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Investigation of the effect of watery and alcoholic extract of *Calendula officinalis* on the growth of fungal agents isolated from skin infections caused by burns

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ABSTRACT

Discovering new antifungals, is essential to saving modern medicine. Therefore, this study aimed to determine the antifungal effects of extracts of *Calendula officinalis* on the growth of fungal agents isolated from skin infections caused by burns. In this experimental study, watery and alcoholic extracts of *C. officinalis* were prepared by the maceration method. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of extracts were evaluated against yeast isolates (*Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, Candida *krusei*, *Candida glabrata*, *Trichosporon asahi*, *Rhodotorula mucilaginosa*, and *Rhodotorula dirnensis*) and filamentous fungi (*Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Rhizopus oryzae*, *Alternaria alternata*, *Fusarium oxysporum*, and *Cladosporium cladosporioides*). All tested yeast isolates were resistant to the watery extract of *C. officinalis*, while all (except *C. tropicalis*) were sensitive to the alcoholic extract. Among filamentous fungi, only *Aspergillus* spp. were sensitive to the watery extract of *C. officinalis*. In this study, only *A. alternata* and *C. cladosporioides* were resistant to the alcoholic extract of *C. officinalis*. The results of this study showed that the alcoholic extract of *C. officinalis* can be a suitable alternative for antifungal drugs in the treatment of burn wound infections caused by fungal agents.

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1. Introduction

Burns are a type of painful skin wound caused by thermal or other acute trauma, such as electricity, radiation chemicals, or friction. They are considered an important injury in low and middle-income countries [1]. They involve threats such as a decrease in quality of life, increasing disability, and death. The moist burn wound environment encourages adhesion, microbial growth, and infection development [2]. Fungal pathogens are among the important microorganisms which can infect burn wounds. These infections more frequently occur after the use of broad-spectrum antibiotics [3].

The most common cause of fungal burn infections includes *Aspergillus, Candida, Mucor, Fusarium, Rhizopus, Microsporia*, and *Alternara* spp. Deadly non-albicans *Candida* spp. such as *Candida tropicalis and Candida krusei* are also becoming more common [4, 5]. In contrast to other (non-filamentous) mycotic infections discovered in burn patients, *Aspergillus* spp. are independently linked to an odds-ratio of death that is about 12-fold higher. This is most likely a reflection of the mold's propensity for angioinvasion [6].

Results of a study by Bahar et al. at Tehran, Iran's Shahid Motahari Burn Hospital revealed that 13% of the study's participants had a fungal infection. The most prevalent fungus was found to be *Candida albicans* (45%), which was followed by *Aspergillus fumigatus* (35%), *Penicillium* (8%), *Aspergillus niger* (5%), and other fungi (7%) [7]. Additionally, just one specimen out of 111 patients in research by Kameshki et al. at the burn center in Isfahan, Iran, was positive, and the prevalence of fungal burn infection was 0.9%. They showed that the low rate of positive fungal cultures was caused by the early consideration of antifungals following burn injury [8].

On the other hand, due to the small number of systemically accessible antifungal drugs, antifungal resistance poses a significant clinical problem to clinicians treating invasive fungal diseases. Additionally, drug-drug interactions and major side effects/toxicities may be a limitation of currently available medications, preventing their extended usage or dosage escalation [9]. Due to the rising occurrence of infections caused by fungal species in various parts of the world and the higher prevalence of resistance to the widely used azole in many institutions, fluconazole resistance in non-*Candida albicans* species is of special concern. It has been shown that *Candida glabrata* resistance to echinocandins is increasing at a number of US institutions, and a greater proportion of these isolates may also be azole resistant. Worldwide, it has also been discovered that *Aspergillus fumigatus* isolates that are azole resistant can result in invasive infections with significant fatality rates due to clinical and environmental exposure to this class of drugs. Additionally, some *Aspergillus* species and other molds, like some types of *Scedosporium* and *Fusarium,* are less susceptible to or completely resistant to therapeutically accessible antifungals [9]. Because of this, therapeutic failure is a significant clinical issue that affects patients with burn wound infections. Emerging resistance strains, limited bioavailability, the inability of topical antifungals to penetrate nonviable tissue, and medication interactions have all been attributed for this failure. For these reasons, finding new treatments or substances that have an antifungal effect is noticeable.

The plant species known as *Calendula* (*Calendula officinalis*) belongs to the daisy family. Due to its nourishing effects on the skin and its calming and antibacterial qualities, it is applied topically to treat wounds [10, 11]. Sesquiterpenes, flavonoid glycosides, triterpene saponins, triterpene alcohols, flavonoids, carotenoids, xanthophylls, phenolic acids, steroids, mucilage, tocopherol, and calenduline are among the chemical substances present in this species. Since the 12th century, *C. officinalis* extract has been used extensively throughout Europe as a topical anti-inflammatory [10, 11].

