GC/MS analysis and antifungal activity of M. Chamomilla extracts against molecularly identified keratinophilic fungi

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ABSTRACT

The objective of this study was to isolate and genetically identify keratinophilic fungi associated with humans, test their susceptibility to nystatin, and evaluate the effectiveness of Methanol Extract (ME) from chamomile flowers against these fungi. No cytotoxic effects were observed in the extract cytotoxicity assay using human blood solution. The antifungal activity of the chamomile extract varied among different fungal isolates and extract concentrations. The extract exhibited a strong inhibitory effect on fungal growth, depending on the type of fungal strain and concentration. Statistical analysis revealed significant differences between the effects of the extract and the conventional antifungal medication (nystatin) on specific fungi. GC/MS analysis of the chamomile extract identified several bioactive compounds, including flavonoids, phenolic compounds, and essential oils. The presence of these bioactive components in the extract likely contributes to its high antifungal activity against the tested fungal infections.

Keywords: GC/MS, nystatin, keratinophilic fungi

INTRODUCTION

The skin is the largest organ in the body, a haven for microbiota, and the first line of defense against noxious substances from the environment and pathogenic bacteria. In addition to serving as a physical barrier, the skin's resident immune system also forms a dynamic system that is essential for preventing infections, repairing damage, and preserving tissue homeostasis. Dermatophytoses (ringworms), which are
among the most common skin infections in humans, are believed to have a global incidence of 20 to 25 percent in the healthy population [1-3]. These infections are brought on by filamentous fungi, which were once keratin-digesting creatures of the soil but have since evolved to live as parasitic microbes on both animals and people. Therefore, subacute or chronic infections with varying degrees of inflammation in immunocompetent persons are caused by hyphae superficial penetration into the skin, hair, and nails in dermatophytoses. Dermatophytes are divided into five genera according to recent taxonomic changes: Microsporum, Arthroderma, Trichophyton, Epidermophyton, and Nannizzia [4].

Additionally, different species within them have been classified as geophilic, zoophilic, and anthropophilic fungi according to their adaptation to particular ecological niches and hosts. Trichophyton rubrum and Epidermophyton floccosum are two anthropophilic species that are well adapted to people and frequently cause chronic infections with no clinical signs. Contrarily, dermatophytes from animals or soil (Nannizzia gypsea/Microsporum gypseum) are frequently isolated from patients with mild to highly inflammatory dermatophytosis but with lesions that are prone to spontaneous resolution [4]. These dermatophytes include Microsporum canis, Trichophyton/Arthroderma benhamiae, Trichophyton mentagrophytes, etc [5].

Contrarily, individuals who are immunosuppressed, particularly those who have cell-mediated immunity deficiencies due to conditions like HIV/AIDS, transplant, neoplasia, diabetes, or corticosteroid therapy, are more vulnerable to these infections and frequently develop extensive superficial lesions that do not respond to conventional antifungal medication [6]. A considerable rise in treatment-recalcitrant, recurring, and chronic dermatophytosis was recently noted in India, perhaps as a result of the careless use of antibiotic and corticosteroid medication combinations [7].

The prevalence of serious fungal infections caused by Candida spp. has increased worldwide in recent years, particularly in immunocompromised individuals [8], and these infections can present superficially or cause serious systemic disease [9]. The limited number of antifungal classes [10], the rise in serious infections caused by Candida spp [11], and the rise in microbial resistance to drugs [12] frequently as a
result of repeated or long-term therapies [13] are driving forces in this trend. It has been noted that innovative alternative treatments for these fungi infections are urgently needed[14]. Compared to chemical and synthetic pharmaceuticals, natural goods are preferred as biocompatible and non-toxic drugs without side effects in medicine. Natural extracts with biological and therapeutic qualities that are given to humans and animals as dietary ingredients or in particular pharmaceutical preparations have drawn more attention in recent years. Promising treatments for various ailments are thought to be derived from medicinal plants. Many plants' biological and pharmacological characteristics, however, remain unknown [15].

