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Original Article

The Antifungal Mechanism of Ethanolic and Methanolic Extracts of *Teucrium polium* Against *Candida albicans and Candida parapsilosis*

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Abstract

Background and Aim: The prevalence of drug resistance in fungi reveals the importance of introducing more efficient and less toxic drugs. Given the anti-inflammatory and antimicrobial qualities of *Teucrium polium*, this study aimed to examine the antifungal mechanism of ethanolic and methanolic extracts of this plant against *Candida albicans* and *Candida parapsilosis*.

Materials and Methods: First, the methanolic and ethanolic extracts of *Teucrium polium* were prepared by the Soxhlet method. The antifungal effects of the extracts were determined by the agar well diffusion and MIC methods. Glucose, sodium, and potassium were identified in the tubes with minimum inhibitory concentration by autoanalyzer and flame photometry methods. Moreover, amino acids were measured by HPLC. Yeast cell wall alterations were studied by scanning electron microscopy (SEM).

Results: The MICs of the ethanolic extract of *Teucrium polium* on *Candida albicans* and *Candida parapsilosis* were 125 and 31.25 mg /ml, respectively, while the MICs of the methanolic extract of the plant against the test organism were 125 and 62.5 mg /ml, respectively. For both types of candida spp., ethanolic and methanolic extracts leaked out less sodium, potassium, glucose, and amino acids than amphotericin B did. Utilizing HPLC analysis, the most amino acids in tubes with the minimum inhibitory concentration of *Teucrium polium* extracts were glutamine, threonine, and alanine. SEM showed damage in the cell wall of *Candida albicans* and *Candida parapsilosis*.

Conclusion: This study showed that *Teucrium polium* is a strong source of antifungal compounds. This plant has a mode of action similar to that of amphotericin B on the fungal cell membrane, by creating a pore in the membrane. Furthermore, based on the release rate of glucose, sodium, and potassium, the methanolic extract was more efficient than the ethanolic extract.

Keywords: Antifungal effect, Scanning electron microscopy, High-performance liquid chromatography, *Candida albicans. Candida parapsilosis, Teucrium polium*

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Introduction

In recent years, yeasts and Candida species, particularly the latter, have been the most widespread fungi isolated from human infections, the prevalence of which has grown dramatically over the past two decades (1, 2). Candidiasis is one of the most significant and common diseases of opportunistic fungi in humans. Pathogenic species of Candida include *Candida albicans (C. albicans), C. glabrata, C. tropicalis, C. krusei, C. parapsilosis, C. pseudotropicalis, C. stalactite,* and *C. guilliermondii* (3, 4). The most important one, *C. albicans*, is a natural resident of the gastrointestinal tract, oral mucosa, and vagina (5). Today, resistance of microorganisms to antimicrobial agents is reported worldwide, increasing the cost and length of treatment (6, 7). Thus, currently, researchers are seeking new antimicrobial compounds with fewer side effects (8). Consequently, various plant species were studied in the form of extracts or essential oils for the treatment of fungal infections (9). One of them, Mary Chickpea, or Teucrium with the scientific name of *Teucrium polium* (*T. polium*), is a perennial herbaceous plant. It is the largest genus of the *Lamiaceae* family in the Mediterranean region, with long, slender leaves covered with cotton hairs (10). This plant has over 300 species around the world and approximately 12 species in Iran. Plants of the genus of *Teucrium* have gradually developed in nature through natural hybridization and selection, exhibiting significant changes in their natural habitat, growth traits and characteristics, and aromatic compounds (11, 12). Extracts of this plant include diterpenoids, 7-5 glycosides, 6-methoxy guanine, thymol, carvacrol, and volatile essential oils. Phytochemical research has shown that *T. polium* has traditionally been used in Mediterranean countries for its antispasmodic and hypoglycemic activities. Moreover, this plant has insulinotropic, anti-inflammatory, antimicrobial, and antioxidant properties (12, 13).

