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# Antifungal Activity of Tea Tree Oil Against *Candida albicans* Isolated from Oral Samples

Antifungal Activity of Tea Tree Oil Against *Candida albicans* Isolated from Oral Samples

Murtadha Jasim Jafar[1], Majid Kadhim Al-Alshibly [2]

[1, 2] Department of Biology, College of Education, University of Al-Qadisiyah, Al-Diwaniyah, Iraq.
*Corresponding author email: bio.edu.posta24.49@qu.edu.iq

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**Abstract**
Candida albicans is among the commonest causes of oral candidiasis, a disease that is mostly related to the mouth. The growing resistance of Candida species to traditional antifungal drugs has highlighted that the development of useful non-pharmacological options is needed. The purpose of this study was to isolate and identify Candida albicans in the oral and peri-dental samples and to test the antifungal effects of Tea Tree Oil as a natural antifungal agent.
One hundred and forty-six samples in total of oral and peri-dental samples were collected and grown in Sabouraud Dextrose Agar. Chromogenic Candida Agar and microscopic analysis were done to identify phenotypes which were further confirmed by VITEK 2 Compact system. Tea Tree Oil was chemically characterized by Gas Chromatography- Mass Spectrometry and Fourier Transform Infrared spectroscopy. The agar well diffusion method was used in the evaluation of antifungal activity and also the minimum inhibitory concentration and minimum fungicidal concentration.
Out of 146 collected samples, 53 (36.3%) showed positive growth for *Candida* species. Among these isolates, *Candida albicans* was the predominant species, identified in 36 samples (67.9% of positive isolates).
The gas chromatography analysis indicated that the terpinen-4-ol was the most significant constituent of Tea Tree Oil, and infrared analysis proved that the functioning groups of bioactive terpene compounds were present. The dose dependent antifungal effect of Tea Tree Oil was demonstrated with inhibition zones that matched with the traditional antifungal agents.

**Keywords:** Candida albicans; Tea Tree Oil; Antifungal activity; GC -MS.

## 1. Introduction

One of the most prevalent opportunistic fungal infections of the oral cavity is oral candidiasis, which is common in immunocompromised persons including diabetics, a cancer patient, denture wearers and patients on long-term antibiotic or corticosteroid therapy [1-4]. Candida albicans is still the most common etiological agent of oral candidiasis due to its capacity to adhere to the cells of the oral epithelium, to develop biofilms and to modify morphogenesis transitions to increase the virulence [5–7].

Azoazoles and polyenes are the most common antifungal agents that have been used in the treatment of oral candidiasis, but the development of anti-fungal resistance, undesired side effects and repeat infections have increasingly undermined their effectiveness [8-10]. Reports on other parts of the world such as in the Middle East have also recorded decreased susceptibility of C. albicans to the commonly used antifungals, thus necessitating alternative therapeutic approaches [11–13].

The natural products have received significant interest as possible sources of new antifungal agents because of their diversification in chemistry, biological activity, and relatively low toxicity [14–16]. Of these, essential oils have shown a wide-spectrum antimicrobial effect, and especially when it comes to fungal pathogens [17,18]. One of the most studied essential oils is Tea Tree Oil (TTO) which is a derivative of the leaves of Melaleuca alternifolia, which is characterized by strong antimicrobial, anti-inflammatory, and antioxidant properties [19-21].

Antifungal properties of Tea Tree Oil have been credited to its abundance in monoterpenes and oxygenated monoterpenes especially terpinen-4-ol, which have been reported to interfere with the enzymatic activity, disrupt fungal cell membranes and form biofilms [22-24]. A number of in vitro experiments have established the effectiveness of Tea Tree Oil against Candida species even in the presence of species which are resistant to standard antifungal agents [25-27]. In addition, Tea Tree Oil chemical characterization methods have been extensively used to associate the biological activity with the chemical composition of the oil through such methods as Gas ChromatographyMass Spectrometry (GCMS) and Fourier Transform Infrared Spectroscopy (FTIR) [28-30].

