Pharmacogn. Res., 2025; 17(1):70-77.
https://www.phcogres.com

Original Article

Assessment of the Antifungal Effect of *Camellia sinensis* Extracts on *Candida albicans* and *Candida parapsilosis* Strains: An *in vitro* Study

Florin Iacob[1], Lavinia Cosmina Ardelean[2,*], Alexandra Roi[1], Ioana Tomoiaga[1], Delia Muntean[3], Laura-Cristina Rusu[1]

[1]University Clinic of Oral Pathology, Multidisciplinary Center for Research, Evaluation, Diagnosis and Therapies in Oral Medicine, "Victor Babes" University of Medicine and Pharmacy Timisoara, 2 Eftimie Murgu Sq, Timisoara, ROMANIA.
[2]Academic Department of Technology of Materials and Devices in Dental Medicine, Multidisciplinary Center for Research, Evaluation, Diagnosis and Therapies in Oral Medicine, "Victor Babes" University of Medicine and Pharmacy Timisoara, 2 Eftimie Murgu Sq, Timisoara, ROMANIA.
[3]Academic Department of Microbiology, Multidisciplinary Research Center on Antimicrobial Resistance, "Victor Babes" University of Medicine and Pharmacy Timisoara, 2 Eftimie Murgu Sq, Timisoara, ROMANIA.

ABSTRACT

Background: Oral candidiasis is a common infection caused by *Candida* species, *Candida albicans* and *Candida parapsilosis* being the most prevalent strains. **Objectives:** To assess the antifungal effects of *Camellia sinensis* (green tea) extracts on standardized *Candida albicans* and *Candida parapsilosis* strains, depending on the extraction solvent and extract concentration. **Materials and Methods:** Extracts with concentrations of 25 and 50% were prepared, using methanol and ethanol as solvents. A 0.5 McFarland concentration and approximate $2x10^6$ CFU/mL suspension of each *Candida* strain was prepared. The four types of extracts were applied on unimpregnated paper discs placed on four Petri dishes with Sabouraud Glucose with Chloramphenicol Agar culture medium. A Dimethyl sulfoxide solution was used as negative control, and a fluconazole impregnated disc was used as positive control. After a 24 hr incubation period, the inhibition zones were measured. The minimum inhibitory concentration was determined. **Results:** The largest inhibition zone was measured for the positive control, while the negative control showed no inhibitory effect. The 50% concentration extracts showed larger inhibition zones compared to 25% concentration, for both ethanol and methanol solvents and for both *Candida albicans* and *Candida parapsilosis* strains, but lower compared to the positive control. No significant differences were determined when comparing the antifungal effects of same concentration extracts prepared with methanol and ethanol as solvents. **Conclusion:** The green tea extract shows the ability to inhibit the growth of standardized *Candida albicans* and *Candida parapsilosis* stains. Its efficacy increases with concentration, and is not dependent on the solvent type.

Keywords: Antifungal, *Camellia sinensis*, *Candida albicans*, *Candida parapsilosis*, Green Tea, Oral Candidiasis.

Correspondence:
Prof. Lavinia Cosmina Ardelean
Academic Department of Technology of Materials and Devices in Dental Medicine, "Victor Babes" University of Medicine and Pharmacy Timisoara, 2 Eftimie Murgu Sq, 300041 Timisoara, ROMANIA.
Email: lavinia_ardelean@umft.ro

Received: 26-08-2024;
Revised: 29-10-2024;
Accepted: 03-12-2024.

INTRODUCTION

Medicinal plants and herbs with pharmacological properties have been used since ancient times for treating various pathologies, including those related to oral health. The use of natural products predates the pharmaceutical industry and continues to be widely utilized at present. Despite the fact that synthetic medication is commonly used worldwide, its excessive administration may lead to the emergence of severe side effects. For this reason, most pharmaceutical companies started investing a lot of financial resources and time in developing natural formulas, derived from plant extracts, to produce effective remedies, such as essential oils, teas, syrups, creams, or pills.[1] The main benefits of plant-based medicines are safety and accessibility compared to their synthetic alternatives, withal offering considerable therapeutic benefits. Following the current trend towards developing medicines that combine efficacy with low toxicity, high safety, and lower production costs, including traditional herbal medicine in clinical practice does help achieving the "health for all" goal.[2]

The second most popular and consumed beverage in the world is tea, an infusion made of dried leaves, which is surpassed by water only. One of the most important types of teas is green tea, obtained by infusion of the *Camellia sinensis* (green tea) leaves.

