Bioactivities of Anethole, Astragalin and Cryptochlorogenic Acid Extracted from Anise Oil and Moringa oleifera on the Keratinase Gene Expression of Trichophyton rubrum

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Abstract

Trichophyton rubrum is the most common cause of dermatophyte skin infections in humans worldwide. The aim of the current study is to search for effective and safe antifungal agents by investigating the bioactivity of Pimpinella anisum and Moringa oleifera extracts, including the impact on the keratinase gene expression of T. rubrum. Astragalin and cryptochlorogenic acid were detected in M. oleifera leaf extracts by Gas chromatography-mass spectrometry (GC-MS), whereas anethole was extracted from P. anisum oil. The expression of the keratinase gene was assessed in two strains of T. rubrum (KP979791 and KP979787) in Sabouraud Dextrose Broth (SDB) medium (containing 10\% keratin) separately supplemented with plant extracts fractions. The results showed that cryptochlorogenic acid and astragalin were active compounds (molecular weights of 353.26 and 449.35 g/mol, respectively that considerably down-regulated the keratinase genes in both strains of T. rubrum. M. oleifera crude extract, astragalin, cryptochlorogenic acid, anise oil, and anethole resulted in the down-regulation of the keratinase genes of T. rubrum strain KP979791 by 0.048, 0.291, 0.001, 0.007, and 0.009 (fold-change), respectively, and that of strain KP979787 by 0.151, 0.42, 0.16, 0.079, and 0.092 as compared to the control (1.00). Nearly all of these outcomes are novel findings that may aid in the identification of novel effective drug targets and antifungal agents.

Keywords: Trichophyton rubrum, Moringa oleifera, Pimpinella anisum, Chromatography, keratinase gene

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INTRODUCTION

Dermatophytes are a group of parasitizing filamentous fungi that infect the keratinized tissues of the nails, skin, and hair as keratin is utilized as the only carbon energy source, which normally results in universal cutaneous mycoses. The prevalence of superficial fungal infection under different living circumstances in Babylon Province, Iraq, was investigated. T. rubrum is a major etiological agent of dermatophytosis and other recurring fungal infections in humans. T. rubrum and T. mentagrophytes account for 27%–76% and 4%–41% of chronic mycotic infections of humans, respectively. In Iraq, five strains of T. rubrum have been identified to date, which have fairly similar cultural features, but considerably different growth requirements and proteolytic activities; their virulence were also wholly studied.

T. rubrum is currently a serious epidemic and, at times, an endemic human pathogen. The major mechanism of the pathogenicity of dermatophytes is the production of proteases which makes it possible to penetrate the human skin via keratinized tissues. The proteolytic enzymes produced by dermatophytes are activated under different environmental conditions (i.e., neutral, alkali, and acidic), which have significant roles in the proteolytic activity, pathogenesis, and particularly, the invasion of T. rubrum. The proteolytic enzymes produced and secreted by this pathogen are major virulence factors. However, the uneven expression of the protease genes is responsible for the variances in the capacity to cause infections. Keratinases are considered the most prominent virulence factors of dermatophytes. Trichophyton species have exceptionally high keratinase activities, and the level of keratinase activity can be used in indirectly estimating the strength of fungal pathogenicity. It is therefore essential to investigate the expression profiles of genes involved in the virulence of T. rubrum.

Previous gene expression analysis demonstrated that trans-chalcone in commercial antifungals suppresses the expression of virulence-related genes of T. rubrum. There are three classes of commercially available drugs that are known to interfere with the plasma membrane: azoles and triazoles, which inhibit the enzyme sterol 14 α-demethylase, and allylamines, which inhibit squalene epoxidase and ergosterol biosynthesis. Allylamines also lead to the accumulation of lanosterol, a toxic intermediary compound of the ergosterol biosynthesis pathway. Sharma et al. mentioned that of six antifungal agents, dermatophytes (T. mentagrophytes and T. rubrum) were most resistant to fluconazole and clotrimazole, and voriconazole was the most effective. The failure of any treatment lies in the complexity of the host–fungus interactions and the virulence of the species and the ability to avoid the host immune response. Several recent studies have improved our understanding of the molecular basis of antifungal resistance. However, the identification of new antifungal targets and the development of novel antifungal compounds from natural as well as synthetic products are needed to promote the development of innovative strategies for antifungal therapies.