Although the evidence of Tea Tree Oil antifungal activity is increasingly growing worldwide, there are yet to be found extensive data on the activity of Tea Tree Oil against oral Candida albicans isolates used by Iraqi patients. Thus, more research into its efficacy is justified to assess it with well-characterized clinical isolates and standardized methods of analysis.

The objective of the current research was to isolate and identify Candida albicans in oral and peri-dental samples, chemically profile Tea Tree Oil with the help of GC-MS and FTIR, and to determine its antifungal properties against identified isolates through agar well diffusion, MIC and MFC tests.

## 2. Materials and Methods

### Specimen Collection

A total of 146 oral and peri-dental swab samples were collected from patients attending hospitals in the Baghdad Governorate. Specimens were obtained aseptically using sterile cotton swabs and were immediately transported to the microbiology laboratory for further mycological investigations.

### Ethical Approval

The study protocol was approved by the Institutional Research Ethics Committee of the College of Education, University of Al-Qadisiyah (Approval No: 231). Written informed consent was obtained from all participants prior to sample collection in accordance with the Declaration of Helsinki.

### Primary Culturing, Isolation and Purification.

All the specimens were inoculated on the Sabouraud Dextrose Agar (SDA) plates and incubated at 37 o C during a period of 24-48 h. Colonies with creamy appearance, smooth surface and distinct margins were observed after incubation that were yeast-like. Pure isolates were done by picking out single colonies and subculturing the colonies several times on new SDA plates. The isolates were then purified and subjected to phenotypic, biochemical identification.

### Phenotypic Identification

The purified isolates were put on Chromogenic Candida Agar and incubated at 37 o C with a period of 24-48 hrs. Identification was done presumptively using color and morphological characteristics of colonies.
To analyze the shape of the yeast cell and the pattern of budding under the light microscopy, the gram staining was conducted.

**Identification with VITEK 2 Compact System.**
Ten randomly selected isolates representing different chromogenic patterns were confirmed using the VITEK 2 Compact system (BioMérieux, France). The selection was performed to validate the phenotypic identification results. Due to resource limitations and the high cost of automated identification cards, only representative isolates were subjected to automated confirmation. The remaining isolates were identified presumptively based on colony morphology on Chromogenic *Candida* Agar and microscopic examination.
Yeast suspensions were prepared in sterile saline and adjusted to 0.5 McFarland standard. YST identification cards were inoculated and incubated automatically, and results were interpreted using VITEK 2 software.

**Preparation of Tea Tree Oil**
In this paper, Tea Tree Oil (Melaleuca alternifolia) was applied as a natural antifungal agent. The antifungal testing of the oil was performed in the correct form prepared. The oil had good physical characteristics such as homogeneity and stability making it capable of being utilized in the further experimental procedures.

**GC–MS Analysis**
Tea Tree Oil had its chemical composition determined by Gas Chromatography-Mass Spectrometry (GC-MS). Analysis was done through a GCMS-QP2010 Plus system (Shimadzu, Japan) with an HP-5MS capillary column (30 m long × 0.25 mm internal diameter × 0.25 u thickness) analysis.
The carrier gas was helium and was maintained at the flow rate of 1.0 mL/min. This injection was performed in split mode, and the temperature of the injector was kept at 250 o C. The initial temperature program of the oven was 80 o C, followed by a gradual increase to 300 o C at which the adequate holding time was adjusted. The mass spectra were taken in electron ionization (EI) mode at 70 eV with a scan range of m/z 40-800. The compounds were identified by matching the mass spectra obtained to the mass spectral library in NIST.

**FTIR Analysis**
The functional groups that were present in the Tea Tree Oil were determined using Fourier Transform Infrared (FTIR) spectroscopy. FTIR analysis was performed with the help of the FTIR spectrophotometer (Shimadzu, Japan). The characteristics were collected in the infrared spectrum recorded at 4000400 cm -1 allowing identification of the characteristic absorption bands of the chemical constituents of the oil.

**Antifungal Activity Assays**
To determine the true and valid assessment of Tea Tree Oil to inhibit Candida albicans, the antifungal activity of the oil against Candida albicans isolates was assessed using various in vitro techniques.

