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Malva sylvestris inhibits *Candida albicans* biofilm formation

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ABSTRACT

Introduction: Candidiasis-associated biofilm formed by *Candida* species complicates treatment and contributes to unacceptable high mortality rates. We performed the aqueous and ethanol extracts of the different parts of *Malva sylvestris, Dorema aucheri, Ferulago angulata* and *Citrullus colocynthis* plants to identify best plant extract that inhibits growth of *Candida albicans* or *Candida krusei*, and conducted a series of follow-up studies to examine the inhibitors of *C. albicans* biofilm formation of the identified plant extract.

Methods: The antifungal activities of the aqueous and ethanol extracts of the different parts of *M. sylvestris*, *D. aucheri*, *F. angulata* and *C. colocynthis* plants were evaluated in vitro using disk diffusion test and broth microdilution test against *C. albicans* and *C. krusei*. The crystal violet assay, morphological response and expression pattern of hyphal wall protein 1 (*HWP1*) gene were carried out to investigate the biofilm-inhibitory properties of the best plant extract tested in *C. albicans.*

Results: The screen identified ethanol extract of *M. sylvestris* root that largely represented antifungal activity among the tested extracts. *M. sylvestris* root inhibited *C. albicans* biofilm formation. Ethanol extract of *M. sylvestris* root demonstrated significant reduction in *C. albicans* biofilm formation ($P < 0.005$). Moreover, morphological observation of ethanol extract of *M. sylvestris* root treated cells confirmed a decrease in biofilm thickness and cellular density. Finally, ethanol extract of *M. sylvestris* root displayed significant down-regulation of *HWP1*.

Conclusion: These results provide proof of concept for the implementation of ethanol extract of *M. sylvestris* root as inhibitor of *C. albicans* biofilm formation.

Implication for health policy/practice/research/medical education:
The antifungal properties of *Malva sylvestris* may have clinical relevance as a new method to treat candidiasis.
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Introduction

Candida species are the most prevalent fungal pathogens in human, causing opportunistic fungal infection worldwide. Fungal infections, including candidiasis represent serious threats, particularly in immunocompromised patients (1,2). *Candida* species such as *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* and *Candida krusei* account for ~90% of invasive candidiasis (2,3). *C. albicans* is still the most common pathogen; its incidence is declining with the increasing prevalence of other species (2,4).

There are significant expansions of virulence factors, such as the ability of this fungus to switch from yeast to filamentous forms (true hyphae or pseudohyphae), molecules which mediate adhesion to and invasion into host cells, phenotypic switching (white-opaque transition), contact sensing and thigmotropism, production of tissue-damaging hydrolytic enzymes such as proteases, phospholipases and haemolysin, biofilm formation and a range of fitness attributes in pathogenic species (3,5,6). Arguably the best studied member of the *Candida* species in terms of pathogenesis is *C. albicans* which has a specialized set of adhesins. One important adhesin of *C. albicans* is Hwp1, hypha-associated GPI-linked protein; this adhesin serves as a substrate for mammalian transglutaminases and induces a covalent bond between hyphae and the host cell. Hwp1 also plays a complementary role in biofilm formation. *C. albicans* biofilms readily forms on any biotic or abiotic moist surfaces. A biofilm is defined a complex three-dimensional structured microbial community and displays extensive spatial heterogeneity. The complex architecture of biofilms, the effects of the biofilm matrix,

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increased expression of drug efflux pump genes and metabolic plasticity provide protection by preventing the penetration of host immune factors and antifungals agents into the extracellular matrix in comparison to planktonic cells (3,6-10).

The ability of *C. albicans* to form drug-resistant biofilms has important clinical repercussions. The increasing prevalence of drug-resistant strains of *Candida* species necessitates searches for new targets for new antifungal agents. Among the alternative therapies, the use of natural medicines obtained from the plants has attracted great attention because, according to the World Health Organization (WHO), these would be the best source for obtaining a wide variety of drugs that could benefit a large portion of the patient population (3,9,10).

