Original Article

The Special Antifungal Properties of Sugar Beet Leaves Against Fungal Species

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Abstract

Background: Research on antifungal agents for infectious diseases caused by multidrug-resistant microorganisms, particularly in immunocompromised patients, has continued. Mucormycosis, candidiasis, aspergillosis, and cryptococcosis are mortal and need more attention due to their spreading potential. Natural products with long-time usage as preservatives, food additives, and medicine in traditional and local resources have been investigated for too many purposes. Some medicinal herbs show antimicrobial properties. This study focused on the antifungal properties of Sugar beet (*Beta vulgaris* subsp. *vulgaris*), Yarrow (*Achillea wilhelmsii* K. Koch), Cinnamon (*Cinnamomum zeylanicum*), and Den or Don (*Oliveria decumbens* Vent.) against *Candida albicans*, *Cryptococcus neoformans*, *Mucor circinelloides*, and *Aspergillus flavus*.

Materials and Methods: The minimum inhibitory concentration (MIC) of hydroethanolic crude extract of Sugar beet leaves (*Beta vulgaris* subsp. *vulgaris*), aerial parts of Yarrow (*Achillea wilhelmsii* K. Koch), Cinnamon barks (*Cinnamomum zeylanicum*), and Den or Don aerial parts (*Oliveria decumbens* Vent.) against clinical and standard isolates of *Candida albicans*, *Cryptococcus neoformans*, *Mucor circinelloides*, and *Aspergillus flavus*, determined according to CLSI M60 and CLSI-M38-A2 for yeasts and filamentous strains, respectively in comparison to Fluconazole and Amphotericin B as positive controls.

Results: Sugar beet extract revealed the best minimum inhibitory concentration (MIC) values against clinical/standard *Candida albicans* (1 µg/ml and 0.5 µg/ml), respectively, but was insufficient against other species. Other herbal extracts showed a higher MIC range compared to controls.

Conclusion: These findings suggest more research in in-vivo studies, finding safe herbal products with antifungal properties.

Keywords: *Candida albicans*, Filamentous fungi, Herbal medicine, Yeast

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Introduction

Despite all improvements in pathogens diagnosis and antimicrobial medicines, invasive fungal diseases cause high mortality due to immunosuppressive conditions[1]. Global microbial drug resistance, access limitations to proper tests, and chronic adverse effects of conventional antifungal agents would also be other reasons to investigate for better active pharmaceutical ingredients[1].

Yeast and Filamentous Pathogens: *Candida* species, the fourth most common nosocomial infection in patients with long-time hospitalizations[2], could cause septicemia with 25-54% mortality and morbidity in neonates[3]. Like *Candida albicans* (*C. albicans*), another highly mortal (41%-61% in HIV patients) fungus in the WHO fungal priority pathogens list is *Cryptococcus neoformans* (*C. neoformans*) which affects the lung first, then leads to cryptococcaemia and cryptococcal meningitis[4]. *Aspergillus flavus* (*A. flavus*), which is predominantly found in hot-arid climates such as Asia, the Middle East, and Africa[5], could dangerously affect different organs like cutaneous and subcutaneous tissues, joints and bones, cardiovascular, respiratory, and central nervous systems[6]. Mucormycosis, another fatal fungal infection caused mainly by *Rhizopus*, *Mucor*, and *Lichtheimia*, is another challenge in treating immunosuppressed patients with high mortality due to necrosis, tissue extension, and vascular invasion[7].

Medicinal Plants; Therapeutic Benefits and Potential Health Effects: Background on using herbal extracts as food preservatives, medication, and cosmeceutical agents has prompted researchers to investigate their properties based on needed applications; they are biodegradable, less toxic, and have low adverse reactions compared to conventional preservatives and antibiotics[8].