Recent research suggests that plants are natural sources of new antibacterial substances with a wide range of medicinal applications [14]. The efficiency of plant extracts against microorganisms has been demonstrated in numerous research [16].

The purpose of this study was to isolate, genetically identify, assay the susceptibility of keratinophyllic fungi connected to humans to Nystatin (NY), and assess the effectiveness of Methanol Extracts (ME) from Matricaria chamomilla (chamomile) flowers against the isolated fungi. Additionally, investigate the ME chemical composition using Gas Chromatography/Mass Spectrometry (GC/MS) analysis to identify active chemicals.

**MATERIALS AND METHODS**

**Ethical Approve**

This research was registered in University of Basrah, College of Education/ Qurna. Human ethical approval was obtained from Iraqi Ministry of Health, Directorate of Health in Basra in their book No. 724 on September 19, 2019. The research did not violate the patient's rights or privacy, nor did the research address personal information such as patient pictures, etc. Informed consent was obtained from each patient included in the study

**Sampling, Isolation and DNA extraction**

Twenty samples were taken from the skin, hair, and nails of people who had fungal infections. Dermatologist Dr. Laith Globe Lazim Al-Kinani (Master of Dermatology, PhD in Dermatology, Iraqi Ministry of Health, Al-Fayhaa Teaching Hospital) aided in the diagnosis of clinical cases. The samples were delivered to the clinical
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mycology laboratory at the College of Sciences at the University of Basrah.

Using a sterile scalpel, samples were taken from patients and kept in sterile plastic bags until they were cultured on Sabouraud Dextrose Agar (SDA), HiMedia Co., India, and incubated at 37 °C for seven to thirty days to identify the samples molecularly and determine their susceptibility to Nystatin and other natural products. The genomic DNA for the 12 positive samples was extracted using Al-Mahmoud [17] methodology. The DNA samples were kept at -80 °C pending PCR amplification after electrophoresis in 0.5 % agarose gel stained with ethidium bromide (Biotech Co, Canada).

Polymerase Chain Reaction (PCR) product

Two universal primers, ITS1(F-5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4(R-5'-TCC TCC GCT TAT TGA TAT GC-3'), were used in the PCR created by White et al. [18] to amplify the Internal Transcribed Spacer region (ITS 1-5.8S-ITS2). The PCR Master Mix (5 μl, Bioneer Co, Korea), DNA template (5 μl, Bioneer Co, Korea), and Nuclease Free Water (25 μl, Bioneer Co, Korea) were combined to create the DNA amplification solution.

Antifungal activity of M. Chamomilla extracts

DNA fragments were separated in a 2 % agarose gel, and the sizes of the products were compared with molecular markers (100–2000 bp, Bioneer Co., Korea). The fragments were then inspected with a UV transilluminator and compared to ladder fragments.

PCR Product Sequencing of ITS1-5.8S–ITS2 rDNA region

Macrogen Co, South Korea, sent the PCR products for sequencing. The NCBI's "BLAST" was used to identify the PCR sequencing products of fungi.

Creation of M. Chamomilla Methanol Extract (ME)

Weighed and ground into a powder, dry chamomile flowers were used to make a 5 % w/v suspension in a flask by adding methanol alcohol. The temperature was then held at 37 °C for 4 hours while the flask was shaken at 200 rpm. After shaking, the suspension was run through a series of Whatman Filter Papers before the flask was cooled to ambient temperature run through a 0.22 micron filter at the end. According to Srivastava and Gupta [19], the filtered ME was dried at ambient temperature and kept at -20 °C until use.
**Gas Chromatography/Mass Spectrometry (GC/MS) Analysis**

One mL of ME was taken for GCMS/MS (Shimadzu Co GCMS-QP2010 Ultra, Japan) analysis. GC-MS analysis was carried out at the Basra oil company Laboratory, by using an Agilent Technologies, 7890B GC system coupled to an Agilent Technologies 5977A MSD with El Signal detector, using HP-5ms 5% phenyl, 95 % methyl siloxane (30m*250um*0.25), the oven temperature was set at 40 °C hold for 5 min then raised to 8 °C/min to 300 °C for 20 min, Helium carrier gas flow rate was 1 ml/min and purge flow 0f 3 ml/min. The injection mode was pulsed Splitless with injection temperature 290 °C and the injection sample volume was 0.5 micro letter. The mass spectrometer used Ion Source Temperature 230 °C, With scan speed 1562(N2), and the mass range 44-750 m/z. Data was run through the NIST 2014,2020 Library data base as an additional tool to confirm identity of compounds. The name, Molecular Weight (MW), Retention Time (RT), peak area and structure of the active constituents of ME we ascertained.

