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Original Research

Application of New Syncretic Antifungal Formulations against *Candida* Species Associated with Oral Cancer

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Abstract

Background: Human oral microbiomes constitute from diverse and multifaceted structure of microorganisms including fungi and bacteria. That complex structure is dynamic and plays important roles in human health and disease. They might influence the progress of infectious diseases like oral cancer. Some of oral mycobiota may transform to bloodstream causing severe systematic diseases and a greater risk of sickness and death up to 79% of oral cancer patients. Oral cancer isolates were detected to be more virulent compared with other studied isolates including non-cancer isolates.

Aims: The aims of this study were to investigate the *in vitro* effects of ZnO NPs and apple cider vinegar (ACV) independently against Iraqi isolates of *Candida albicans* and *Candida tropicalis* isolated from oral cancer patients and healthy individuals. Dual and triple combinations of ZnO NPs or/and ACV combined with fluconazole (Flu) and Amphotericin B (AmphB) were tested against *Candida* isolates and first applied as alternative therapies in Iraq and in some application points around the world.

Methods: The two most common types *C. tropicalis* (P128), (H25) and *C. albicans* (P111), (H10) were selected against a range of concentrations of ZnO NPs, Flu and amphB (0.01-0.06 and 0.1 µg/ml) and six concentrations of ACV (50,000-780µg/ml) using micro dilution method. The minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) for each antifungal agent and their interactions among these drugs were also detected.

Result: The oral cancer isolates were more resistance to tested antifungals and their combinations compared with non-cancer isolates. Dual and triple combinations were synergistic and showed significant reductions in fungal growth in a dose dependent matter. The triple combination of ZnO NPs and ACV with Flu or AmphB revealed notable reduction in MICs and MFCs values.

Conclusion: These concentration-dependent interactions may have critical effects that need further study in animal models of investigated species.
The dual combination of ZnO NPs and ACV had a significant similarity with triple antifungal combinations of antifungal activity against *Candida tropicalis.*

Keywords: Apple cider vinegar, *Candida* isolates, Oral cancer, syncretic formulations, ZnO NPs.

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Introduction

Human oral cavity is resident with diverse and multifaceted structure of microorganisms related to a heterogenous nature of oral cavity and is microbial entry into the body (Hora & Patil, 2022). Fungi are important component of the oral microbiota, known as mycobiota. The oral mycobiota has been recently linked to health and disease states with development of microbial identification techniques (Defta et al., 2024; Al Anbagi et al., 2025). Mycobiota play a crucial role and mutually interact with other microorganisms to maintain many physiological functions in the human body particularly in immune compromised individuals (Radaic & Kapila, 2021). They might influence the progress of infectious diseases especially among immunocompromised patients, such as cancer patients (Defta et al., 2024). For example, the oral thrush or candidiasis is an important example that common as secondary feature to oral cavity cancer (Zhang et al., 2020a). However, there are still technique limitations and theoretical gaps in current understanding of the intricate relationship between microbes and humans. Thus, the assortment and functionality of inhabitant fungi in the human body is being interested area for researchers, recently.

The core oral commensal mycobiota is *Candida* as well as many other fungal species that have been recorded in both health and disease states (Defta et al., 2024; Al Anbagi et al., 2025). Head and neck cancers represent a growing global health challenge, as the microbiological makeup of those affected differs significantly from that of healthy individuals. Some of oral mycobiota may transform to bloodstream causing severe systematic diseases and a greater risk of sickness and death up to 79% of oral cancer patients (Hora & Patil, 2022). From that microbial structure, *Candida* emerge as a key opportunistic component of this makeup, prompting research into their response to traditional antifungal agents versus newer alternatives (Lalla et al., 2010). Both *Candida albicans* and *Candida tropicalis* were prevalent in oral premalignant and malignant lesions. Oral cancer isolates were detected to be more virulent compared with other studied isolates including non-cancer isolates (Castillo et al., 2018). The widespread interest in the fungus *C. tropicalis* is not only due to its high disease-causing capacity, but also to its unique ability to form biofilms that give it greater invasiveness and virulence (Meng et al., 2025a). Several studies reported that patients with weakened immune systems are more susceptible to candidiasis due to structure of oral mycobiota isolated (Defta et al., 2024; Al Anbagi et al., 2025). Oral candidiasis can colonize cancer patients with colonization levels ranged 30%-50% (Lakshmy et al., 2016). The *C. albicans* isolates of oral cancer patients showed prevalent in antifungal resistance genes with significant resistance to fluconazole and itraconazole (Abd Ulrahman Majeed et al., 2024). Increasing incidents of *Candida* colonization in cancer patients suggest decreasing sensitivity to fluconazole, amphotericin B and nystatin, and needing to find newer antifungal drugs (Jabalameli et al., 2017).

To overcome multidrug-resistant mechanisms and provide more effectiveness therapeutic applications, new approaches, including the application of nanotechnology are applied (Fayed et al., 2025). Recent research has focused on exploring mechanisms that enhance the efficacy of traditional antifungal drugs or work synergistically with them to reduce toxicity and increase inhibitory effectiveness (Li et al., 2025). The zinc nanoparticles (ZnO NPs) biogenically or chemically synthesized, for example, have been proved their effectiveness against prokaryotic and eukaryotic microorganisms due to their biocompatibility and multifunctional properties (Fayed et al., 2025; (Lopez Venditti et al., 2025; Safaei et al., 2025). Other antifungal agents have been applied by limited studies including apple cider vinegar with its rich composition of acetic acid and phenolic compounds (Mota et al., 2015; Ousaaid et al., 2021). Recently, combinatorial strategies for resisting invasive fungal infections have been suggested (Spitzer et al., 2017) and applied for ZnO NPs with some traditional antifungal drugs (Chand et al., 2021; (Yassin et al., 2023). At the same time, that application may

not only potentially target antifungal cells but also selectively kill cancer cells due to its effect on different cancer cell lines (Hadi et al., 2025). Thus, the combination therapy and applications of low cost and toxicity led to decrease the required doses of antifungal drugs, and the host side effects of experimented antifungals in some studies (Yassin et al., 2023; Hadi et al., 2025).

The current study aimed to detect independently the *in vitro* effects of ZnO NPs and apple cider vinegar against Iraqi isolates of *Candida albicans* and *Candida tropicalis* isolated from oral cancer and healthy participants against tested antifungal drugs using a micro dilution method. Also, antifungal interactions of dual combination of ZnO NPs or apple cider with fluconazole and amphotericin B, ZnO NPs and apple cider. Triple combinations of ZnO NPs/apple cider /fluconazole and ZnO NPs/apple cider /amphotericin B were evaluated against species isolates. These treatments were applied using different concentrations of tested antifungal agents and drugs. Dual and triple combinations were first applied as an alternative therapy in Iraq and in some application points around the world.

Materials and Methods

Fungal isolation and inoculum preparation of fungal isolates

Saliva samples were collected from both oral cancer and oral health participants and cultured on the CHROM agar (HI media, India) for strain distribution and morphological identification. The strains were purified on the Sabouraud dextrose agar medium (SDA, HI media, India). The maintained temperature for incubation was consistently at 30°C for 48 hours. Based on their distribution across clinical isolates of the investigated sets, *Candida albicans* and *Candida tropicalis* were selected. After being ensured from their purity and growth activity on previous culture media, the species identification was confirmed genetically, following the methodology published in in Al Anbagi et al., 2025.

The fungal suspensions and following process were prepared according to Espinel-Ingroff et al. (2007) with some modifications. Briefly, the purified cultured colonies of *C. albicans* and *C. tropicalis* were selected and ensured each had a diameter close to 1 mm to prepare fungal suspensions from selected isolates. These strains involve *C. albicans* strains isolated from oral cancer (P111) and oral healthy (H10) individuals and *C. tropicalis* strains isolated from oral cancer (P128) and oral healthy (H25) individuals. The fresh inoculums were obtained after sub culturing the previous strains on SDA medium and incubated at 28ºC overnight. Then, the isolate colonies of tested species were separately immersed in sterile saline solution (0.89%). The turbidities of the *candida* suspensions were then standardized using the 0.5 McFarland standards at a wavelength of 530 nm and obtain the final concentration of $1x10^8$ CFU /ml.

Suspension preparation of antifungal agents

The suspensions of antifungal agents were prepared and diluted independently. The stock solutions were prepared independently from ZnO NPs (purity 99.8**%**, Sky spring, Houston, Texas, USA) and apple cider vinegar (ACV) concentration 5**%** and purity 100**%**, USA). Additionally, two different classes of antifungal drugs: azoles including fluconazole (Flu., Pfizer, USA) and polyenes involving amphotericin B (AmpB., Sigma, USA); both drugs with concentration 750 µg/mg and 99.9 purity. The stock solutions of tested agents were dissolved in sterilized deionized water (DW, milli Q). The stock solutions of powdered agents were vortexed for one minute and sonicated on 40 KHZ for 1 hour before use. The final concentrations ranged from 0.01-0.06, and 0.1µg/ml for (ZnO NPs, AmpB, and Flu) and 50,000µg/ml, 25,000µg/ml, 12,500µg/ml, 6,250µg/ml, 3,125µg/ml, 1,562µg/ml and 781µg/ml for apple cider vinegar (Mota et al., 2015a).