DOI: 10.5530/pres.20251913

Green tea is widely regarded as a health-promoting beverage, not only due to its anti-inflammatory effects, but also to its beneficial antioxidant role, mainly attributed to its polyphenol content, especially catechins. Additionally, its mineral and vitamin content enhance the antioxidant potential of green tea. The efficiency of polyphenol extraction from green tea leaves depends on the extraction method, the contact time with the solvent, the solvent composition, and tea form (i.e., packaged or loose). The cathechines content is also credited for the antifungal effect of the tea.[3-5]

Candida is one of the commensal organisms belonging to the normal oral flora, inhabiting the oral cavity of 30-60% of the adult population, and 45-65% of the infants.[6-8] Oral candidiasis is a common infection caused by *Candida* species, *Candida albicans* being most commonly isolated from the lesions (more than 80%). Oral candidiasis is most often associated with immunocompromising and metabolic diseases, chronic systemic steroid and antibiotic treatment, radiation therapy, organ transplantation, malnourishment, and salivary gland hypofunction.[9,10] It frequently occurs in neonates and infants, as well as in elderly patients, commonly associated with the use of dentures. It has been reported to be found in 81.7% of denture attached plaque.[11-13] Although much less common, other implicated species in oral candidiasis include*Candida glabrata, Candida parapsilosis, Candida tropicalis, Candida kruesi, Candida guillermondii, Candida lusitaniae, Candida pseudotropicalis*, and *Candida stellatoidea.*[14-16]

Topical antifungal drugs such as nystatin, miconazole, clotrimazole, and ketoconazole are usually used to treat mild oral candidiasis, while for moderate to severe cases, orally administrated fluconazole is recommended.[17] Promising new approaches in the treatment of oral candidiasis were also made by using herbal medicines and natural bioactive compounds, such as honey, probiotics, garlic, green tea, propolis, curcumin, licorice root, cinnamon, resveratrol, ginger, and berberine.[18,19]

The antimicrobial effect of green tea has been widely studied, and its antifungal role was previously investigated and confirmed.[20-24] The aim of this *in vitro* study is to investigate the antifungal effects of two different concentrations of green tea extracts (25% and 50%) on *Candida albicans* and *Candida parapsilosis* standardized cultures, prepared by using two different solvents, namely ethanol and methanol. The primary objective is to determine if using different solvents and extract concentrations has an influence on the antifungal efficacy.

MATERIALS AND METHODS

Preparation of the extracts

Two hundred grams of *Camellia sinensis* leaves were harvested from a tea tree grown in well-drained acidic soil with a light texture (the nursery of the University of Life Sciences "King Mihai I" of Timisoara). The leaves were washed under tap water, stored under optimal conditions, in a bright place, without direct sunlight exposure, and dried in a ventilated environment, at room temperature, for one month. The leaves were then ground into a fine powder.

Extraction with methanol and ethanol was carried out, these particular solvents displaying a non-selective capacity to extract a wide range of antioxidants. Extraction was performed in the same ratio of 10:1, for both methanol and ethanol. The following protocol was used for preparing the extracts: for every 10 mg of dried and grinded leaves, 100 mL of high-quality methanol or ethanol with a concentration of 80% were added, and the mixture, contained in a round-bottom flask, was stirred (Figure 1). Subsequently, the flasks were sealed with a Parafilm type isolation foil, to prevent evaporation and left to soak for 15 min. The flasks were transferred to the LBS2 10 LT (FALC, Treviglio, Italy) ultrasound water bath and processed for 30 min at 40°C and 40 kHz, in order to reduce the reaction time and increase its efficiency.