**Cytotoxicity test**

The produced ME underwent a biocompatibility test against human fresh blood using the Nair et al. method [20].

**Antifungal activity of M. Chamomilla extracts**

**Evaluation of the efficacy of ME and antifungals against isolated fungi**

A portion of the colony was taken and mixed with 5 ml of physiological saline solution (normal saline), which was placed on the dishes until dry. The mixture was then added to the extract in wells that had been prepared with a diameter of 6 mm, as it was filled with 50 microliters. This method, known as the agar well diffusion method [21], was used to study the sensitivity of ME. Based on the same steps above and the same concentrations, the antifungal (Nystatin, Sapha Co, Iraq) was tested on isolated keratinophilic fungi.

**Statistical description**

Data were analysis by the ANOVA test at significant level 0.05.

**RESULTS**

**Molecular Identification**

The molecular results of sequencing the ITS region revealed the presence of multiple fungal species among the isolates. The identified species included Scopulariopsis cordiae, Scopulariopsis stercoraria, Scopulariopsis brevicaulis, Trichophyton rubrum, Trichophyton mentagrophytes, and Microsporum canis. Among these species, our research showed that Scopulariopsis brevicaulis was the
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Most prevalent (Table 1, Figure 1). Using this method, we were able to register four new keratinocyte fungal isolates in DNA Data Bank of Japan (DDBJ) for the first time ever, as fungi had certain point mutations that were found in the sequence data.

**Cytotoxicity**

Various amounts of a human blood solution were used to investigate the cytotoxic activity of ME. The outcome shown that no turbidity development took place after adding 100 l of flavonide M. chamomile extract to all concentrations of human blood solution after 15, 30, and 60 min. This implies that the study's fungal extract did not have a cytotoxic effect (Figure 2).

<table>
<thead>
<tr>
<th>Code</th>
<th>Molecular identification</th>
<th>Source</th>
<th>Accession number</th>
<th>strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td><em>Trichophyton rubrum</em></td>
<td>skin</td>
<td>LC369522.1</td>
<td>TWCC 57922</td>
</tr>
<tr>
<td>b</td>
<td><em>Trichophyton mentagrophytes</em></td>
<td>skin</td>
<td>EF631618.1</td>
<td>BMU03104</td>
</tr>
<tr>
<td>c</td>
<td><em>Microsporum canis</em></td>
<td>skin</td>
<td>MT423731.1</td>
<td>19/600310</td>
</tr>
<tr>
<td>d</td>
<td><em>Scopulariopsis brevicaulis</em></td>
<td>nails</td>
<td>LC639848.1</td>
<td>9AFRODET*</td>
</tr>
<tr>
<td>e</td>
<td><em>Scopulariopsis brevicaulis</em></td>
<td>nails</td>
<td>LC639860.1</td>
<td>37AFRODET*</td>
</tr>
<tr>
<td>f</td>
<td><em>Scopulariopsis brevicaulis</em></td>
<td>nails</td>
<td>LR983931.1</td>
<td>CCF 6434</td>
</tr>
<tr>
<td>g</td>
<td><em>Scopulariopsis cordiae</em></td>
<td>nails</td>
<td>LC638843.1</td>
<td>2FRAFAF*</td>
</tr>
<tr>
<td>h</td>
<td><em>Scopulariopsis stercoraria</em></td>
<td>nails</td>
<td>LC638844.1</td>
<td>3AFFRAF*</td>
</tr>
</tbody>
</table>

**Table 1.** Molecular identification for keratinophilic fungi in our study (*the new keratinocyte fungal isolates in (DDBJ) for the first time ever*)
Figure 1. Phylogenetic tree of keratinophilic fungi (**our study strains).