Thus, with the proliferation of multidrug-resistant *Candida* species and the declining number of available drugs, it has become necessary to search for new sources of antifungal drugs and compounds that inhibit this resistance (14). Although there are currently several synthetic and natural product-based drugs for the treatment of candidiasis, they are not effective enough in treating pathogenic yeast infections in a coordinated manner. Fungal resistance to most of these drugs has also been reported (15, 16). Hence, there remains the need for new antifungal drugs belonging to a variety of building categories that selectively affect new targets with fewer side effects. The purpose of this investigation was to ascertain how *T. polium* inhibits the growth of *C. albicans* ATCC 10231 and *C. parapsilosis* ATCC 22019.

Materials and Methods

Preparation of Samples

In a descriptive-comparative study, the stems, leaves, and flowers of *T. polium* were purchased from Isfahan Agricultural and Natural Resources Research and Training Center (Herbarium No. 15124) in May 2016. *C. albicans* ATCC 10231 and *C. parapsilosis* ATCC 22019 were investigated in this study.

Preparation of the Extracts

Stems and leaves of the plant were washed and dried at room temperature. Extraction of *T. polium* was done by the Soxhlet extraction method. Briefly, 25 g of the mixture of powders was wrapped in a filter paper and extracted with 250 ml of 80% ethanol and 80% methanol. Then, by removing the solvent, the extracts were dried and their dry weights were measured (17).

Determination of the Minimum Inhibitory Concentration (MIC) and the Minimum Fungicidal Concentration (MFC) of Alcoholic Extracts of *T. polium*

According to the procedure of the approved Macro-dilution method of antifungal susceptibility testing (M-27-A) of the NCCLS, MIC and MFC were determined for Candida spp. (106 CFU/ml) (18).

To prepare the serial dilution of the extracts, 2 ml of Sabouraud Dextrose Broth (SDB) (Merck, Germany) was added to 5 sterile tubes. Then, one gram of the extract was dissolved in 4 ml of 10% dimethyl sulfoxide to dilute 250 mg/ml. From this sample, 2 ml was added to the next tube containing 2 ml of SDB. The dilution of the second tube was equal to 125 mg/ml by repeating this operation, and serial dilutions of 31.25, 62.5, 125, and, 250 mg/ml were prepared. The tested fungi were cultured on Sabouraud Dextrose Agar (SDA) (Merck,

Germany). A fungal suspension with a turbidity of 0.5 McFarland was prepared from the grown colonies. After adding 20 µl of the fungal suspension, the tubes were incubated for 48 h at 35°C. Subsequently, the turbidity of the tubes and the growth of the fungi were evaluated visually in comparison with the controls. The tube with the lowest concentration of the extract that had no fungal growth, and no turbidity was considered for the MIC. To determine the MFC, 20 µl of MIC wells and 2 wells before were then cultured on SDA and incubated at 35°C for 24 h (19).

Determination of the Susceptibility of the Fungi to the Extracts Using the Well Diffusion Method

For this purpose, in SDA agar medium two wells were created at a distance of 20 mm from each other and 100 µl of each extract was poured into the wells separately. Subsequently, 20 µl of yeast (0.5 McFarland grade) was cultured on medium. After 48 hours of incubation at 25-27°C, the presence or absence of a growth inhibition zone around the wells was investigated (19).

Scanning Electron Microscopy (SEM)

The anti-candida activities of the ethanolic and methanolic extracts of *T. polium* against *C. albicans* ATCC 10231 and *C. parapsilosis* ATCC 22019 were investigated using SEM observations at a concentration of 250 mg/ml in *in vitro* conditions. For this purpose, the bacteria were adjacent to the extract, and the sample was centrifuged at 2000 rpm for four minutes. The centrifuged sample and its supernatant were discarded again. Gluter aldehyde precipitate of 5.2% in the sample buffer was sent to the Biochemistry-Biophysics Research Center of University of Tehran and the Iranian Research Organization for Science and Technology (IROST).

Determination of the Presence of Sodium, Potassium, and Glucose

The presence of sodium and potassium was determined quantitatively by flame photometry using standard solutions (20). Glucose level in the extracts was measured using the auto analyser (BT-3000) as explained by the manufacturer (21).

