

# Antifungal activity of herbal extracts against *Malassezia* species

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## INTRODUCTION

*Malassezia* spp. is an opportunistic dimorphic Basidiomycetous fungi<sup>1</sup>. These lipophilic fungi are normal components of the skin biota. Analysis of the morphological characteristics of *Malassezia* and investigation of the structures of 20 different compounds have been recognized from 14 species of *Malassezia*. *Malassezia* spp. is associated with a

**Background:** *Malassezia* spp. is an opportunistic dimorphic Basidiomycetous fungi associated with a variety of diseases including dandruff, atopic eczema, pityriasis versicolor, seborrheic dermatitis and folliculitis. It also causes systemic infections in immune compromised patients. The aim of this study was to evaluate the antimicrobial activity of nettle leaves (*Urtica dioica*), colocynths fruits (*Citrullus colocynthis*), green tea (*Camellia sinensis*), burdock root (*Arctium lappa*) extracts and ketoconazole against 26 isolates of *Malassezia* spp., isolated from patients with *Malassezia* infections.

**Method:** Twenty-six (26) *Malassezia* spp. identified by RFLP-PCR, were isolated from patients with *Malassezia* infections. The antifungal activity was evaluated by micro broth dilution assay.

**Result:** *M. globosa* (50%), *M. furfur* (46%) and *M. slooffiae* (4%) were the isolated strains. There was no significant difference between the minimal inhibitory concentration (MIC) values for colocynth extracts and ketoconazole. Also, the antimicrobial activity of green tea and burdock extracts against *Malassezia* spp. was not significantly different ( $P>0.05$ ). The minimal fungicidal concentration (MFC) values for green tea, colocynth extracts and ketoconazole against *Malassezia* spp. were the same ( $P>0.05$ ).

**Conclusion:** Further clinical studies are required to determine the efficacy of *C. colocynthis* ethanol extract, in the treatment of *Malassezia* skin disorders.

**Keywords:** azole, fungi, genetic analysis, herbal drug, *Malassezia*, mycology

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variety of diseases including dandruff<sup>2</sup>, atopic eczema, pityriasis versicolor<sup>3</sup>, seborrheic dermatitis<sup>4</sup> and folliculitis. It also causes systemic infections in immune compromised patients<sup>5,6</sup>. The conditions that cause *Malassezia* related infections in humans are not fully understood but researchers have been able to determine the role of different factors including genetic and environmental factors, imbalance in skin normal biota, immune suppression, and

profuse sweating as agents for these infections. *Malassezia* infections are cured by azole drugs and usually do not respond properly to treatment and after treatment have recurrence. Thus, there is an increase in azole resistant *Malassezia*<sup>7-9</sup>. In addition to prevalence of resistant *Malassezia* to azoles, their adverse effects on humans are reasons for finding new sources of antifungal agents. In this regard, medicinal plants including essential oils and extracts are being analyzed by researchers. In traditional medicine, some medicinal plants are used for the treatment of different kinds of infectious diseases. From these plants, nettle, burdock, green tea and colocynth extracts were used in this study to evaluate their antimicrobial activity against *Malassezia* spp.

*Urtica dioica* or nettle is a herbaceous flowering plant from the *Urticaceae* family. Nettle is traditionally used for treatment of diarrhea, vaginal discharge, as well as internal and external bleeding<sup>10</sup>. Pharmacological activities of nettle extracts such as antibacterial, antifungal activities<sup>11</sup>, anti-diabetic<sup>12</sup>, anti-inflammatory<sup>13</sup>, and anti-hyperglycemic effects<sup>14</sup> were confirmed.

*Citrullus colocynthis* schrad or colocynth (Cucurbitaceae family) roots are traditionally used to treat urinary tract infections. The anti-inflammatory activities<sup>15</sup>, the antibacterial and antifungal effects<sup>16-18</sup>, hypoglycemic activity<sup>19</sup> were the subjects of many studies in the past.

The anti proliferative activity of leaves and roots<sup>20,21</sup>, antioxidant effect<sup>21</sup>, antimicrobial activity<sup>22</sup>, anti diabetic effect<sup>23</sup> of *Arctium lappa* (burdock) from the *Asteraceae* family has been confirmed. Burdock roots are used as food in many East-Asian countries.

*Camellia sinensis* or green tea (*Theaceae* family) is known for many identified effects such as antioxidant activity<sup>24</sup>, anti-helminthic, and antimicrobial activities<sup>25,26</sup>.