Medicinal plants are valuable sources of antifungal compounds. Moringa oleifera is a precious source of bioactive compounds with different pharmacological activities. Extracts of M. oleifera reportedly have potential antifungal activities against dermatophytic fungi with good remedial properties and few side effects as compared with synthetic antibiotics. In addition, cell lysis was recognized in fungal tissues of T. rubrum grown on Sabouraud’s dextrose agar (SDA) and treated with anise (Pimpinella anisum) essential oil (EO) and anethole, suggesting that both extracts possessed antimicrobial activities against T. rubrum. The use of natural products, such as plant-derived EOs, as an alternative to synthetic chemical agents is regarded as an environmentally safe alternative for disease control.

Therefore, the aim of the present study is to obtain a secure, efficient, naturally acquired, antifungal agent from P. anisum EO and leaf extracts of M. oleifera. These natural products were screened for antifungal effects against T. rubrum, which is responsible for most human dermatophyte infections. Furthermore, the effects of the phytoconstituents of selected medicinal plants (i.e., anise oil and M. oleifera), especially anethole, astragalin, and cryptochlorogenic acid, on the expression of the keratinase gene of T. rubrum were investigated, since this gene is known to contribute to the
pathogenesis of \( T. \) rubrum, and that would aid in the design of effective therapeutics without causing any serious adverse reactions, which may provide future insights into the virulence of this clinically important pathogen.

**MATERIALS AND METHODS**

**Source of \( T. \) rubrum isolate**

\( T. \) rubrum strains KP979791 (isolate N05 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and KP979787 isolate N01 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene were obtained from Kadhim et al., 2015b\^\, Department of Biology, Advanced Mycology Laboratory, University of Babylon (Iraq), both strains were previously identified at the molecular level\.

**Preparation and maintenance of \( T. \) rubrum**

SDA medium was prepared according to the manufacturer’s instructions by dissolving 64 g of SDA in 1000 mL of distilled water. The medium was sterilized by autoclaving prior to culturing and sub-culturing of the \( T. \) rubrum isolates\:

Briefly, a colony of \( T. \) rubrum (diameter, 0.5 cm) was placed at the center of a Petri plate (diameter, 9 cm) containing sterilized SDA supplemented with cycloheximide (0.5 g/L) and chloramphenicol (0.05 g/L) at pH of 5.6 and incubated at 28°C ± 1°C for 14 days and then stored in a refrigerator at 5°C. The fungus was maintained on SDA slants at 4°C and subcultured monthly throughout the study period.

**Plant Materials**

The leaves of \( M. \) oleifera and the seeds of \( P. \) anisum were collected to obtain crude plant extracts according to Obaid et al., 2017\^\, extracted anethole was used as it was described previously\.

**Preparation of active materials from the leaves of \( M. \) oleifera**

The active material from the crude extract was processed by preparative HPLC. The preparative reversed-phase HPLC was performed using column C18-ODS (25 cm × 4.6 mm × 5μm). The mobile phases were determined based on the method adopted by Porel et al.\^\, and these phases include (A) Methanol: Acetic acid: Distilled water (10:2:88) and (B) Methanol: Acetic acid: Distilled water (90: 3: 7). The gradients are 40% of A, 60% of B for 4 min, 50% of A, 50% of B for 5-8 min and 60% of A, and 40% of B for 8-10 min. The injection volume was 100μL with a flow rate of 1.0 ml/min, and the detection wavelength was 280 nm\.

**Detection of the active compounds of \( M. \) oleifera extract by liquid chromatography with tandem mass spectrometry (LC-MS/MS)**

LC-MS/MS analyses of the \( M. \) oleifera protease inhibitors were performed using a Nexera HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a C18 reversed-phase Inertsil ODS-4 analytical column (3μm, 150 × 2.1 mm) coupled to a tandem MS instrument. The column temperature was fixed at 40°C. The elution gradient consisted of a mobile phase (A) of water: formic acid (99:1) and a mobile phase (B) of methanol: formic acid (99:1). The flow rate was 0.1 mL/min. Elution was performed at a starting concentration of B of 10%, which was increased to 70% over a period of 30 min. The solvent flow rate was maintained at 500μL/min, and the injection volume was 4μL. Detection was performed using an LCMS-8040.
Triple Quadrupole system (Shimadzu Corporation) equipped with an electrospray ionization (ESI) source operating in both positive and negative ionization modes. LC-MS/MS data were collected and processed using LabSolutions software (Shimadzu Corporation). Multiple reaction monitoring was used to quantify the results. Analysis of the investigated compounds was performed following two or three transitions per compound, i.e., the first time for quantitative purposes and the second and/or third time for confirmation to ensure accuracy. The optimum ESI conditions were specified as follows: desolvation line temperature, 250°C; heat block temperature, 400°C; nebulizing gas (N₂) flow rate, 3 L/min; and drying gas (N₂) flow rate, 15 L/min²⁸. The active material that was detected was Astragalin and cryptochlorogenic acid.