The current experiment included tested individually varying concentrations of ZnO NPs, ACV, AmpB, and Flu against studied isolates. Then, different concentrations of each antifungal class were combined separately with variable concentrations of ZnO NPs and ACV. Finally, a concentration-dependent ternary system was

designed. That system included distinctive concentrations of each antifungal drug was tested in combination with concentration of ZnO NPs and ACV.

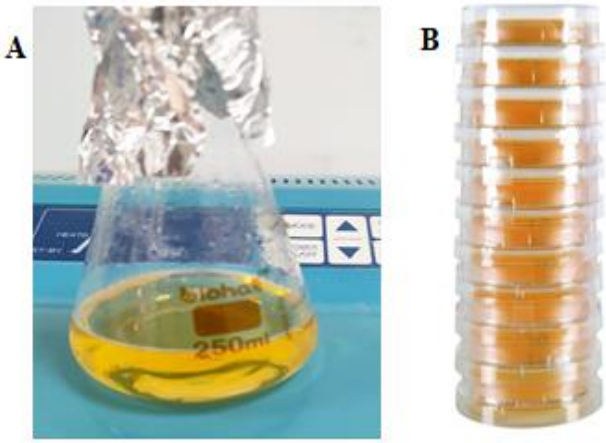

Figure 1. (A) Preparation and sterilization of Sabouraud dextrose agar (B) Pouring of Sabouraud dextrose agar medium in Petri dishs

Evaluation of antifungal activity in vitro

The broth micro dilution experiments were conducted in 96-well microtiter plates in a sterile environment inside safety cabinet based on the standardized CLSI M27 (2017). To evaluate the antifungal activity of tested agents against *Candida* strains, the micro dilution plate was filled with 100 µl SD broth (2x), the upper horizontal column, and with 100 µl distal water (DW, blank). Then, the rest of wells were load with 100 µl SD broth. The columns 1 - 4 were individually loaded with 60 µl DW and 20 µL concentrations of the AmpB, Flu, ZnO NPs, and ACV respectively. The columns 5-8 were allocated to the concentrations of combined treatments consisting of 20 µl AmpB or Flu and 20 µl ZnO NPs or ACV with 40 µl DW. The column 9 was loaded with 20 µl of mix of ZnO NPs and ACV with 40 µl DW. Columns 10-11 were assigned for ternary combinations consisting of concentrations of 20 µl from AmpB or Flu, ZnO NPs and ACV with 20 µl DW. Subsequently, all wells with 20ul the previously prepared fungal suspension except the upper horizontal column blank wells. The last column of microtiter plates had only 100 µl growing medium with 20 µl fungal suspension diluted with 80 µl deionized water (negative control for growth colonies). The microtiter plates of tested antifungal agents against Candidal strains were incubated at 30°C for 48 hours.

Thereafter, the turbidities of *Candida* suspensions treated with different concentrations of treatments including controls were analyzed using spectrophotometer at 530 nm after a period of 48 h. The obtained results were used to investigate the effect of different concentrations of treatments on the growth of the *Candida* strains. The growth inhibitions of investigated strains were also detected visually according to Espinel-Ingroff et al. (2007).

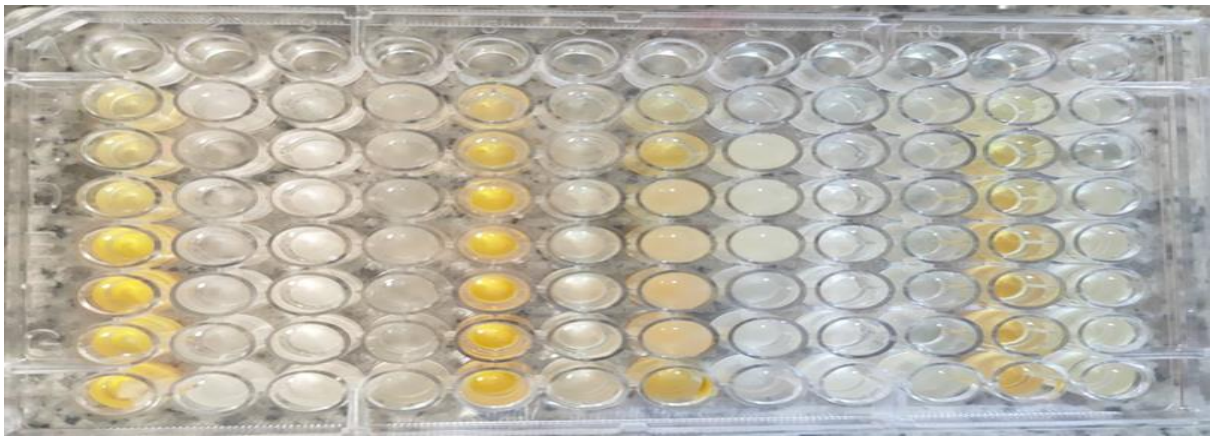

Figure 2. Micro titer plate of 96-well SD broth micro dilution model of *Candida tropicalis* after treatment with (a) AmphB; (b) Flu; (c) ZnO NPS; (d) ACV; (e) combination of AmphB with ZnO NPs; (f) combination of Flu with ZnO NPs; (g) combination of AmphB with ACV; (h) combination of Flu with ACV; (i) combination of ZnO NPs with ACV; (j) triple combination of Flu, ZnO NPs and ACV; (k) triple combination of Amph, ZnO NPs and ACV; (l) positive control included incubated *C. tropicalis* only ; (m) negative control include the culture medium only.

Detection of minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs)

The minimum inhibitory concentrations (MICs) were detected visually and spectrophotometrically for each tested strain isolated from oral cancer and healthy participants. The MICs were referred as the lowest concentrations of antifungal agents that prevent a visible growth of investigated strains. Moreover, the minimum fungicidal concentrations (MFCs) which are the lowest concentration of antifungals revealing no candidal growth were assessed. For determination of these concentrations, fifty microliters taken from MIC wells and the two other successive concentrations of different treatments were transferred onto freshly prepared SDA plates. Similarly, fifty microliters were transferred from control wells and cultured on SDA plates. Subsequently, the cultured plates were incubated at 30°C for 24 h. After incubation period, the lowest concentrations that did not show any *Candida* growth of the tested isolates were considered as MFCs (Espinel-Ingroff et al. 2007).

Statistical analysis

Statistical analysis was performed using SPSS statistical software. An analysis of variance (ANOVA) test was performed to identify any significant differences among the means of the compared treatments and isolates. The data was present using means of optical densities (ODs), slandered errors (SEs) and statical differences presented by letters. For pairwise comparations between groups, the Tukey HSD test was performed. Accordingly, obtained values were considered statistically significant when the P value was less than 0.05 (Al Anbagi et al., 2025).

Results

In the current results, two isolates per each *Candida albicans* and *Candida tropicalis*, those were dominant in both oral cancer and oral health participants (Figure 3). These were taxonomy identified based on their morphological characteristic on chrome agar. The species identification was confirmed by molecular identification of their sequences of ITS regions deposited in the Gen Bank under the PX920981-PX920984 accessions (Figure-3). Later, the identified isolates of these species from both oral cancer and oral health participants were utilized for susceptibility testing.

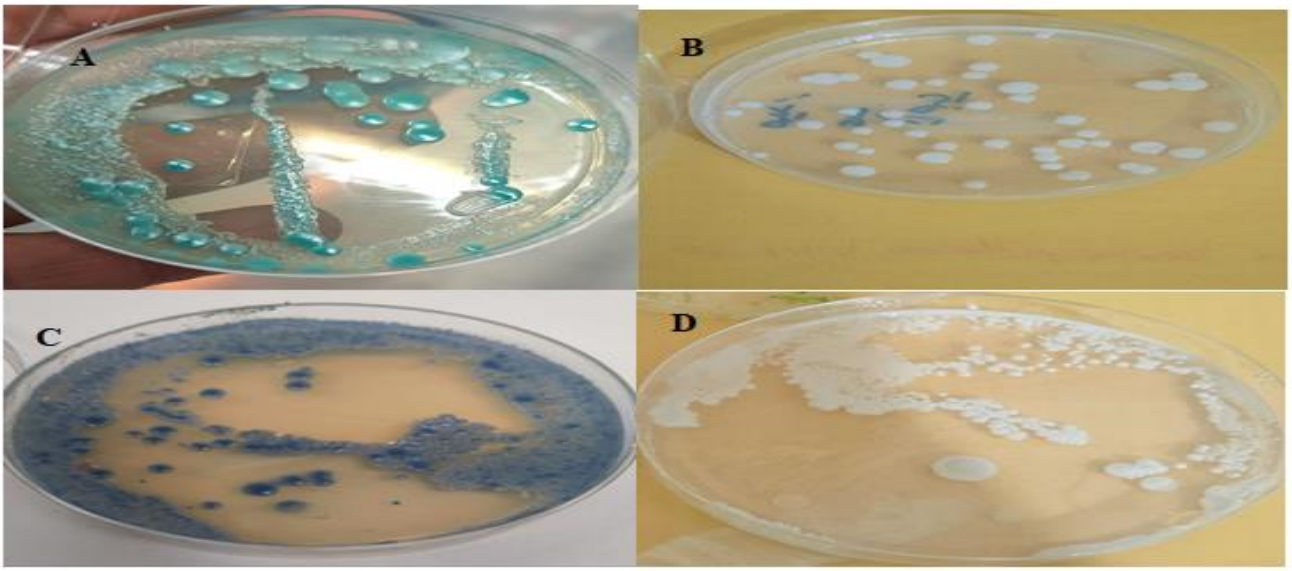

Figure 3. Morphological examinations of *Candida albicans* (A and B) and *Candida tropicalis* (C and D) on Chrome agar and SDA, respectively