**Agar Well Diffusion Method**
The antifungal property of Tea Tree Oil was first determined by agar well diffusion method. Candida albicans suspensions were placed on Sabouraud Dextrose Agar plates in standardized working suspensions. The agar was then prepared and the wells loaded with various concentrations of Tea Tree Oil. Plates were incubated at 37 o C, 24-48 h later the diameter of inhibition zones was measured in millimeter.

**Determination of Minimum Inhibitory Concentration**

The MIC was determined using the broth microdilution method according to CLSI guidelines (M27-A3). Sabouraud Dextrose Broth was used as the test medium. Serial two-fold dilutions of Tea Tree Oil were prepared using 0.5% Tween 80 as a solubilizing agent to enhance oil dispersion. Each concentration was tested in triplicate. Fungal inoculum was adjusted to 0.5 McFarland standard (approximately $1 \times 10^6$ CFU/mL). MIC was defined as the lowest concentration that showed no visible fungal growth after incubation at 37°C for 24–48 hours. Ketoconazole was used as a positive control.

**Determination of Minimum Fungicidal Concentration (MFC)**

For MFC determination, aliquots from wells showing no visible growth were subcultured onto Sabouraud Dextrose Agar plates and incubated at 37°C for 24–48 hours. The lowest concentration that showed no colony growth was recorded as the MFC value.

**Statistical Analysis**

All experiments were performed in triplicate, and results were expressed as mean ± standard error (SE). Statistical analysis was conducted using one-way analysis of variance (ANOVA) followed by the Least Significant Difference (LSD) post hoc test. A P-value $< 0.05$ was considered statistically significant.

## 3. Results

**Separation, Culturing, and Preliminary Identification of *Candida* spp. from Oral Samples**

A total of 146 oral and peri-dental swab samples were collected from patients attending hospitals in the Baghdad Governorate. All specimens were inoculated onto Sabouraud Dextrose Agar (SDA) and incubated under appropriate conditions to permit fungal growth. After incubation, 53 samples (36.3%) demonstrated yeast growth, characterized by smooth, creamy to whitish colonies with distinct margins suggestive of *Candida* species (Figure 1A), whereas 93 samples (63.7%) showed no growth of *Candida*.

Pure cultures were obtained by subculturing individual colonies onto fresh SDA plates. This purification step was necessary to eliminate mixed growth and to ensure accurate phenotypic and biochemical identification. For preliminary species differentiation, purified isolates were subsequently cultured on Chromogenic *Candida* Agar (Figure 1B). The chromogenic medium produced characteristic colony color patterns, allowing presumptive differentiation of *Candida* species. Among the positive isolates (n = 53), *Candida albicans* was the predominant species, isolated from 36 samples (24.7% of total samples; 67.9% of positive isolates), followed by *Candida krusei* (12 samples; 8.2% of total samples; 22.6% of positive isolates) and *Candida tropicalis* (5 samples; 3.4% of total samples; 9.4% of positive isolates). These findings demonstrate the usefulness of chromogenic media as a rapid presumptive identification method based on colony color and morphology.

