Journal of Biosciences and Medicines, 2019, 7, 29-43
http://www.scirp.org/journal/jbm
ISSN Online: 2327-509X
ISSN Print: 2327-5081

Biochemical and Molecular Effects of *Phoenix dactylifera* and *Ziziphus spina-christi* Extracts on *Candida albicans*

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How to cite this paper: Al-Ali, S. and Al-Judaibi, A. (2019) Biochemical and Molecular Effects of *Phoenix dactylifera* and *Ziziphus spina-christi* Extracts on *Candida albicans*. *Journal of Biosciences and Medicines*, **7**, 29-43.
https://doi.org/10.4236/jbm.2019.73004

Received: January 21, 2019
Accepted: March 3, 2019
Published: March 6, 2019

Abstract

Our overall knowledge of the medicinal uses of plants suggests that natural compounds could be useful in the treatment of cutaneous fungal infections in tropical regions. Furthermore, the possibilities of treatment using plant extracts may be even broader than is already known when one considers plants that have not been extensively studied in this context, such as the regional species *Phoenix dactylifera* and *Ziziphus spina-christi*. This study compared the antimicrobial activity of *Phoenix dactylifera* and *Ziziphus spina-christi* extracts in terms of their biochemical and molecular effects on *Candida albicans* (ATCC CA 10231). These effects included altered levels of intracellular sterols, changes in the permeability of the cell membrane, and changes in the *TEF*1: *QRTTEF*1, *CaERG*1: *ERG*1, *CdERG*12: *CdERG*1, and *ERG*25: *ERG*25 genes. Scanning electron microscopy (SEM) was used to identify morphological characteristics, and energy-dispersive X-ray spectroscopy (EDAX) analyses were conducted. In treated samples, the SEM and EDAX analyses showed cell cavities and shrinkage of the cell wall, and the number of cells was reduced to only a few abnormal cells as compared with that in the untreated samples. Yttrium was detected in the cells treated with *Z. spina-christi*, and high levels of osmium were detected in the cells treated with *P. dactylifera*. Compared with control cells, cells exposed to the concentration 150 µl/ml of *Z. spina-christi* extract had an average sterol concentration that was nearly 3 times higher, while the concentration was 5.5 times higher for cells treated with the 150 µl/ml of *P. dactylifera* extract. The ethanol extracts affected the permeability of *C. albicans* cell membrane. Gene sequencing showed gaps and mismatches in the *ERG*1, *ERG*12, and *ERG*25 genes after treatment with *P. dactylifera* and *Z. spina-christi* extracts compared with that in the controls. The results were highly significant ($p \leq 0.01$). We conclude

that the ethanol extracts of *P. dactylifera* and *Z. spina-christi* have antimicrobial activity through several mechanisms in the yeast cell.

Keywords

Sterols, Cell Permeability, EDAX Analyses, *Phoenix dactylifera*, *Ziziphus spina-christi*, Molecular Characterization

1. Introduction

Medicinal plants have been used as a source of medicines for many centuries. Such plants are reliable sources for the treatment of various health problems. It is reasonable to expect that plants will contribute to treating future health challenges as well [1]. A large proportion of medicinal compounds has been discovered with the aid of ethno-botanical knowledge of their traditional uses. More than 35,000 plant species are known to have been used for medical purposes in various human cultures around the world [2]. The medicinal properties of plants can be attributed to different plant parts including leaves, roots, bark, fruit, seeds, and flowers. Our overall knowledge of the medicinal uses of plants suggests that natural compounds could be useful in the treatment of cutaneous fungal infections in tropical regions [3] [4].

The effects of plant extracts on microorganisms have been studied by numerous researchers worldwide [5]-[11]. Furthermore, the possibilities of treatment using plant extracts may be even broader than already known when one considers plants that have not been extensively studied in this context, such as the regional species *Phoenix dactylifera* and *Ziziphus spina-christi.*

In Saudi Arabian folk medicine, plant components such as sidr (*Z. spina-christi*) and Ajwa date (*P. dactylifera*) seeds are used to heal wounds and treat skin diseases, inflammatory conditions, sores, ringworm, fevers, gonorrhea, and ulcers. A decoction of the bark and fresh fruits is used to promote the healing of fresh wounds and as a body wash, while the fruits are used to treat dysentery [12] [13].