The current results of the strains of *C. albicans* and *C. tropicalis* isolated from oral cancer and healthy participants showed variable susceptibility to the tested compounds using broth micro dilution assays. For *C.albicans* strains, the present Figure (4) showed the comparison of mean optical density (OD) values between strain P111 obtained

from an oral cancer patient and H10 obtained from an oral healthy individual. The isolate P111 exhibited a higher mean OD value than isolate H10, indicating greater fungal growth under the same experimental conditions of antifungal susceptibility testing. This difference suggests variation in growth behavior and antifungal susceptibility between the two tested isolates.

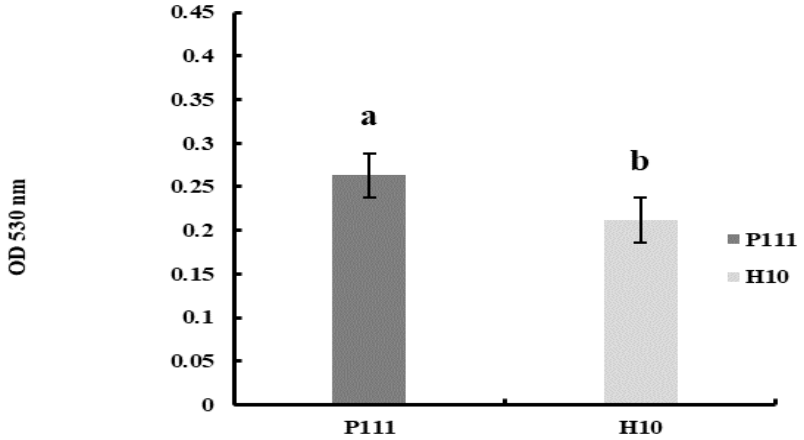

Figure 4. Comparison of mean optical density (OD) values between *C. albicans* isolates from patients of oral cavity cancer (P111) and healthy individuals (H10).

As shown in the (Figure 5), the mean OD values varied among antifungal treatment combinations. The control group demonstrated the highest OD value, reflecting maximal fungal growth. Single antifungal agents, including AmphB and Flu, showed relatively high OD values, indicating limited inhibitory activity. In contrast, the individual treatment of ZnO NPs and ACV resulted in lower OD values. Combination therapies further reduced fungal growth. However, the combination of ZnO NPs and ACV was significantly more effective in inhibiting growth of the isolates than the treatment efficacy obtained by adding up the efficacies of the two constituents separately or other combinations. The combination of the two antifungal drugs with ZnO NPs or ACV didn't differ significantly although the AmphB and ACV showed obvious growth reduction from the previous combinations. The lowest and significant mean OD values were observed in triple combinations containing ZnO NPs with ACV and the AmphB or Flu, indicating superior antifungal efficacy.

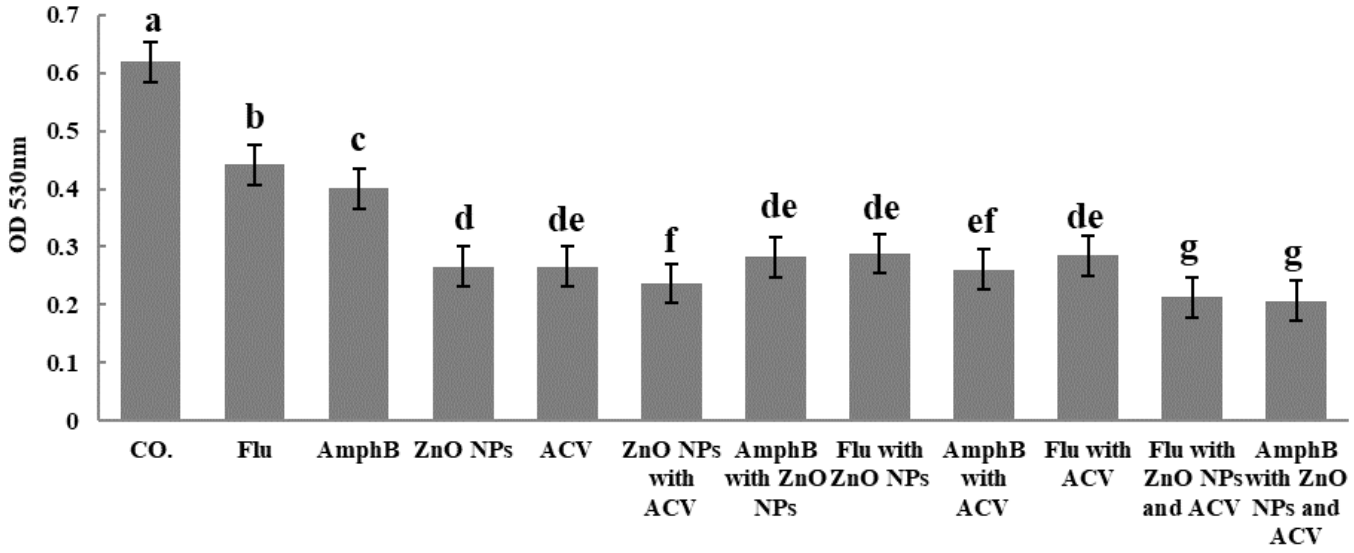

Figure 5. Mean optical density (OD) values for antifungal agents alone and their combinations with ZnO NPs and apple cider vinegar against *C. albicans* strains. Labels: AmphB- Amphotericin B, Flu-Fluconazole, ZnO NPs- Zinc Oxide nano particles, ACV-Apple cider vinegar, OD-Optical density and CO-Control.

In this study, the mean OD values of antifungal treatments showed significant inhibitory effects on *C. albicans* isolates in a dose dependent manner (Figure 6). The experiment showed that mean OD values increased steadily as antifungal concentration decreased across all treatments. The highest concentration tested (consisted of combination from 0.1 µg/ml and 50,000 µg/ml) resulted in the lowest mean OD value. In contrast, the lowest concentration (consisted of combination from 0.01µg/ml and 781µg/ml) were corresponded to the highest OD values, triple and combination treatments produce the lowest OD, especially at higher concentrations. The control group consistently showed the highest OD values across all concentrations.

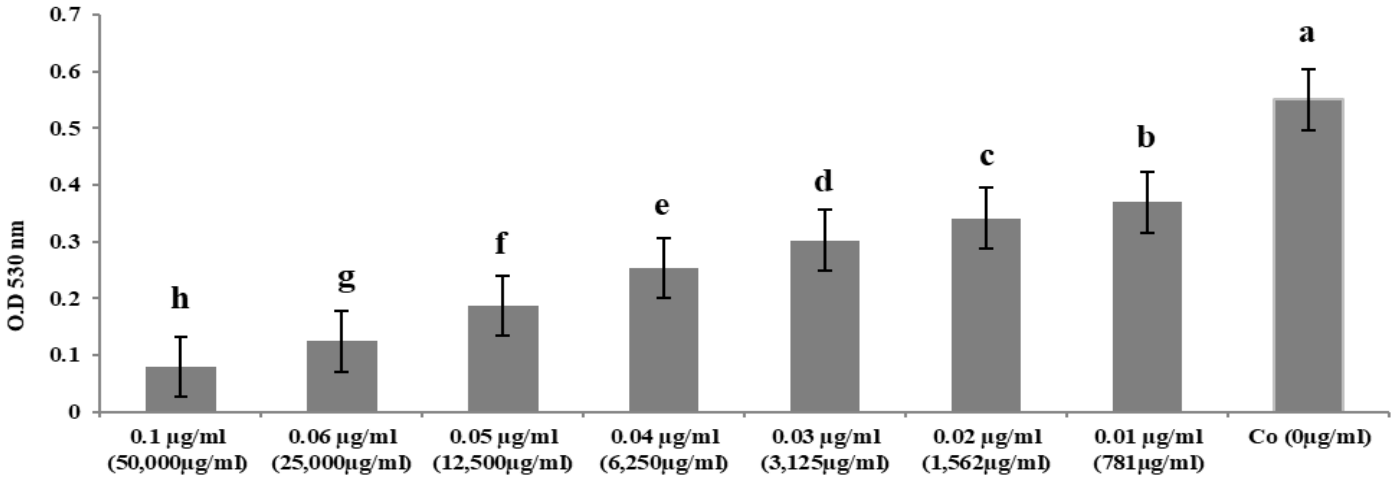

Figure 6. The relations between the concentrations of antifungal treatments and mean optical densities and standard errors (SEs) of *Candida albicans* strains isolated from oral cancer(P111) and health participants (H10). Levels connected by different letters are significantly different.

