Research Article

Antifungal activity of combination of medicinal plant extracts with terbinafine through regulating subtilisin virulence genes in *Microsporum canis*

Maryam Q. H. Khazia[1], Jawad K. Abood Al-Janabi[2]*

ABSTRACT

Background: *Microsporum canis* is a zoophilic dermatophyte global distribution resulting in human as well as animal dermatophytosis. Different proteases have been associated with potential virulence factors of the pathogen. The current study aimed to investigate the antifungal and synergistic effects of *Pimpinella anisum* and *Moringa oleifera* with terbinafine on growth and the presence of subtilisin proteases (SUB1-3 genes) in *M. canis*. **Methodology:** Subtilisin proteases (SUB1-3 genes) were assayed by polymerase chain reaction (PCR) amplification techniques under different combinations of treatment with specific primers were conducted. **Results:** The current study revealed the substantial effect of *P. anisum* and *M. oleifera* and/or antibiotic (terbinafine) on the growth of the pathogen during the experiment period when *P. anisum* and *M. oleifera* and/or antibiotic (terbinafine) were combined. There was a low growth of *M. canis* in the combination of *M. oleifera*, *P. anisum,* and terbinafine. It was reported as (M+P+T)-(3.4 cm) then it was followed by P+T (3.6 cm) and M+T (3.8 cm) then T treatment which was (4.1 cm) and finally P+M (5.5 cm) when compared with untreated one (7.1 cm). Three subtypes of subtilisin genes (SUBs), coding for serine proteases, were identified by giving positive results of PCR products that observed in different fragments of SUB1(609bp), SUB2(1120bp), and SUB3(1324bp). **Conclusion:** From these study findings, the combinatorial use of medicinal plant extracts with a known antifungal agent, terbinafine for exhibiting a potential antifungal property, and developing control mechanisms concerning SUBS by interacting with subtilisin virulence genes in *M. canis* DNA in particular with SUB1 and SUB3.

KEY WORDS: Antifungal activity, *Microsporum canis, Moringa oleifera, Pimpinella anisum,* Subtilisin genes

INTRODUCTION

The global cutaneous mycoses infections of the hair, nails as well as skin are caused by the dermatophytes (filamentous fungi) that utilize keratin.[1] Almost 25% of the skin mycoses are formed by superficial fungal infections, which are associated with common types of infectious diseases globally.[2] Dermatophytosis is known as the superficial mycotic infection, which results from three types of the keratinophilic fungi such as *Epidermophyton* which affects the skin and nails, then *Trichophyton* that infects the nails, hair, and skin, and finally, microsporum that affects the skin and hair. They have the greatest agents of superficial cutaneous fungal infections.[3,4]

Access this article online	
Website: jprsolutions.info	ISSN: 0975-7619

Rarely do dermatophytes enter epidermis, similarly, in immunocompromised hosts systemic infections, there would be a penetration that is more profound, which increases the brutality of the infection and at the same time producing adversarial impacts that were not expected.[5,6]

Identify *Microsporum canis* virulence factors have focused on proteases, including keratinases. A family of three subtilisins,[7] a family of five metalloproteases[7,8] and two dipeptidyl peptidases (GenBank accession nos. DQ286524 and DQ286525) have been identified, but so far no definitive role in fungal virulence has been assigned to these enzymes, although the *in vivo* secretion of several of them has been demonstrated.[7,9] The role of secreted proteases in virulence has, however, been demonstrated in *Candida albicans*. Pathogenic *M. canis* has the ability to attack keratinized structures.[10] The main families

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Received on: 11-06-2019; Revised on: 17-07-2019; Accepted on: 22-08-2019

of proteases secreted by fungi are subtilisins (serine proteases) and fungalisins (metalloproteases).[11] The role of secreted subtilisin has been identified, during the infection steps.[12] SUBs coding serine proteases on *M. canis* give it the ability to digest keratinized tissues (skin and hair) in humans and animals and zoonotic attributes.[13]

The protease gene is one of the dermatophyte genes that have been identified as a virulence factor. For example, SUB1, SUB2, and SUB3 that encode a subtilisin family of serine protease is produced by *M. canis* during the invasion of keratin.[14]

Since subtilisin is an extracellular-located enzyme that is connected with the cell wall, it generally contributes to the fungal virulence and facilitates tissue colonization. There is a considerable correlation between their activity and the severity of infections.[15]

Subtilisins play a key role in pathogenicity of *M. canis* against humans, as they are responsible for initial contact and adherence of fungi to host cells,[13] since the pathogenicity of *M. canis* is associated with the SUB3 protease that is needed to adhere to the epidermis.[16]

Although several antibiotics presently used to cure mycoses, their cellular targets are limited, and fungi may reveal resistance or tolerance to these agents.[17]