Nature has been a source of medicinal products for centuries, *Malva sylvestris, Dorema aucheri, Ferulago angulata* and *Citrullus colocynthis* are important traditional medicinal plants. *M. sylvestris* (11-13), *D. aucheri* (14,15), *F. angulate* (16,17) and *C. colocynthis* (18-21) have inhibitory activities against microorganisms. Moreover, *M. sylvestris* (11,12), *F. angulata* (16) and *C. colocynthis* (19,20) extracts exhibited in vitro antifungal activity against some *Candida* species.

Here we performed the aqueous and ethanol extracts of the different parts of *M. sylvestris*, *D. aucheri*, *F. angulata* and *C. colocynthis* plants to identify best plant extract that inhibits growth of *C. albicans* or *C. krusei*, and conducted a series of follow-up studies to examine the inhibitors of *C. albicans* biofilm formation of the identified plant extract.

Methods

Microorganisms

Three clinical isolates of *C. albicans* and *Candida krusei* from Shahid Beheshti hospital, Yasooj, Iran were obtained. All samples were isolated from patients with systemic candidiasis.

Candida albicans ATCC 10231 and *C. krusei* ATCC 6258 as reference quality-control strains were employed. After all the isolates were identified by conventional methods (22) and using CHROM agar *Candida* medium (CHROM agar Company, France), all the isolates maintained as sterile 20% (v/v) glycerol stocks and subcultured on sabouraud dextrose agar with chloramphenicol (SDA, Difco Laboratories, Detroit, Michigan) at 35–37°C for 24–48 hours to ensure viability and purity prior to testing.

Plants materials

Malva sylvestris, D. aucheri, F. angulata and *C. colocynthis* plants were harvested in Yasooj, Iran. Plant materials were transported to the lab immediately and voucher specimens were verified by Zardband Pharmaceuticals Company, Yasooj. Different parts of the plant were immediately cleaned and further processed according to the method described by Khodavandi et al (23), with slight modifications. Different parts of the plant were washed with sterile distilled water, sliced and dried in the oven for at least 2 days. One gram of tissues powder was mixed with 5 mL of sterile distilled water or ethanol and incubated at 37°C for 72 hours. The tissues extract was undergone a sequential extraction using soxhlet. The tissues extract was filter-sterilized (0.22-µm durapore, Millipore).

Effect of *Malva sylvestris, Dorema aucheri, Ferulago angulata* and *Citrullus colocynthis* on growth of *Candida albicans* and *Candida krusei*

The Clinical and Laboratory Standards Institute guidelines (CLSI) (24) were used for the antifungal disk diffusion susceptibility test. The experiments were conducted in triplicate in 2 independent assays. The plant extracts were subjected to broth microdilution antifungal susceptibility test using CLSI reference method for yeast (25), with end points determined at 24 hours post inoculation.

Effect of ethanol extract of *Malva sylvestris* root on *Candida albicans* biofilm formation

Candida albicans ATCC 10231 was used to form biofilm according to the method described by Khodavandi et al (26). Briefly, a suspension of *C. albicans* cell with a density of 1×10^6 cells/mL was added to 100 µL of plant extract at different concentrations based on minimum inhibitory concentration (MIC) (1/4 MIC, 1/2 MIC, 1 MIC and 2 MIC) using 96-well microplate (Brand 781660, Wertheim, Germany) and incubated at 35°C for 90 minutes. Subsequently, the mixture was incubated at 35°C for 24 hours with gentle shaking.

Quantitative and qualitative analysis

The cultures were used for quantitative and qualitative analysis using crystal violet (CV) assay and morphological response. The protocol of CV assay by Khodavandi et al (26) was adopted. The biofilm was fixed for 15 minutes by adding 100 µL of 99% methanol to each well and then the supernatants were ejected. Further, the cells were air-dried and then stained with CV solution (1:50 from stock solution, Sigma). The extra CV was removed and the wells were filled with 150 µL of acetic acid 33% (Sigma, USA). The absorbance was measured at 630 nm using ELISA microplate reader. We employed light microscopy method as a qualitative assay to investigate the anti-biofim activity of plant extract against *C. albicans*. The *C. albicans* biofilms were prepared as previously described (26). Thereafter, the media were discarded and coverslips were washed twice by PBS and viewed with a light microscope (Leica, DMRA II, Germany).