The results of the comparing antifungal treatment responses between *C. albicans* strains P111 and H10 revealed a significant decrease in OD values compared to control treatments (Figure 7). Overall, the isolate H10 showed lower mean OD values across most antifungal combinations and compared to the isolate P111, indicating greater susceptibility. The combination of ZnO NPs and ACV considerably and effectively reduced the OD value for P111 compared to other binary and triple combinations of the same isolate. However, the most pronounced reduction in OD values was for H10 and observed with triple combination treatments in addition to the combinations of antifungal drugs with ZnO NPs or ACV and combined ZnO NPs with ACV. Noticeably, the most pronounced reduction in OD values for both isolates was observed with triple combination treatments, demonstrating strong inhibitory effects regardless of isolate type.

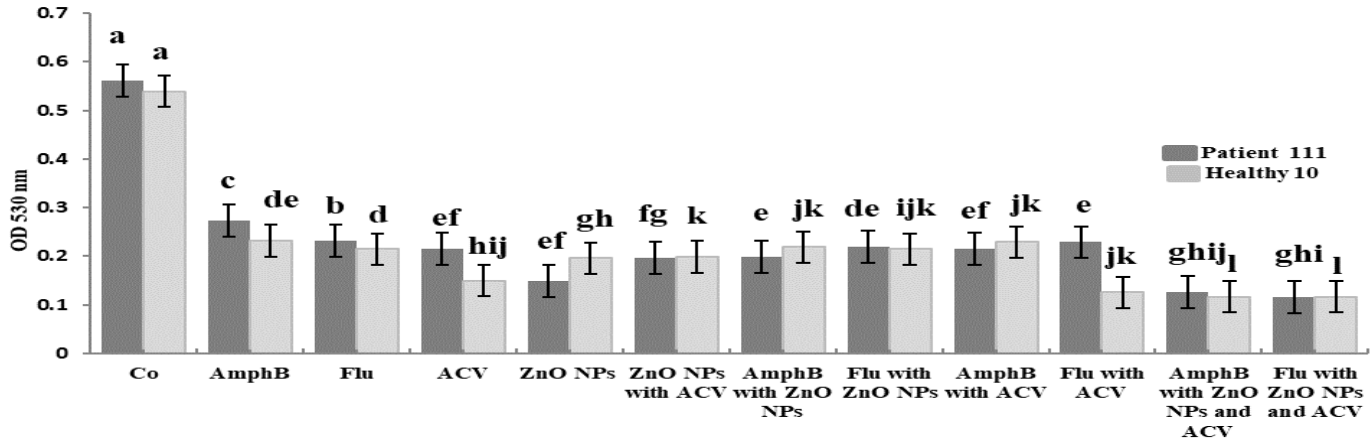

Figure 7. Comparison of mean optical density values for antifungal combinations between *C. albicans* isolates P111 and H10. Labels: AmphB- Amphotericin B, Flu-Fluconazole, ZnO NPs- Zinc Oxide nanoparticles, ACV-Apple cider vinegar and OD-Optical density

The effects of antifungal concentrations of ZnO NPs or ACV alone and in combination with AmphB or Flu., or ACV as well as the triple combination of ZnO NPs, ACV and either AmphB or Flu on fungal growth of studied isolates were illustrated in the Figure 8. A clear concentration-dependent trend in optical density (OD) was observed depending on the type of treatment, where increasing antifungal concentrations resulted in progressively lower OD values.

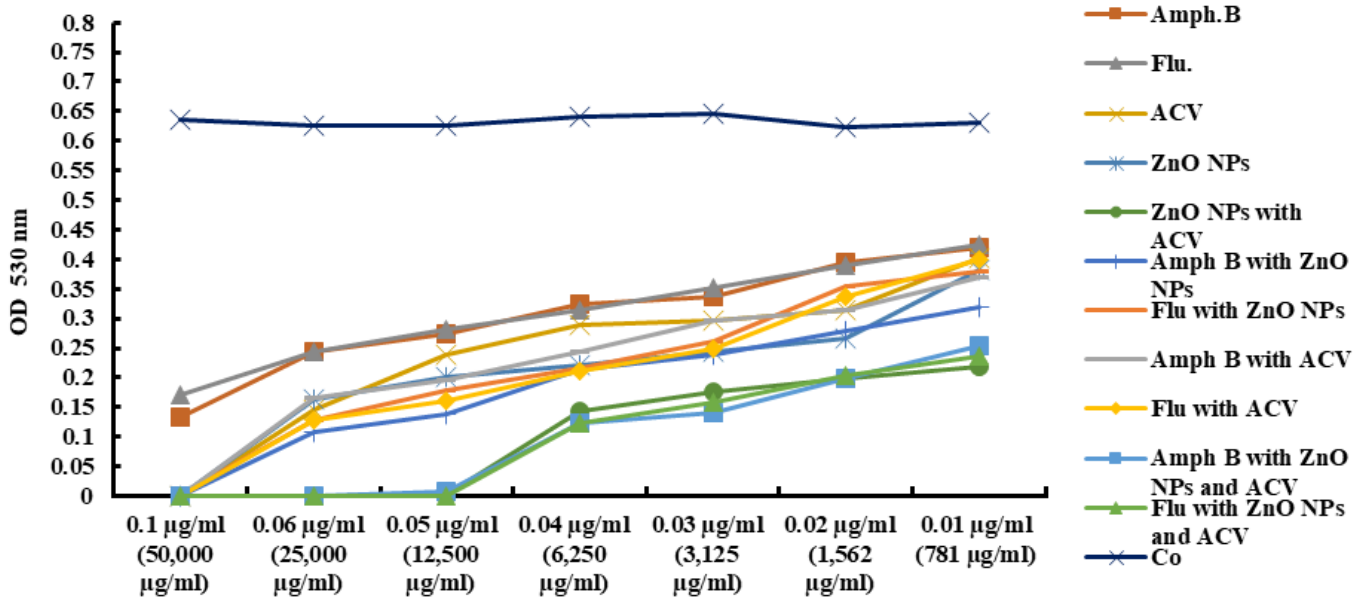

Figure 8. Mean optical density (OD) values of different concentration of antifungal agents alone and their combinations with ZnO NPs and ACV against *C. albicans* strains. Labels: AmphB-Amphotericin B, Flu-Fluconazole, ZnO NPs- Zinc Oxide nanoparticles, ACV-Apple cider vinegar and OD-Optical density.

The current results of *C. tropicalis* strains, P128 isolated from the oral cancer patient and H25 isolated from the oral healthy individual showed variable susceptibility to the tested compounds using broth micro dilution assays. The OD values of P128 were significantly less affected by the concentrations of treatments including antifungal drugs, ZnO NPs and apple vinegar and their combinations compared to the OD values of H25 (Figure 9).

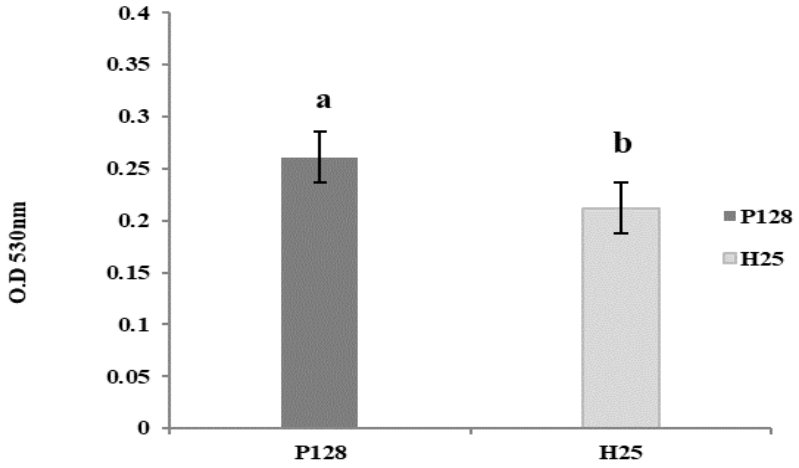

Figure 9. Comparison of mean optical density OD values between *C. tropicalis* isolates of patients with oral cavity cancer (P128) and healthy individuals (H25).