This study evaluated the antifungal activity of *Urtica dioica*, *Arctium lappa*, *Camellia sinensis* and *Citrullus colocynthis* ethanolic extracts against clinical isolates of *Malassezia* spp. from the skin of patients with *Malassezia* infections.

## MATERIALS AND METHODS

### Microbial strains

Twenty-six (26) *Malassezia* spp. were isolated

from patients with *Malassezia* infections. The samples were cultured on modified Dixon agar and incubated at 32°C for 14 days. The morphology was examined on Leeming and Notman agar after incubation at 32°C for 7 days. The isolates were identified by physiological characteristics such as catalase reaction, Tween assimilation test, cremophor EL assimilation, splitting of esculin and pigment production<sup>27</sup>.

### Detection of *Malassezia* species by RFLP-PCR

Chromosomal DNA extraction was performed by the phenol-chloroform method. For identification of different species of *Malassezia* by the RFLP-PCR method, primers with specification 5'-TAACAAGGATTCCCCTAGTA-3' for the Forward strand and 5'-ATTACGCCAGCATCCTAAG-3' for the Reverse strand were used. The PCR products were digested by *Cfo I* and the different product patterns were compared and *Malassezia* species were identified<sup>9</sup>.

### Plant extracts

In this research, plant extracts with these specifications were used:

- 1- *Urtica dioica* ethanol extract (50%:50%) from leaves standardized to 0.98 mg/ml chlorogenic acid.
- 2- *Citrullus colocynthis* ethanol extract (95%) from fruits standardized to 0.81 mg/ml total phenolic content.
- 3- *Camellia sinensis* leaves ethanol extract (50%) standardized to 36.6 mg/ml total catechin and 57 mg/ml total phenolic content.
- 4- *Arctium lappa* roots ethanol extract (25%) standardized to 0.224 mg/ml chlorogenic acid.

These extracts were prepared and standardized by the Phytochemistry Department, Medicinal Plant Research Center of Barij Essence, Kashan, Iran. Plant extracts were prepared by the percolation method. The powdered dried parts of each plant were mixed with each solvent at the ratio of 1:10 (w/v) for 24 h at ambient temperature. The mixture was filtered using Whatman filter paper No. 2, the residue was rinsed with the same solvent and the extracts were dried under vacuum and kept in a cool place until the examination<sup>28</sup>.

## Antimicrobial evaluation

The antimicrobial evaluation was performed by micro broth dilution assay. In brief, one colony of *Malassezia* spp. on modified Dixon Agar was suspended in normal saline containing 0.05% Tween 60. The turbidity of the fungal suspension was adjusted to  $1-4 \times 10^6$  by Neubauer Lam.

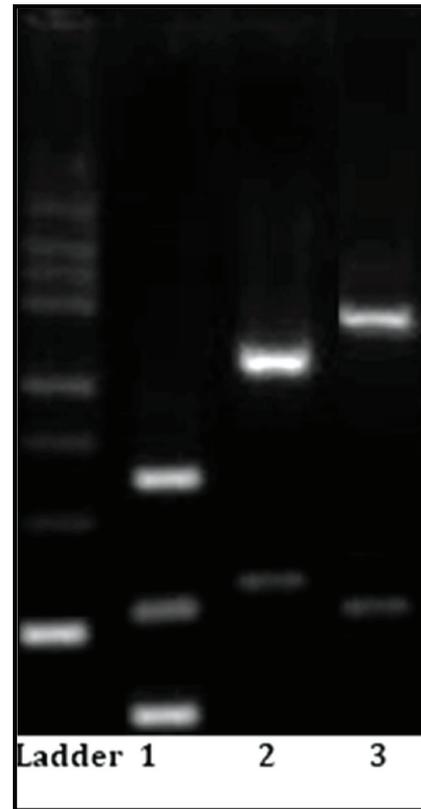
To determine the antimicrobial potential of plant extracts, the MIC and MFC values were determined. The extract was diluted in distilled water by serial dilution in the ranges of 0.125-32 mg/ml. Ketoconazole (Sigma Aldrich) was used as drug control in the ranges of 16-0.03 µg/ml. Positive and negative control wells were used in each extract. 100 µl of diluted extracts were added to each well. Then, 100 µl of diluted fungal suspension in modified Dixon broth ( $1-4 \times 10^3$  CFU/ml) was inserted into each well and incubated at 32°C for 4 days. After that, the first well that exhibited 90% growth inhibition of *Malassezia* spp. was defined as the MIC value, after observing the plates under a stereomicroscope. The MFC value is defined as the dilution that inhibits growth on modified Dixon agar, after incubation at 32°C for 7-10 days<sup>29</sup>.