The methanol extract of *Z. spina-christi* showed antifungal activity against dermatophytes in *Trichophyton rubrum*, *T. mentagrophytes*, *Microsporum canis*, and *Aspergillus fumigatus* when tested by the agar diffusion method [14]. In the 2000-2006 period, approximately 50% of the new chemical molecules extracted from natural products demonstrated an important role in the development of drugs in the treatment of infectious diseases [15]. *P. dactylifera* is known to be effective against fungi and yeasts. This activity appears to depend upon the total phenolic content and flavonoids present in the fruit [16]. *In vitro* studies have shown that flavonoids possess antifungal activities against *Candida albicans* and their presence in an extract may explain observed antifungal effects [17] [18]. Shraideh *et al.* [19] reported that treatment of *C. albicans* with Barhi date extract caused distortion, weakening, and partial collapse of the cell wall. At high

concentrations, cell lysis, leakage of cytoplasmic material, and eventual cell death were observed.

The antifungal activity of *P. dactylifera* leaves and pits using different solvents has also been reported. This research showed that water, acetone, and methanol extracts induced varying degrees of growth inhibition in *Fusarium* spp., *Fusarium oxysporum*, *F. soloni*, *Alternaria* spp., *Aspergillos flavus*, *A. alternate*, and *Trichoderma* spp. [20]. These results collectively suggest that phytochemicals in date extract may have multiple effects on *Candida*, and further study could reveal therapeutic uses. Doddanna *et al.* [21] found that plant extracts in alcohol, including onion leaves, tea leaves, onion bulb, aloe vera, and mint leaves, inhibited the growth of *C. albicans*. The ethanolic extract of ginger powder has pronounced inhibitory activities against *C. albicans* [22]. Pomegranate (*Punica granatum*) methanol extract also demonstrated an effect against *C. albicans* [23].

Medicinal plants have a promising future owing to the need for new antimicrobial drugs due to continuous development of drug resistance; in addition, natural antimicrobials are of utmost importance owing to safety issues and availability [24] [25]. The aim of new antifungal strategies is to develop drugs that combine sustainability, high efficacy, and restricted toxicity, safety for humans, animals, host plants, and ecosystems while maintaining a low production cost. This study was designed to investigate the effect of *P. dactylifera* seeds and *Z. spina-christi* extracts on several biochemical activities of *C. albicans*, including metabolism of glucose uptake and cell wall permeability, morphology, and structure; moreover, we studied the molecular characteristics of the *TEF*1: *QRTTEF*1, *CaERG*1: *ERG*1, *CdERG*12: *CdERG*1, and *ERG*25: *ERG*25 genes.

2. Material and Methods

2.1. Test Organism

An antibiotic-resistant strain of *C. albicans* (ATCC CA 10231) was obtained from American Type Culture Collections (ATCC; Rockville, Md, USA), and grown at 27˚C ± 2˚C for 48 h in an aerobic incubator. The yeast was cultured on Sabouraud Dextrose Agar medium (SDA), Sabouraud Dextrose Broth (SDB), Mueller Hinton Agar (MHA), and Mueller Hinton Broth (MHB); (HiMedia, India).

2.2. Study Specimens and Extraction

Z. spina-christi (sidr) leaves were collected from the Jeddah region in Saudi Arabia during autumn 2013. *P. dactylifera* (Ajwa date) seeds were collected from Almadina Almonawara City, Saudi Arabia during Autumn 2013. Plant identification was confirmed in the Department of Biological Sciences, Botany Section at King Abdulaziz University, Jeddah KSA.

Ajwa date seeds and sidr leaves were thoroughly washed and then dried in shade at 30˚C ± 2˚C, for 4 days. Specimens were powdered by a grinding machine (IKA A10 basic); powdered plant samples were placed in ethanol (1:10 w/v)

in conical flasks and shaken at 120 rpm at 30˚C for 3 days. Flask contents were filtered through Whatman no. 1 filter paper (Whatman No. 1), and the filtrates were dried under reduced pressure at 40˚C. The extracts were weighed, and the yields were calculated as percentages based on the weight of the initial material used for extraction. Each extract was then dissolved in dimethyl sulfoxide (Sigma-Aldrich, USA) at 50 µg/ml and filtered through a 0.22-µm pore filter (Millipore, Billerica, MA, USA). Filtrates were stored in closed vials at 4˚C.