Beta vulgaris (*B. vulgaris*, from the Chenopodiaceae family) is a biennial edible root plant[9]. Two classified groups related to the *Beta* genus are wild maritime and cultivated. *B. vulgaris* subsp. *cicla* (Leaf beets), *B. vulgaris* var. *crassa* (Forage beets), *B. vulgaris* subsp. *vulgaris* (Sugar beet) and *B. vulgaris* L. ssp. *vulgaris* var. *rubra* (Beetroot) are related to the last one[10]. As a colorant, red beetroots have water-soluble nitrogenous phytochemicals such as betacyanins (with a red-violet color) and betaxanthins (with a yellow color)[11-13]. Betavulgaroside°I to Betavulgaroside°IV, Betavulgaroside°VI to Betavulgaroside°VIII, and Chikusetsusaponin°IVa were found in roots of Sugar beet, however, in the leaves, reported there are Betavulgaroside°V, Betavulgaroside°IX, and Betavulgaroside°X[14, 15].

Achillea wilhelmsii K. Koch (*A. wilhelmsii* K. Koch), from the Asteraceae family, mostly found in Iran, is a rich source of flavonoids and sesquiterpene lactones with antimicrobial, antihyperlipidemic, and antihypertensive properties[16]. Traditionally, it is used for gastrointestinal diseases such as *Helicobacter pylori*, spasms, and ulcers[17].

Cinnamon, which was commonly used as a spice and herbal medicine in the past[18], is the bark of two species of the genus *Cinnamomum* (*C. verum* J.Presl and *C. zeylanicum* Blume) related to the Lauraceae family[19]. There are too many studies on the biological effect of Cinnamon essential oils on cardiovascular disease, cognition, reducing inflammation, and antimicrobial properties[20]. In Ayurvedic medicine, there were some indications for Cinnamon usage in respiratory, gastrointestinal, and gynecological disorders[20]. Reported effects may be related to its secondary metabolites such as trans-cinnamaldehyde, cinnamaldehyde, eugenol, and linalool[21].

Oliveria decumbens Vent. (*O. decumbens*), a member of the Compositae family is an endemic plant in the Iranica flora[22]. It has traditionally been used for GI disturbance, diarrhea, and febrility[23]. Also, the effects of its phenolic compounds on the immune system as a natural booster and antioxidant have been informed[24].

To the best of our knowledge, there was no pointed report on the effect of Sugar beet leaves extract on *Mucor circinelloides* (*M. circinelloides*) and resistant *C. albicans*; also, most studies deal with Beetroot (*Beta vulgaris* L.). In this study, the antifungal effects of the hydro-ethanolic extract of *A. wilhelmsii* K. Koch (flowers), *B. vulgaris* (Sugar beet leaves), *C. zeylanicum* Blume (barks), and *O. decumbens* Vent. (aerial parts) have been investigated on the mentioned resistant microorganisms.

Methods

Preparation of Herbal Extracts: The maceration was the selected method for preparing crude extracts, according to the other relevant studies with agreeing and disagreeing results[25-34]; however, there was a change in the solvents ratios. 50 g Cinnamon cortex (SBMU-8053), purchased from a local market (2023

November, Isfahan_Iran), 50 g Sugar beet leaves (SBMU-8301), purchased from farmland in Khatoonabad (2023 November, Isfahan_Iran), 25 g aerial parts of *A. wilhelmsii* K. Koch (SBMU-1001). Moreover, 25 g *O. decumbens* Vent. (SBMU-1029), collected from Kazeroon (2024 July, Fars_Iran) were prepared. Mr. M. Kamalinejad (pharmacy school in Shahid Beheshti University of Medical Sciences) confirmed all herbs. Voucher specimens were stored at the herbarium of Shahid Beheshti University of Medical Sciences (Tehran, Iran). After cleaning and drying, liquid extraction was done at room temperature with 500 ml solvent, composed of a 50:50 proportion of deionized water and 96% ethyl alcohol (Jahan Khorma Processing Co.) for each plant separately, until 48 hours. After filtration with Whatmann filter paper grade 44, concentrative extract was prepared by heating on bain marie. Gained dried extracts are as follows: 7.75 g from Sugar beet, 2.57 g from *C. zeylanicum*, 4 g from *A. wilhelmsii* K. Koch., and 3.6g from *O. decumbens* Vent.°. Each of the extracts was kept in sterile vials at 4°C.