**Effect of *M. oleifera* crude extract, astragalin, cryptochlorogenic acid, anise oil and anethole on the keratinase gene expression of *T. rubrum***

Cultures of *T. rubrum* strains KP979791 and KP979787 at turbidities of 0.5 McFarland standard were prepared in SDB containing 10% keratin. The active materials of the crude extracts of *M. oleifera*, anise oil, astragalin, cryptochlorogenic acid, and anethole were added to the medium at concentrations of 50, 7.8, 20, 20, and 3.9μg/ml respectively, to assess their effects on keratinase gene expression.

**RNA extraction**

The medium was inoculated with *T. rubrum*. Following incubation and centrifugation at 5,000×g for 10 min at 4°C, the supernatant was collected for isolation of the fungal cells. Total RNA was extracted using a FAVORGEN® total RNA extraction kit (Fisher Biotec, Wembley, WA, Australia). The extracted RNA was stored at −70°C to keep it stable along the time of experiment until analysis.

**Real-time polymerase chain reaction (RT-PCR)**

RT-PCR primers

Primers for keratinase mRNA expression were designed with reference to the GenBank database (https://www.ncbi.nlm.nih.gov/nucleotide/XM_003235234.1). Moreover, the 18S primer pair was designed by Shanghai ShineGene Molecular Biotech, Inc. (Shanghai, China). The primer sequences for the keratinase gene were similar to those in the GenBank database (accession number 003235234): (forward) 5′-CTC TCC TCT ACG AAT GCC AAA G-3′ and (reverse) 5′-GAA TGT GAA CTG GTT GCG TTT C-3′, which produced an amplicon of 159 bp. The following primer sequences were used for amplification of the reference 18S gene (accession number 01000074.1): (forward) 5′-AAA CGG CTA CCA CAT CC A-3′ and (reverse) 5′-CAC CAG ACT TGC CCT CCA-3′, which produced an amplicon of 166 bp. The fungal samples were treated with chemical compounds and plant extracts.

**cDNA synthesis**

DNase I-treated RNA samples were used to produce the cDNA of the keratinase (target) and 18S genes with the use of FicoScript® Moloney murine leukaemia virus reverse transcriptase (M-MLV RT; Shanghai ShineGene Molecular Biotech, Inc.) in accordance with the manufacturer’s instructions. Each reaction included 5 × RT buffer, 100 ng/μL of primer, M-MLV RTase (200 U), RNase inhibitor, RNA, and DEPC water at volumes of 4, 1, 1, 1, 6, and 7μL, respectively. The conditions for cDNA synthesis were 37°C for 20 s and 95°C for 3 min and the RT-PCR reaction conditions were 95°C for 20 s, 59°C for 20 s, and 72°C for 25 s.

**Quantitative reverse transcriptase (qRT)-PCR data analysis**

The relative gene expression levels (fold-change) were determined by analyzing the qRT-PCR data of the housekeeping and target genes using the ΔΔCT method as described by Livak and Schmittgen²⁹ as follows:

\[
\Delta \text{CT target gene} = \text{Ct}_{\text{target gene}} - \text{Ct}_{\text{reference gene/target}} \quad \text{(1)}
\]

\[
\Delta \text{CT control} = \text{Ct}_{\text{control}} - \text{Ct}_{\text{reference gene/control}} \quad \text{(2)}
\]

Upon determining the ΔCT values of the target and controls, the following equation was used to determine the ΔΔCT value:

\[
\Delta \Delta \text{CT} = \Delta \text{Ct}_{\text{target gene}} - \Delta \text{Ct}_{\text{control}} \quad \text{(3)}
\]

The \(2^{-\Delta \Delta \text{CT}}\) method was then used to determine the fold-change in gene expression with the data presented in Table 1.