Quantitative Measurement of Amino Acids in the Fungal Solution by HPLC

We used high-performance liquid chromatography (HPLC) developed by Knauer Azura, Germany. We used a UV detector at a wavelength of 254 nm at room temperature and an isocratic system with a solvent flow rate of 1mm/min. Each sample was injected three times. The steps included preparing the mobile phase, preparing the buffer solution to dilute the sample, and preparing the amino acid extract from the fungal solution, to which 15 ml of distilled water and 4 ml of the buffer solution were added. Then, the solution was poured into the tubes and centrifuged at 30 rpm for 30 minutes. The high clear solution was passed through a 0.0.45-microfilter and collected in a separate container, to be used for injection into the HPLC machine (22, 23).

Statistical Analysis

The results were analyzed using SPSS version 19 by Mann-Whitney and Kruskal-Wallis test. A significant level of $p > 0.05$ was used to interpret the data.

Results and Discussion

The Anti-Candida Effect of the Ethanolic and Methanolic Extracts of *T. polium* by Agar-Well Diffusion Method

The mean diameter of the growth inhibition zone of the ethanolic and methanolic extracts of the stem and leaves of *T. polium* at concentrations of 250, 125, 62.5, and 31.25 mg/mm against *C. albicans* and *C. parapsilosis* has been shown in Table 1. The mean diameter of the growth aura increased with as the concentration increased. The mean halo diameter at four concentrations of the extract was larger than the negative control and smaller than the positive control. The Kruskal-Wallis test was used to compare the diameter of the growth inhibition zone between the four concentrations of the extracts and positive as well as negative controls in the two *Candida* species. In both *Candida* species, a noticeable distinction was observed between the diameter of the no-growth halo at various concentrations and positive as well as negative controls ($P \leq 0.05$). A comparison of no-growth halo diameters of 31.25 and 6 mg/ml extracts against *C. albicans* and *C. parapsilosis* revealed no significantly difference ($P \leq 0.05$). Moreover, the diameter of the growth inhibition zone at 31.25 and 62.5 mg/ml was significantly lower than the concentrations of 125 and 250 mg/ml≤ 0.05). Furthermore, comparing the diameter of the ethanolic extract growth inhibitor zone between the two *Candida* species using the Mann-Whitney test

Table 1: The Mean and Standard Seviation (M ± SD) of the Growth Inhibition Zone of *T. polium* extracts against *C. albicans* and *C. parapsilosis*.

Extracts	Concentration (mg / ml)	*Candida parapsilosis* ATCC 22019(M±SD)	*Candida albicans* ATCC 10231 (M±SD)
Ethanolic	31.25	13.00±2.00	10.33±1.53
	62.5	15.67±1.15	13.00±1.00
	125	18.00±1.73	15.33±0.58
	250	20.00±1.00	18.00±1.00
Methanolic	31.25	15.33±0.58	00.00±1.14
	62.5	18.33±0.58	00.00±1.16
	125	20.67±0.58	15.67±1.18
	250	23.00±1.00	21.00±1.00
Positive control	-	45.23±0.00	38.21±0.00
Negative control	-	0.00±0.00	0.00±0.00

indicated that there was a significant difference between the halo diameter of the two *Candida* species (P≤0.05) only at 62.5 and 125 mg/ml. The halo in *C. parapsilosis* was significantly larger than the diameter of the halo in *C. albicans*.

Determination of MIC and MFC

The mean MIC and MFC results of each of the ethanolic and methanolic extracts of *T. polium* against C. *albicans* and *C. parapsilosis* have been shown in Table 2.

Table 2: MIC and MFC of *T. polium* Extracts against *C. albicans* and *C. parapsilosis*.