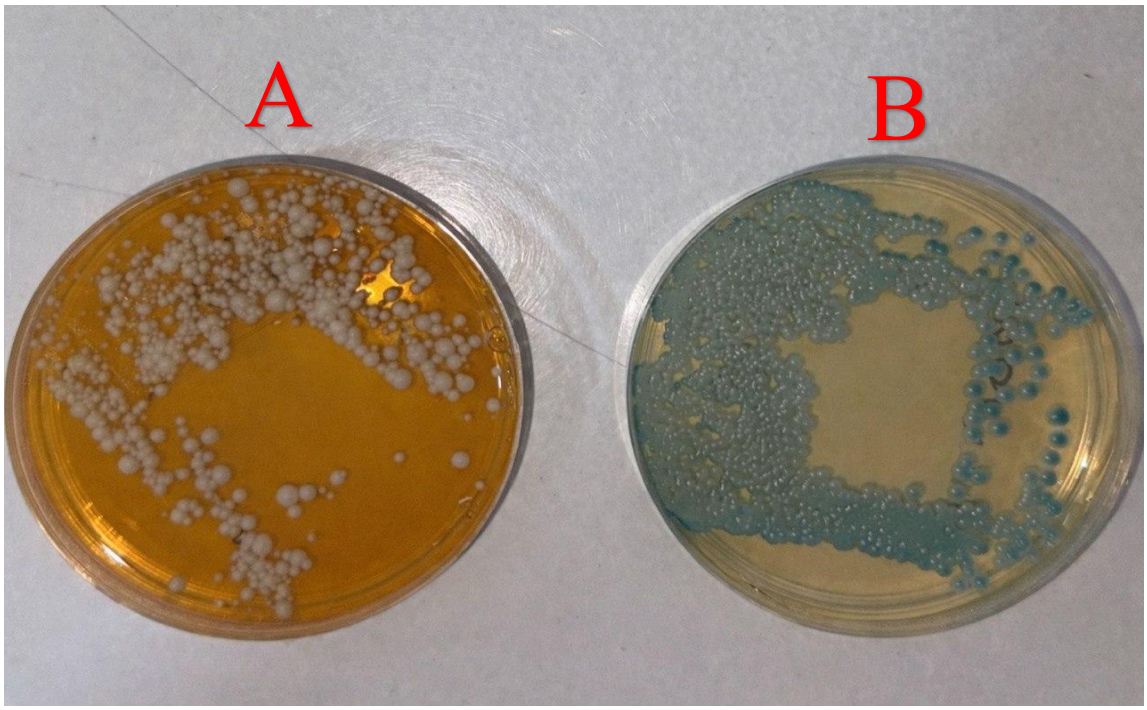


Figure (1) presents representative images of purified *Candida* isolates, showing their macroscopic appearance on Sabouraud Dextrose Agar (A) and the corresponding colony coloration on Chromogenic Candida Agar (B).

**Candida Isolates: Microscopic Analysis.**

Gram-stained smears prepared off purified isolates under microscopic observation displayed oval to spherical yeast cells which were observed in single forms as well as in budding forms; both of which are typical of Candida species. The purity of the cultures was proved by the absence of bacteria cells on the smears under investigation (Figure 2).

The results obtained with the help of these microscopic observations were additionally supportive of the findings made with the help of cultural features on Sabouraud Dextrose Agar and chromogenic identification, which strengthened the correct identification of Candida isolates.

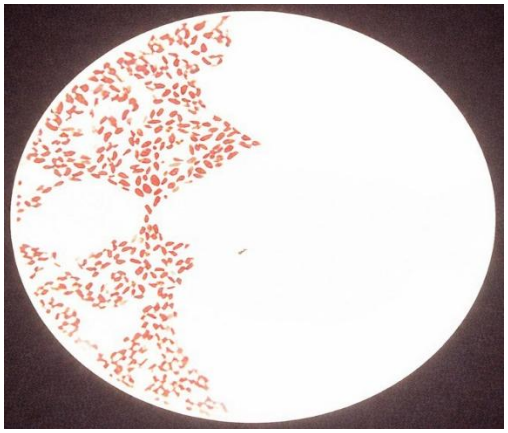

Figure 2 illustrates the typical Gram-stained appearance of *Candida* cells observed under light microscopy.

**Determination of Candida Isolates with VITEK 2 Compact System.**

Final identification of ten representative Candida isolates was done through VITEK 2 Compact system. These findings indicated that the isolates (100% of them) were all detected as Candida albicans. Probabilities of identifying were 94-99% with which the accuracy of the results is highly confident (Table 1).

Some slight differences in some biochemical reactions were also seen in some of the tested isolates but these did not influence the eventual species identification. The findings demonstrated by the VITEK 2 Compact system were in good consensus with those of the phenotypic characterization, microscopic and chromogenic media identification.