## Statistical analysis

Each parameter was tested in triplicate. Conventional statistical methods were used to calculate the means and standard deviation (means ± SD). Statistical analysis (ANOVA) was applied to determine the differences ( $P < 0.05$ ). Significant differences between the extracts were determined by Tukey test. Statistical data analysis was performed by SPSS software (version 17, Chicago, Illinois, USA).

## RESULTS

Twenty six clinical isolates were separated from patients with *Malassezia* infections. The RFLP- PCR



**Figure 1.** 26S rDNA PCR products after digestion with CfoI: Lanes 1: *M. furfur*, with 250, two ~107-113 bp (as overlapping) fragments. *M. furfur* has multiple fragments (59, 30, 21, 2bp) not distinguishable after gel electrophoresis, lanes 2: *M. globosa*, with 129 and 455 bp fragments, lane 3, *M. slooffiae*, with lane 508, 107 bp; Ladder: 100 bp ladder.

method was used to show the frequency of different species as follows: *M. globosa* (50%), *M. furfur* (46%), and *M. slooffiae* (4%) (Table 1, Figure 1).

The results of the antifungal activity of extracts against 26 *Malassezia* spp. are summarized in Table 1. The results of this study show that different species of *Malassezia* spp. had different sensitivity to ketoconazole. The MIC values of ketoconazole for different *Malassezia* spp. including *M. globosa*, *M. furfur* and *M. slooffiae* were  $0.1 \pm 0.1$ ,  $0.1 \pm 0.1$  and  $0.6 \pm 0$  µg/ml; while the MFC values were  $0.2 \pm 0.2$ ;  $0.2 \pm 0.2$ ; and  $0.12 \pm 0.0$  µg/ml, respectively. The means of MIC values for *C. sinensis*, *U. dioica*, *C.*

**Table 1.** The sensitivity of different species of *Malassezia* spp. to plant extracts in comparison to Ketoconazole

<i>Malassezia</i> sp	n	ketoconazole		<i>C. sinensis</i>		<i>U. dioica</i>		<i>C. colocynthis</i>		<i>A. lappa</i>	
		MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
<i>M. globosa</i>	13	$0.1 \pm 0.1$	$0.2 \pm 0.2$	$1.1 \pm 1$	$2.4 \pm 2$	$1.5 \pm 1.5$	$3.7 \pm 3.3$	$0.6 \pm 0.5$	$1.5 \pm 1.3$	$0.6 \pm 0.6$	$1.8 \pm 1.7$
<i>M. furfur</i>	12	$0.1 \pm 0.1$	$0.2 \pm 0.2$	$1.4 \pm 1$	$4.4 \pm 2.8$	$1.6 \pm 1.2$	$6.6 \pm 5.7$	$0.9 \pm 0.5$	$3.4 \pm 2.9$	$1.4 \pm 1$	$8.2 \pm 6.2$
<i>M. slooffiae</i>	1	$0.6 \pm 0$	$0.12 \pm 0$	$0.125 \pm 0$	$0.25 \pm 0$	$0.25 \pm 0$	$0.5 \pm 0$	$1 \pm 0$	$2 \pm 0$	$1 \pm 0$	$2 \pm 0$
Total	26	$0.1 \pm 0.08$	$0.2 \pm 0.2$	$1.2 \pm 1.1$	$3.3 \pm 2.7$	$1.5 \pm 1.3$	$5 \pm 4.87$	$0.7 \pm 0.5$	$2.5 \pm 1.5$	$1.4 \pm 0.8$	$5 \pm 4.0$

*colocynthis* and *A. lappa* were  $1.2 \pm 1.1$ ,  $1.5 \pm 1.3$ ,  $0.7 \pm 0.5$  and  $1.4 \pm 0.8$  mg/ml, respectively. The MFC values were  $3.3 \pm 2.7$ ,  $5 \pm 4.87$ ,  $2.5 \pm 1.5$  and  $5 \pm 4.0$  mg/ml, respectively. On the basis of MIC and MFC values, different species of *Malassezia* had lower values for *C. sinensis* and *C. colocynthis*. Although the *A. lappa* extract had a lower MIC than the *U. dioica* extract, but higher MFC values were observed in the *A. lappa* extract group (Table 1). When the antimicrobial effects were compared, there was no significant difference between the antimicrobial activities of *C. sinensis* and *A. lappa* extracts. On the basis of the MFC values of different extracts and analysis by the Tukey HSD Test, the fungicidal activities of *C. sinensis*, *C. colocynthis* and ketoconazole did not show any significant difference.