qRT-PCR analysis of *Candida albicans* hypha-specific gene

A suspension containing different concentrations of antifungal agents and 1×10^6 cells/mL of *C. albicans* ATCC 10231 were prepared as previously described (26). Total RNA was extracted from biofilm sample using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quantity and purity of the isolated RNA were measured by Nano Drop

Spectrophotometer ND-1000 (NanoDrop Technologies Inc., Wilmington, DE) and its integrity was checked on formaldehyde-denaturing agarose gel electrophoresis. To avoid DNA contamination, the RNA samples were treated with RNAse-free DNase I (Fermentas), according to manufacturer's protocol. Single stranded cDNA was synthesized from 0.5 µg total RNA using Moloney-Murine Leukemia Virus reverse transcriptase (Fermentas, USA). *C. albicans* hypha-specific gene *HWP1* was amplified from the synthesized cDNA with primer as described by Khodavandi et al (26). The amplification condition contained 26 cycles and PCR products were performed by gel electrophoresis and visualized via Molecular Imager Gel Doc XR System (Bio-Rad, USA). The PCR products were quantitated in terms of intensity of bands by comparing to known standard volumes (concentration of DNA mass standard [MassRuler Low Range DNA Ladder, Ready-to-Use, Fermentas]) according to manufacturer's operating instructions of Quantity One 1-D Analysis software (Bio-Rad, USA, version 4.6.5). The PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen). The identity of the purified product was confirmed by sequencing analysis (First BASE Laboratories Sdn. Bhd., Malaysia). The sequence similarity was queried via GenBank database in the NCBI by BLASTN (27).

Data analysis

Three technical replicates in 2 independent assays were performed for each test. Results are expressed as mean value ± standard deviation (SD) and analyzed by using the software SPSS 20.0 for windows (SPSS Inc. Chicago, IL, USA).

Results

Effect of *Malva sylvestris, Dorema aucheri, Ferulago angulata* and *Citrullus colocynthis* on growth of *Candida albicans* and *Candida krusei*

In order to identify inhibitors of growth of *C. albicans* and *C. krusei* isolates we performed a primary screen using different part of *M. sylvestris*, *D. aucheri*, *F. angulata* and *C. colocynthis* extracts. As a screening assay, we employed antifungal disk diffusion susceptibility test. Our initial screen tested the effect of exposure to 20, 50 and 100 µg/mL of each of the tested extracts on *C. albicans* and *C. krusei* isolates. From these results we noticed that ethanol extract of *M. sylvestris* root was largely represented antifungal activity among the tested extracts. This study demonstrated various degrees of the antifungal potency of the aqueous and ethanol extracts of the different parts of plants against *C. albicans* and *C. krusei*. Table 1 shows the antifungal activity (assessed in terms of inhibition zone) of the tested extracts against *C. albicans* and *C. krusei* at concentration of 100 µg/mL. Results from primary screen were subjected to quantitative assay using broth microdilution antifungal susceptibility test. Table 2 shows the MIC values of tested extracts for *C. albicans* and *C. krusei*. Ethanol extract of *M. sylvestris* root was the most potent; with an MIC of 0.78 mg/mL against *C. albicans* and *C. krusei*. Aqueous extract of *M. sylvestris* leaf had inhibitory activity against *C. albicans* and *C. krusei*.

Table 1. Antimicrobial activities of *Malva sylvestris, Dorema aucheri, Ferulago angulata* and *Citrullus colocynthis* extracts against isolates of *Candida albicans* and *Candida krusei* at a concentration of 100 mg/mL