Table (1) provides a summary of the detailed identification results achieved with the VITEK 2 Compact system.

| Isolate No. | Identified organism | Probability (%) | Status |
|---|---|---|---|
| 1 | *Candida albicans* | 99 | Final |
| 2 | *Candida albicans* | 99 | Final |
| 3 | *Candida albicans* | 99 | Final |
| 4 | *Candida albicans* | 99 | Final |
| 5 | *Candida albicans* | 98 | Final |
| 6 | *Candida albicans* | 99 | Final |
| 7 | *Candida albicans* | 94 | Final |
| 8 | *Candida albicans* | 95 | Final |
| 9 | *Candida albicans* | 96 | Final |
| 10 | *Candida albicans* | 94 | Final |

**Natural Alternative that was used in the Research.**

In the current research, Tea Tree Oil was employed in the natural substitute to determine the antifungal activity of the compound on Candida albicans. Tea Tree Oil is an essential oil derived out of the leaves of Melaleuca alternifolia and is commonly renowned with its complex chemical structure and extensive biological functions specifically its antimicrobial/antifungal action.

Tea Tree Oil has been chosen in this study as one of the possible antifungal agents that could be studied as having an inhibitory action against Candida albicans isolates found in oral samples.

**Tea tree oil extract Preparation.**

Tea Tree Oil (Melaleuca alternifolia) was purchased from a local commercial supplier in Erbil, Kurdistan Region, Iraq. The product was labeled as 100% pure essential oil. The oil was stored in dark amber glass bottles at 4°C until use to preserve chemical stability and minimize oxidation. Prior to antifungal assays, the oil was equilibrated to room temperature and homogenized to ensure uniform dispersion.

**GC–MS Results Description**

GC–MS analysis revealed seven major compounds in Tea Tree Oil (Table 2). Terpinen-4-ol was the predominant component (53.24%), followed by γ-terpinene (27.36%) and terpinolene (11.54%). Minor constituents included α-pinene (0.30%), 1,8-cineole (1.89%), terpinen-4-ol acetate (2.73%), and α-terpineol (2.45%). The chemical profile is consistent with previously reported compositions of Melaleuca alternifolia essential oil and supports its antifungal activity.

Table (2). Significant organic compounds found in Tea Tree Oil using GC 1.

| Peak No. | Retention Time (min) | Compound Name | Molecular Formula | Area % |
|---|---|---|---|---|
| 1 | 3.516 | α-Pinene | $C_{10}H_{16}$ | 0.30 |
| 2 | 4.809 | Terpinolene | $C_{10}H_{16}$ | 11.54 |
| 3 | 5.071 | Eucalyptol (1,8-Cineole) | $C_{10}H_{18}O$ | 1.89 |
| 4 | 5.572 | γ-Terpinene | $C_{10}H_{16}$ | 27.36 |
| 5 | 5.896 | Terpinen-4-ol acetate | $C_{12}H_{20}O_2$ | 2.73 |
| 6 | 7.914 | Terpinen-4-ol | $C_{10}H_{18}O$ | 53.24 |
| 7 | 8.028 | α-Terpineol | $C_{10}H_{18}O$ | 2.45 |