In various studies conducted in Iran and the world, the antibacterial properties of *C. officinalis* and its role in healing burn wounds have been proven [11-15], but so far there is no comprehensive study evaluating the antifungal effect of this plant in the treatment of fungal infections according to the type of fungus (either yeast or saprophytic fungi). Also, the present investigation is the first study conducted on this issue in Iran. We conducted an in-depth investigation to fill this gap because Iranian patients lacked knowledge about the antifungal susceptibility profiles of fungi causing fungal burn wound infection against *C. officinalis*, despite the economic value of *C. officinalis* as an herbal medicine and its use in cosmetics, perfumery, pharmaceutical preparations, and food. Therefore, the present study aimed to assess the antifungal effects of extracts of *C. officinalis* on the growth of fungal agents isolated from skin infections caused by burns in Iran. Clarifying this aspect would improve clinical care and enable the selection of the most effective treatment regimens.

2. Materials and Methods

2.1 Plant material

The leaves of *C. officinalis* 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.

2.2 Preparation of extracts

The collected leaves were cleaned, dried by air in the shade, and then pulverized using a mechanical grinder after being cut into small pieces and put through a 20-mesh screen. The extraction process was conducted using 96% ethanol (for alcoholic extracts) and distilled water (for watery extract). For preparing alcoholic extract a powdered leaf (100 g) was added to 500 mL 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 [16, 17].

2.3 Fungal isolates

The antifungal activity was carried out against *C. albicans*, *C. tropicalis*, *C. krusei*, *C. glabrata*, *Candida parapsilosis*, *Trichosporon asahi*, *Rhodotorula mucilaginosa*, *Rhodotorula dairenensis*, *Aspergillus flavus*, *A. niger*, *A. fumigatus, Rhizopus oryzae*, *Alternaria alternate;*, *F. oxysporum*, and *Cladosporium cladosporioides*. All the mentioned isolates were previously collected from clinical specimens of patients with fungal burn infection hospitalized in Velayat Burn Hospital in Rasht city, Guilan, Iran and were recognized previously to the species level through sequencing of the partial b-tubulin gene for *Aspergillus* isolates and the internal transcribed spacer (ITS1-5.8s-ITS2) gene foe the other fungal isolates (ethical code: IR.TUMS.SPH.REC.1401.017).

Patients with localized fungal wound infections were included, including those with eschar separation, partial thickness burns that became full thickness burns, blackening of tissue, worsening of the wound with cellulitis or necrotizing fasciitis, and fever despite taking broad-spectrum antibiotics for more than 7 to 15 days. Patients who had taken any systemic antifungal medications before to participation were disqualified from the research in order to prevent false-negative outcomes [18].

2.4 Screening for antifungal activity

According to the procedures outlined in the Clinical and Laboratory Standards Institute (CLSI) recommendations, document M38-A2 for filamentous fungi [19] and document M27-A3 for yeasts [20], *in vitro* antifungal susceptibility testing was carried out against isolated strains. Briefly, by employing 24 hours cultures of yeast isolates on sabouraud dextrose agar (SDA; Difco; USA) Spectrophotometric measurements of homogenous yeast conidial solutions were made at 530 nm with a percent transmission between 75 and 77%. In RPMI 1640 medium (GIBCO, UK) buffered at pH 7.0 with 0.165 M morpholino propanesulfonic acid (MOPS, Sigma-Aldrich, St. Louis, MO, USA), the final inoculum suspension was adjusted to 105 conidia/mL. The microdilution plates were incubated at 35 °C for 48 hours after adding 100 µL of the inoculum suspension; the plates were then visually read in accordance with the suggestions made in the CLSI M27-A3 documentFor filamentous fungi, sterile cotton swabs soaked in sterile saline solution with Tween 40 (0.05%) were used to gently scrape the surface of mature colonies to create inoculum suspensions, which were then produced on potato dextrose agar (MERCK, Germany) for 2-3 days. The quantitative colony count used to quantify the viable CFU/per milliliter was used to adjust the supernatants spectrophotometrically to an OD range of 0.09-0.13 (0.5106 to 3.1106 CFU/mL) at a wavelength of 630 nm. Conidial suspensions, the majority of which were conidia, were adjusted to 10^6 conidia/mL after being diluted 1:50 in RPMI 1640 medium (GIBCO, UK). Then, 100 µL of the diluted conidial inoculum suspension was used to inoculate the microdilution plates, which were then incubated for 48 hours at 35 °C. The CLSI M38-A2 document's instructions were followed when visually reading the plates. For quality control, reference strains of *Candida parapsilosis* (ATCC 22019) and *C. krusei* (ATCC 6258) were employed. The minimum inhibitory concentration (MIC) was understood to be the sample's lowest concentration, which revealed clear fluid devoid of the formation of turbidity.