Tested extracts/Isolates			*Ca-1*	*Ca-2*	*Ca-3*	*Ck-1*	*Ck-2*
M. sylvestris	Ethanol extract	Leaf	6.00 ± 0.00	6.00 ± 0.00	7.40 ± 0.57	7.38 ± 0.57	6.00 ± 0.00
		Stem	6.00 ± 0.00	6.00 ± 0.00	7.21 ± 0.34	6.00 ± 0.00	6.00 ± 0.00
		Root	7.89 ± 0.73	7.76 ± 0.73	6.45 ± 0.73	7.53 ± 0.73	6.00 ± 0.00
	Aqueous extract	Leaf	6.35 ± 0.62	7.88 ± 0.62	6.00 ± 0.00	6.94 ± 0.62	6.00 ± 0.00
		Stem	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
		Root	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
D. aucheri	Ethanol extract	Leaf	6.00 ± 0.00	7.85 ± 0.55	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
		Stem	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
		Root	6.44 ± 0.22	6.44 ± 0.22	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
	Aqueous extract	Leaf	6.55 ± 0.55	7.27 ± 0.55	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
		Stem	6.00 ± 0.00	6.65 ± 0.34	7.05 ± 0.34	6.00 ± 0.00	6.00 ± 0.00
		Root	6.44 ± 0.22	6.37 ± 0.44	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
F. angulata	Ethanol extract	Leaf	6.63 ± 1.43	6.00 ± 0.00	6.38 ± 0.11	6.00 ± 0.00	6.00 ± 0.00
		Stem	6.00 ± 0.00	6.44 ± 1.43	7.15 ± 0.43	6.00 ± 0.00	6.00 ± 0.00
		Root	6.00 ± 0.00	6.30 ± 0.53	7.25 ± 0.53	6.00 ± 0.00	7.58 ± 0.53
	Aqueous extract	Leaf	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
		Stem	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
		Root	6.94 ± 0.27	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
C. colocynthis	Ethanol extract	Skin of fruit	7.21 ± 0.30	7.10 ± 0.40	6.84 ± 0.40	6.99 ± 0.40	6.00 ± 0.00
		Seed	6.50 ± 0.11	6.54 ± 0.15	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
	Aqueous extract	Skin of fruit	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
		Seed	6.00 ± 0.00	6.00 ± 0.00	6.40 ± 0.98	6.00 ± 0.00	6.00 ± 0.00
Fluconazole			33.01 ± 0.01	32.09 ± 0.11	33.11 ± 0.21	33.31 ± 0.22	32.00 ± 0.13

Ca: Clinical isolates of *C. albicans; Ck*: Clinical isolates of *C. krusei.*

Data are means ± standard deviation of three independent experiments.

Table 2. Relative MIC values of *Malva sylvestris, Dorema aucheri, Ferulago angulata* and *Citrullus colocynthis* extracts against isolates of *Candida albicans* and *Candida krusei*

Tested extracts/Isolates				*Ca-1*	*Ca-2*	*Ca-3*	*Ck-1*	*Ck-2*
*M. sylvestris	Ethanol extract	Leaf	MIC_{80}	-	-	12.5	6.25	-
			MIC_{50}	-	-	0.78	0.047	-
		Stem	MIC_{80}	-	-	6.25	-	-
			MIC_{50}	-	-	0.19	-	-
		Root	MIC_{80}	0.78	0.78	0.78	0.78	-
			MIC_{50}	0.19	0.19	0.047	0.047	-
	Aqueous extract	Leaf	MIC_{80}	3.12	25	-	3.12	-
			MIC_{50}	0.19	0.39	-	0.39	-
		Stem	MIC_{80}	-	-	-	-	-
			MIC_{50}	-	-	-	-	-
		Root	MIC_{80}	-	-	-	-	-
			MIC_{50}	-	-	-	-	-
*D. aucheri	Ethanol extract	Leaf	MIC_{80}	-	12.5	-	-	-
			MIC_{50}	-	0.39	-	-	-
		Stem	MIC_{80}	-	-	-	-	-
			MIC_{50}	-	-	-	-	-
		Root	MIC_{80}	6.25	6.25	-	-	-
			MIC_{50}	0.047	0.047	-	-	-
	Aqueous extract	Leaf	MIC_{80}	6.25	25	-	-	-
			MIC_{50}	0.095	0.19	-	-	-
		Stem	MIC_{80}	-	3.12	6.25	-	-
			MIC_{50}	-	0.39	0.095	-	-
		Root	MIC_{80}	6.25	6.25	-	-	-
			MIC_{50}	0.095	0.047	-	-	-