Type of Extract	*Candida* Spices	MFC (mg/ml)	MIC (mg/ml)
Ethanolic	*Candida albicans* ATCC 10231	250	125
Methanolic	*Candida albicans* ATCC 10231	250	125
Ethanolic	*Candida parapsilosis* ATCC 22019	125	62.5
Methanolic	*Candida parapsilosis* ATCC 22019	62.5	31.25

Evaluation of Sodium, Potassium, and Glucose in the Ethanolic and Methanolic Extracts of *T. polium*

The amount of released sodium and glucose of *C. albicans* and *C. parapsilosis* in the methanolic extract of *T. polium* was higher than that of the ethanolic extract (P <0.05) and lower than Amphotericin B (Table 3). A comparison of sodium and glucose levels in the ethanolic and methanolic extracts and Amphotericin B in two *Candida* species by the Kruskal-Wallis test revealed significant differences between sodium and glucose levels in the three groups in *C. albicans* and *C. parapsilosis* (P ≤0.05). Released glucose and sodium levels for *C. albicans* were significantly lower than *C. parapsilosis* (P ≤0.05).

Qualitative Identification and Quantitative Measurement of Amino Acids in Fungal Solutions by the Ninhydrin Method and HPLC

The resuls of the comparison of amino acid concentrations in *C. parapsilosis* and *C. albicans* against the methanolic extract and amphotericin B (positive control) have been shown in Tables 4-7. In this study, the amount of each amino acid was determined by HPLC for (1) amphotericin B as the positive control, (2) *T. polium* and fungus as the treatment group, and (3) *T. polium* as the negative control. To confirm the damage caused by the *T. polium* on yeast, the amount of each amino acid related to extracts of *T. polium* was subtracted from the sample of *T. polium* plus fungus.

Scanning Electron Microscopy

Figure 1 illustrates SEM images of the damage against *C. albicans* and *C. parapsilosis* by the ethanolic and methanolic extracts of *T. polium*. The extract caused the cell surface to shrink and created holes in the yeast. Dispersion of yeast cells was also observed. A form of damage to yeast was also shown by amphotericin B, with more shrinkage and perforation, but the cells were still densely packed.

Some plant species display an antifungal activity which is considered a treatment option. This activity also involves preventing the spread of resistance and speeding up the treatment (24).

As a polyene antifungal, amphotericin B binds to ergosterol in fungal cell membranes, creating holes in the membrane, and allowing cell constituents to leak out, which leads to cell death. In this study, the antifungal mechanism of the ethanolic and methanolic

Table 3: The Mean and Standard Deviation (M ± SD) of Sodium and Glucose Contents in Extracts of *T. polium* against *C. albicans* and *C.parapsilosis*.

	Mean and standard deviation (M ± SD) of sodium content		**Mean and standard deviation (M ± SD) of glucose content**	
Type of Extract	***Candida parapsilosis* ATCC 22019 (M±SD)**	***Candida albicans* ATCC 10231 (M±SD)**	***Candida parapsilosis* ATCC 22019(M±SD)**	***Candida albicans* ATCC 10231 (M±SD)**
Ethanolic	19.00±1.00	14.33±2.08	213.67±10.21	184.00±12.49
Methanolic	25.33±1.53	19.00±2.65	220.33±7.02	193.00±3.61
Amphotericin B	51.33±3.51	44.67±1.53	291.67±3.06	279.67±3.06