Filtration was performed using a vacuum pump, followed by rotary evaporation using Laborota 4000 eco (Heidolph, Schwabach, Germany) at a temperature of 40°C, 150 rpm and a pressure of 250 bar. The pressure was gradually decreased until the concentrated extract was obtained, by removing the vapour phase. Both methanol and ethanol green tea extracts were diluted

Figure 1: The concentrated green tea extracts.

Figure 2: The macroscopic and microscopic appearance (optical microscopy, 10x magnification), following methylene blue staining, of the *Candida colonies*.

to concentrations of 25% and 50% respectively, resulting in four types of solutions, as shown in Table 1. A Dimethyl Sulfoxide (DMSO) solution was used to dilute the extract, further on used as negative control, as well. Impregnated paper discs for antifungal susceptibility tests, containing a precise amount of 25 µg Fluconazole solution (FCZ), were used as positive control (Table 1).

The *Candida* strains

The study material consisted of *Candida albicans* ATCC 90028 and *Candida parapsilosis* ATCC 22019 reference strains, which conform to the ATCC (American Type Culture Collection) standard. Direct inoculation Culti-Loops (Thermo Fisher Scientific, Waltham MA, USA), containing viable fungal species, stabilized in a gel matrix, were used. The loops, individually packaged, are intended for single use, and are ready for immediate use.

The experimental protocol

The standardized fungal strains, *Candida albicans* ATCC 90028 and *Candida parapsilosis* ATCC 22019, were inoculated onto 90 mm diameter Petri dishes, containing a ready to use Sabouraud Glucose with Chloramphenicol Agar culture medium (Oxoid, Basingstoke, UK). The inoculation loops were directly applied onto the medium for 10-15 sec, with linear, uniform movements, in multiple directions. The inoculated Petri dishes were incubated at 30°C for 24 hr, using Incubator I (Memmert, Schwabach, Germany). After the termination of the incubation period, the presence of *Candida* colonies was macroscopically detectable, and was subsequently confirmed by methylene blue staining. The staining procedure was performed as follows: a sample was collected and aseptically transferred onto a dry, degreased glass slide, then mixed with aqueous methylene blue. The smear was allowed to air dry, then examined and analysed using Primo Star

Table 1: **The extracts and controls used for the test.**

Sample labeling	Solution	Extract concentration
1/25	Green tea extract in ethanol	25%
1/50	Green tea extract in ethanol	50%
2/25	Green tea extract in methanol	25%
2/50	Green tea extract in methanol	50%
M	DMSO	Negative control
FLU 25	FCZ	Positive control

Table 2: **The inhibition zones measured after incubating the culture plates for 24 hr.**

Extract	*Candida albicans* ATCC 90028 (mm)		*Candida parapsilosis* ATCC 22019 (mm)	
FCZ	39	35	40	45
DMSO	6	6	6	6
1/25	24	21	18	27
1/50	27	30	32	30
2/25	24	26	26	21
2/50	32	28	30	29

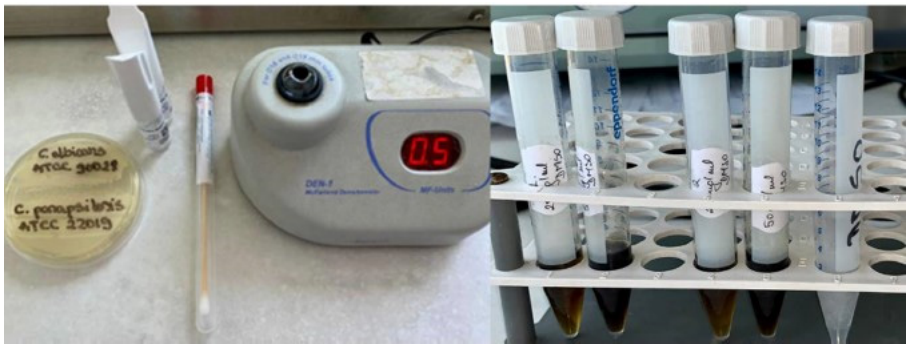

Figure 3: Preparing the suspensions to a 0.5 McFarland standard, the diluted green tea extracts (1/25, 2/25, 1/50 and 2/50) and the DMSO solution used as negative control.