2.3. Inoculum Preparation

C. albicans inoculums were prepared by seeding SDA (HiMedia, Mumbai, India) with 100 µl of the yeast containing $(1 - 5) \times 10^6$ colony-forming units (cfu).

2.4. Cell Wall Sterol Measurement

Total intracellular sterols were extracted as reported by Breivik and Owades [26] with slight modifications. Briefly, a single *C. albicans* colony from an overnight SDA plate culture was used to inoculate 5 ml of SDB (HiMedia) containing 50, 100, or 150 mg of *P. dactylifera* and *Z. spina-christi* extracts per milliliter. The cultures were incubated for 48 h with shaking at 27˚C. The stationary-phase cells were harvested by centrifugation at 120 rpm (Eppendorf 5424 refrigerated bench top centrifuge, USA) for 5 min and washed once with sterile distilled water, and the net wet weight of the cell pellet was then determined. Three milliliters of 25% potassium hydroxide-alcohol solution (25 g of KOH in 35 ml of sterile distilled water brought to 100 ml with 100% ethanol) was added to each pellet. After vortex mixing for 1 min, the resultant cell suspensions were transferred to 16- by 100-mm sterile borosilicate glass screw-cap tubes and incubated in an 85˚C water bath for 1 h. Tubes were allowed to cool to 25˚C ± 2˚C, and sterols were then extracted by addition of a mixture of 1 ml of sterile distilled water and 3 ml of n-heptane followed by vigorous vortex mixing for 3 min. The n-heptane layer containing the sterol fraction was then transferred to a clean borosilicate glass screw-cap tube and stored at −20˚C for 24 h. A 20-ml aliquot of this fraction was diluted fivefold in 100% ethanol and scanned spectrophotometrically between 240 and 300 nm with a Genesys 20 spectrophotometer (Thermo Fisher Scientific) [27]. Each treatment was performed in triplicate.

2.5. Confocal Scanning Laser Microscopy (CSLM)

CSLM was used to evaluate the effect of the altered permeability of cell membranes in *C. albicans*. *C. albicans* was grown in SDB with 150 µl/ml of *P. dactylifera* and *Z. spina-christi* extracts, and cells were then harvested and incubated for 45 min at 37˚C in 4 mL of phosphate-buffered saline (PBS) containing the fluorescent stain ethidium bromide (10 mM) (excitation wavelength 543 nm and 560 nm longpass emission filter). This stain attaches to DNA in dead cells, forming cylindrical orange-red intravacuolar structures. After incubation, the cells were placed in a 35-mm-diameter glass-bottom Petri dish (MatTek Corp.,

Ashland, MA, USA). Stained cells were observed with a Zeiss LSM510 confocal scanning laser microscope equipped with argon and HeNe lasers and mounted on a Zeiss Axiovert100 M microscope (Carl Zeiss Inc. Germany). The objective used was a water immersion C-Apochromat lens (403; numerical aperture of 1.2) (Carl Zeiss Inc., Germany). Depth measurements were taken at regular intervals across the width of the device. Confocal images of red (ethidium bromide) fluorescence were obtained simultaneously using a multitrack mode. Planktonically grown *C. albicans* cells were used as comparators in these studies [28]. Samples were scanned in the Center of Nanotechnology at King Abdulaziz University.

2.6. Scanning Electron Microscopy (SEM) and Energy-Dispersive X-Ray Spectroscopy (EDAX) Analyses

To assess the morphological characteristics of selected treated yeast, SEM was used. Yeast was incubated with ethanol extracts of *P. dactylifera* and *Z. spina-christi* (150 µl/ml) for 48 h in MHB as described by Al-Wathnani *et al.* [29]. The treated and control cultures were incubated at 27˚C and then centrifuged at 120 rpm to separate the yeast cells. A thin film of cells from each group was spread on a copper stub. The samples were then coated with gold by cathodic spraying (Polaron gold) and dried under a mercury lamp for 5 min. The morphology of the *C. albicans* cells was observed with a scanning electron microscope (JEOL, JSM-7600F) [30] [31]. Samples were scanned in the Center of Nanotechnology at King Abdulaziz University.