2.5 Statistical analysis

For clinical and environmental samples, MIC values were computed, and the strains were compared. A Chi-square test of homogeneity was carried out for statistical analysis at a significance level of 5% [21].

Table 1: The minimum inhibitory concentration (MIC) of watery and alcoholic extracts of *C. officinalis* on yeast species studied in the present investigation by broth microdilution method

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

+: Positive fungal growth, -: Negative fungal growth

Table 2: The minimum inhibitory concentration (MIC) of watery and alcoholic extracts of *C. officinalis* on filamentous fungi studied in the present investigation by broth microdilution method

Type of extract	Filamentous fungi	The Concentration of watery and alcoholic extracts of *C. officinalis* (µg/mL) in 96-well microplates										Positive control	Negative control
		4096	2048	1024	512	256	128	64	32	16	8		
Watery	*A. flavus*	-	-	-	-	-	-	-	-	+	+	+	-
Watery	*R. oryzae*	+	+	+	+	+	+	+	+	+	+	+	-
Watery	*A. niger*	-	-	-	-	+	+	+	+	+	+	+	-
Watery	*A. fumigatus*	-	-	-	-	-	-	-	-	+	+	+	-
Watery	*A. alternata*	+	+	+	+	+	+	+	+	+	+	+	-
Watery	*F. oxysporum*	+	+	+	+	+	+	+	+	+	+	+	-
Watery	*C. cladosporioides,*	+	+	+	+	+	+	+	+	+	+	+	-
96% ethanol	*A. flavus*	-	-	-	-	-	-	-	-	-	+	+	-
96% ethanol	*R. oryzae*	-	-	-	+	+	+	+	+	+	+	+	-
96% ethanol	*A. niger*	-	-	-	-	-	-	-	-	+	+	+	-
96% ethanol	*A. fumigatus*	-	-	-	-	-	-	-	-	-	+	+	-
96% ethanol	*A. alternata*	+	+	+	+	+	+	+	+	+	+	+	-
96% ethanol	*F. oxysporum*	-	-	-	+	+	+	+	+	+	+	+	-
96% ethanol	*C. cladosporioides,*	+	+	+	+	+	+	+	+	+	+	+	-

+: Positive fungal growth, -: Negative fungal growth

3. Results

In the present study, MIC and minimum fungicidal concentration (MFC) of watery and alcoholic extracts of *C. officinalis* on the growth of yeasts (*C. albicans, C. tropicalis, C. parapsilosis, C. krusei, C. glabrata, T. asahi, R. mucilaginosa,* and *R. dirnensis*) and filamentous fungi (*A. flavus, A. fumigatus, A. niger, R. oryzae, A. alternata, Fusarium oxysporum,* and *C. cladosporioides*) isolated from skin infections caused by burns were evaluated using broth microdilution method.

Table 1 presents the MIC of the watery extract of *C. officinalis* against yeasts isolated from skin infections caused by burns (*C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krosei*, *C. glabrata*, *T. asahi*, *R. mucilaginosa*, and *R. dirnensis*). The results demonstrate that all the mentioned yeasts against the watery extract of *C. officinalis* showed resistance. Also, according to the results mentioned in Table 1 all the tested yeasts against the alcoholic extract of *C. officinalis* were susceptible except for *C. tropicalis*.

MIC and MFC of the alcoholic extract of *C. officinalis* for *C. albicans* and *C. parapsilosis* were 16 µg/mL, for *C. krusei* and *C. glabrata* were 32 µg/mL, for *R. mucilaginosa* and *R. dirnensis* were 256 µg /mL, and for *T. asahi* were 512 µg/mL (Table 1).

Table 2 presents the MIC of the watery extract of *C. officinalis* against filamentous fungi isolated from skin infections caused by burns (*A. flavus, A. fumigatus, A. niger, R. oryzae, A. alternata, F. oxysporum, and C. cladosporioides*). The results demonstrate that watery extract of *C. officinalis* had an inhibitory effect on the growth of *A. flavus, A. fumigatus,* and *A. niger* isolates, but *R. oryzae, A. alternata, F. oxysporum, and C. cladosporioide* showed resistance to the watery extract of this plant. The MIC and MFC of the watery extract of *C. officinalis* for *Aspergillus flavus* and *A. fumigatus* were 32 µg/mL and for *A. niger* were 512 µg/mL.