Interactions between antifungal treatments and *C. tropicalis* isolates revealed significant reductions in the fungal growth among all individual antifungal treatments and their combinations compared to controls regardless of the isolates. The individual treatments with antifungal drugs against isolates showed significantly lower in OD means compared to controls, and higher in OD means compared to other treatments. No significant differences in OD means were recorded between the combinations of the two antifungal drugs with ZnO NPs or AVC. However, the OD means of the combined ZnO NPs and AVC were significantly reduced compared to other treatments and did not significantly differ from the ternary combinations in both investigated isolates (Figure 10).

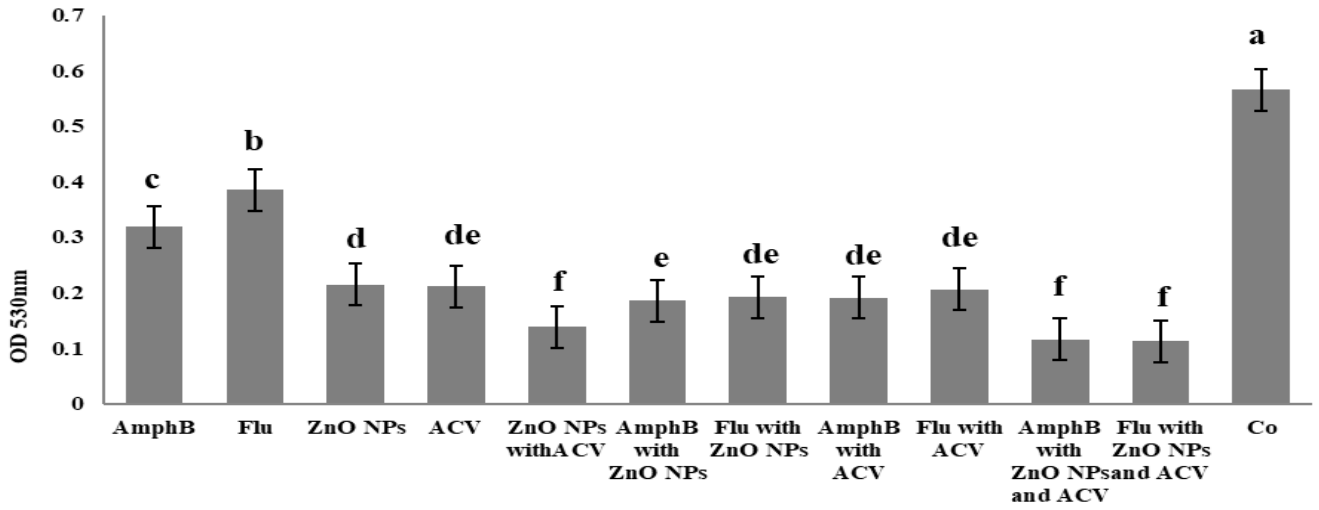

Figure 10. Comparison of mean optical density values for antifungal combinations between *C. tropicalis* isolates patients of oral cavity cancer (P128) and healthy individuals (H25). Labels: AmphB- Amphotericin B, Flu-Fluconazole, ZnO NPs- Zinc Oxide nanoparticles, ACV-Apple cider vinegar and OD-Optical density and Co-Control.

In this study, the mean OD values for all antifungal treatments showed significant inhibitory effects on the growth of *C. tropicalis* isolates in a dose dependent manner (Figure 11). The OD values increased steadily as antifungal concentration decreased across all treatments. The highest concentration tested (consisted of combination from 0.1µg/ml and 50,000µg/ml) resulted in the lowest mean OD value (0.079). In contrast, the lowest concentration (0.01 µg/ml and 781µg/ml) corresponded to the highest OD mean (0.369 ± 0.003).

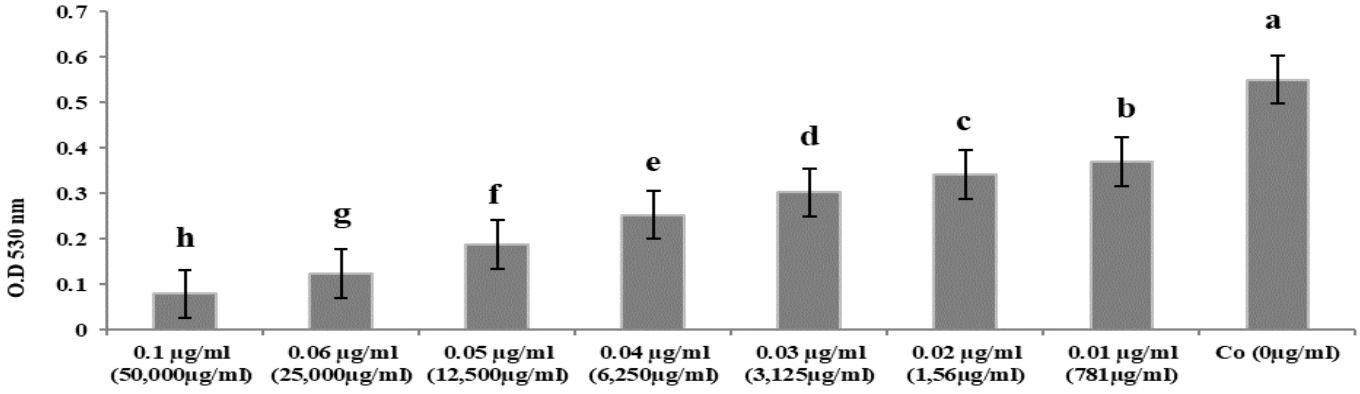

Figure 11. The relations between the concentrations of antifungal treatments and mean optical densities and standard errors of *Candida tropicalis* strains isolated from oral cancer (P128) and health participants (H25). Levels connected by different letters are significantly different.

Interactions between antifungal treatments and *C. tropicalis* isolates P128 and H25 revealed significant reductions in the fungal growth between all individual antifungal treatments and their combinations compared to controls in both investigated strains (Figure 12). However, the isolate H25 was more antifungal susceptibility than P128 across all antifungal treatments. The binary combination treatments of ZnO NPs and/or ACV with Flu and AmpB obviously reduced the fungal turbidity compared with the individual treatments of the antifungal drugs in both investigated strains. Remarkably, the effects of individual treatment by ZnO NPs or ACV of isolates didn't significantly differ than the most binary combination effects. However, the ternary combinations, Flu or AmpB, ZnO NPs and ACV, had the best influence on both fungal growths of *C. tropicalis* strains. Interestingly, the combined ZnO NPs and AVC against H25 didn't significant differ on the ternary combinations. For the isolate P128, the trinary combinations of ZnO NPs, ACV and Flu revealed significant reduction in the OD value compared to binary combination included ZnO NPs and ACV and trinary combination, ZnO NPs, ACV and AmphB.