2.7. Molecular Characterization

To determine the effect of *P. dactylifera* and *Z. spina-christi* ethanol extracts on the *TEF*1: *QRTTEF*1, *CaERG*1: *ERG*1, *CdERG*12: *CdERG*1, *and ERG*25: *ERG*25 genes in *C. albicans*, yeast DNA was isolated by using a Qiagen DNA extraction kit (Germany), following the protocol for yeast by Karthy *et al.* [32].

For genomic DNA isolation a Qiagen kit was applied as described; 10 µl of cells from a yeast colony on an overnight agar plate at 27˚C was transferred to a 1.5-ml Eppendorf tube. After centrifugation the pellet was resuspended in 180 µl of ATL (A Tissue Lysis) buffer, and 20 µl of proteinase K was added. The tube contents were then thoroughly mixed by vortexing, and the samples were incubated at 56˚C until the tissue was completely lysed. The samples were mixed by vortexing for 15 s, 200 µl of ATL buffer was added, and the samples were again mixed by vortexing. Then, 200 µl of ethanol (96% - 100%) was added, followed by vortex mixing. The mixtures were pipetted into DNeasy Mini spin columns and placed in 2-ml collection tubes, which were centrifuged at 9800× g for 1 min. The DNeasy Mini spin columns were placed in new 2-ml collection tubes, 500 µl of buffer AW1 was added to each, and the mixtures were centrifuged for 1 min at 9800× g. The DNeasy Mini spin columns were then placed in new 2-ml collection tubes, 500 µl of buffer AW2 was added to each, and the mixtures were centrifuged for 3 min at 9800× g. The DNeasy Mini spin columns were then placed

in clean 2-ml microcentrifuge tubes, 200 µl of buffer AE was pipetted directly onto the DNeasy membrane, and the membranes were incubated at 28˚C ± 2˚C for 1 min. They were next centrifuged for 1 min at 9800× g to elute. The isolated DNA samples were stored at −20˚C as per the manufacturer's protocol.

The genes *TEF*1: *QRTTEF*1, *CaERG*1: *ERG*1, *CdERG*12: *CdERG*1 and *ERG*25: *ERG*25 were amplified by PCR using the following primers:

Forward	*TEF*1	*QRTTEF1 F*	CCACTGAAGTCAAGTCCGTTGA
	*CaERG*1	*ERG1 F*	ACTAATGTTCCACCATTGGCTCT
	*CdERG*12	*CdERG1 F*	ACTAATGTTCCACCATTGGTTCT
	*ERG*25	*ERG25 F*	GCTCATCCAGTTGAAGTTGCC
Reverse	*TEF*1	*QRTTEF1 R*	CACCTTCAGCCAATTGTTCGT
	*CaERG*1	*ERG1 R*	CACATGACCTTTGCCCTTAGCT
	*CdERG*12	*CdERG1 R*	CACATGACCTTTGCCCTTGGCT
	*ERG*25	*ERG25 R*	GCAAGTTACCAGTGATAAGACACCA

The primers for the amplification of the above genes were designed based on the conserved regions in the *TEF*1: *QRTTEF*1, *CaERG*1: *ERG*1, *CdERG*12: *CdERG*1, and *ERG*25: *ERG*25 genes from the *C. albicans* ATCC 10231 genome sequences [33] [34]. The extracted DNA was sequenced by Macrogen (https://www.macrogenusa.com/). The sequenced data were analyzed by T-COFFEE (http://tcoffee.crg.cat/).

2.8. Statistical Analysis

Data on microbial growth and cell counts (cfu/ml) were collected, summarized, and tabulated. Statistical analyses were performed using the Statistical Package for the Social Sciences, IBM SPSS 20 (SPSS Inc., Chicago, IL, USA). The results are expressed as the mean ± standard deviation (mean ± SD). The significance of the differences between samples and the homogeneity between groups were determined by analysis of variance (ANOVA). Results were considered significant at $p \leq 0.05$ and highly significant at $p \leq 0.01$.

2.9. References

The references were performed by using EndNote Thomson Reuters software version X7. References are in APA format.