Tested extracts/Isolates				*Ca-1*	*Ca-2*	*Ca-3*	*Ck-1*	*Ck-2*
*F. angulata	Ethanol extract	Leaf	MIC_{80}	1.56	-	3.12	-	-
			MIC_{50}	0.047	-	0.047	-	-
		Stem	MIC_{80}	-	3.12	6.25	-	-
			MIC_{50}	-	0.39	0.095	-	-
		Root	MIC_{80}	-	1.56	3.12	-	-
			MIC_{50}	-	0.19	0.39	-	-
	Aqueous extract	Leaf	MIC_{80}	-	-	-	-	-
			MIC_{50}	-	-	-	-	-
		Stem	MIC_{80}	-	-	-	-	-
			MIC_{50}	-	-	-	-	-
		Root	MIC_{80}	3.12	-	-	-	-
			MIC_{50}	0.39	-	-	-	-
*C. colocynthis	Ethanol extract	Skin of fruit	MIC_{80}	6.25	6.25	6.25	6.25	-
			MIC_{50}	0.39	0.047	0.047	0.047	-
		Seed	MIC_{80}	3.12	3.12	-	-	-
			MIC_{50}	0.047	0.047	-	-	-
	Aqueous extract	Skin of fruit	MIC_{80}	-	-	-	-	-
			MIC_{50}	-	-	-	-	-
		Seed	MIC_{80}	-	-	100	-	-
			MIC_{50}	-	-	0.19	-	-
**Fluconazole			MIC_{80}	1	4	1	32	32
			MIC_{50}	0.5	2	0.5	16	16

*mg/mL; **µg/mL.
Ca: Clinical isolates of *C. albicans; Ck*: Clinical isolates of *C. krusei.*
Data are means ± standard deviation of three independent experiments.

Table 3. CV assays results for biofilm-associated *Candida albicans* ATCC 10231 treated with antifungal agents in different concentration based on MIC

Concentration of antifungal agents	Means absorbance at 630 nm ± SD	
	Ethanol extract of *M. sylvestris* root*	Fluconazole**
¼ × MIC	1.19 ± 0.06[a]	0.22 ± 0.01[a]
½ × MIC	0.95 ± 0.07[b]	0.21 ± 0.01[a]
1 × MIC	0.63 ± 0.08[c]	0.19 ± 0.01[a]
2 × MIC	0.45 ± 0.07[d]	0.18 ± 0.00[a]
Untreated control	2.42 ± 0.00[e]	2.42 ± 0.00[b]

*mg/mL; **µg/mL.
[a-e] Means ± SD in a column with different superscript differ significantly ($P < 0.005$) using Duncan test. The results were performed in 3 independent experiments.

Effect of ethanol extract of *Malva sylvestris* root on *Candida albicans* biofilm

On the basis of these analyses, we selected ethanol extract of *M. sylvestris* root as the most potent antifungal one for further biofilm-inhibitory properties. Findings from the biofilm-inhibitory properties of ethanol extract of *M. sylvestris* root on growth of *C. albicans* ATCC 10231 exhibited significant reduction in biofilm growth ($P < 0.05$) compared to fluconazole-treated and also growth control cells. As shown in Table 3, the antifungal agents could significantly ($P < 0.005$) reduce the *C. albicans* biofilm. Treatments with the ethanol extract of *M. sylvestris* root at 1 MIC and 2 MIC doses were found to be effective in reducing biofilm formation in *C. albicans* ATCC 10231, whilst the fluconazole reduced biofilm formation at all concentrations.

The ability of fluconazole and the ethanol extract of *M. sylvestris* root to prevent biofilm formation visually verified by microscopic examination. Light microscopy indicated that incubation in the presence of ethanol extract of *M. sylvestris* root and fluconazole led to a decrease in biofilm thickness and cellular density as compared with biofilms formed in the untreated control (Figure 1). Moreover, as shown in Figure 1, the ability of antifungal agents to reduce *C. albicans* biofilm formation occurred in a dose-dependent manner.

Effect of ethanol extract of *Malva sylvestris* root on the expression of hypha-specific gene

Ethanol extract of *M. sylvestris* root down regulated the expression of the *HWP1*. Figure 2 exhibits the representative gel electrophoresis for RT-PCR products of *HWP1* gene from *C. albicans* treated with ethanol extract of *M. sylvestris* root in different concentrations based on MIC. We found that the relative quantification of *HWP1* expression displayed significant down-regulation ($P < 0.05$) compared to untreated control after treatment of *C. albicans* with ethanol extract of *M. sylvestris* root in different concentrations based on MIC (Figure 3). The fold changes relative to untreated control in terms of