Microbial Isolates Selection: This study was conducted on four archived clinical isolates (from previous studies)[35, 36] and four standard isolates of *C. albicans* (ATCC 10261), *C. neoformans* (ATCC 90112), *A. flavus* (ATCC 16872), and *M. circinelloides* (ATCC 15242) strains. Three rows of a microplate were considered for the contact of Sugar beet leaves extract with fungal strains. Amphotericin B (AMB; Sigma-Aldrich; USA) and Fluconazole (FLC; Sigma-Aldrich; USA) were used as a control.

Yeast Suspensions and Antifungal Activity: An 18-hour culture of yeasts (*C. albicans* and *C. neoformans*) on Sabouraud Dextrose Agar (SDA; Merck, Germany) was used for yeast suspension preparation. Then, colonies were inserted into 3 ml distilled water in a tube. The suspensions' optical density (OD) with a spectrophotometer (536 nm) showed a transmittance value of about 75%-77%, revealing the presence of 5×10^3 CFU/ml yeast. Then, the suspensions were diluted 1:10 (1 ml of the primary suspension plus 9 ml of distilled water). The suspensions were diluted 1:100 by Roswell Park Memorial Institute 1640 medium (RPMI; Invitrogen, Gibco) addition[37]. Then, RPMI and Sugar beet leaves were added to the yeast suspensions, and the number of yeast cells was 2.5×10^3 cells/ml.

The broth microdilution method was made for antifungal activity. 100 µl of RPMI medium was added to all microplate wells. Next, 100 µl of Sugar beet leaves extract (500 µg/ml concentration) was added to the first well of row A. Then,100 µl of the content of the first well was transferred to the second well, and so on. Finally, 100 µl of the 10th well was discarded. So, concentrations of Sugar beet leaves extract were obtained at 500, 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9, 1.9, 0.95, and 0.47 mg/ml. The culture medium with yeast suspension was the positive control (11th well). To control for the potential antifungal effect of the extraction solvent (50% ethanol), the solvent alone was also considered a negative control (12th well). After, 100 µl of the yeast suspensions were added to all wells. Then, microplates were incubated (24, 48, and 72 h), and the minimum inhibitory concentration (MIC) was read. The test was performed in triplicate. The MIC breakpoints of FLC were MIC $\leq$ 2 µg/ml and MIC $\geq$ 8 µg/ml as a susceptible and resistant strain, respectively. For CLSI M60. 1st ed[38]. *Candida parapsilosis* (ATCC 22019) was used as a quality control.

Filamentous Suspension and Antifungal Activity: *A. flavus* and *M. circinelloides* were cultured on Potato Dextrose Agar (PDA; Merck, Germany) for filamentous suspension. With the saline solution and tween 80, inoculum suspensions were prepared from covering cultures of two strains, according to CLSI, M38-A2)[39]. The suspensions were inserted into a saline solution tube (2 ml). The final density of inoculum was 1×10^3 to 3×10^3 CFU/ml with a spectrophotometer (530 nm). The inoculum was diluted 1:50 in RPMI. First, 100 µl of RPMI was inserted into a 96-well microplate, and 100 µl sugar beet leaves extract (64 mg of crude extract in 1 ml distilled water) was added to the first well. The contents were mixed, 100 µl was transferred to a second well, and so on. 100 µl of content was discarded from the 10th well. The concentrations ranging of plant extract were prepared from 0.00625-32 µg/ml. Then 100 µl of fungal inoculum was added to wells (1 to 10), and the contents were mixed. The microplates were incubated at 30°C for 72 h. The tests

Table 1. MIC values (µg/ml) against yeasts and filamentous fungi.