3. Results

3.1. Sterol Levels (Mg/Ml) Increase in the *C. albicans* Cell Wall after Treatment with *Z. spina-christi* and *P. dactylifera* Extracts

Table 1 shows the increase in sterol levels in the *C. albicans* cell wall after treatment with *Z. spina-christi* and *P. dactylifera* extracts at various concentrations.

The results were highly significant ($p < 0.01$). *C. albicans* showed increased sterol levels (0.588% and 1.096%) at the end of the incubation period with *Z. spina-christi* and *P. dactylifera*, respectively. The results were highly significant ($p < 0.01$). Therefore, in this assay the extract of *P. dactylifera* had a greater effect on the yeast than the *Z. spina-christi* extract.

3.2. Confocal Scanning Laser Microscopy

The results in Table 2 and Figure 1 show increased numbers of dead cells with increasing concentrations of *Z. spina-christi* and *P. dactylifera* extracts compared with that in the untreated cells. After treatment with 150 µl/ml of *Z. spina-christi* and *P. dactylifera* extracts, the dead cell count was increased to 150.33 cfu/ml and 348 cfu/ml, respectively, compared with that in the untreated cells at 52 cfu/ml.

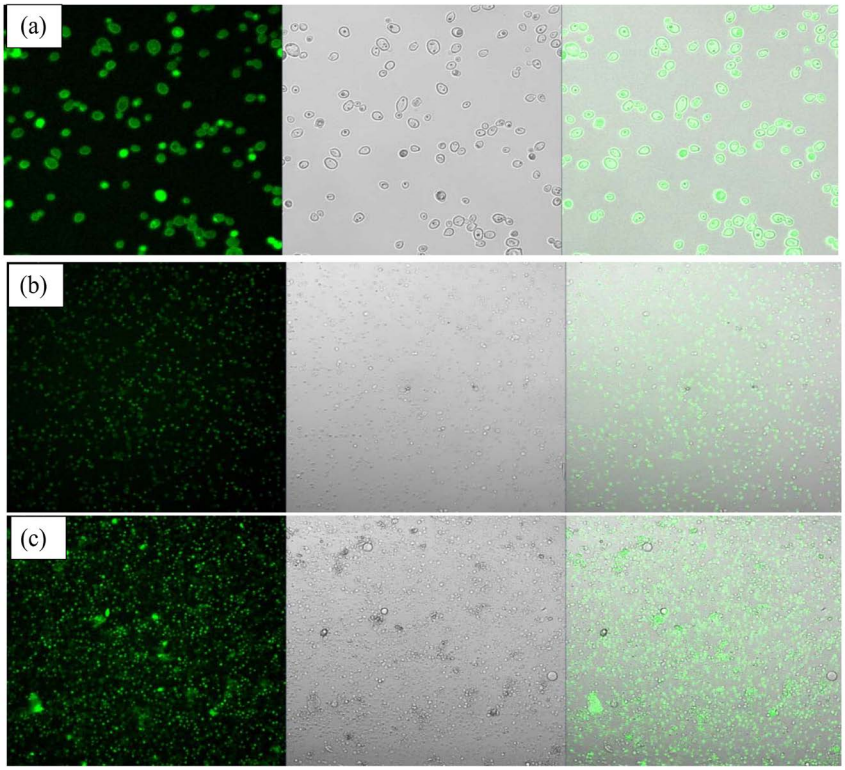

Figure 1. Confocal scanning laser microscopy of *C. albicans* shows the effect of the 150 µl/ml *Z. spina-christi* (B) and *P. dactylifera* (C) extracts on cell wall permeability and cell vitality, compared with untreated cells (A).

Table 1. Sterol levels (mg/ml) in the *Candida albicans* cell wall after treatment with *Ziziphus spina-christi* and *Phoenix dactylifera* extracts (µl/ml) and 48-h incubation (mean ± SD).

	Treatments						
		Z. spina-christi			*P. dactylifera*		
C. albicans	Control	50	100	150	50	100	150
	0.198	0.092 ± 0.0049**	0.146 ± 0.0062**	0.588 ± 0.0085**	0.632 ± 0.0049**	0.845 ± 0.0062**	1.096 ± 0.0085**

**$p \leq 0.01$, *$p \leq 0.05$.

Table 2. *Candida albicans* cell wall permeability after treatment with 150 µl/ml of *Z. spina-christi* and *P. dactylifera* extracts and 48-h incubation (mean ± SD).

