JCOMS eISSN:2783-3127 https://journalofcoms.com

Journal of

Current Oncology and Medical Sciences

Vol. 3, No.2

Original Free Access

Investigation of the effect of watery and alcoholic extract of *Arnebia euchroma* on the growth of *Candida* species isolated from patients with COVID-19 associated oral candidiasis using microdilution method

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Abstract

Introduction: Conventional antifungals used to treat fungal infections are no longer as effective, leading to increased mortality. On the other hand, there is an emergence of multidrug-resistant (MDR) fungal strains and for this reason, finding new treatments or substances that have an antifungal effect is noticeable. Therefore, this study aimed to determine the antifungal effects of extracts of *Arnebia euchroma* on the growth of *Candida* species isolated from patients with COVID-19-associated oral candidiasis.

Materials and Methods: Conventional antifungals used to treat fungal infections are no longer as effective, leading to increased mortality. On the other hand, there is an emergence of multidrug-resistant (MDR) fungal strains and for this reason, finding new treatments or substances that have an antifungal effect is noticeable. Therefore, this study aimed to determine the antifungal effects of extracts of *Arnebia euchroma* on the growth of Candida species isolated from patients with COVID-19-associated oral candidiasis.

Results: The results of the present study showed that all the investigated isolates were sensitive to watery and alcoholic extracts of *Arnebia euchroma*. The MIC and MFC of *Arnebia euchroma* watery extract for *Candida albicans* were 512 µg/mL and for *Candida glabrata* were 1024 µg/m, as well as the MIC and MFC of this extract for Candida tropicalis, *Candida parapsilosis*, and *Candida krusei* were 2048 µg/mL. Whereas the MIC and MFC of the *Arnebia euchroma* alcoholic extract for Candida albicans were 0.015625 µg/mL and for *Candida glabrata* were 256 µg/mL, also the MIC and MFC of this extract for *Candida tropicalis* and *Candida parapsilosis* were 512 µg/mL and for *Candida krusei* were 1024 µg/mL.

Conclusion: All the studied *Candida* isolates were sensitive to both types of *Arnebia euchroma* root extract, and the alcoholic extract, compared with the watery extract, inhibited the growth of the tested *Candida* isolates at a lower concentration

Keywords: COVID-19, *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*

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Received: 2023.4.6, Accepted: 2023.6.18

Introduction

The immune dysregulation triggered by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has been hypothesized as a causal pathway for the increasingly reported oral manifestations associated with coronavirus diseases (COVID-19), especially the ones of fungal origin. Oral candidiasis is a common opportunistic fungal infection of the oral cavity caused by an overgrowth of *Candida* species (1,2). In healthy individuals, *Candida* exists harmlessly in mucus membranes such as the ears, eyes, gastrointestinal tract, mouth, nose, reproductive organs, sinuses, skin, stool, and vagina, but in some patients, it can overgrow and cause symptoms (3). Oral candidiasis causes creamy white lesions, usually on the tongue or inner cheeks. Sometimes it may spread to the roof of the mouth, the gums or tonsils, or the back of the throat (1).

The most common cause of COVID-19-associated oral candidiasis includes *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, and *Candida tropicalis* (4,5). *Candida albicans* are recovered from 60% of dentate patients' mouths over the age of 60 years.

On the other hand, antifungal resistance represents a major clinical challenge to clinicians responsible for treating oral candidiasis due to the limited arsenal of available antifungal agents. In addition, current drugs may be limited by drug–drug interactions and serious adverse effects/toxicities that prevent their prolonged use or dosage escalation (6). Changes in *Candida* spp. distribution may impact treatment recommendations due to differences in susceptibility to antifungal agents among different spp. Antifungal agents available for the treatment of oral candidiasis are restricted to polyenes, azoles, and the most recent echinocandin class. The emergence of multidrug-resistant strains that are insensitive to several classes of antifungals is a major concern worldwide (6,7). For these reasons, finding new treatments or substances that have an antifungal effect is noticeable.

One of the most common herbal drugs that are used in traditional medication is Abukhals (*Arnebia euchroma*) from the family of Boraginaceae. This plant is herbaceous, with sharp silver pubes and the flower is cluster shaped with stretched and alternate leaves (8-12). One of the most common habitats of this plant is Iran, especially Rasht. The root of this plant was used in reducing the swellings and had anticancer activity. It caused mild constipation and was used in nourishing the liver, kidneys, and spleen. New studies have shown that its extracts contain shikonin which is used in the treatment of burns and dermatitis, proliferation of skin's stem cells, improving arthritis, and inhabitation of inflammation by its antibacterial and antifungal effects (8-12).