Figure 2. Toxicity test results for ME.
Antifungal Activity
Depending on the extract content and the source from which the sample was taken, the isolates had varying degrees of fungal growth inhibition. This implies that various fungi may be more or less susceptible to the extract, and that the origin of the sample may also affect how they react.

The methanolic extract showed a substantial inhibitory action against fungus in the efficacy test results. S. brevicaulis, one of the tested fungi, showed the most inhibition with a diameter of 26 mm at a dosage of 1000 g ml\(^{-1}\), whilst M. canis showed the lowest inhibition with a diameter of 9 mm at a concentration of 100 g ml\(^{-1}\). According to statistical analysis, there were highly significant differences between the concentrations utilized and the particular fungus species (p< 0.05), emphasizing the extract's varied effects. Particularly, S. brevicaulis demonstrated the extracts maximum receptivity (Table 2, Figure 3, Figure 4). The M. chamomilla plant extract concentration of 1000 g ml\(^{-1}\) produced the maximum inhibition zone parameter. Additionally, statistical examination of the antifungal Nystatin indicated not important variations in the quantities utilized for most species.

GC-MS Profile of ME
In the current investigation, a sizable number of chemicals were found in the chamomile plant GC/MS analysis (Table 3) provides a summary of the significant elements and their percentages. There are at least 11 chemical compounds in M. chamomilla.
Table 2. Effectiveness of ME and the antifungal (Nystatin) on keratinophilic fungi isolates

<table>
<thead>
<tr>
<th>Code</th>
<th>Control</th>
<th>Inhibition zone of ME (mm)</th>
<th>Inhibition zone of Nystatin (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Concentrations g ml⁻¹</td>
<td>Concentrations g ml⁻¹</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>500</td>
</tr>
<tr>
<td>a</td>
<td>0</td>
<td>13.00</td>
<td>15.00</td>
</tr>
<tr>
<td>b</td>
<td>0</td>
<td>10.00</td>
<td>12.90</td>
</tr>
<tr>
<td>c</td>
<td>0</td>
<td>6.00</td>
<td>12.00</td>
</tr>
<tr>
<td>d</td>
<td>0</td>
<td>18.00</td>
<td>22.00</td>
</tr>
<tr>
<td>e</td>
<td>0</td>
<td>18.00</td>
<td>22.00</td>
</tr>
<tr>
<td>f</td>
<td>0</td>
<td>17.00</td>
<td>20.00</td>
</tr>
<tr>
<td>g</td>
<td>0</td>
<td>10.00</td>
<td>13.90</td>
</tr>
<tr>
<td>h</td>
<td>0</td>
<td>13.90</td>
<td>16.00</td>
</tr>
</tbody>
</table>
Figure 3. Effectiveness of ME on keratinophilic fungi isolates using some concentrations.

Figure 4. Inhibition Zone (IZ) and mechanism of action potential for ME and NY on keratinophilic fungi.
### Table 3. Identifications, formulas, area, Retention Time (RT), Molecular Weight (MW) and chemical structures of GC-MS profile for ME