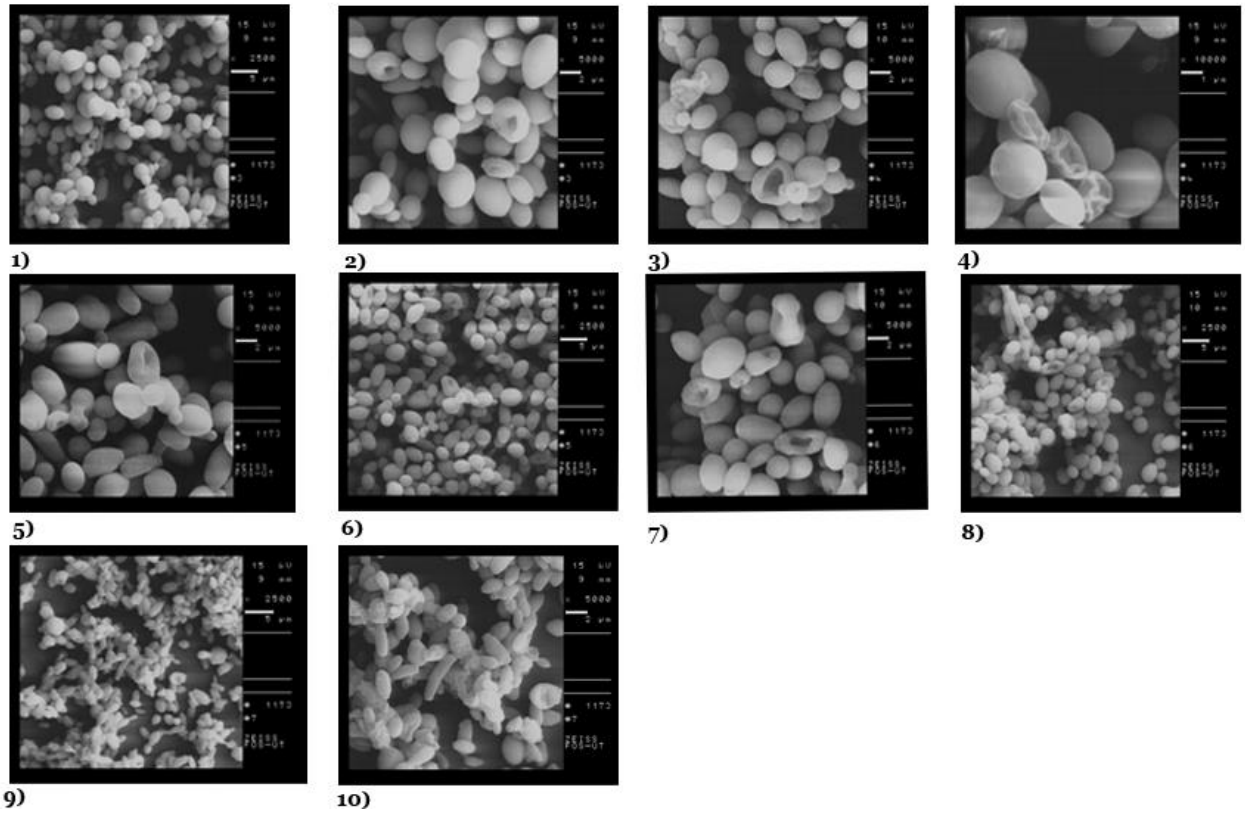

Figure 1. Damage caused to *C. albicans* and *C. parapsilosis* by *T. polium* extracts shown by SEM for: (1) and (2) the methanolic extract on *C. albicans*, (3) and (4) the ethanolic extract on *C. albicans*, (5) and (6) the methanolic extract on *C. parapsilosis*, (7) and (8) the ethanolic extract on *C. parapsilosis*, *and,* (9) and (10) amphotericin B on C. albicans.

extracts of *T. polium* against *C. albicans and C. parapsilosis* was investigated towards the Amphotericin B antifungal mechanism. The MIC of the methanolic extract on *C. parapsilosis* and *C. albicans* was 31.25 and 125 mg/ml, respectively, and the MIC of the ethanolic extract was 62.5 and 125 mg/ml, respectively, which indicates a notably better effect of the *T. polium* methanolic extract on *C. parapsilosis*. For both *candida spp.*, some sodium, potassium, glucose, and amino acids in the ethanolic and methanolic extracts were exposed, though less than amphotericin B. According to the results of SEM,

effects of the ethanolic and methanolic extracts of *T. polium* were similar to those of Amphotericin B.