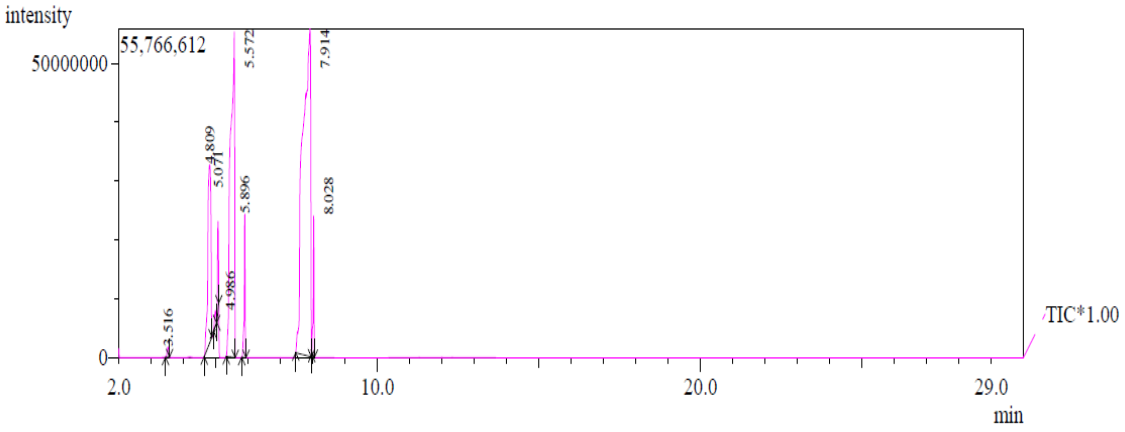


Figure 3. GC–MS Chromatogram of Tea Tree Oil

**FTIR Description**

Figure 4 illustrates the FTIR spectrum of Tea Tree Oil indicates that there are a few typical absorption bands that confirm that it is a chemical compound comprising of terpene essential oil. The general absorption band at 3458 cm -1 is ascribed to the presence of the OH stretching vibration, therefore, showing that there are alcohol groups present in the sample, which are characteristic of oxygenated terpenes, including terpinen-4-ol, a significant bioactive constituent of tea tree oil.

The C-H stretching vibrations of aliphatic -CH 3 and -CH 2 resonances at the strong bands at around 2962 cm -1 support the existence of hydrocarbon chains, as found in monoterpenes. Peaks found in the region 27292603 cm -1 could be associated with weak CH stretching of aldehydic or other similar functional groups. The presence of an absorption band close to 16511600 cm -1 relates to C=C stretching vibrations, which reveals the existence of unsaturated bonds in the terpene structures. The 14561373 cm -1 bands are ascribed to bending vibrations of methyl and methylene groups, which once again proves the teppene backbone of the oil.

Moreover, the band between 1300 and 1000 cm -1 indicates C-O stretching vibrations, which prove the presence of alcohols and ether functional groups. The lower wavenumber peaks found below 1000 cm minus one are ascribed to the out-of-plane C -H bending vibrations, which are typical of unsaturated terpene compounds.

All in all, FTIR data prove that the Tea Tree Oil is packed with oxygenated and non-oxygenated monoterpenes and this is why the product has established antimicrobial and therapeutic effects.