<table>
<thead>
<tr>
<th>NO.</th>
<th>Name and Identification</th>
<th>Formula</th>
<th>Area (%)</th>
<th>RT (min)</th>
<th>M.W (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2-Butoxynaphthalene</td>
<td>C_{14}H_{16}O</td>
<td>6.123</td>
<td>15.539</td>
<td>200.28</td>
</tr>
<tr>
<td>2</td>
<td>4-methylumbelliferone</td>
<td>C_{10}H_{8}O_{3}</td>
<td>5.123</td>
<td>16.049</td>
<td>176.17</td>
</tr>
<tr>
<td>3</td>
<td>Methyl hexadecanoate</td>
<td>C_{17}H_{34}O_{2}</td>
<td>5.83</td>
<td>17.724</td>
<td>270.5</td>
</tr>
<tr>
<td>4</td>
<td>oleic acid</td>
<td>C_{18}H_{34}O_{2}</td>
<td>8.232</td>
<td>18.505</td>
<td>282.5</td>
</tr>
<tr>
<td>5</td>
<td>4,4'-Methylenediphenol</td>
<td>C_{13}H_{20}O_{2}</td>
<td>5.83</td>
<td>19.411</td>
<td>200.23</td>
</tr>
<tr>
<td>6</td>
<td>Citronellyl tiglate</td>
<td>C_{15}H_{26}O_{2}</td>
<td>38.47</td>
<td>20.815</td>
<td>238.37</td>
</tr>
<tr>
<td>7</td>
<td>bis(1-methylpiperidin-4-y1)amine</td>
<td>C_{12}H_{25}N_{3}</td>
<td>1.3045</td>
<td>21.042</td>
<td>211.35</td>
</tr>
<tr>
<td>8</td>
<td>2-Tridecenal</td>
<td>C_{13}H_{2}O</td>
<td>1.1965</td>
<td>22.829</td>
<td>196.33</td>
</tr>
<tr>
<td>9</td>
<td>Fisetin</td>
<td>C_{15}H_{10}O_{6}</td>
<td>22.159</td>
<td>23.010</td>
<td>286.24</td>
</tr>
<tr>
<td>10</td>
<td>m-Cresol, 6-propyl-</td>
<td>C_{10}H_{14}O</td>
<td>2.411</td>
<td>23.665</td>
<td>150.22</td>
</tr>
<tr>
<td>11</td>
<td>Dextran</td>
<td>C_{18}H_{2}O_{16}</td>
<td>3.321</td>
<td>29.875</td>
<td>504.4</td>
</tr>
</tbody>
</table>
DISCUSSION
You used molecular techniques to identify the fungal isolates in our study. Due to its precision and dependability, this method is chosen above conventional morphological ones [22]. Given that it relies on visual observations of physical traits that could differ between species or be challenging to distinguish, morphological identification can be time-consuming and inaccurate [23].

You decided to use molecular techniques, which offer a straightforward and accurate way of identification, to get around these restrictions. We amplified and sequenced the ITS region of the fungal isolates in particular. Between the rRNA genes in the fungal genome is the conserved ITS region, which is frequently used to identify fungi[24]. By amplifying and sequencing the ITS region, we were able to obtain precise and consistent results for identifying the fungal species. The ITS region serves as a molecular marker that contains both highly conserved regions, which facilitate amplification, and variable regions, which allow for species-specific identification [25].

The simplicity and dependability of molecular identification are improved by the availability of ITS region sequences in generic databases. In order to accurately identify the fungal species, these sequences are used as a reference for comparing and matching the acquired sequences [26].

Our study used molecular techniques and focused on the ITS region to genetically identify fungal isolates. This method allowed us to precisely and reliably identify the species that were present during our investigation. The findings illustrated the importance of genetic diagnosis using the ITS region in comprehending fungal variety and prevalence by demonstrating the prevalence of Scopulariopsis brevicaulis among the isolated fungi.

A number of infections, including nail infections, onychomycosis, and invasive fungal diseases in immunocompromised people have been linked to Scopulariopsis species, including Scopulariopsis brevicaulis [27,28]. Therefore, worries regarding the possible influence on human health are raised by the high incidence of Scopulariopsis brevicaulis found in this study.

Additionally, it is important to note the presence of dermatophyte species like Trichophyton rubrum, Trichophyton mentagrophytes, and Microsporum canis
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because these fungi are the main culprits behind common fungal infections in humans like dermatophytosis (ringworm), tinea infections, and others [29]. These infections, which can affect the skin, hair, and nails, may have an impact on public health, particularly in areas where these fungi are common [29].

In general, the development of suitable diagnostic and treatment approaches can benefit from understanding the prevalence and distribution of fungus through molecular identification [30]. Based on the susceptibility patterns of the detected species, it enables medical professionals to precisely identify the fungi that are the cause of fungal illnesses and select the most effective antifungal treatments [31].