Figure 4: Pipetting the green tea extract and the Petri dishes and the tubes following incubation.

(Zeiss, Oberkochen, Germany), optical microscope, with 10x magnification, and low light intensity (Figure 2).

Subsequently, for each of the two studied *Candida* strains, suspensions were prepared, using 0.85% NaCl physiological saline solution (bioMérieux, Marcy-l'Etoile, France), according to the McFarland standard. This standard is used as a reference point to adjust the microorganism suspensions turbidity, so that the number of studied microorganisms falls within a specific range for standardized testing. A sterile inoculation loop was used to touch the *Candida albicans* ATCC 90028 and *Candida parapsilosis* ATCC 22019 colony, respectively, at several points, and then immersed in 5 mL of sterile saline solution. The density of this suspension was adjusted to a 0.5 McFarland standard, by adding microorganisms if case of a too light suspension, or by diluting it with physiological saline in case of a higher density. The DEN-1 (Biosan, Riga, Latvia) McFarland densitometer was used to assess the characteristics of the suspension (Figure 3).

Two tubes with a 0.5 McFarland concentration and approximate $2x10^6$ CFU/mL (Colony-Forming Units) of each *Candida* strain were obtained (Figure 3). A sterile swab was dipped into the prepared suspension, pressed against the side of the tube to remove excess liquid, then streaked onto the Petri dish with linear (back and forth), close proximity movements. After the dish was rotated, streaking was repeated, in order to create a uniform distribution. Two Petri dishes from each type of *Candida* suspension were inoculated.

Five unimpregnated paper discs (BioMaxima, Lublin, Poland), with a diameter of 6 mm, were subsequently applied on each dish, under strict aseptic conditions, using a sterile forceps, approximately 10 min after the plates were inoculated.

The four types of green tea extracts previously obtained (1/25, 1/50, 2/25 and 2/50) and the DMSO solution used as negative control (Figure 3), respectively, were pipetted onto the unimpregnated paper discs, until completely soaked. One FCZ disc, used as positive control, was applied on each dish, as well. The discs were applied at a distance of at least 24 mm between each other, a total of six discs being applied on a 90 mm Petri dish, to ensure an accurate and precise measurement of the inhibition zone diameters.

The dishes were incubated (Incubator I, Memmert, Schwabach, Germany) at 30°C for 24 hr. During incubation, the antifungal agent is transferred from the disc to the medium. The sensitivity was assessed by measuring the inhibition diameters.

Determination of the minimum inhibitory concentration

The Minimum Inhibitory Concentration (MIC) represents the lowest concentration of an antibacterial agent, in this case, the green tea extract, expressed in mg/mL, which, under carefully controlled *in vitro* conditions, completely restricts the visible growth of the tested strains.

1 mL of the working suspension, previously diluted to a density of 0.5 McFarland, with 2×10^6 CFU/mL of *Candida*, was pipetted into 10 mL of Mueller Hinton broth (Oxoid, Basingstoke, UK), over which different amounts of green tea extract were subsequently applied, namely 100 µL, 50 µL, and 25 µL (Figure 4).

As control, 0.5 mL of DMSO was added to a tube containing 1 mL of working suspension and 10 mL of Mueller Hinton medium; the growth of the microorganism in this tube validated the test. After incubating the tubes at 30°C for 24 hr, the MIC was determined to be 0.06 mg/mL or 60 mg/mL.