Antifungal agent	*Candida albicans*		*Cryptococcus neoformans*		*Mucor circinelloides*		*Aspergillus flavus*	
	S[a] ATCC 10261	**C[b]**	**S ATCC 90112**	**C**	**S ATCC 15242**	**C**	**S ATCC 16872**	**C**
Sugar beet (leaves)	**0.5**	**1**	4	16	2	16	1	8
FLC[c]	**1**	**2**	2	8	1	8	0.5	4
AMB[d]	**1**	**2**	1	4	0.5	4	0.25	2
Achillea wilhelmsii K. Koch.	4	8	8	8	4	16	2	8
FLC	4	8	4	8	8	16	4	8
AMB	8	16	4	16	4	8	8	16
Cinnamomum zeylanicum	4	16	8	16	4	16	4	16
FLC	2	8	4	16	4	8	4	8
AMB	4	8	8	16	4	8	2	8
Oliveria decumbens Vent.	8	16	4	8	8	16	8	16
FLC	4	8	8	16	8	8	4	4
AMB	8	16	16	32	8	16	4	8

a: Standard Strain, b: Clinical Strain, c: Fluconazole, d: Amphotericin B.

were performed in triplicate. All archived clinical strains were isolated from patients with otomycosis, and that project received approval from the Ethics Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1400.559).

Results

According to the broth microdilution technique performed, the MIC values for the hydroalcoholic extract of *A. wilhelmsii* K. Koch., *O. decumbens* Vent., Cinnamon cortex, and Sugar beet leaves are as follows in Table 1.

FLC shows a range of MIC values between 4-16 µg/ml for all clinical strains except *C. albicans*, which was better inhibited (2-8 µg/ml). Clinical *A. flavus* isolates were better inhibited by FLC (4-8µg/ml) than *M. circinelloides* (8-16µg/ml).

AMB shows MIC range values for clinical strains as follows: 2-16 µg/ml for *C. albicans*, 4-32 µg/ml for *C. neoformans*, 4-16 µg/ml for *M. circinelloides* and 2-16 µg/ml for *A. flavus*.

MIC values for Sugar beet leaves extract (0.5 µg/ml & 2 µg/ml) were better than FLC and AMB for *C. albicans* strains. Other strains inhibited with the higher MIC value for all herbal extracts.

Discussion

In this study, antifungal properties of *B. vulgaris* subsp. *vulgaris* (Sugar beet leaves), *A. wilheimsii* K. Koch. (aerial parts), *C. zeylanicum* (barks), and *O. decumbens* Vent. (aerial parts) which their extracts were prepared by maceration method (EtOH: H2O_50:50), have been surveyed against clinical and standard strains *C. albicans*, *C. neoformans*, *M. circinelloides*, and *A. flavus*. The optimal efficacy against *C. albicans* was related to Sugar beet leaves extract. There was a question mark about the chance of non-volatile antifungal secondary metabolites in the aromatic plants that could be extracted with an ethanolic solution. Results showed it could be possible. Finally, we focused on *B. vulgaris* and its effect on *C. albicans*.

Resistance to Conventional Antibiotics; The Major Challenge in the Management of Infectious Diseases: The First-line treatments for *Candida* infections are triazoles and polyenes. Regardless of chronic side effects, overuse or misuse of antifungal agents leads to the emergence of resistant *C. albicans* strains[30]. Echinocandins, usually used in invasive candidiasis, are limited in access in most countries[4]. FLC and AMB are also used in localized and disseminated cryptococcosis; however, the reduced antifungal effect of FLC and clinical breakpoint for AMB has also been reported[4]. On the other hand, there are not enough randomized clinical trials for Mucoral infections. Treating patients is based on experience and practical data[40]. It Is reported that Mucorales are innately not susceptible to echinocandins and azoles such as Fluconazole and Voriconazole[4]. Except for some isolates, AMB has good potency on Mucorales with low MIC values[4]; However, various acute and chronic complications have been attributed[41]. Widespread azole-drug usage in medical purposes and agricultural settings against *Aspergillus* species leads to failure in getting adequate treatment response[42-44].