The extract can be regarded as safe and possibly employed as a therapeutic substitute for antifungal drugs that might have negative host-related side effects. These results concur with research done by Osman et al in 2016 [32]. It's likely that the study in question supported the idea that the fungus extract utilized in the current study is safe and might be a beneficial therapeutic alternative.

The fact that the extract used in this study can be described as a therapeutic alternative to antifungals with adverse side effects on the host" indicates that the M. chamomile extract is regarded as safe and may one day be employed as a therapeutic option for treating fungal infections. The extract may have advantages over traditional antifungal drugs, which frequently have unfavorable host side effects.

To learn more about the effectiveness, safety, and possible therapeutic uses of the M. chamomile extract in treating fungal infections, additional research, such as in vitro and in vivo investigations, would also be necessary. Last but not least, the M. chamomile extract used in the study is safe and might be used as a therapeutic substitute for antifungals with negative side effects. However, more in-depth research is required to support this claim and provide a thorough understanding of its potential advantages and disadvantages.

The acquired results show that, within the permitted dose range, all tested fungal isolates responded differently to various doses of the flavonoid extract taken from M. chamomilla. Al-Dabbagh et al. [33] findings are consistent with the ME ability to effectively control the fungus.

Antibiotics should never be used without contacting a doctor, as doing so can result
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in fungal colonization of the injured area. This may account for changes in the fungi's sensitivity and resistance patterns. Antibiotics can be poisonous to fungi, but they can also create resistance in them to lessen their effects [34].

Overall, these results highlight the methanolic extract's strong inhibitory action against the investigated fungus. Higher concentrations of the extract typically result in bigger inhibition zones. The concentration of the extract had a substantial impact on the inhibition. The varying susceptibility of the fungal species also emphasizes how crucial it is to take into account the precise target organisms when evaluating the effectiveness of antifungal medicines. According to the findings, S. stercoraria and S. cordiae are specifically sensitive to methanolic extract and Nystatin, respectively. These results advance our knowledge of the potential uses of organic plant extracts and antifungal medications to treat fungus infections. To understand the underlying mechanisms and improve their application in clinical or agricultural contexts, more investigation and study are required.

Various mold species, including *aspergillus*, *fusarium*, *alternaria*, *rhizoctonia*, and *chaetomium*, have been documented to be susceptible to the antifungal effects of natural extracts and essential oils from aromatic and medicinal plants [35,36]. There has previously been information about a natural peptide from *M. chamomilla* flowers with broad-spectrum antifungal activity against human pathogenic molds and yeasts [37]. The rise of antifungal drug resistance in fungal species is considered to be an increasing challenge for the public health.

Chemical compounds, related to essential oils and phenolic and flavonoid, prevent fungal growth through the inhibition of mycelia, conidial production, and sporulation of fungi [38]. Essential compounds from different plants have been reported to inhibit the fungal pathogens of *F. moniliforme*, *F. oxysporum*, *Rhizoctonia solani*, *A. solani*, and *Aspergillus sp* [39]. Flowers of *M. chamomilla* have a blue essential oils content, ranging from 0.2 % to 1.9 %, which has been used in various applications [39].

Extracts and essential oils of *M. chamomilla* exhibit a wide range of biological activities, such as antimicrobial, antioxidant and anti-inflammatory properties [40]. Plants and other naturally
occurring sources typically contain natural chemicals with biological activity [41].

The results of the current study showed that ME extract for M. chamomilla flowering contains a number of major bioactive compounds, which are believed to be responsible for its potent antifungal activity against all of the test fungal pathogens [42-45].

**CONCLUSION**

Our study emphasizes the value of molecular identification in comprehending the diversity and frequency of fungi. Our study's discovery of pathogenic fungus stresses the potential harm to human health and the demand for efficient diagnostic and therapeutic methods. The M. chamomilla extract demonstrated encouraging findings as a secure and effective substitute for conventional antifungal medications, but additional study is necessary to fully understand its therapeutic potential and mechanisms of action. In general, our research adds to the corpus of information on identifying fungi and creating antifungal tactics.

**ACKNOWLEDGMENT**

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