	Treatments		
	Control	*Z. spina-christi* (150 µl/ml)	*P. dactylifera* (150 µl/ml)
Dead cells	52	150.33 ± 28.50**	348.00 ± 1.73**

**$p \leq 0.01$.

3.3. Scanning Electron Microscopy and EDAX Analyses

Figure 2 shows the SEM results, which reveal changes in the yeast morphology and structure in response to the *Z. spina-christi* and *P. dactylifera* extracts. Treatment with the *P. dactylifera* extract resulted in cell cavities and shrinkage of the cell wall, and the number of cells was greatly reduced to a few abnormal cells compared with that in the control samples. *C. albicans* was more affected by the *P. dactylifera* treatment than the *Z. spina-christi* extract, to which the cells appeared to have some resistance. However, both treatments reduced cell counts compared with the untreated cells. Budding was obvious in the untreated cells but was unclear in treated cells.

EDAX analyses of *C. albicans* showed differences between the treated and untreated cells. As shown in Figure 3, the results clarified the different elemental compositions of the ethanol extracts of *Z. spina-christi* and *P. dactylifera*. Both ethanol-based extracts contained carbon (C) and oxygen (O). Yttrium (Y) was detected in the cells treated with *Z. spina-christi* and high levels of osmium (Os) was detected in the cells treated with *P. dactylifera*.

3.4. Effect of *P. dactylifera* and *Z. spina-christi* Extracts on Genes in *C. albicans*

The aim of this study was to observe changes in the nucleotide position of the translation elongation factor activity (1-alpha) (TEF1) gene and ERG genes, which are involved in ergosterol biosynthesis. The results in Figures 4(A)-(E); Figures 5(A)-(E); and Figure 6(A-1), Figure 6(A-2), Figure 6(B-1), and Figure 6(B-2) show changes in the gene sequences of *C. albicans* treated with *P. dactylifera* and *Z. spina-christi* extracts in comparison to that in the untreated cells. Figure 4; shows the results of *C. albicans* after treatment with *P. dactylifera* extract. Changes included; *ERG*1 gene 28 mismatches and 7 gaps (Figure 4A and Figure 4B), *ERG*12 gene 41 mismatches (Figure 4C and Figure 4D), and one mismatch and 7 gaps in the *ERG*25*F* gene (Figure 4E). Figure 5 shows the results for *C. albicans* after treatment with *Z. spina-christi* extract, Changes included; 43 mismatches in the *ERG*1 gene (Figure 5A and Figure 5B), 44 mismatches in the *ERG*12 gene (Figure 5C and Figure 5D), and 13 mismatches and 2 gaps in the *ERG*25*F* gene (Figure 5E). The *TEF*1 gene was more stable, with few changes that could cause mutation or change the gene expression. These results are presented in Figure 6(A-1), Figure 6(A-2), Figure 6(B-1), and Figure 6(B-2); there were 12 mismatches (Figure 6(A-1) and Figure 6(A-2)), and 15 mismatches (Figure 6(B-1) and Figure 6(B-2)) in the *TEF*1 gene after treatment