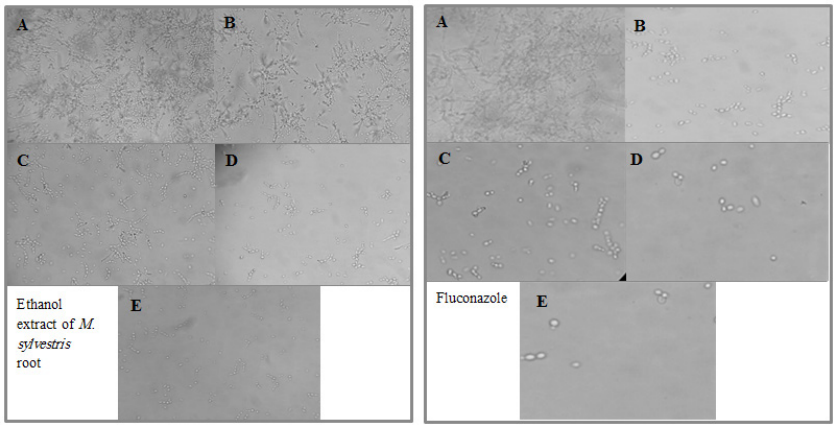

Figure 1. Light microscopic view of the antifungal effect of ethanol extract of *Malva sylvestris* root and fluconazole at different concentrations based on MIC on the *Candida albicans* ATCC 10231 after 24 hours. (A) Untreated control (B) ¼ MIC (C) ½ MIC (D) MIC (E) 2 MIC. Magnification ×40, Bar = 50 µm.

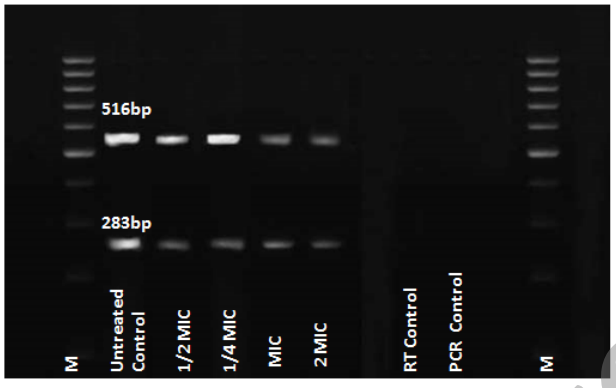

Figure 2. Expression analysis of *HWP1* gene in *Candida albicans* ATCC 10231 biofilm treated with different concentrations of ethanol extract of *Malva sylvestris* root after 24 h. RT-PCR amplification products were separated by electrophoresis in 1.7% agarose and stained with ethidium bromid.

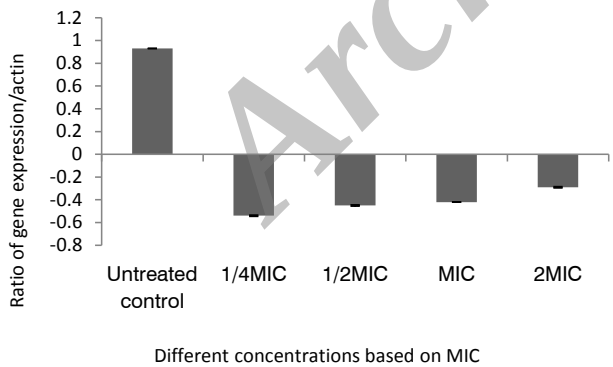

Figure 3. Relative quantitation of *HWP1* gene expression (normalized to house-keeping gene, actin) in *Candida albicans* ATCC 10231 biofilm treated with different concentrations of ethanol extract of *Malva sylvestris* root after 24 h. Data are means ± standard deviation from three independent experiments amplified in triplicates.

HWP1 expression for ¼ MIC, ½ MIC, 1 MIC and 2 MIC of ethanol extract of *M. sylvestris* root were 0.58 ± 0.012, 0.48 ± 0.006, 0.46 ± 0.006 and 0.31 ± 0.006, respectively. The ability of ethanol extract of *M. sylvestris* root to reduce the expression level of *HWP1* occurred in a dose-dependent manner. The authenticity of the amplification products was verified by DNA sequencing. The sequences displayed high similarity analyzed via the non-redundant nucleotide sequences from GenBank and confirmed to have 99% identity with the respective gene sequences.