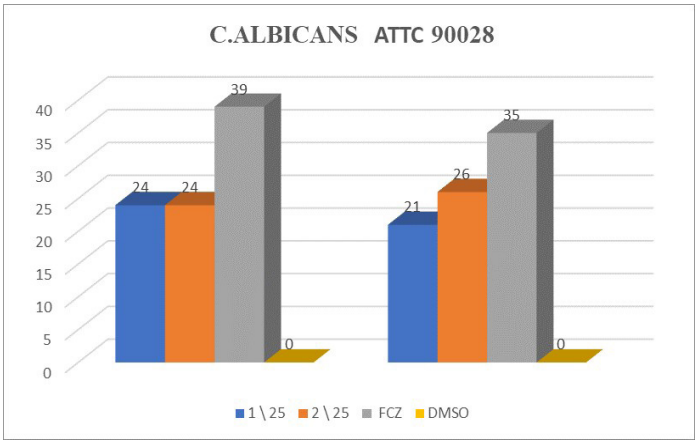

Figure 5: Comparative results for *Candida albicans* ATTC 90028 treated with a 25% green tea extract prepared in ethanol (1) and methanol (2).

RESULTS

After a 24-hr incubation period, the samples were processed, following the same protocol, and data were stored.

The antifungal activity of the samples was assessed using the agar disc diffusion method and broth macro dilution method according to the Clinical Laboratory and Standards Institute (CLSI) guidelines.[25] The dimensions of the inhibition zones were measured using a ruler, with the note that the diameter of the disc was included in the measurement.

All measurements were conducted manually and analysed visually, while observing the Petri dish from the back, in natural light. In cases where the zone boundaries did not allow for direct measurement of the diameter, the radius was measured from the centre of the disc, then multiplied by 2, in order to determine the inhibition zone more accurately. The data were analysed at a single time point, immediately after removing the dishes from the incubator (24 hr). The results are shown in Table 2 and Figures 5-8.

In Figure 9, the mean values of the inhibition zones obtained for the two Petri dishes of each *Candida* strain, treated with the same extract type and dilution are being represented.

The colonies of *Candida albicans* ATTC 90028 and *Candida parapsilosis* ATCC22019, treated with green tea extract prepared in ethanol, at a concentration of 25% (1/25), showed equal mean inhibition diameters of 22.5 mm. The colonies treated with green tea extract prepared in methanol, at a concentration of 25% (2/25), recorded a slightly increased average value in case of *Candida albicans* (25 mm, compared to 23.5 mm in case of *Candida parapsilosis*).

The mean results obtained for the colonies treated with green tea extract prepared in ethanol at a concentration of 50% (1/50) are 28.5 mm for *Candida albicans* and 31 mm for *Candida parapsilosis*. In case of methanol 50% (2/50), the mean values are very similar, 30 mm for *Candida albicans*, compared to 29.5 mm for *Candida parapsilosis* (Figure 9).

DISCUSSION

Plants are an important source of natural therapeutic products and bioactive compounds, being valuable components of traditional medicines.[26] Based on the knowledge offered by traditional medicine, exploration of medicinal plants to treat a wide range of diseases and manufacturing phytopharmaceutical products is on a rising trend.[27] According to Wangchuck, 73% of the currently used pharmaceutical products are derived from natural products, while 85-90% of the world's population depends on the traditional medicine for primary health services.[28]

Widely used worldwide, the benefits of green tea include demonstrated anti-inflammatory, antibacterial, antiviral, antifungal, antimutagenic, and anti-aging properties.[29-34] According to an *in vivo* study of Rahayu *et al*. a green tea extract with a concentration of 1.25% exhibited immunomodulatory effects in case of immunocompromised Wistar rats infected with *Candida albicans*.[35]

Based on the growing interest in developing natural remedies, this *in vitro* study aimed to determine the antifungal effect of green tea extracts obtained by extraction from *Camellia sinensis* dried leaves, using ethanol and methanol as solvents. The sensitivity of standardized *Candida albicans* ATCC 90028 and *Candida parapsilosis* ATCC 22019 strains to 25% and 50% green tea extract concentrations has been analysed. The antimicrobial activity of the extracts was evaluated using the agar disk diffusion method and analysed in relation to a FCZ positive control, and a DMSO negative control.