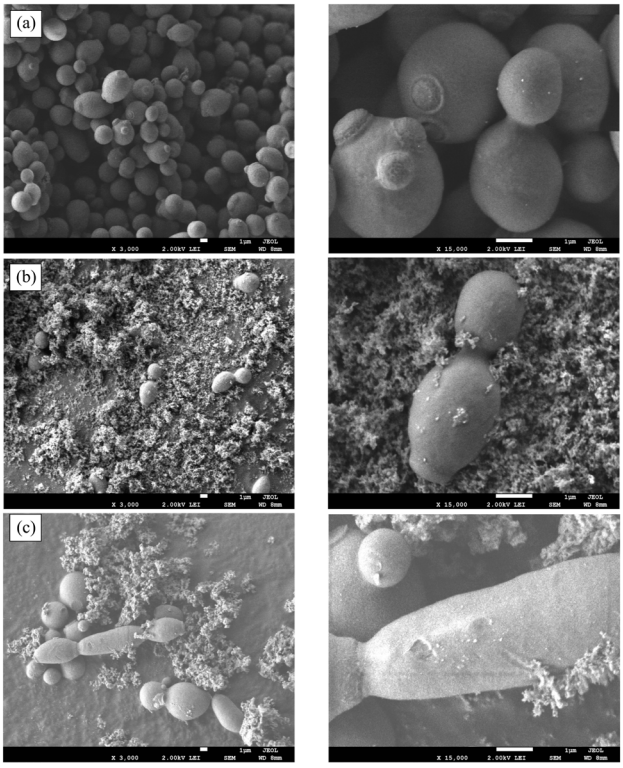

Figure 2. Scanning electron microscopy of *C. albicans* shows the effect of the 150 µl/ml *Z. spina-christi* (b) and *P. dactylifera* (c) extracts on cell wall morphology, compared with untreated cells (a).

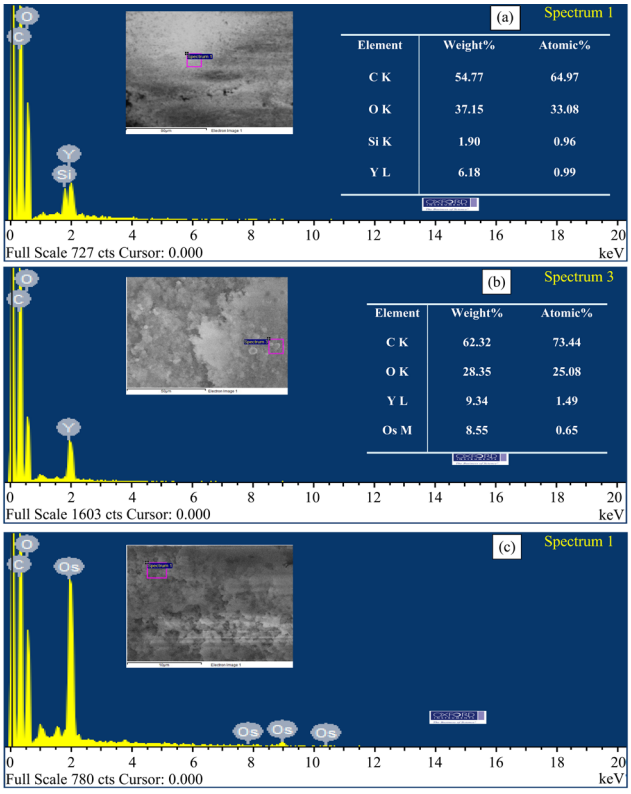

Figure 3. Energy-dispersive X-ray spectroscopy of *C. albicans* shows the effect of the 150 µl/ml *Z. spina-christi* (b) and *P. dactylifera* (c) extracts, compared with untreated cells (a).

Figure 4. *C. albicans* F and R in ERG genes detection after treatment with 150 µl /ml of *P. dactylifera* ethanol extract.

Figure 5. *C. albicans* F and R in ERG genes detection after treatment with 150 µl/ml of *Z. spina-christi* ethanol extract.