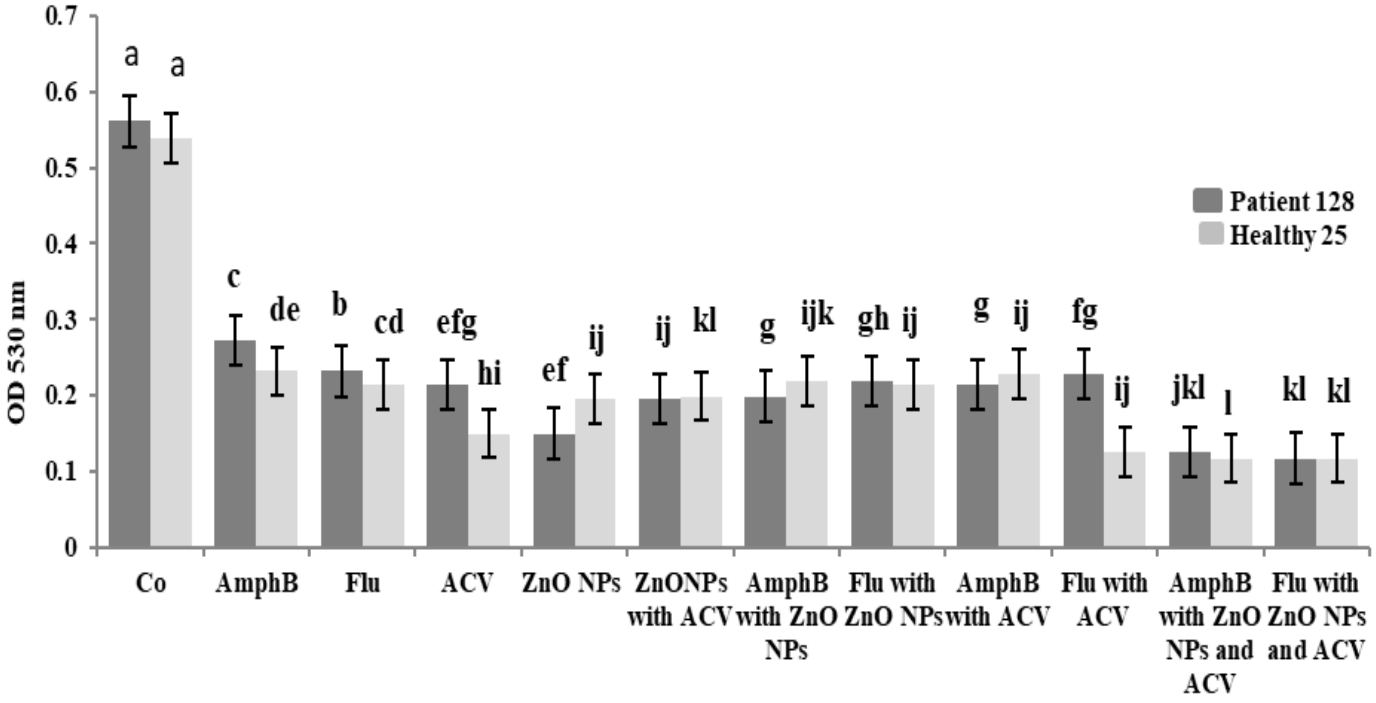

Figure 12. Comparison of mean optical density values for antifungal combinations between *C. tropicalis* isolates patients of oral cavity cancer (P128) and healthy individuals (H25). Labels: AmphB- Amphotericin B, Flu-Fluconazole, ZnO NPs- Zinc Oxide nano particles, ACV-Apple cider vinegar and OD-Optical density.

A significant concentration-dependent reduction in OD values related to the type of treatment was documented (Figure 13). The highest concentration tested produced the greatest inhibition of fungal growth, while lower concentrations were associated with higher OD values, indicating reduced antifungal activity. The growth inhibition was obviously started at 0.05µg/ml and 12.500µg/ml for both the binary combination of ZnO NPs with ACV and triple combinations containing of ZnO NPs, ACV and the AmphB or Flu.

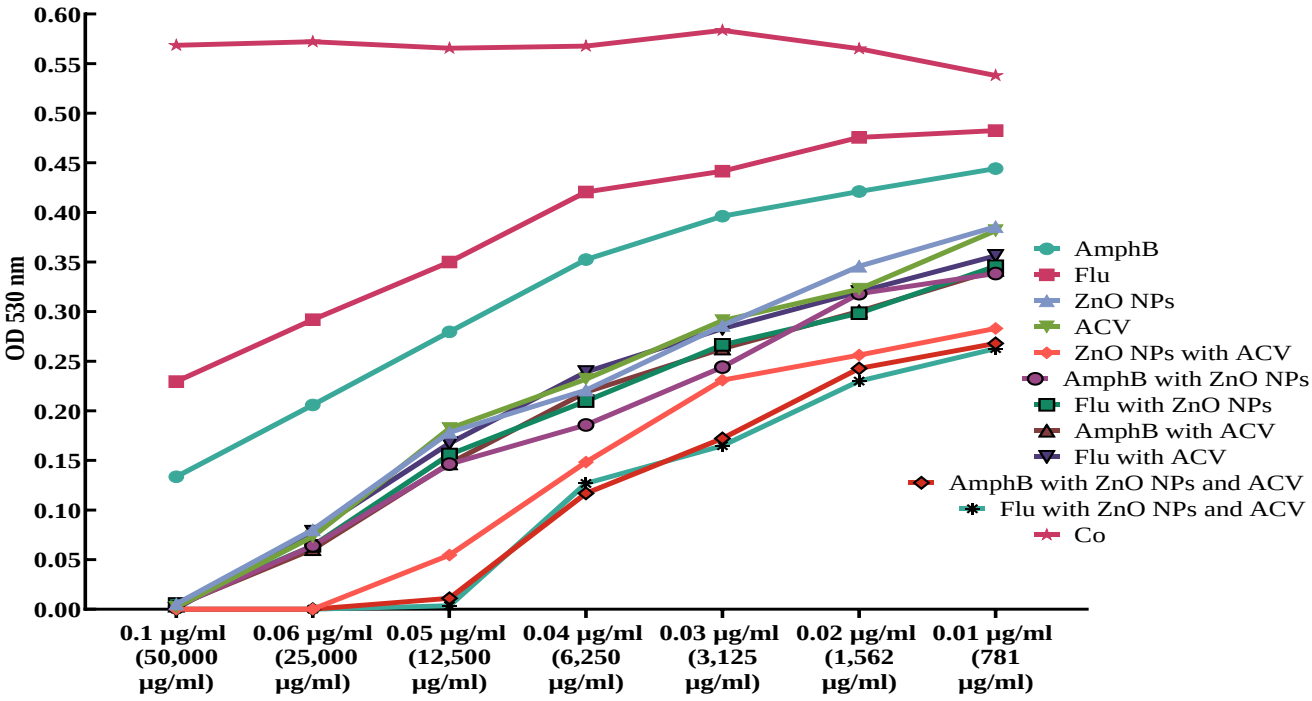

Figure 13. Changes in optical density of *Candida tropicalis* isolates across increasing antifungal concentrations for all treatments compared to control. Labels: AmphB- Amphotericin B, Flu- Fluconazole, ZnO NPs- Zinc Oxide Nano Particles, ACV- Apple Cider Vinegar and OD- Optical Density.

Estimation of minimum inhibition concentration (MICs) and minimum fungicidal concentration (MFCs)

The results of *candida* isolates treated with the different concentrations of investigated treatments to detect the MICs and MFCs showed decrease the number of colonies formed on SDA or no growth recorded as the concentrations of treatments increased compared to controls (Figure 14). Based on the MICs and MFCs results, all treatments show antifungal activities against test isolates of *C. albicans* and *C. tropicalis* after incubation at 30 °C for 48 h. Generally, results showed unexpected resistances in the oral cancer isolates to typically effective drugs including AmphB and Flu in both species. Their activities were increased when being combined together or each with AmphB or/and Flu lowering the required doses for MIC or MFC values compared to using either agent alone in both isolates (Tables 1 and 2).

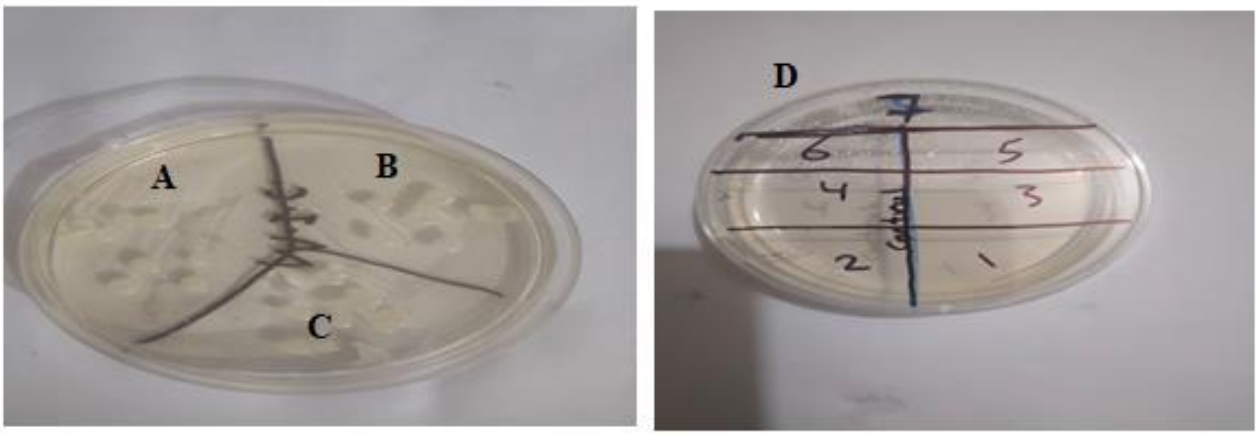

Figure 14. (A, B, C) Examples of various phenotypic susceptibility results for *Candida tropicalis* isolates showed the minimum inhibition concentrations (MICs) results for triple combination ZnO NPs, ACV and Flu against isolates at (0.04 µg/ml: 6,250 µg/ml) indicating MICs values significant inhibition of *Candida tropicalis* growth and high activity of antifungal drugs. (D) No recorded fungal growth in blank growth media in all plate wells.

The oral cancer isolates of *C. albicans* showed intermediate resistance at the highest test concentration 0.1µg/ml compared to the oral health isolate when using antifungal drugs alone. Clearly, treatments of ZnO NPs or ACV show potent efficacy against both test isolates compared to AmphB and Flu. Among the binary treatments, the isolate obtained from an oral cancer patient exhibited the highest MICs (0.06µg/ml; 25,000 µg/ml) and MFCs (0.1 µg/ml; 50,000 µg/ml) values in the combination of ZnO NPs and ACV compared with isolate derived from healthy individual (Table 1). Moreover, the MICs (0.04µg/ml; 6,250µg/ml) and MFCs (0.06µg/ml; 25,000µg/ml) values were higher in oral cancer isolates compared to treatments of healthy isolates in the triple combinations of ZnO NPs, ACV, and AmphB or Flu.