While *Candida albicans* is accounted for more than 80% cases of oral candidiasis, other *Candida* species, such as *Candida tropicalis, Candida glabrata, Candida krusei*, and *Candida parapsilosis*, may produce clinical infections, some of which can be particularly

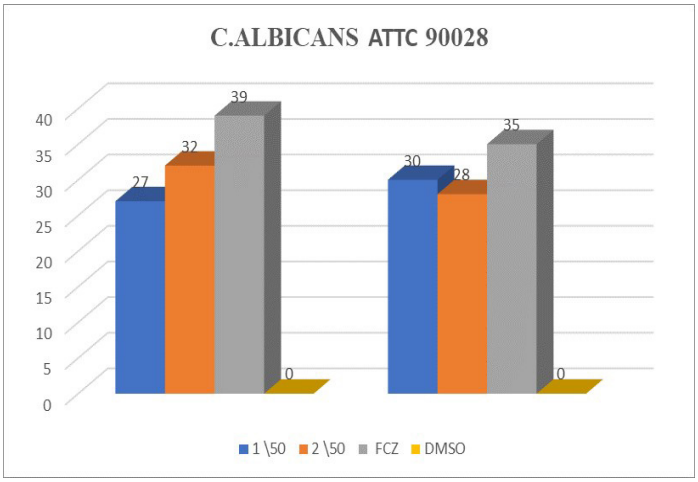

Figure 6: Comparative results for *Candida albicans* ATTC 90028 treated with a 50% green tea extract prepared in ethanol (1) and methanol (2).

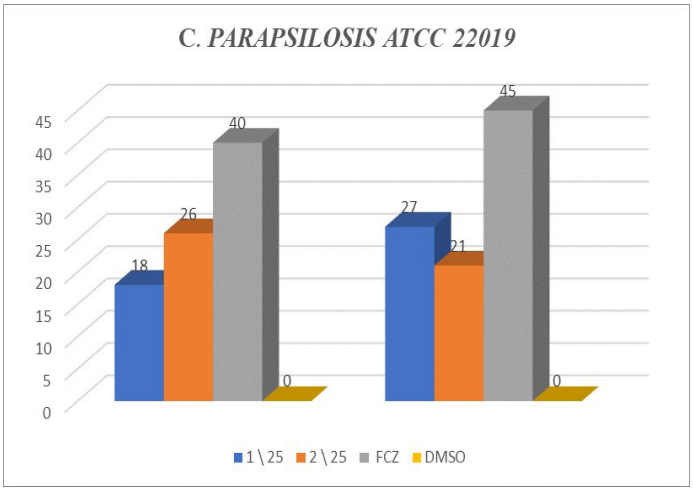

Figure 7: Comparative results for *Candida parapsilosis* ATTC 22019 treated with a 25% green tea extract prepared in ethanol (1) and methanol (2).

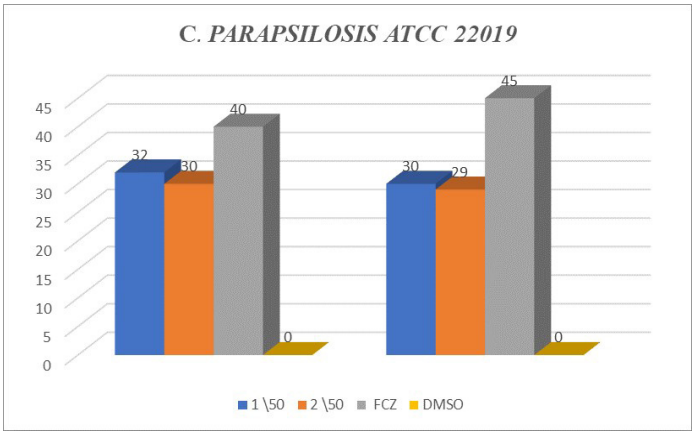

Figure 8: Comparative results for *Candida parapsilosis* ATTC 22019 treated with a 50% green tea extract prepared in ethanol (1) and methanol (2).