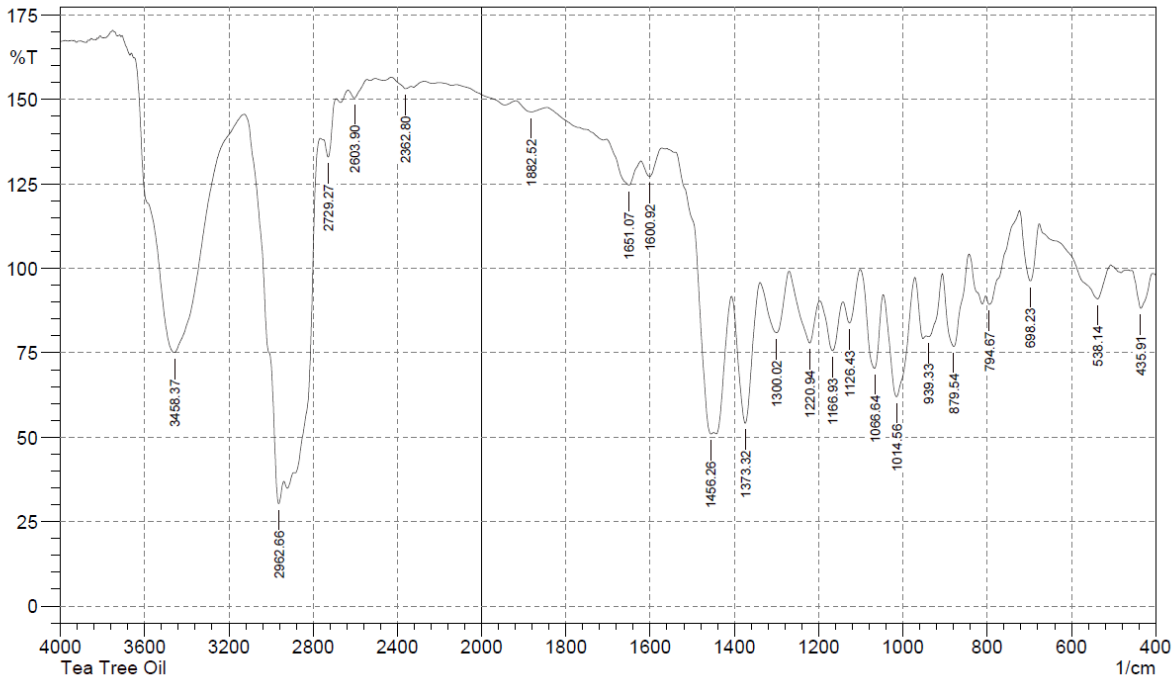


Figure (4) Identification of Functional Group of Tea Tree Oil by FTIR

**Antifungal Effect of Tea tree Oil.**

The antifungal property of the Tea Tree Oil was tested at the various concentrations (0.5%, 1%, and 2%) and was compared with some standard antifungal agents. These findings have demonstrated a dose dependable antifungal activity of Tea Tree Oil with increasing levels of inhibition as the concentration rose (Table 3 and Figure 5).

With the highest concentration of 2%, Tea Tree Oil had the greatest antifungal activity with a mean inhibition zone of 16.19 and this was statistically similar to the antifungal drug, ketoconazole (14.09 ± 1.54). There were moderate antifungal activities at 1% (11.47) and the lowest activity was at 0.5% (9.80). The negative control (DMSO) was not antifungal.

The statistical analysis revealed that there was a highly significant difference ($P < 0.0001$) between treatments with letters used to indicate significant differences at $P < 0.05$, so Tea Tree Oil is proven to be effective against fungal isolates.

Also, minimum inhibitory concentration (MIC) and Minimum Fungicidal Concentration (MFC) were determined (Table 4). The findings showed that the MIC value of Tea tree oil was 90 + -18.7 mg/mL and the MFC value was 1200 + -252 mg/ mL, which showed a high antifungal potential against the isolates.

Table (3) Antifungal activity of different concentrations Tree oil and a number of antifungal drugs

| Concentration (%) | Mean ± SE |
|---|---|
| 0.5% | 9.80cd |
| 1% | 11.47bc |
| 2% | 16.19a |

| | |
|---|---|
| DMSO | 0±0e |
| Nystatin (disc) | 7.42±1.98d |
| amphot.B (disc) | 12.66±2b |
| Fluo (Disc) | 9.38±2.49cd |
| Keto. (disc) | 14.09±1.54a |
| Calculated P value | <0.0001 |
| LSD(P<0.05) | 2.81 |

Different letters between any two means denote to the significant difference

Table (4) MIC and MFC values of Tea Tree Oil (For 5 isolates)

| Test | Mean ± SE |
|---|---|
| MIC value | 90±18.7 |
| MFC value | 1200±252 |

Figure 5. Antifungal Activity of Tea Tree Oil Against *Candida albicans* Using the Agar Well Diffusion Method

## 4. Discussion

The current paper examined antifungal activity of Tea Tree Oil against Candida albicans isolates that were obtained orally and peri-dentally, as well as, the overall phenotypic, microscopic, biochemical, and chemical characterization. Fungal growth of Candida species was detected in 53 out of 146 samples (36.3%), which showed that the samples were collected appropriately and that the fungal growth occurred well. Much higher rates of Candida spp. isolation of oral samples have been reported in the past, especially in patients with predisposing factors contributing to improved fungal colonization [1,2].

The morphological analysis of SDA showed colonies that were creamy and smooth and resembling yeast which is typical of Candida species. Purification was done to make sure that mixed growth was removed and

is necessary to identify it. Moreover, presumptive identification was made possible through the application of the use of Chromogenic Candida Agar, since isolates that had been purified showed colony colorations that were characteristic of C. albicans. This is in agreement with other studies that have indicated that chromogenic media is a powerful tool in preliminary identification [31-33].

Gram-stained smears showed purity of culture through the microscopic observation of oval-shaped to spherical budding yeast cells with no bacteria contamination. The morphology complies with the classical description regarding Candida spp. and reinforces the results of the phenotypic identification [34].