**Table 1. qRT-PCR data analysis**

<table>
<thead>
<tr>
<th>Gene Entry</th>
<th>Test (Treatment group)</th>
<th>Cal. (Control group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target gene</td>
<td>CT (target, test)</td>
<td>CT (target, cal)</td>
</tr>
<tr>
<td>Reference gene</td>
<td>CT (ref, test)</td>
<td>CT (ref, cal)</td>
</tr>
</tbody>
</table>

*This table was adopted from reference²⁹.*
Statistical Analysis
SPSS (25) was used for statistical analysis. Variation among the groups was analysed using one-way (ANOVA) test, monitored by LSD’s tests. A minimum range of variance $P < 0.05$ was reflected significant.

RESULTS AND DISCUSSION

HPLC of M. oleifera extract

As shown in the chromatograph presented in Fig. 1, there were two main peaks. Two compounds in the extracts of the M. oleifera protease inhibitor sample were obtained by HPLC (Fig. 1), with retention times of 4.784 and 5.705 min for the first and second compounds, respectively. Therefore, LC-MS/MS analysis was performed to further characterize the detectable compounds.

LC-MS/MS analysis of the M. oleifera active compounds

The LC-MS/MS results show that the protease inhibitor (extracted from M. oleifera leaves) after purification and isolation with HPLC had two predominant peaks: cryptochlorogenic acid ($C_{18}H_{18}O_{11}$; molecular weight, 353.26 g/mol; retention time, 5.61 min; Fig. 2A) and astragalin ($C_{28}H_{30}O_{14}$; molecular weight, 449.35 g/mol; retention time, 9.19 min; Fig. 2B). The inhibitory effects on the protease enzyme can be attributed to the presence of these two compounds.

Lin et al.$^{30}$ reported that cryptochlorogenic acid and astragalin were two of the basic compounds present in M. oleifera leaf extracts with molecular weights of 355 and 447, respectively, which is consistent with the results of the present study. Cryptochlorogenic acid possesses inhibitory effects against proteases and many other enzymes, including hepatic glucose-6-phosphatase.$^{31}$ In addition, Rohn et al.$^{32}$ found that the inhibitory activities of cryptochlorogenic acid against alpha-amylase, trypsin, and lysozyme were deactivated. Inhibition of this enzyme is dependent on the reactivity of the phenolic and related ingredients tested and on the type of the substrate. The decreased activities were accompanied with reductions in the amounts of free amino and thiol groups as well as tryptophan residues, which resulted from the covalent attachment of phenolic and related compounds to reactive nucleophilic sites of the enzymes.

Flavonoids, such as astragalin, are an important group of natural compounds that can interfere with the activities of various enzymes. Almost all flavonoids inhibit aldehyde oxidase activity, consistent with the results of the present study, which confirmed the inhibitory effect of astragalin.$^{33}$ Braz$^{34}$ found that a number of flavonoids inhibited the enzymatic activity of glyceraldehyde-3-phosphate dehydrogenase.

Gene expression

The results of the RT-PCR analysis showed that the keratinase mRNA levels of T. rubrum were significantly decreased in the treated samples, as compared with the control group. The down-regulation expression of the keratinase gene in T. rubrum by the plant extracts and chemical compounds was evident. The Livak technique was applied to normalize the gene expression of the target gene (keratinase) with the housekeeping gene (18S) expression as a reference gene when determining the relative expression of the keratinase gene in treated and untreated T. rubrum.

In both plots, the target and reference genes as well as the gene expression in the crude isolates (i.e. the control or calibrator) are presented. The target gene-threshold cycle numbers were first normalized to that of the reference gene in the two tested isolates to obtain the $\Delta\Delta CT$ for target gene and control, equation number (1) and (2), the $\Delta\Delta CT$ was calculated as shown in the equation number (3). Then, the $2^{-\Delta\Delta CT}$ can be calculated as the fold change that represented the gene expression.

The results of the calculations demonstrated that the relative expression of the keratinase gene in the T. rubrum (KP979791) treated with M. oleifera crude extract, astragalin, cryptochlorogenic acid, anise oil extract and anethole was 0.048, 0.291, 0.001, 0.007, and 0.009, respectively, while in T. rubrum (KP979787) was 0.151, 0.42, 0.16, 0.079, and 0.092 respectively compared with a high gene expression value in the control which was 1 (Tables 2A and 2B).