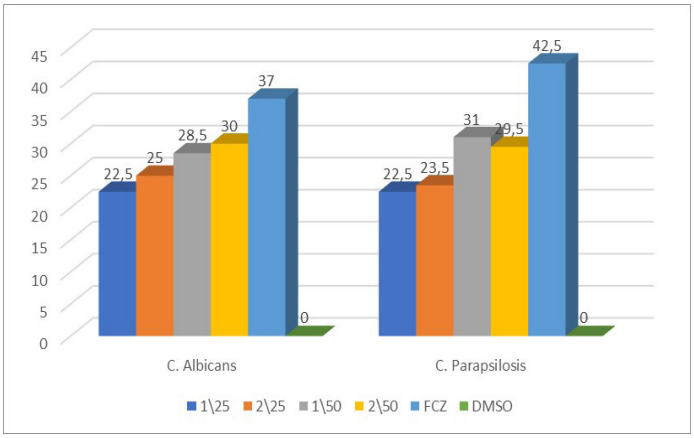

Figure 9: Comparison results of the mean values of both tested colonies.

insensitive or even resistant to antifungal therapy.[36,37] According to Horvath *et al. Candida albicans* and *Candida parapsilosis* are the most prevalent strains to be found in the oral cavity.[38] Based on these literature data, *Candida albicans* ATCC 90028 and *Candida parapsilosis* ATCC 22019 strains were chosen for assessment in this study.

The antifungal effects of green tea and other tea extracts are attributed to their catechins contents and enhanced antioxidant capacity, which is directly correlated with the concentration of the tea extract. However, there is no consensus on the specific dose of catechins or concentration of the tea extract which exhibits efficient antifungal effects.[21,23] Huang *et al.*[21] compared the antifungal effects of three different tea extracts, including green tea and two types of oolong tea, on *Candida albicans*, and concluded that they had a significant effect on its growth in culture medium, but no correlation could have been established between the antifungal activity of the tea extracts and their catechin concentrations. Of the 5 extract concentrations tested (1.25, 2.50, 5.00, 10.00, and 20.00 mg/mL), only 10.0 mg/mL green tea, and 2.5 mg/mL of one oolong teas yielded significant inhibitory effects. According to Mathur *et al.*, who compared the antifungal efficacy of green coffee and green tea extract against *Candida albicans*, both tested extracts started showing antifungal efficacy at 50 mg/mL, the highest antifungal activity being determined at the range of 100-200 mg/mL.[39]

Various concentrations of green tea extracts were previously assessed for their antifungal activity. According to a recent study by Monte *et al.*, a 20% green tea extract showed antifungal activity against *Candida albicans* and other non-albicans *Candida* strains, both in biofilm and planktonic form.[40] According to Madhura *et al.* a statistically significant increase in the zone of inhibition was noticed at both 25% and 50% green tea extract, tested on *Candida* species sampled from human's oral cavity.[41]

Chen *et al.* also reported that green tea extracts exhibit antifungal activity. They tested the effect of 23 different green, black, white and oolong teas and tea catechins on *Candida albicans, Candida glabrata, Candida krusei, Candida parapsilosis, Candida tropicalis*, and *Aspergillus fumigatus* and concluded that catechins have differential antifungal activity *in vitro* against *Candida glabrata, Candida albicans* and *Candida parapsilosis*, and that the fungicidal effect is more pronounced at higher concentrations. They also stated that the mechanism of action on which the antifungal activity of tea and tea polyphenols is based is not clear.[42]