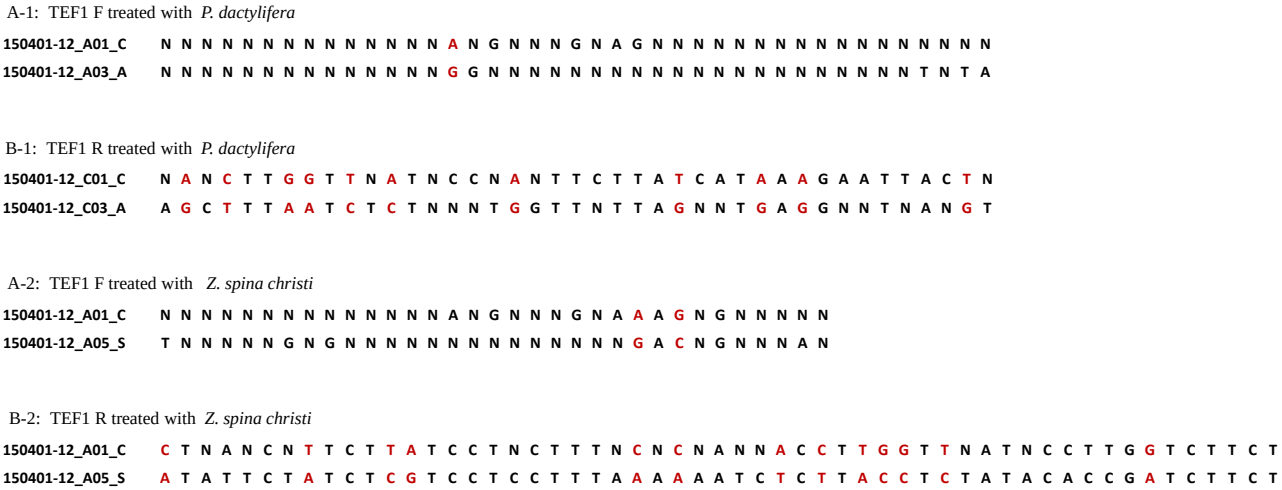

Figure 6. *C. albicans* F and R in TEF1 genes detection after treatment with 150 µl/ml of *P. dactylifera* and *Z. spina-christi* ethanol extracts.

with *P. dactylifera* and *Z. spina-christi* respectively.

4. Discussion

Bioactive compounds in antifungal agents inhibit fungal and yeast growth. These compounds include secondary metabolism components, such as alkaloids, peptides, terpenes, pigments, and sterols [35]. Plant secondary metabolism is influenced by environmental conditions, including temperature, sunlight, and dryness. It is also affected by the presence of organic compounds and essential oils, such as flavonoids, sugar alcohols (sorbitol, ribitol, inositol), soluble sugars (saccharose, raffinose, stachyose, trehalose), and nitrogenous compounds (proline, glycine, betaine) [36].

Extracts of *Z. spina-christi* and *P. dactylifera* have previously been shown to affect *C. albicans* biomass, as reflected by the cells' dry weight and glucose uptake. The decreased dry weight compared to that of untreated cells and increased glucose leakage may be due to the cell wall damage and subsequent sterol leakage from the cell wall [37].

As cellular energy requires an energy source, when glucose uptake provides this energy to the cell, the cell can resist the inhibitory effect of *Z. spina-christi* and *P. dactylifera* extracts. However, when this energy supply is lost, the cell loses the ability to block this inhibition. This explanation is confirmed by the results indicating sterol leakage following exposure to 150 µl/ml extracts and the mismatches found in the *ERG*1, *ERG*12, and *ERG*25 genes as well as the *TEF*1 gene. Our results agreed with the findings from previous research [38] [39].

Damage to the cell wall could be seen in the electron micrographs in our study. The detection of elements in the *Z. spina-christi* and *P. dactylifera* extracts may be attributable to osmotic stress on the cells or failure of cell membrane regulation. The results confirmed those of the EDAX analysis, which revealed a loss of sterols from the treated cells compared with that in the untreated cells. Further, yttrium (Y) was detected in the cells treated with *Z. spina-christi* and *P. dactylifera* extracts, while osmium (OS) was detected in the cells treated with *P. dactylifera*. These results are consistent with those of [40]-[47].

The evaluation of the *Z. spina-christi* and *P. dactylifera* extracts indicates that they may be used to develop novel antibiotics with several mechanisms of action. We found that the crude extracts downregulate several areas of the yeast cell, including the cell wall, cytoplasmic membrane, and genomic DNA. In addition to the development of new drugs, we recommend further study of *Z. spina-christi* and *P. dactylifera* to determine the fractions of the complex extracts that contain the active compounds against *C. albicans* and yeast in general.

5. Conclusion

The results presented in this study demonstrate the importance and promise of the antifungal and antimicrobial activities of *P. dactylifera* and *Z. spina-christi* extracts for novel drug development that inhabited the biochemical activities,

SEM and EDAX analyses, and the expression of *ERG*1, *ERG*12, and *ERG*25 genes of *C. albicans*. Further research into the properties of these extracts is needed to isolate the bioactive compounds, and toxicity testing on mammalian cells or higher eukaryotes is another necessary avenue for research.

Acknowledgements

Funding: This work was supported by the Deanship of Graduate Studies at King Abdulaziz University, Jeddah, Saudi Arabia.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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