Studies by other researchers have also indicated the inhibitory effect of the ethanolic and methanolic extracts of *T. polium* on various microorganisms. The effects of the antibacterial activity of 21 species of *Teucrium*, including *T. polium*, on Gram-positive bacteria as higher than that of Gram-negative bacteria were described in a study conducted by Orhan who reported the antifungal and bacterial activities of this plant associated with phenolic compounds and tranpoids (25). Darabpour *et al.* showed that the ethanolic extract at 400 and 200 mg/ml and the methanolic extract at 400 and 600 mg/ml exhibited high antibacterial activity. Moreover, the ethanolic extract at 400 mg/ml and the methanolic extract at 600 mg/ml inhibited the growth of Gram-negative bacteria. *T. polium* was effective on *Proteus mirabilis* only at 10 mg/ml of ethanolic extract. They explained that this resistance was due to the permeability of the cell membrane (26). In a study conducted by Belmekki *et al.*, the essential oil of *T. polium* was investigated against Gram-positive, Gram-negative bacteria, and fungi at different concentrations, and its antimicrobial properties were reported. Due to hydrophobicity, these essential oils and their components may break down cell membrane lipids, making cell membranes and mitochondria more permeable. Consequently, the leakage of ions and cell contents might occur (27). Nadimi *et al.* indicated that the essential oil of *T. polium* had an inhibitory effect on most Gram-positive and Gram-negative bacteria and could be more influential than gentamicin. According to the findings, aqueous and ethanolic extracts of *T. polium* do not affect *Malassezia furfur* and *Malassezia globosa* (28). Based on the findings of this study, it seems that the aerial parts of *T. polium* are strong sources of antifungal agents. Sevindik *et al.* reported the inhibitory activity of *T. polium* on methicillin-resistant *Staphylococcus aureus* (MRSA), *Bacillus cereus*, *Escherichia coli*, and *Pseudomonas aeruginosa* (29). Moreover, Akin *et al.* confirmed the antimicrobial effect of essential oils of some species belonging to the growing *Lamiaceae* family, including *T. polium*, against *Staphylococcus aureus* and *Bacillus cereus* (30). The antimicrobial effect of *T. polium* in Iran was compared with the rest of the world by Mahmoudi *et al.* (30), who showed that *T. polium* essential oils had a noticeable effect against *Salmonella typhimurium*, which is a significant determinant in food poisoning.

Table 4: Amounts of Amino Acids Released from *C. parapsilosis* in the Methanolic Extract and Amphotericin B *(mol/L)* Obtained Using HPLC.

Amino acid	Amphotericin B (Positive control)	Methanolic extract (250mg/ml) and *C. parapsilosis* (Treatment)	Methanolic extract (mg/ml) (negative control)	Differences between treatment and methanolic extract
Aspartic acid	216	124	24	100
Glutamic acid	463	315	152	163
Serine	261	92	50	42
Glutamine	3124	1619	1606	13
Glycine	463	178	156	22
Alanine	230	71	62	9
Tyrosine	544	206	88	118
Methionine	149	95	86	9
Valine	156	62	48	14
Phenylalanine	310	399	101	298
Isoleucine	230	112	84	28
Leucine	214	82	72	10

Table 5: Amounts of Amino Acids Released from *C. albicans* in the Methanolic Extract and Amphotericin B (mol/L) Obtained Using HPLC.

Amino acid	Amphotericin B (Positive control)	methanolic extract (250mg/ml) and *C. parapsilosis* (Treatment)	methanolic extract (mg/ml) (negative control)	Differences between treatment and methanolic extract
Aspartic acid	216	26	11	6
Glutamic acid	463	386	279	107
Serine	261	81	62	19
Glutamine	3124	2253	2118	135
Glycine	463	353	290	63
Threonine	230	36	20	16
Alanine	544	274	248	26
Tyrosine	149	61	52	9
Methionine	156	48	35	13
Valine	310	214	160	54
Phenylalanine	230	90	81	9
Isoleucine	213	63	53	10
Leucine	271	86	64	20

Thus, distinctions in the results of different geographical areas might be due to differences in plant collection location, collection time, and plant variety, differences in methods such as extraction methods, differences in the quality or composition of plant species, or distinctions in environmental conditions and genetic changes (31). Talib *et al.* investigated the effect of *T. polium* extract on bacteria, *C. albicans*, and *Aspergillus niger* (32). Moderate antibacterial and antifungal activities of *T. polium* were also reported by Lograda *et al.* who examined it in eastern Algeria in 2014 (33). Vahdani *et al*. indicated that the essential oils of *T. polium* in Iran had antimicrobial activity against *Bacillus cereus* and *Staphylococcus aureus, Bacillus subtilis, Micrococcus luteus*, and *Paracoccus pantotrophus* , while they did not affect *Escherichia coli* (34). Alreshidi *et al.* also reported the antifungal activity of the methanolic extract of *T. polium* against *C. albicans* ATCC 10231, *C. vaginalis*, and *C. neoformans* ATCC 14116, and its low activity against filamentous fungi, including *A. fumigatus* ATCC 204305 and *A. niger*p in Saudi Arabia (35).