From the results, all fractions (M. oleifera extract, astragalin, cryptochlorogenic acid, anethole and anise oil) had significantly down-regulation effects on the keratinase gene expression of T. rubrum KP979791 (Fig. 3 A) and KP979787 (Fig. 3B) strains. Gene expression in
KP979791 was substantially higher than that in KP979787 compared with that in control (1). There is a significant difference in ability of *M. oleifera* extract, astragalin, cryptologic acid, anise oil and anethole to decrease gene expression for KP979791 by 95%, 70%, 99%, 99% and 99% and for KP979787 by 84%, 58%, 84% 92% and 90% respectively compared to that in control (0%). From these results, the anise oil and anethole have the greatest ability to inhibit keratinase

![Fig. 1. HPLC chromatogram of the *M. oleifera* protease inhibitor. Two main peaks with retention times of 4.784 and 5.705 min were detected.](image)

![Fig. 2. LC-MS/MS chromatograms of: A. cryptochlorogenic acid (molecular weight, 353.26), B. astragalin (molecular weight, 449.35).](image)
**Table 2A.** The analysis of the Keratinase gene’s relative gene expression for the *T. rubrum* KP979791 (ΔΔCT) Technique

<table>
<thead>
<tr>
<th>Treatments</th>
<th>CT (Keratinase)</th>
<th>CT (18S)</th>
<th>ΔCT</th>
<th>ΔΔCT</th>
<th>2-ΔΔCT</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. oleifera</em> crude extracts</td>
<td>19.80333333</td>
<td>16.72666667</td>
<td>3.08</td>
<td>4.36</td>
<td>0.048</td>
</tr>
<tr>
<td>Astragalin</td>
<td>16.37111111</td>
<td>15.87111111</td>
<td>0.5</td>
<td>1.78</td>
<td>0.291</td>
</tr>
<tr>
<td>Cryptochlorogenic acid</td>
<td>20.29444444</td>
<td>12.12666667</td>
<td>8.17</td>
<td>9.45</td>
<td>0.001</td>
</tr>
<tr>
<td>Anise oil</td>
<td>20.25444444</td>
<td>14.49333333</td>
<td>5.76</td>
<td>7.04</td>
<td>0.007</td>
</tr>
<tr>
<td>Anethole</td>
<td>19.83888889</td>
<td>14.42333333</td>
<td>5.41</td>
<td>6.69</td>
<td>0.009</td>
</tr>
<tr>
<td>Control</td>
<td>18.67555556</td>
<td>19.95333333</td>
<td>-1.28</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 2B.** The analysis of the Keratinase gene’s relative gene expression for the *T. rubrum* KP979787 (ΔΔCT) Technique

<table>
<thead>
<tr>
<th>Treatments</th>
<th>CT (Keratinase)</th>
<th>CT (18S)</th>
<th>ΔCT</th>
<th>ΔΔCT</th>
<th>2-ΔΔCT</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. oleifera</em> crude extracts</td>
<td>18.28666667</td>
<td>17.13444444</td>
<td>1.15</td>
<td>2.72</td>
<td>0.151</td>
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<tr>
<td>Astragalin</td>
<td>19.48777778</td>
<td>19.8</td>
<td>-0.32</td>
<td>1.25</td>
<td>0.420</td>
</tr>
<tr>
<td>Cryptochlorogenic acid</td>
<td>19.85666667</td>
<td>18.79666667</td>
<td>1.07</td>
<td>2.64</td>
<td>0.160</td>
</tr>
<tr>
<td>Anise oil</td>
<td>18.79666667</td>
<td>16.70555556</td>
<td>2.09</td>
<td>3.66</td>
<td>0.079</td>
</tr>
<tr>
<td>Anethole</td>
<td>19.90111111</td>
<td>18.03222222</td>
<td>1.87</td>
<td>3.44</td>
<td>0.092</td>
</tr>
<tr>
<td>Control</td>
<td>16.83222222</td>
<td>18.37555556</td>
<td>-1.57</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

**Fig. 3.** The effects of plant extracts (Control, *M. oleifera*, Astragalin, Cryptochlorogenic acid, Anise oil and Anethole) on the keratinase gene expression of *T. rubrum* KP979791 (A) and KP979787 (B).
compared to *M. oleifera* extract, Astragalin and cryptochlorogenic acid. In contrast, the astragalin showed the lowest ability (70 %) in KP979791 and 58% in KP979787.