In various studies conducted around the world, the antiviral and antibacterial properties of *Arnebia euchroma* have been proven (13,14), but so far there is no comprehensive study evaluating the antifungal effect of this plant in the treatment of oral candidiasis. The economic value of *Arnebia euchroma* as an herbal medicine and its use in cosmetics, food, and personal care products, and the lack of knowledge about antifungal susceptibility profiles of fungal elements causing oral candidiasis against *Arnebia euchroma* among Iranian patients prompted us to conduct a comprehensive study to fill this gap. It is important to have antifungal agents that will treat fungal infections without leading to increased resistance, though the use of azoles and echinocandin antifungal drugs against *Candida* species has seen this happen. As changes are seen in the resistance of fungi to antifungal drugs, in the present study we aimed to assess the antifungal effects of extracts of *Arnebia euchroma* on the growth of fungal agents isolated from COVID-19-associated oral candidiasis in Iran as a new antifungal agent.

Materials and Methods

Collection of plant materials

The roots of *Arnebia euchroma* were collected from the local areas of Rasht, north of Iran. It was authenticated from the proper source and a voucher specimen No: 01 was deposited in the Department of Pharmacognosy, Guilan University of Medical Sciences, Rasht, Iran.

Preparation of Plant Extracts

Collected roots were dried on mats in the shade and at room temperature, spread into thin layers that were not mixed over the 10-day drying period. The extraction process was conducted using 96% ethanol (for

alcoholic extracts) and distilled water (for watery extracts). For preparing alcoholic extract a powdered leaf (100 g) was added to 500 mL of ethanol and for preparing watery extract 100 g of the powder was added to 500 mL of distilled water. The extraction was carried out for 72 hours at room temperature with mild shaking. The extracts were filtered and concentrated at 37° C for 48 hours (15,16).

Fungal species

The antifungal activity was carried out against *C. albicans*, *C. tropicalis*, *C. krusei*, *C. glabrata*, and *C. parapsilosis* clinical isolates. All the mentioned isolates were previously collected from clinical specimens of patients with COVID-19-associated oral candidiasis hospitalized in Razi Hospital in Rasht City, Guilan, Iran (ethical code: IR.TUMS.SPH.REC.1400.030) and were recognized previously to the species level through sequencing of the internal transcribed spacer (ITS1-5.8s-ITS2) gene. Also, the standard strains of *Candida albicans* (ATCC 10231), *Candida glabrata* (ATCC 48465), *Candida krusei* (ATCC 2159), *Candida tropicalis* (ATCC 750), and *Candida parapsilosis* (ATCC 22019), which were obtained in lyophilized from the microbial collection of Iranian Biological Resource Center (IBRC, No. 80, West Hoveizeh St, North Sohrevardi Ave, Tehran, Iran) were included in the study.

Antifungal Activity Assessment

In vitro, antifungal susceptibility testing was performed against isolated strains according to the protocols described by the Clinical and Laboratory Standards Institute (CLSI) guidelines, document M27-A3 for yeasts (17). Briefly, by employing 24 hours cultures of yeast isolates on sabouraud dextrose agar (SDA; Difco) homogeneous yeast conidial suspensions were spectrophotometrically measured at the 530 nm wavelength and a percent transmission within the range of 75- 77%. The final inoculum suspension was adjusted to 10^5 conidia/mL in RPMI 1640 medium (GIBCO, UK) buffered at pH 7.0 with 0.165 M morpholino propane sulfonic acid (MOPS, Sigma-Aldrich, St. Louis, MO, USA). For the determination of antimicrobial activities against all of the studied microorganisms, the concentration of each plant extract was diluted two-fold from 4096 µg/mL to 0.00390625 µg/mL. After adding 100 µl of the inoculum suspension the microdilution plates were incubated at 35°C for 48 h; the plates were read visually according to the recommendations proposed by the CLSI M27-A3 document. The microdilution plates were inoculated with 100 µl of the diluted conidial inoculum suspension and incubated at 35 °C for 48 h. The plates were read visually according to the recommendations proposed by the CLSI M27-A3 document. Reference strains of *C. parapsilosis* (ATCC 22019) and *C. krusei* (ATCC 6258) were used for quality control purposes. MIC was interpreted as the lowest concentration of the sample, which showed clear fluid without the development of turbidity.

Ethics Statement

The study was approved by the Research Ethics Committee of Guilan University of Medical Sciences (the number of ethics committee protocol: IR.GUMS.REC.1401.526).

Statistical analysis

MIC values were calculated for clinical and standard samples and the strains were compared. For statistical analysis, a Chi-square test of homogeneity was performed at a significance level of 5 % (18).

Results

In the present study, the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of watery and alcoholic extracts of *Arnebia euchroma* on the growth of *Candida* species isolated from patients with COVID-19 associated oral candidiasis (*C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, and *C. glabrata*) were evaluated using broth microdilution method.