Nevertheless, the combination of pure natural compounds with conventional antibiotics may grasp better capacity for promptly giving reasonable treatment options. Certainly, some combinational antibiotic therapies are clinically accessible.[18]

So far as we know, no specific studies were focused on combinatorial use of antibiotics like terbinafine which has a fungicidal mode of action through its ability to inhibit the enzyme squalene epoxidase, leading to a fungicidal accumulation of squalene within the fungal cell[19] and natural products as *Moringa oleifera* extracts which showed potential antifungal activity against dermatophytic fungi such as *Microsporum gypseum*, having good healing properties without side effects when compared with synthetic antibiotics.[20] Furthermore, cell lysis was recognized in fungal tissues of *Trichophyton rubrum* grown in Sabouraud dextrose agar (SDA) treated with anise essential oil and anethole; both extracts could be a potential source of new antimicrobial agents against *T. rubrum*.[21,22]

It was reported that terbinafine has a fungicidal mode of action through its ability to inhibit the enzyme squalene epoxidase, leading to a fungicidal accumulation of squalene within the fungal cell.[19] Combination therapy can offers advantages in increased synergistic action with enhanced spectrum activity and could lead to the implementation of new therapeutic tools aimed at preventing the adherence virulence factors like subtilisins. Therefore, the current study aimed to explore the antifungal and synergistic effects of *Pimpinella anisum* and *M. oleifera* with terbinafine on growth and the incidence of subtilisin proteases (SUB1-3 genes) in *M. canis*.

MATERIALS AND METHODS

Plants

Moringa oleifera leaves were collected from Hill country sides of Babylon Province, Iraq, in September 2018. In addition, matured seeds of *P. anisum* were bought from the nearby local market of the herbs. The materials were thoroughly washed, shade and dried then they were ground in powder form using an electric grinder, and it was packed in polythene bags until extraction then stored for analysis

Source of *M. canis*

The clinical isolate *M. canis* obtained from patients with tinea capitis from Dr. Ali Abdul-Hussain Al-Janabi, College of Medicine, Karbala University, Iraq. The preparations of the media were done according to manufactures that were fixed on their containers after being sterilized by autoclave at 15 psi/inch^2 in 121°C for 15 min, sterilized media of 15 ml were poured in disposable Petri dishes and incubated overnight at 25±1°C to ensure surety then stored at 4°C.

Growth and Maintenance of *M. canis*

20 ml of sterilized SDA medium was also prepared and poured in the Petri dishes and left to solidify. Then, the medium in the Petri dishes was incubated at for 14 days at 25±1°C and later stored in the refrigerator at 5°C. Throughout this study, the fungus was subcultured and maintained on SDA on a monthly basis.

Preparation of Terbinafine and its Effect on the Growth of *M. canis*

The solution was prepared using antibiotic terbinafine by dissolving 250 µg/ml from the drug in 100% dimethyl sulfoxide and 100 ml distilled water. Different concentrations were also prepared to start from 0.2 µg/ml, 4 µg/ml, and 6 µg/ml to 8 µg/ml, and the sterile SDA was planned in the 9 cm diameter dishes. Every concentration was kept for 30 min to allow the spreading of antibiotic in the medium and it was inoculated with *M. canis* centrally by taking discs of 0.5 mm diameter from the colonies of the pathogen. Each had three replicates for every concentration which were incubated for 10 days at 25±1°C.

Calculations for the growth of *M. canis* by taking two intersecting lines from the center of the dishes

after every 2 days, it lasted for 10 days.[23] Then, the mean diameter of the fungal growth was taken in centimeters.

Preparation Aqueous Extract of *Moringa oleifera* and *P. anisum* and their Effect on Growth of *M. canis*

Soluble extracts were prepared with some modifications[24,25] powdered seeds of *P. anisum and M. oleifera* leaves were also extracted separately in 100 ml of deionized distilled water in a 250 ml conical flask. The mouth was well covered with an aluminum foil then shaken at 150 rpm/min for 24 h to allow dissolution of active materials. The suspension was then filtered twice with a Whatman NO.1 paper then clarified with centrifugation at ×7000 g for 30 min. Thereafter, the extracts were filtered 2 times. The supernatant was then dried under the oven at 50°C. One gram of every extract was dissolved in the 5 ml of distilled water to get 200 mg/ml, sterilization which was done by sieving the suspension using the filter paper.[26] Each extract was left to freeze before being used. Then, the SDA was supplemented differently with the extracts of *P. anisum*, *M. oleifera* at 10, 20, and 40 mg/ml based on the equation (N1V1=N2V2), that was placed in the Petri plates and was left for 1 h to harden before inoculation. The control treatment included only distilled water for every treatment, and three replicates were maintained. Growth measurement, inoculation, and incubation were done and were described as above in terbinafine treatment.