## DISCUSSION

Antifungal treatments have problems of toxicity, low efficacy and development of resistant strains<sup>30</sup>. The increase in resistant microorganisms, is the major cause of spreading infections, and an extension in time is necessary for treatment of infections. Consequently, many patients stop treatment before being cured. Traditional Iranian Medicine is an important source of new antimicrobial agents, especially for opportunistic fungi such as *Malassezia* spp. In this regard, at first, clinical isolates of *Malassezia* spp. were isolated from infected patients and different species were identified. In this study, *M. furfur* and *M. globosa* were significantly more common in patients.

A study conducted in Yazd Province (Iran) on 200 persons (100 patients with skin lesions and 100 healthy volunteers) showed the presence of *M. globosa* (38.3%), *M. furfur* (29.4%), *M. sympodialis* (14.9%), *M. pachydermatis* (9.6%) and *M. slooffiae* (5.3%) as the most commonly isolated species from skin lesions of patients while *M. furfur* (37.2%), *M. globosa* (25.6%), *M. sympodialis* (16.3%), *M. pachydermatis* (13.9%) and *M. slooffiae* (4.6%) were the common types from healthy volunteers<sup>31</sup>. *M. globosa* (55.8%), *M. furfur* (32.5%), *M. restricta* (9.1%), *M. sympodialis* (1.3%) and *M. japonica* (1.3%) were also isolated from Iranian seborrhoeic dermatitis patients<sup>32</sup>.

Although, recent research has shown that *M. globosa* is the most common agent of infections

and *M. furfur* is responsible for a small number of cases<sup>33</sup>, the finding of this study suggests that *M. furfur* presents the main species in *Malassezia* infections and *M. globosa* as the second agent of importance. *M. slooffiae* was less isolated as previously reported<sup>8,34</sup>.

The literature review revealed that many studies have evaluated the antifungal activities of plant extracts against *Malassezia* spp. The antifungal activity of *Phyllanthus emblica*, *Hibiscus rosa sinensis*, *Acacia concinna*<sup>35</sup>, *Teucrium polium* L.<sup>36</sup> and *Jasminum sambac*<sup>37</sup> extracts were confirmed against *Malassezia* spp. While investigating other similar studies, a study was revealed the antifungal activity of *C. colocynthis*, and *U. dioica* extracts against *Alternaria alternata*, *Fusarium oxysporum*, *Fusarium solani*, and *Rizoctonia solani*. *A. alternata* was the most sensitive fungi to the *U. dioica* extract, and at a concentration of 0.9% *U. dioica* extract, the growth of *A. alternata* was completely inhibited. At 0.9%, the *C. colocynthis* extract completely inhibited *A. alternata*, and *R. solani*<sup>38</sup>. The result of this study showed that the *C. colocynthis* extract had a higher antifungal activity compared to the *U. dioica* extract, against saprophytic fungi. The findings of this study suggest a higher anti-*Malassezia* activity for *C. colocynthis* extract compared to the *A. lappa* extract, followed by the *C. sinensis* and *U. dioica* extracts. The antimicrobial activity of the *C. colocynthis* ethanolic extract was confirmed against *Bacillus subtilis*, *Bacillus pumilus*, *Micrococcus luteus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Escherichia coli*, *Candida albicans*, *Aspergillus niger*, *Penicillium chrysogenum* and *Trichosporon begelli*<sup>39</sup>.

The presence of flavonoids, saponins, alkaloids, phenolic content, riboflavin, thiamin and ascorbic acid were identified in *C. colocynthis* extracts. Total phenolic compounds depending on phenolic type, have long been identified for their antimicrobial activities. Phenolic compounds disrupt the membrane proton motive force, dissolve and leak the intracellular constituents, disturb cell homeostasis, inhibit the enzymes involved in electron transport and oxidative phosphorylation, as well as coagulate the cytoplasmic constituents and biosynthetic process<sup>40</sup>. The antimicrobial activity of flavonoids is caused by inhibition of nucleic acid synthesis, inhibition of cytoplasmic membrane function and inhibition of energy metabolism<sup>41</sup>. Furthermore, the different kinds of chemical compounds are

responsible for the antifungal activity of the *C. colocythis* ethanolic extract against *Malassezia* spp.

Further clinical studies are required to ascertain the efficacy of *C. colocythis* ethanolic extract from ripe fruits, in the treatment of *Malassezia* skin disorders. The study suggests that the *C. colocythis* ethanolic extract might find application in anti-dandruff formulations.

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