In the present study, only A. alternata and C. cladosporioides were resistant to the alcoholic extract of C. officinalis and the other tested filamentous fungi were susceptible to the alcoholic extract. MIC and MFC of the alcoholic extract of C. officinalis for A. *flavus* and *A. fumigatus* were 16 µg/mL, for *A. niger* were 32 µg/mL and for *R. oryzae* and *F. oxysporum* were 1024 µg/mL (Table 2).

4. Discussion

The healing of burn wound infections with the use of medicinal plants is of great interest due to fewer side effects, 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 in Iran and the world, the antibacterial properties of *C. officinalis* and its role in healing burn wounds have been proven [11-15], but so far there is no comprehensive study evaluating the antifungal effect of this plant in the treatment of fungal infections according to the type of fungus (either yeast or saprophytic fungi). For this reason, the purpose of the present study was to investigate the antifungal effects of *C. officinalis* on different fungal species (yeast and filamentous fungi) isolated from patients with burn wound infection. Determining the possibility of using the watery and alcoholic extract of this plant as an antifungal product in the treatment of fungal burn infections was another purpose of this study.

The findings of the present study demonstrate that all the tested yeasts against the watery extract of *C. officinalis* showed resistance. Whereas, all the studied yeast isolates against the alcoholic extract of *C. officinalis* were susceptible except for *C. tropicalis*. Therefore, the alcoholic extract of *C. officinalis* showed more antifungal effect on the yeasts isolated from the burn wound infection

than the watery extract of this plant.

Among filamentous fungi, only *Aspergillus* spp. were sensitive to the watery extract of *C. officinalis*, while all (except *A. alternata and C. cladosporioides*) were sensitive to the alcoholic extract. Therefore, based on the results of this study, in addition to the yeast isolates, the alcoholic extract of *C. officinalis* showed more antifungal effect than the watery extract on filamentous fungal agents isolated from burn wounds.

A study by Efstratiou et al. was carried out in England to evaluate the antifungal activity of methanol and ethanol extracts of *C. officinalis* petals against fungal pathogens (*C. albicans* 0103 (UUC collection), *C. albicans* ATCC 90028, *C. krusei* ATCC 6258, *C. glabrata* ATCC 2001, *C. parapsilosis* ATCC 22019, *A. flavus* GC 6158, *A. fumigatus* 27.5, *A. niger* 27.5 and *Exophiala dermatitidis* GC 7895) using disc diffusion assay. When compared to Fluconazole, the results revealed that both methanol and ethanol extracts had outstanding antifungal activity against the tested strains of fungus [11]. Also, in a study conducted by Kasiram et al in India the in vitro antifungal activity of *C. officinalis* flower chloroform, acetone, and ethanolic (95%) extracts has been investigated against *A. niger*, *Rhizobium japonicum*, *C. albicans*, *C. tropicallis* and *Rhodotorula glutinis* using agar well diffusion assay. The extracts of *C. officinalis* showed a high degree of activity against all test fungi [22]. The difference in the findings of the present study with other studies could be due to the fact that 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.

It should be emphasized that the current study is the first one ever to use the broth microdilution method in accordance with the CLSI protocol to assess the antifungal activity of *C. officinalis* extracts. The creation of a quantitative result (the MIC), the reproducibility and convenience of having preprepared panels, and the economy of reagents and space that happens owing to the test's miniaturization are all benefits of the microdilution technique. If an automatic panel reader is employed, it can help with the computerized report generation as well.

Also, the probability of a multi-targeting effect of the extract, not enough knowledge of the active compounds, and the possibility of antagonistic or synergistic interactions between components in a mixture were limitations of the current study.

In general, the findings of the present study showed that the alcoholic extract of *Calendula* has a greater antifungal effect than its watery extract on the growth of fungal isolates causing burn wound infections and can be a suitable alternative for antifungal drugs for the treatment of fungal burn wound infection (caused by yeasts and filamentous fungi). Further, it is necessary to conduct more studies in *in vitro* conditions in order to introduce this extract as a natural and new antifungal agent.

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Authors' contributions

GM: Investigation, Data curation. DR: Conceptualization, Methodology, Project administration. ZR: Conceptualization, Methodology, Project administration, Writing - original draft, Resources, Visualization, Data curation. Writing - review & editing. MM: Methodology, Investigation. All authors contributed to the article and approved the submitted version.

Conflict of interests

The authors have no conflict of interest to declare.

Ethical declarations

The Guilan University of Medical Sciences Research Ethics Committee (the number of ethics committee protocol: IR.GUMS.REC.1401.185) approved the study design.

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