Herbal Extracts Anti-Fungal Efficiency; Other Related Studies: Dissimilarity in reported results related to the antifungal profile of *B. vulgaris* species could be related to different plant varieties, types of used plant parts, extraction methods, and tested microbial strains. For instance, Ahmadi *et al.*[25] found that hydro-methanolic extract of beetroot leaves has better antifungal activity than essential oil and aqueous extracts. However, they concluded that different fungus genera and species lead to dissimilar responses. In the Ugwuokpe *et al.* study[34], methanolic extract (95%(v/v)) of beetroot leaves showed 1.64 µg/ml MIC against *C. albicans*. It may confirm that hydroalcoholic solvents could better extract effective metabolites of *B. vulgaris*. In another related study, testing Ag-Cu Bimetallic Nanoparticles based on an aqueous beetroot extract on four clinical FLC-susceptible and FLC-resistant *C. albicans* showed significant MIC compared to FLC. Ag-Cu nanoparticles and FLC show 0.5-2 µg/ml and 0.25-0.5 µg/ml MIC for susceptible strains, respectively. However, nanoparticles show 8 µg/ml and 16 µg/ml MIC, compared to FLC with 16-64 µg/ml for resistant strains[30]. Femina *et al.*[28] showed the synergism between silver and beetroot aqueous extract on *C. albicans* growth inhibition. Interestingly, beetroot extract had less inhibition zone (18 mm) than gentamicin (20 mm). In another study, less inhibition zone diameter on standard *C. albicans* strains (11 mm) for Hematin-nanoparticles concluding beetroot, compared to gentamicin (15 mm), has been observed[45]. Amin *et al.*[46] reported that essential oils of *O. decumbens* showed 57.8 µg/ml MIC value for *Aspergillus niger* and *C. albicans*, compared to nystatin as a positive control (13 µg/ml, 1.6 µg/ml respectively). Silver nanoparticles based on *A. wilhelmsii* K.°Koch aqueous extract showed 50 µg/ml MIC and 100 µg/ml minimum fungicidal concentration against *C. albicans* in the Chahrdoli *et al.*[27] study. In another paper, MIC values for *C. zeylanicum* bark essential oils against *A. flavus* and *C. albicans* have been reported as 20 µl/l and 40 µl/l respectively[47].

On the other hand, the antifungal activity of ZnO nanoparticles composed of *B. vulgaris* on standard *C. albicans* strains has been reported as insufficient[33]. In Mirahmad *et al.*[32] study, none of the polar (H_2O, n-butanol) and non-polar (n-hexane, dichloromethane, ethyl acetate) fractions of hydroethanolic extract (70%(v/v)) of *O. decumbens* were active against tested fungi such as *C. albicans*. Additionally, according to other studies, it seems that essential oils of aromatic plants (*A. wilhelmsii* K. Koch. and *O. decumbens* Vent.) would show better MIC on *C. albicans*, rather than hydroalcoholic extracts[26, 29, 31]; however, aqueous extraction may not be sufficient for eliciting active ingredients[27]. As other papers show, this conclusion may be relevant to Cinnamon[33].

Other plants, such as Curcumin, have an anti-candida effect. Hajifathali *et al.*[48] tested the antifungal properties of Curcumin against 10 resistant *C. albicans* and a standard one (ATCC 10261); they then found that the resistant one inhibited in MIC values between 7.8 µg/ml and 32.25 µg/ml, and the standard strain in 62.5 µg/ml.

As mentioned above, Sugar beet is a biennial plant that can be harvested in the middle of the spring and the autumn. Also, *O. decumbens* Vent. and *A. wilhelmsii* K. Koch grow in the first half of summer. Also, there was difficulty in collecting dried hydroalcoholic extracts because of their high viscosity and sticky form; It was solved with mild heating during the process. Another limitation was maceration time; It may be solved with particle size reduction, sonication, stirring during maceration, etc. These were our research challenges and limitations.

Conclusion

Due to the importance of fungal multidrug-resistant, different life-threatening complications, as well as high-cost procedures and research to find effective synthetic lead compounds, natural products will be the center of investigation for antimicrobial properties because of being safe, less toxic, and biodegradable, with historical use. Sugar beet leaves showed significant antifungal effects against resistant *C. albicans*. This finding prompted us to do other research on animal models and clinical trials in the next studies.

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Conflict of interest

The authors further declare that they have no conflict of interest.

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