A great deal of variation has been demonstrated using the PCR technique to determine the dermatophyte’s detection rate (Fig. 4). This detection is determined by the target gene employed in the analysis. Since the experimentation of dermatophytes using traditional laboratory approaches is a difficult problem and in most cases inadequate as a result of fungal phenotypic inconsistency and pleomorphism, the use of established molecular diagnostics provides an efficient method for the rapid analysis of pathogenic dermatophytes with unusual specificity, sensitivity and accuracy.

The analysis and sequencing of many dermatophyte genomes, as well as developments in the methods of gene therapy of dermal cells, provide tools, which make it much easier for these diffusion species to clarify the direction of virulence. Understanding the basic virulence contributing factors to skin dermatitis can help design effective treatments and help develop new therapeutic strategies.

The plant extracts and chemical compounds investigated in this study down-regulated the keratinase gene in *T. rubrum* strains KP979791 and KP979787. The results revealed that all fractions in this experiment had inhibitory impacts on gene expression by down-regulating the keratinase gene expression of *T. rubrum* strains. Turkoglu *et al.* demonstrated that the analysis...
of gene expression proved that the *Ficus carica* leaf extract had a significant down-regulation of SRD5A2, IL-1α, TNF-α, and VEGF compared to the untreated cells. Additionally, a qRT-PCR study was performed using essential oils to distinguish the extent of the gene expression, focusing on the production of ochratoxin A biosynthesis and two regulatory genes: IaeA and vea. All six essential oils reduced the expression of the investigated genes, specifically the ackps that was down-regulated by 99.2%, which in this study is the maximum.\(^{19}\)

The results of the current study and other related studies found contrasting capabilities of plant extracts (EO or leaf extract) because of the nature of the plant extract, the concentration in the medium, and the active material, which may be related to the virulence and physiology of the microorganism. These factors can explain the discrepancy in the effects of the plant materials on the gene expression profiles of different microorganisms. Liu *et al.*\(^{40}\) reported a high difference in genes expression of strain of *T. rubrum* that grow under the same condition.

However, other studies have revealed that treatment with lemongrass EO resulted in the up-regulation of *acpP*, whereas the increase of the amount of lemongrass EO had a profound impact on the expression levels of *acpP*, *hly*, and *inlJ*. However, *M. oleifera* extracts considerably down-regulated the mRNA expression levels of PPARα1, PPARγ, and HMG-CoAR\(^{41}\).

The excellent antifungal agent has been assumed as the compound that represses possible virulence factors, affects fungi physiology, and also acts on particular fungal cells. Moreover, these natural compounds may be more effective relative to common antifungal agents, thus synergistically improving their antifungal activity and acting as chemosensitizers to overcome fungal resistance. Therefore, the natural sources may expand the possibilities to develop antifungal therapies\(^{16}\). Our results revealed that kp979791 strain was more sensitive to *M. oleifera* extract, Astragalin, Cryptochlorogenic acid, Anise oil and Anethole than that in kp979787 by inhibiting a keratinase gene which led to down-regulation of the keratinase genes of kp979791 strain more than kp979787. More recent studies\(^{42}\) suggested that *M. oleifera* could be a source of metabolites with terbinafine to be used against *Micsporum canis*, particularly during combination with a distinguished antifungal agent (terbinafine) which interacts with subtilisin virulence genes in *M. canis*\(^{43}\). Also, when culture filtrates of *Marasmius palmivorus* and *Pleurotus ostreatus* were combined with terbinafine, the mixture exhibited downregulation of citrate and isocitrate genes in *T. rubrum*\(^{43}\). Moreover, *M. palmivorus* is a strong antagonist fungus and could be used as bioagent against various phytopathogenic fungi\(^{44}\).

**CONCLUSIONS**

We concluded from the aforementioned study that *M. oleifera* leaf extract, cryptochlorogenic acid, Anise oil and Anethole had pharmaceutical effects against the virulence factors (keratinase gene) of *T. rubrum*. These findings should prove tremendously helpful in the development of new regimens for the treatment of *T. rubrum* infections, especially in Babylon Province, Iraq, which has a fairly high rate of such infections. To the best of our knowledge, this is the first report on the impact of astragalin, cryptochlorogenic acid, and anethole on the keratinase gene as a target for *T. rubrum* infections.

**SUPPLEMENTARY INFORMATION**

Supplementary information accompanies this article at https://doi.org/10.22207/JPAM.14.1.64

Additional file: Additional Table A1.

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**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.
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AUTHORS' CONTRIBUTION
All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

DATA AVAILABILITY
All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT
This article does not contain any studies with human participants or animals performed by any of the authors.

REFERENCES