Table 1. The efficiency of the tested antifungal agent, either alone or in combination with dual and triple formulations, against *Candida albicans* isolates isolated from the oral cavity cancer (P111) and healthy participants (H10).

C. albicans **(The patient isolate)**	**MIC (three antifungal agents tested) (µg/ml); ACV (µg/ml)**	**MFC(three antifungal agents tested) (µg/ml); ACV (µg/ml)**
Flu	> 0.1	> 0.1
AmphB	> 0.1	> 0.1
ZnO NPs	0.06	0.1
ACV	25,000	50,000
ZnONPs with ACV	0.05; 12,500	0.1; 50,000
Flu with ZnONPs	0.06	0.1
Flu with ACV	0.06; 25,000	0.1; 50,000
AmphB with ZnONPs	0.06	0.1
AmphB with ACV	0.06; 25,000	0.1; 50,000
Flu with ZnONPs and ACV	0.04; 6,250	0.06; 25,000
AmphB with ZnONPs and ACV	0.04; 6,250	0.06; 25,000
C. albicans **(The healthy isolate)**		
Flu	0.06	0.1
AmphB	0.06	0.1
ZnO NPs	0.05	0.06

ACV	12,500	25,000
ZnONPs with ACV	0.03; 3,125	0.05; 12,500
Flu with ZnONPs	0.04	0.05
Flu with ACV	0.04; 6,250	0.05; 12,500
AmphB with ZnONPs	0.04	0.05
AmphB with ACV	0.04; 6,250	0.05;12,500
Flu with ZnONPs and ACV	0.01; 781	0.03; 3,125
AmphB with ZnONPs and ACV	0.01; 781	0.03; 3,125

Labels: AmphB- Amphotericin B, Flu- Fluconazole, ZnO NPs- Zinc Oxide Nano Particles and ACV- Apple Cider Vinegar.

The isolate of *C. tropicalis* isolated from the oral cancer patient showed more resistance in some antifungal treatments compared with this isolated from healthy individual. For example, the MIC was 0.05; 12,500 µg/ml for the combination of ZnO NPs and ACV respectively in the isolate obtained from oral cancer patient, compared to 0.04; 6,250 µg/ml for the same combination in the isolate isolated from healthy individual. Similar observations for these formulations were recorded in MFCs where their values were 0.06; 25,500 µg/ml and 0.05; 12,500 µg/ml for the isolates obtained from oral cancer and healthy individuals respectively. Triple combinations had the best effect on the fungal growth by lowering the doses required for MFC values into 0.03; 6,250 µg/ml in investigated isolates regardless of the source of the fungal isolates (Table 2). These findings suggest both binary and triple combinations were overcoming resistance mechanisms.

Table 2. The efficiency of the tested antifungal agent, either alone or in combination with dual and triple formulations, against *Candida tropicalis* isolates isolated from the oral cavity cancer (P128) and healthy participants (H25).

C. tropicalis **(The patient isolate)**	**MIC (three antifungal agents tested) (µg/ml); ACV (µg/ml)**	**MFC(three antifungal agents tested) (µg/ml); ACV (µg/ml)**
Flu	> 0.1	> 0.1
AmphB	> 0.1	> 0.1
ZnO NPs	0.06	0.1
ACV	25,000	50,000
ZnO NPs with ACV	0.05; 12, 500	0.06; 25,000
Flu with ZnO NPs	0.05	0.1

Flu with ACV	0.05 ; 12,500	0.1
AmphB with ZnO NPs	0.05	0.1
AmphB with ACV	0.05; 12,500	0.1; 50,000
Flu with ZnO NPs and ACV	0.03; 3,125	0.05 ; 12,500
AmphB with ZnO NPs and ACV	0.03; 3,125	0.05; 12,500
C. tropicalis **(The healthy isolate)**		
Flu	0.1	> 0.1
AmphB	0.06	0.1
ZnO NPs	0.05	0.06
ACV	12,500	25,000
ZnO NPs with ACV	0.04; 6,250	0.05; 12,500
Flu with ZnO NPs	0.05	0.06
Flu with ACV	0.05; 12,500	0.06; 25,000
AmphB with ZnO NPs	0.05	0.06
AmphB with ACV	0.05; 12,500	0.06; 25,000
Flu with ZnONPs and ACV	0.03; 3,125	0.05; 12,500
AmphB with ZnONPs and ACV	0.03; 3,125	0.05;12,500

Labels: AmphB- Amphotericin B, Flu- Fluconazole, ZnO NPs- Zinc Oxide Nano Particles and ACV- Apple Cider Vinegar.

Discussion

The current results showed that *C. albicans* and *C. tropicalis* were dominant species in oral cavities of cancer patients and healthy individuals. These results are similar to other findings who found a notable prevalence in isolation rats of both previous species in medical departments (Meng et al., 2025); (Cornely et al., 2025).

Diverse yeast form has been documented in the oral cavity of the Iraqi population in both health and disease states (Al Anbagi et al., 2025). That microenvironment able to be altered based on the human body state. Recently, several studies have been linked between the oral cancer and the mycobiome. The structure of human mycobiome may impact on various aspects of cancer including a pathogenesis, treatment, and prognosis. The weekend immune system may offer conditions to showcase the virulence of ubiquitous mycobiome (Monteiro et al., 2024). When fungal infections occurred, they cause serious health problems

especially in immunosuppressant. Recently, the incidence of invasive *Candida* infections including *C. tropicalis and C. albicans* has been increased leading to attracted clinical attention. Both species became noticeable pathogens in systemic regions and have unique pathogenicity and drug resistance worldwide (Lima et al., 2022; Iyer et al., 2022).

The wide use of azoles including fluconazole in the hospital as a primary treatment for greatest fungal infections and as a prophylactic therapy for high-risk patients leads to increase resistance especially in *Candida* spp. With their long-term use, unlike azoles, resistant evolution to polyense, including amphotericin B remines extremally rare (Spitzer et al., 2017).

Although the findings of *C. tropicalis and C. albicans* related to antifungal testing are closely similar (Figures 2-11), their isolates recovered from oral cavity cancer patients and healthy individuals were significantly in their susceptivity to the different drugs and their combinations. Notably, the isolates recovered from oral cancer patients were significantly lower susceptible to the tested antifungals (Figures ex..2,5, 7and 10). Increased Fluconazole and Amphotericin B resistance was observed in the current investigated Iraqi isolates in contrast to previous studies, for example *C. tropicalis* are showing worrisome resistance trends at worldwide. Antifungal resistance mechanisms are mainly related to the mutations of specific genes such as mutations in the ergosterol biosynthesis pathway genes and the increase expression of that genes as well as efflux pumps hyper expression (Branda et al., 2025; Lima et al., 2022b).

Cleary, isolates isolated from oral cancer patients showed highest resistance among all treatments compared to isolates from healthy individuals. these findings agreed with previous observations that recorded resistances either to specific drugs such as fluconazole or to amphotericin B in isolates obtained from different types of cancers including head-neck cancer (Schelenz et al., 2011); Monsen et al., 2023). These resistances are frequently associated with disturbing of fungal cell membrane. For example, amphotericin B usually targets ergosterol resulting in cytoplasmic membrane instability and pore formation. Similarly, Azoles including Fluconazole, lead to qualitative or quantitative alterations in the target enzyme lanosterol 14 q-demethylase leading block the synthesis of cell membrane-stabilizing ergosterol (Woods et al., 1974; Smith & Lee, 2022).

In current study, the individual treatments of ZnO NPs or ACV resulted in lower OD values in both species compared to controls and antifungal drugs. Similar to antifungal drugs, the isolates of *C. albicans* and *C. tropicalis* obtained oral cancer patients were a high susceptibility to ZnO NPs or ACV treatments. The Zinc oxide nanoparticles including ZnO NPs and AgNPs have been effectively reduction in *Candida* growth as well as inhibiting virulence factors (Marie et al., 2023). With nanoparticle size and large surface area, ZnO NPs have ability to penetrate the fungal membrane by diffusion and endocytosis and disrupt mitochondrial activity inside the cytoplasm (Chauhan et al., 2020); (Yassin et al., 2023) Additionally, ergosterol biosynthesis was significantly reduction in strains of *C. albicans* compared to untreated control cells as described (Abomuti et al., 2021).