Hence, the results of the present research are consistent with previous studies regarding the antibacterial and antifungal impacts of *T. polium*. According to the the results of this research, ethanolic and methanolic extracts of *T. polium*, sampled in Iran, could exhibit antifungal properties against *C. albicans* and C. *parapsilosis*.

This might be explained as follows. The mentioned extracts somehow cause the formation of bowl-shaped depressions and folds on the inner surface of the membrane, increasing the permeability of the membrane and causing the leakage of monovalent ions from the width of the membrane. Destruction of the fungal cell wall leads to the leakage of cellular contents and the death of the fungal cell. Effectiveness of these compounds depends on the dose and time of their activation. The release of limited amounts of these substances may be tolerable for the bacterium, though it is effective on its bioavailability. The release of large amounts of cellular contents or the release of vital ions and molecules could lead to cell death. At a concentration of 250 mg/ml, we saw a greater increase in the diameter of the stunted halo and the concentration of sodium, potassium, and glucose compared with

Table 6: Amounts of Amino Acid Released from *C. parapsilosis* in the Ethanolic Extract and Amphotericin B (mol / L) Obtained Using HPLC.

amino acid	amphotericin B (Positive control)	methanolic extract **(250mg/ml) and** *C. parapsilosis* **(Treatment)**	methanolic extract(**mg/ml**) **(negative** control)	Differences between treatment and methanolic extract
Aspartic acid	237	17	11	6
Glutamic acid	343	296	279	17
Serine	261	73	62	11
Glutamine	2287	2181	2118	63
Glycine	487	330	290	40
Threonine	273	31	20	9
Alanine	285	263	248	15
Tyrosine	149	100	52	48
Methionine	99	57	35	22
Valine	306	194	160	34
Phenylalanine	244	102	81	21
Isoleucine	233	70	53	17
Leucine	263	73	64	9

lower concentrations. As a result, a longer time span is needed to produce a similar antifungal effect at lower concentrations. Although *T. polium* has been used orally for a long time, its medicinal properties have not been seriously considered. The results obtained in this study revealed the significance of this medicinal plant. Further studies are required to evaluate the effects of other extracts such as aqueous, acetone, and hexane extracts of *T. polium* on *Candida* or other yeast species. Moreover, analysis of the active compounds of *T. polium* and determination of the relationship between these compounds and their antifungal effect can be helpful for their utlization as drug alternatives.

Conclusion

This study revealed that the ethanolic and methanolic extracts of *T. polium* could exhibit antifungal effects against *C. albicans* and *C. parapsilosis* by causing changes in the cellular structure of these fungi. Furthermore, it was found that this plant could act with the same mechanism as Amphotericin B, which is an antifungal drug. It creates holes in the cell membrane of the fungus that releases glucose, amino acids, sodium, and potassium from the fungus, leading to the death of the fungus. Consequently, the results can pave the way for more comprehensive research on the use of this plant to enhance and expedite the treatment of certain fungal infections.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Table 7: Amounts of Amino Acid Released from *C.albicans* in the Ethanolic Extract and Amphotericin B (mol / L) Obtained Using HPLC.

Amino acid	**A**mphotericin B (Positive control)	**M**ethanolic extract **(250mg/ml) and** *C. parapsilosis* **(Treatment)**	**Methanolic extract (mg/ml) (negative** control)	Differences between treatment and methanolic extract
Aspartic acid	237	17	11	6
Glutamic acid	343	296	279	17
Serine	261	73	62	11
Glutamine	2287	2181	2118	63
Glycine	487	330	290	40
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Alanine	285	263	248	15
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Valine	306	194	160	34
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Isoleucine	233	70	53	17
Leucine	263	73	64	9

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