida* to commonly used antifungals, and the high cost of antifungal agents [36].

In the present investigation, the therapeutic potential of *P. dioscoridis* was assessed directly against the *Candida* spp. isolated from different patients with clinical manifestations. The results of *P. dioscoridis* were extremely promising and showed good consistency with strong inhibitory potential towards the clinical isolates of *C. albicans*, with a MIC value of only 30 mg/mL. This was in agreement with Metwally *et al*. [37], who reported the antifungal activity of *P. dioscoridis* extract against *Aspergillus niger*. A time-kill assay was utilized in this study to verify MIC findings and to evaluate the ability of *P. dioscoridis* extract to eliminate *C. albicans* growth *in vitro*. Similar to our results, Prabhakar *et al*. [38] reported that the ethanolic extracts of *Syzygium jambolanum* and *Cassia siamea* exhibited anticandidial activity at 50 mg/mL, whereas *Odina wodier* was at 100 mg/mL. Ketoconazole, a well-known commercial antifungal drug, was chosen to be a positive control to compare and evaluate the *P. dioscoridis* extract activity in the present assay. The choice of ketoconazole was in agreement with its *in vitro* activity and MIC against

Figure 4. Quantification of different genes expression using real-time PCR.



ACT: control sample and *Pluchea dioscoridis*-treated sample of phospholipase gene; HLP: control sample and *Pluchea dioscoridis*-treated sample of hemolytic gene; SAP1: control sample and *Pluchea dioscoridis*-treated sample of proteinase SAP1 gene; SAP10: Control sample and *Pluchea dioscoridis*-treated sample of proteinase SAP10 gene.

pathogenic fungi recorded by Mahmoud *et al*. [39]. The succession of anticandidal activity of any plant extract depends on the specific interactions between the bioactive compounds with the cell wall compartments [40]. This interaction with the constituent's target site will aid the penetration of the bioactive compounds into the cells. The presence of phenolics, flavonoids, and tannins in the ethanol extract of *P. dioscoridis* was previously reported by Metwally *et al*. [37]. It is possible that these compounds were mainly responsible for the observed anticandidal effects in this study. Moreover, Paerkh and Chanda [41] showed high antimicrobial activity of *P. dioscordis* leaf extract against all Gram-positive bacteria and yeasts.

Although the antifungal activity of several essential oils and natural components has been reported, the investigations focused mostly on the determination of their cidal activity. Relatively little research concerned the mechanisms of their action, such as impact on the virulence factors [42,43]. This report includes results proving the influence of *P. dioscoridis* extract on possible mechanisms reported to be relevant for *Candida* pathogenesis, namely extracellular production of various enzymes. Our results also indicate that all the isolated strains were pathogenic, and that all strains of *Candida* produced phospholipase, hemolysin, and proteinase as virulence factors. Furthermore, *P. dioscoridis* extract decreased the ability of *Candida* to produce these enzymes, which was in agreement with

the results reported by Ibrahim *et al*. [44]. The virulence of *Candida* species is attributed not to a single factor but to a combination of several factors [45], including proteinase, phospholipase [46], and hemolysin production [47]. These data suggest that the capacity of *C. albicans* to produce these enzymes must have a role in its pathogenic potential.

Morphological changes observed from SEM and TEM studies showed that the extract could completely collapse the yeast cell and inhibit the growth of *C. albicans*. This was in agreement with anticandidal activity of *Vernonia cinerea* methanol extract recorded by Latha *et al*. [48].

Real-time PCR was used to enable the quantification of *C. albicans* virulence genes of phospholipase, hemolysin, and proteinase (SAP1 and SAP10) in both samples treated with and those not treated with *P. dioscoridis*. This is the first reported study on the detection and quantification of expressed virulence genes using quantitative real-time PCR. In this study, suppression of expressed genes in treated samples but not in untreated ones demonstrates the effect of *P. dioscoridis* on these genes, illustrating its inhibitory effect *in vitro*. This was in agreement with Mohseni *et al*. [49], who reported that licorice extract affected *aflR* gene expression of *A. parasiticus*, which was suppressed up to 40% on quantitative RT-PCR analysis. Our results also suggest that SAP1 and SAP10 were the most common genes expressed during infection and carriage; this was similar to the findings of Naglik *et al*. [30]. All these changes in fungal surface behavior, colony appearance, metabolic and molecular parameters leads to the inability of *Candida* to cause infection. Changes in virulence gene expression may further help our understanding of yeast invasion and how *P. dioscoridis* treatment can inhibit fungal growth. This means that the results of real-time PCR confirm the results obtained by enzyme assay. This assumption was confirmed by the production of these enzymes in the treated *Candida* being reduced to low levels *in vitro* and how this disability affected the structure and the morphology of the treated fungal cells when compared with the non-treated cells.

Conclusions

To our knowledge, this is the first detailed study on the anticandidal effects of *P. dioscoridis* extract against *C. albicans* virulence factors. *P. dioscoridis* may be a promising remedy for the development of anticandidal agents in the future.

Changes in phospholipase, hemolysin, and secreted aspartyl proteinase gene expression may further help our understanding of yeast invasion and how *P. dioscoridis* treatment can inhibit *Candida* growth by affecting its genes.

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