Table 1 presents the MIC of the watery and alcoholic extracts of *Arnebia euchroma* against tested *Candida*. The results demonstrate that all the mentioned *Candida* species against the watery and alcoholic extracts of *Arnebia euchroma* showed sensitivity.

The MIC and MFC of *Arnebia euchroma* watery extract for *Candida albicans* were 512 µg/mL and for *Candida glabrata* were 1024 µg/mL, as well as the MIC and MFC of this extract for *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei* were 2048

µg/mL. Whereas the MIC and MFC of *Arnebia euchroma* alcoholic extract for *Candida albicans* were 0.015625 µg/mL and for *Candida glabrata* were 256 µg/mL, also the MIC and MFC of this extract for *Candida tropicalis* and *Candida parapsilosis* were 512 µg/mL and for *Candida krusei* were 1024 µg/mL (Table 1, Figure 1).

Table 1. The minimum inhibitory concentration (MIC) of watery and alcoholic extracts of Arnebia euchroma on the growth of Candida species isolated from patients with COVID-19-associated oral candidiasis by microdilution method.

Type of extract	Yeast	The Concentration of watery and alcoholic extracts of *Arnebia euchroma* (µg/mL) in 96-well microplates											
		4096	2048	1024	512	256	128	64	32	16	8	Positive control	Negative control
Watery	*C. albicans*	-	-	-	-	+	+	+	+	+	+	+	-
Watery	*C.glabrata*	-	-	-	+	+	+	+	+	+	+	+	-
Watery	*C. krusei*	-	-	+	+	+	+	+	+	+	+	+	-
Watery	*C. parapsilosis*	-	-	+	+	+	+	+	+	+	+	+	-
Watery	*C. tropicalis*	-	-	+	+	+	+	+	+	+	+	+	-
96% ethanol	*C. albicans*	-	-	-	-	-	-	-	-	-	-	+	-
96% ethanol	*C. glabrata*	-	-	-	-	-	+	+	+	+	+	+	-
96% ethanol	*C. krusei*	-	-	-	-	+	+	+	+	+	+	+	-
96% ethanol	*C. parapsilosis*	-	-	-	-	+	+	+	+	+	+	+	-
96% ethanol	*C. tropicalis*	-	-	-	+	+	+	+	+	+	+	+	-

Type of extract	Yeast	The Concentration of watery and alcoholic extracts of *Arnebia euchroma* (µg/mL) in 96-well microplates											
		4	2	0.5	0.25	0.125	0.0625	0.03125	0.015625	0.0078125	0. 00390625	Positive control	Negative control
96% ethanol	*C. albicans*	-	-	-	-	-	-	-	-	+	+	+	-

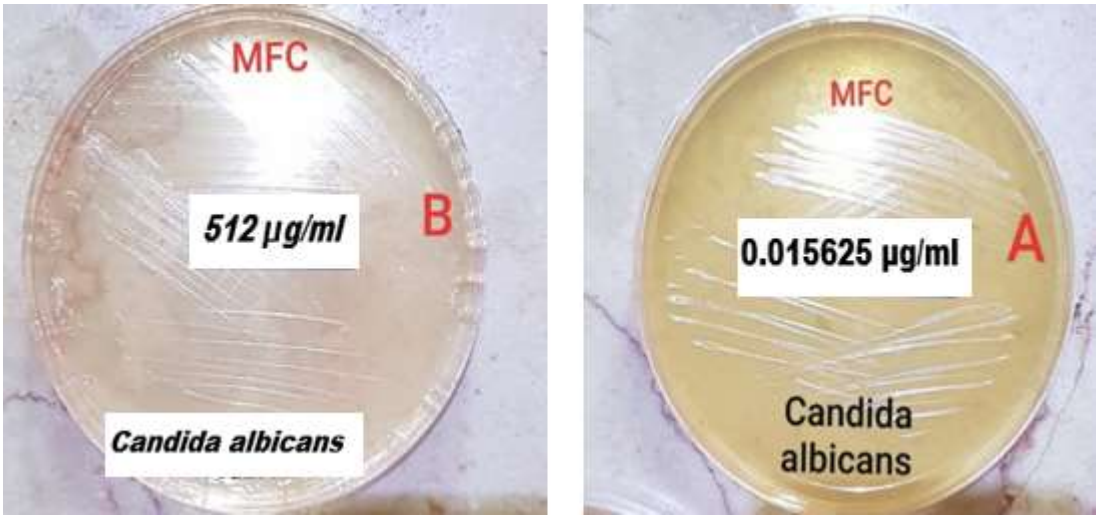

Figure 1. The MFC of the watery (A) and alcoholic (B) extracts of *Arnebia euchroma* against *Candida albicans*.