Based on the available literature data, 25% and 50% green tea concentration extracts were chosen for this study, in order to determine if the fungicidal effect is more pronounced at higher concentrations. According to our knowledge, data comparing the antifungal activity of tea tree extracts obtained by using different solvents are not yet available. We chose to compare ethanol and methanol, as they are both alcohols. Methanol, due to its lower boiling point is presumably to exhibit a better extraction outcome, following the solvent's evaporation, yielding a higher content of bioactive compounds. However, no significant differences were determined when comparing the antifungal effects of same concentration extracts prepared with methanol and ethanol as solvents (Table 2). The mean values for the inhibition zone of tea tree extracts in methanol (2/25 and 2/50) compared to ethanol (1/25 and 1/50) are slightly higher, both for *Candida albicans* and *Candida parapsilosis* strains, excepting the 1/50 values for *Candida parapsilosis*, which are higher than those of 2/50 (Figure 9). Regarding the inhibition-zone measurements, the largest inhibition zone was measured for the FCZ samples (positive control), with a mean value of 37 mm for *Candida albicans* and 42.5 mm for *Candida parapsilosis*. The negative control showed no inhibitory effect. As of differences between the 25% and 50% green tea concentration extracts, the 50% concentration showed larger inhibition zones compared to 25% concentration, for both ethanol and methanol solvents and for both *Candida albicans* and *Candida parapsilosis* strains (Table 2, Figure 9). However, when compared to the FCZ mean values, the 50% extracts, showed mean values of 28.5% for ethanol and 30% for methanol, in case of *Candida albicans* (37 for FCZ) and 31% for ethanol and 29.5% for methanol, in case of *Candida parapsilosis* (42,5), which advocate

the good antifungal effect of green tea on both *Candida* strains. The antifungal efficacy of the extracts is also demonstrated by the low value (0.06 mg/mL) of MIC.

Based on the differences reported in literature regarding the efficient concentration, it is most probable that factors other than catechin concentrations (which are directly related to the extract concentration) influence the antifungal properties of tea extracts. The factors mentioned are variations in the extraction methodology, and the type of tea. It has been pointed out that, even in case of teas originating from the same type of plant, each product may display different taste and chemical profile, due to agricultural conditions and processing methods. The pH value is also mentioned as a potential influence on the antifungal activity of tea extracts.[1,21,43,44] A study by Hirasawa and Takada confirmed the inhibitory effects of green tea catechins on *Candida albicans*, and concluded that their antifungal activity is pH dependent.[45] Harvest time has also been reported as having influence on the catechin content and antifungal activity of the green tea extract.[46]

Within its limitations, the results of this *in vitro* study confirm that green tea extract exhibits antifungal effects that increase with concentration, on both *Candida albicans* and *Candida parapsilosis* strains. Further studies are needed for understanding the parameters and molecular markers in charge of its biological effects.

CONCLUSION

Green tea extract, obtained by processing the *Camellia sinensis* plant material in both ethanol and methanol has the ability to inhibit the growth of standardized *Candida albicans* and *Candida parapsilosis* strains. No significant differences were found when comparing the antifungal effect of green tea on the two *Candida* strains assessed. Increased concentration of the extract resulted in increased inhibition zones, regardless of the type of solvent used. No significant differences were found between the efficacy of same concentration extracts obtained by using different solvents.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

We would like to acknowledge VICTOR BABES UNIVERSITY OF MEDICINE AND PHARMACY TIMIŞOARA for their support in covering the costs of publication for this research paper.

ABBREVIATIONS

DMSO: Dimethyl sulfoxide; **FCZ:** Fluconazole; **ATCC:** American Type Culture Collection; **MIC:** Minimum inhibitory concentration; **CLSI:** Clinical Laboratory and Standards Institute.

SUMMARY

The study is about the antifungal effect of *Camellia sinensis* 25% and 50% extracts, obtained by extraction in methanol and ethanol. The agar disc diffusion method and broth macro dilution method were used and the minimum inhibitory concentration was determined. The results showed that green tea extract, obtained by processing the *Camellia sinensis* plant material in both ethanol and methanol has the ability to inhibit the growth of *Candida albicans* and *Candida parapsilosis* strains. Increased concentration of the extract resulted in increased inhibition zones, regardless of the type of solvent used, and no significant differences were found between the efficacy of same concentration extracts obtained by using different solvents.

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Cite this article: Iacob F, Ardelean LC, Roi A, Tomoiaga I, Muntean D, Rusu LC. Assessment of the Antifungal Effect of *Camellia sinensis* Extracts on *Candida albicans* and *Candida parapsilosis* Strains: An *in vitro* Study. Pharmacog Res. 2025;17(1):70-7.