Discussion

Candidiasis is now the third-to-fourth most hospital acquired infections, as this normal flora of humans causes opportunistic infections particularly in immunocompromised patients. These infections are frequently associated with the formation of biofilms, which contributes to unacceptable high mortality rates (28). Evidence from studies of formation of biofilm in *Candida* has demonstrated adherence of *Candida* to host cells surfaces and Hwp1 interfere with host cells surfaces for formation of the stable complexes (29). Based on current knowledge in developing novel approaches for the prevention and treatment of *Candida* biofilms, it appears that medicinal plants have benefits in the area of human health, particularly in the design of more efficient natural medicines and the development of new antimicrobial agents (3,10). Our data demonstrate that the different part of *M. sylvestris*, *D. aucheri*, *F. angulata* and *C. colocynthis* extracts possessed activity against *C. albicans* and *C. krusei*. This is consistent with previous observations that *M. sylvestris*, *D. aucheri*, *F. angulata* and *C. colocynthis* plants could exhibited antimicrobial activities (11-21). The ethanol extract of *M. sylvestris* root possessed strong activity against *C. albicans*. The ethanol extract of *M. sylvestris* root was able to reduce biofilm formation in comparison with fluconazole. Several lines of evidence previously demonstrated the antifungal property of *M. sylvestris* against *Candida* species (11,12). The traditional use of *M. sylvestris* has been reported since a long-time ago and roots, shoots, leaves, flowers, fruits, and seeds are applied in the pharmaceutical industries. Active components in *M. sylvestris* such as amino acids and protein

derivatives, flavonoids, mucilages, phenol derivatives, enzymes (sulphite oxidase), coumarins, vitamin E, vitamin C, fatty acids and sterols, pigments (chlorophyll A, chlorophyll B, xanthophylls and carotenoids) and terpenoids, including monoterpenes, diterpenes, sesquiterpenes and norterpenes as well as other important pythochemicals possibly affect fungal infections (13,29-33). Several studies have shown that flavonoids and other plant-derived polyphenolic compounds have free radical scavenging potential and antioxidant properties by single-electron transfer (30,32). Cushnie and Lamb (34) considered that flavonoids within plants account for the antifungal activities. The metal chelating properties of flavonoids and the ability to scavenge singlet oxygen, superoxide anions, peroxyl radicals, hydroxyl radicals and peroxynitrite suggest that they may play a role in biological activity (32).

The mechanisms of the antimicrobial actions of plant-derived polyphenolic compounds such as tannins may be related to the ability to inactivate microbial adhesins, enzymes and cell envelope transport proteins (35). The actions of plant natural products on biofilm eradication may involve the inhibition of cell accumulation, interrupting the biofilm structure, destruction of extracellular matrix, dissemination through the biofilm extracellular matrix and subsequent damage due to their powerful antimicrobial activities (36,37).

Our previous reports have shown a high efficacy of allicin and *Allium ascalonicum* to inhibit biofilm formation which was linked to down regulation of *HWP1* in *C. albicans* (23,26). Meanwhile, our new findings have demonstrated that *M. sylvestris* could also significantly reduce the expression of *HWP1* in *C. albicans*. The molecular mechanism of *C. albicans* in response to active components of *M. sylvestris* warrants further investigation. *C. albicans* biofilms constitute a threat to successful antifungal treatment (38). It is thus conceivable that suppression of the yeast-to-hypha transition in *C. albicans* by ethanol extract of *M. sylvestris* root could be a viable antifungal strategy.

Conclusion

In conclusion, ethanol extract of *M. sylvestris* root exhibits anti-biofilm effect selectively against *C. albicans*. In addition, the non-toxic nature of medicinal plants to human cells may have clinical relevance as a new method to treat candidiasis. Further studies are required to determine whether the anti-biofilm effect of ethanol extract of *M. sylvestris* root is applicable in clinical settings.

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Authors' contributions

FA and AK have designed the study, analyzed and interpreted the data. All the authors managed the literature searches and produced the initial draft. All authors read and approved the final manuscript.

Conflict of interests

The authors declare that they have no competing interest.

Ethical considerations

Authors have been entirely regarded ethical issues such as plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.

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