Discussion

The incidence of fungal infections with high morbidity and mortality has increased globally due to the limited antifungal arsenal and the high toxicity of some drugs. Only five antifungal drug classes are available, including polyenes, azoles, and allylamines that target ergosterol in the cell membrane, pyrimidine analogs that target DNA synthesis, and the new echinocandin class that targets β-glucan in the fungal cell wall. The treatment of oral candidiasis with the use of medicinal plants is of great interest due to fewer side effects, a variety of effective compounds in plants, and lower economic costs. Also, due to the increasing resistance of bacteria and fungi to antimicrobial compounds, the attention of researchers to medicinal plants and natural antimicrobial compounds to treat infections has increased. In various studies conducted around the world, the antiviral and antibacterial properties of *Arnebia euchroma* have been proven (13,14), but so far there is no comprehensive study evaluating the antifungal effect of this plant in the treatment of oral candidiasis according to the type of *Candida* species. For this reason, the purpose of the present study was to investigate the antifungal effects of *Arnebia euchroma* on different *Candida* species (*C. albicans, C. tropicalis, C. parapsilosis, C. krusei,* and *C. glabrata*) isolated from patients with COVID-19 associated oral candidiasis. Determining the possibility of using the watery and alcoholic extract of this plant as an antifungal product in the treatment of oral candidiasis was another purpose of this study.

The findings of the present study demonstrate that all the tested *Candida* species against the watery and alcoholic extract of *Arnebia euchroma* showed sensitivity and the alcoholic extract, compared with the watery extract, inhibited the growth of the tested *Candida* isolates at a lower concentration. Therefore, the alcoholic extract of *Arnebia euchroma* showed more antifungal effects on the tested *Candida* species isolated from the patients with COVID-19-associated oral candidiasis than the watery extract of this plant. In a study conducted by Madarshahi et al., (2022) in Mashhad, northeastern Iran, extracts from *Arnebia euchroma* root have been prepared and its antifungal and anti-aflatoxigenic activities against *Aspergillus flavus* have been investigated. The experiment confirmed the antifungal activity of *Arnebia euchroma* and provided evidence for the potential use of these natural compounds against fungi (8). Also, in a randomized controlled triple-blind trial conducted by Mohammadi et al., (2022) in Ahvaz, southwest of Iran, the effectiveness of *Arnebia euchroma* with vaginal cream clotrimazole 1% United States Pharmacopeia (USP) for the treatment of vulvovaginal candidiasis were compared. The Chi-square showed that there was a significant difference between the culture results in both groups ($p = 0.001$). and confirmed that a vaginal cream containing *Arnebia euchroma* could reduce the complaints of vulvovaginal candidiasis (9). In another

study conducted by Doulah et al., (2014) in Ahvaz, southwest Iran, the antifungal activity of methanolic extract of *Arnebia* species was screened using the disc-diffusion (DD) method and the minimal inhibitory concentration (MIC) using the macro dilution broth technique against *Aspergillus niger, Aspergillus flavus*, and *Candida glabrata*. The tested plant showed mild antimicrobial activity against all tested strains. The results obtained indicate that tested plants may become important in the obtainment of noticeable sources of compounds with health-protective potential, antioxidant, and antimicrobial activity (11). Besides, in a study conducted by Sasaki et al., (2000), in Japan, the antifungal activity of *Arnebia euchroma* was investigated *in vitro* against *Candida albicans*. The results showed that the extract inhibited the fungal growth at MIC 15.6 micrograms/ml (RPMI24 h) or 3.9 micrograms/ml (YNB24 h) (12). The difference in the findings of the present study with other studies could be since the composition of plant extracts and then their antimicrobial effects are different under the influence of endogenous and exogenous factors (environmental light, plant growth location, soil pH, plant genetics, temperature, and humidity). In other words, considering that the geographical location is effective on the amount and even the type of plant metabolites, the plant extracts in different geographical locations can have different antifungal activities.

Conclusions

In general, the findings of the present study showed that the alcoholic extract of *Arnebia euchroma* has a greater antifungal effect than its watery extract on the growth of Candida species isolated from COVID-19-associated candidiasis and can be a suitable alternative for antifungal drugs for the treatment of this infection. Further, it is necessary to conduct more studies in *in vitro* conditions to introduce this extract as a natural and new antifungal agent.

Author contribution

FF investigation, Data curation. **DR** conceptualization, methodology, project administration, and funding acquisition. **ZR** conceptualization, methodology, project administration, writing - original draft, resources, visualization, data curation. writing - review & editing. **KD** methodology, investigation. All authors contributed to the article and approved the submitted version.

Acknowledgments

The authors would like to thank all the staff members of the Medical Parasitology and Mycology Laboratory of Guilan University of Medical Sciences, Rasht, Iran.

Conflict of interest

The authors have no conflict of interest to declare.

Funding

There is no funding.

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