The current results of effective apple cider vinegar treatments against both species are in agreement with studies indicating its capability to inhibit growth of both *C. albicans* and *C. tropicalis* due to the existence of organic acids, especially acetic acid and phenolic compounds (Ousaaid et al., 2021). However, rare studies have demonstrated its potential antifungal activity (Mota et al., 2015).The mechanisms of ACV action related to role of organic acids to release protons into cells decreases intracellular pH destruction the microbial membrane cells leading to the protonation of cell macromolecules disrupting the microorganisms and stimulating their death (Zhang et al., 2020). Similarly, phenolic compounds may disturb the integrity of cell membrane resulting to modify their permeability (Botton et al., 1990). The ACV compounds

network with diverse intra-molecular constituents of cells producing complexes and affective the processes of energy and protein synthesis in microorganisms as well as the synergy between its active ingredients (Ousaaid et al., 2021). In current studies, the earlier variations of antifungal resistances of isolates in both investigated species may link to owing some virulence traits such as thicker cell walls and share genetic pathways with virulence (Makled et al., 2024).

The synergistic mode of action between ZnO NPs with ACV was superior to reduce growth of *C. albicans* compared with all other dual combinations except AmphB with ACV. In another hand, combination of ZnO NPs and ACV was superior to reduce fungal growth of *C. tropicalis.* However, that optical growth reduction was significantly varied between the two sets. No previous studies have been tested that combinations on fungal species or even rare studies have investigated other combinations of either ZnO NPs or ACV with. The highly effectiveness of the ZnO NPs and ACV combination or other combinations is clearly related to synergetic effect compared to their effect in alone treatments due to having different cellular targets. That results in an improvement in antifungal effectiveness and suggests integrating in their mechanisms (Chand et al., 2021). Combined ZnO NPs or ACV with AmphB or flu may lead to increase effect of later antifungal agents that are known for their roles in reduction of ergosterol and toxic accumulation of ergosterol precursors subsequent causing severe stress on the cell membrane (Spitzer et al., 2017). Variations between isolates in combination treatments may be explained by morphogenesis in *C. albicans* in response to specific genetic and virulence traits of isolates or environmental conditions inside the host (Vila et al., 2020; Makled et al., 2024). The species ability to switch from a yeast form to a hyphal form and other cell types is usually controlled by the host immune system. That makes cancer patients prone to proliferation, tissue invasion and local infection by highly pathological hyphae (Vila et al., 2020).

The current findings of triple combinations of ZnO NPs, ACV and AmphB or Flu showed noteworthy abilities to inhibit growth of *C. albicans* compared with all other dual combinations. Both AmphB or Flu functioned by directedly interact with membrane ergosterol or targeting the ergosterol biosynthetic enzyme respectively (Spitzer et al., 2017) may contribute to facilitate interring the ZnO NPs, or constitute particles of ACV into the *Candida* cells and start other mechanisms of actions. That combinations showed to be more effective against health isolate than against oral cancer isolate. Though, the addition of any antifungal drugs to the dual combinations of ZnO NPs and ACV didn't differ significantly in *C. tropicalis* even between isolates, indicating to reach fungal cells to maximum inhibition effects that cannot exceeded by adding a third compound. Furthermore, possess a unique resistance mechanism in *C. tropicalis* that are not found in another species could be suggested (Chand et al., 2021).

Recently, several studies have proposed using combination therapy to reduce host toxicity through lowering the standard drug doses and enhancing the efficiency of conventional antifungal drugs (Scorzoni et al., 2017; Chand et al., 2021). Rare studies have experimented triple combinations. However, several suggestions have proposed for drug combinations. For example, delay or even prevent the development of resistance might complete through quickly decreasing the pathogen populace and accumulate several mutations to present resistance for two combined drugs or reverse antibiotic resistance. Based on drug combinations, that combination potentially develops synergistic effect in resistant mutants (Spitzer et al., 2017). Similar to dual combinations, thus, different strategies to converse of antibiotic resistance using triple combinations might be proposed.

Determination of minimum inhibition concentrations (MICs) and minimum fungicidal concentrations (MFCs) for tested isolates

Determining the MICs can be tricky due to 'residual growth', where some *Candida* species like *C. albicans* and *C. tropicalis* show persistent but incomplete growth at high azole concentrations. This stress-response phenomenon can be easily misinterpreted as drug resistance in visual assays (Berkow et al., 2020). In this study, all introduced treatments against *Candida* isolates for both species were effective in a dose dependent manner. To detect the MICs and MFCs, the obtained results of *Candida* isolates treated with the different concentrations of studied treatments showed decrease in the number of colonies or preventing of the *Candida* growth with increased concentrations of treatments. The MIC values for AmphB were highly active against isolates of both *C. albicans* and *C. tropicalis* isolated from healthy isolate with MIC values 0.06µg/ml in agreement with previous studies (Chiu et al., 2006); (de Aquino Lemos et al., 2009). Compared to AmphB, the MIC values for Flu against *C. albicans and C. tropicalis* were 0.1µg/ml in healthy isolates due to the emergence of the resistant trait. Studies have reported fluconazole resistant in *Candida* isolates (Pfaller & Diekema, 2012; (de Aquino Lemos et al., 2009).

MIC values for the isolates of both species obtained from the oral cancer patients were not recorded in this study because it needed to prepare concentrations higher than the highest concentration tested (0.1µg/ml) of drugs AmphB and Flu. The current findings concur with previous suggestion that fungistatic or fungicidal are strain and concentration dependent (de Aquino Lemos et al., 2009). Successfully, independent treatments of ZnO NPs and ACV showed greater antifungal efficacy than drugs AmphB and Flu in the current results, and reduced the MIC values of isolates of both species (Table 1and2) with requirement a high dose of ZnO NPs or ACV in patient isolates. The MFCs of ZnO NPs or ACV alone were obviously reduced the concentrations needed to prevent *Candidal* growth compared to single treatments of antifungal drugs.

The combination of ZnO NPs with ACV reduced the MIC values for both the patient isolate and for non-patient isolate of *C. albicans* compared with the use of each antifungal separately. These results agreed with a previous result revealed that ZnO NPs with Fluocytosin can reduce the antifungal concentration to 25-50% with MIC of the *Trichophyton* spp (Khalifa et al., 2023). Similarly, the MIC values for the binary combinations of ZnO NPs or ACV with AmphB or Flu were obviously decreased for both species and isolate dependent in the present study.

Thus, the current findings indicate that combining ZnO NPs and ACV offers an alternative in combinations therapies against multidrug-resistant fungi, leading to a reduction in the number of antifungals used as being proposed by Chand and Colleagues (2021).

Furthermore, using binary combinations of ZnO NPs or ACV with antifungal drugs were proposed in this study to reduce doses of antifungal drugs and their toxicities. That performed through several mechanisms due to targeted distinct cellular components. For examples, the fluconazole-induced disruption of the cell membrane allowed the ZnO NPs to enter the fungal cells generating reactive oxygen species (ROS) which alter the permeability of the fungal membrane resulting in the breakdown of fungal DNA, enzymes, and proteins. Later, the inhibited cellular processes lead to leakage of cells content and demise of the cells (Yassin et al., 2025).

Similarly, the current results the showed triple antifungal combinations had in excellent growth inhibitions against isolates of both species tested due to the complex interactions occurred between the three antifungal agents. There are little *in vitro* or *in vivo* data available dual combinations of antifungals tested in the current study and even rare on the triple combinations with nanoparticles or apple cider vinegar. However, some studies reported drug combinations were a suitable decision in specific conditions (Vitale et al., 2005). That results may be due to targeting different cellular pathways. It is known that amphotericin B usually bind to ergosterol,

creating pores in the membrane and fluconazole target ergosterol biosynthesis that both work synergically with ZnO NPs and apple cider vinegar. These findings suggest a typical solution for multidrug resistance fungal infections (Mohammed et al., 2021).

In this study, a subset of the most promising drug combinations showing interesting efficacy against strains of *C.albicans* and *C. tropicalis*. Its transform fluconazole and amphotericin B from completely ineffective to highly efficacious when applying both dual and triple combinations. Antifungal resistance is a critical problem, so searching on new antifungals and targets is needed. Also, combination therapy could increase the activity of antimicrobial drugs against candidiasis. Due to non- toxic effects of these combinations to human cells, they can be used as a new application method for fungal infections. Synergism observed in combinations indicates that (ZnO NPs or ACV) and individual drugs (Flu, AmphB) probably share a non-competitive cellular target (Mohammed et al., 2021).

Conclusion

Based on these findings, it is concluded that *C. albicans* and *C. tropicalis* are associated commonly with the oral cavity patients and healthy individuals in Iraq. The susceptivity to antifungal and their combination by the isolates of both species recovered from oral cancer patients was significantly less than that isolated from healthy individual that suggested that they enquired the resistant during the treatment the cancer and their side effects. Also, ZnO NPs and ACV, and their combinations have demonstrated high antifungal activities against *C. albicans* and *C. tropicalis*. They overcome conventional drug resistance strategies and act synergistically with currently available antifungals, making them promising candidates for future therapies. In the future, antifungal therapeutic strategies will be rapidly modified with seeking to improve the used compounds by providing a broader-spectrum formula, safe, less toxicity, and eliminating or reducing antifungal resistance.

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