The VITEK 2 Compact system confirmed all the isolates were Candida albicans with high confidence probabilities of between 94% and 99%. It is a well-reported strong correlation between automated identification systems and traditional phenotypic and microscopic systems [35-37]. The small biochemical deviations found between isolates did not interfere with the identification at the species level and are usually explained by metabolic strain-specific differences [38].

Chemical characterization of Tea Tree Oil using GC–MS revealed that terpinen-4-ol was the predominant component (53.24%), followed by γ-terpinene and terpinolene. This analysis is in line with the international standards and other past reports of high-quality Melaleuca alternifolia oil [22,28]. It is also important that terpinen-4-ol is the most prominent since this is known as the key antifungal component that disrupts fungal cell membranes and causes the release of intracellular elements [23,24].

The FTIR analysis also corroborated the results of the GC-MS analysis by establishing the existence of functional groups that are typical of terpenes and oxygenated monoterpenes. The chemical structure of bioactive compounds that have been observed to be associated with antimicrobial activity are supported by the presence of absorption bands attributed to OH, C H, C equal C, and C O vibrational bands [29, 30]. The GC-MS and FTIR data obtained are confirmatory to both the chemical integrity and therapeutic applicability of the Tea Tree Oil that was utilized in the study.

The antifungal effect of Tea Tree Oil in the current study showed a definite dose-dependent inhibition property of Tea Tree Oil on C. albicans which is highly endorsed by several in vitro studies [22-27]. The results of the agar well diffusion assay indicate that the oil causes very strong inhibitory activities due to the ability to affect the cell membranes of the fungi and disrupt their fundamental cellular processes.

Some studies have indicated that Tea Tree Oil is antifungal by working on various mechanisms, which include blocking of the production of ergosterol, mitochondrial dysfunction, and distorting the activity of enzymes that support the survival of the fungi [20,21,30]. A combination of these mechanisms undermines fungal cells and lowers their ability to grow, which explains the high level of antifungal activity in this paper and other studies with similar procedures [25-27].

The values of MIC and MFC that were obtained, also prove the inhibitory and fungicidal properties of Tea Tree Oil. Similar MIC and MFC range were observed against C. albicans previously, and the observed differences are explained by the variation in fungi strains, oil composition and experimental conditions [26,31,32]. This type of variability should be anticipated when analyzing natural products since other aspects of the product, including geographical origin and chemical composition, may affect biological activity [28, 22].

One of the main strengths of Tea Tree Oil is that it has reported success in the treatment of Candida strains which are resistant to traditional antifungal drugs. It has been reported to be active against azole-resistant C. albicans, with a mechanism of action, is proposed to be independent of antifungal drugs commonly used [16, 24,33], and the cross-resistance. This feature is especially applicable in the current context of the rising rates of antifungal resistance and frequent oral candidiasis.

In addition, Tea Tree Oil was noted to prevent biofilm development in C. albicans, which is a key virulence factor in regard to chronic and treatment-resistant infection [15,23,34]. Because the cells in biofilms are much more resistant to antifungal drugs, the capacity of Tea Tree Oil to have an impact on planktonic and biofilm cells increases its chances of action as a therapeutic agent [5,15].

Besides possessing antifungal effects, Tea Tree Oil is usually considered to be safe in case it is utilized in the right concentrations. There have been few adverse effects that have been reported in toxicological and clinical studies especially when administered topically or as controlled oral formulations [8,34]. This is a positive safety profile accompanied by a high antifungal activity, which means that it can be used as a natural substitute or a supplement to traditional antifungal treatments.

Generally, the results of the current research paper strongly correlate with past findings and give additional testimony that Tea Tree Oil has considerable antifungal potential in oral Candida albicans. Combining chemical characterization with biological appraisal supports the arguments supporting the concept of Tea Tree Oil as a potential natural antifungal agent especially as more antifungal agents become resistant to existing antimicrobial agents.

## Conclusion

Within the limitations of this in vitro study, Tea Tree Oil demonstrated significant antifungal activity against clinical isolates of Candida albicans obtained from oral samples. The antifungal effect may be attributed to its high content of bioactive terpene compounds, particularly terpinen-4-ol. However, further in vivo and clinical studies are required before recommending its therapeutic application in oral candidiasis management.

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