Determination of Minimum Inhibitory Concentration (MIC) of Plant Extract (*M. oleifera* and *P. anisum*) and Terbinafine against *M. canis*

Ranging from the lowest to the highest concentration, the plant extracts and terbinafine were done to get the MIC for the growth of *M. canis*. Different sequences of 20, 40, 60, 80, and 100 mg/mL, for both *M. oleifera* and *P. anisum* plant extracts. Different concentrations between 0.2 µg/ml, 4µg/ml, and 6 µg/ml and 8 µg/ml in case of the terbinafine, every concentration was planned on the surface of the Petri dishes of the SDA by the sterile swap, and then left 30 min to spread the mixture in agar, after that vaccinated with *M. canis*) apart from the three replicates for each concentration and incubated at 25±1°C for 10 days. There was no fungal growth at the lowest concentration because of the lowest inhibitory concentration.[27]

Effect of Combination between Plant Extracts (*P. anisum* and *M. oleifera*) and Terbinafine on Growth of *M. canis*

The diameter

The following treatments were conducted to assess the impacts of the combination of the *P. anisum*, *M. oleifera* and/or terbinafine (1:1) on the growth of *M. canis extracts:*

1. *M. oleifera* (40 mg/ml)+Terbinafine (8 µg/ml)
2. *P. anisum* (20 mg/ml)+Terbinafine (8 µg/ml)
3. *P. anisum* (20 mg/ml)+*Moringa oleifera* (40 mg/ml)
4. *P. anisum* (20 mg/ml)+*M. oleifera* (40mg/ml)+Terbinafine (8 µg/ml).

To achieve this objective, *M. canis* was treated with the above four combinations from every pair. The SDA was alternated with sterilized extracts for every treatment based on the N1V1=N2V2 equation and poured in the Petri dishes under sterilized lab condition and left to harden for 1 h. Thereafter, the growth measurement, inoculation, and incubation were done as shown above with three replicates for each combination, then the growth of *M. canis* was measured every 2 days.[28]

Genomic DNA Extraction

Genomic DNA was extracted from the blood sample using the Favor Prep™ Genomic DNA Mini Kit according to the manufacturer's protocol. First, 1~5×10 of cultures (fungal cells) were placed in a 1.5 ml microcentrifuge tube. Next, FA Buffer (I ml) was added, and the cells resuspended using a pipette. Centrifuging was done at 7500 rpm (×5000 g) for 2 min to descend the cells, after which all the supernatant was disposed. In addition, a 550 µl of fully buffered buffer was used to resuspend the cell pellets, followed by addition 50 µl of lyticase solution, mixed well by vortexing. Further, there was a 30 min incubation period at 37°C where the cells were centrifuged for ten minutes at 500 rpm (×5000 g). The supernatant was completely discarded followed by the addition of 350 µl TG1 buffer and resuspension of the cell pellets by pipetting. The samples were then placed in a bead tube and thoroughly mixed for 5 min using the Plus-vortexing technique. 20 µl were added to the mixture and thoroughly mixed through vortexing. This was followed by a 15-min incubation period at 60°C where vortexing was done for 30 s at 5-min intervals. To descend the cells, the mixture was centrifuged at 7500 rpm (×5000 g) for 60 s, and the resulting supernatant (200 µl) moved to a 1.5 ml microcentrifuge tube. Here, 200 µl of TG2 buffer was put into the tube and mixing done thorough pipetting. Further mixing was done for 10 s through pulse-vortexing, after adding 200 µl of ethanol (96–100%) to the tube. A TG Mini column is placed in the collection tube to collect the sample mixture together with any precipitate obtained. Centrifuging is done for 1 min, and the TG Mini Column is then transferred to a new collection tube where it is cleaned using 450 µl of W1 Buffer in a 1-min centrifuge. Once the flow-through is removed, the TG Mini Column is returned to the collection tube. Further, the TG Mini Column is washed by centrifuge for 1 min using 750 µl of Wash Buffer and returned to the collection tube after discarding the flow-through. Another 3-min centrifuge

is done to dry the mini column which is then put into an Elution Tube. Here, 50 ~ 100 µl of Elution Buffer or ddH2O is added to the membrane center of the column and left to stand for 3 min. This is followed by a 2-min centrifuge to remove the DNA. A NanoDrop 2000 spectrophotometer is used to determine the amount and quality of the DNA. The extracted genomic DNA is kept at −20°C for future use.

Polymerase Chain Reaction (PCR) Reaction Mixture

The PCR used Maxime PCR PreMix Kit (*i*-Taq) (Intronbio/Korea) to augment DNA fragments. *i*-Taq DNA polymerase, reaction buffer, and dNTP mixture were put in a PCR tube. To this tube, forward and reverse primers, template DNA, and RNase-free water were added and everything mixed well, as shown in Table 1.

Molecular Studies for SUB Genes

Sequencing of PCR

Twenty microliters of ITS PCR product sent to Macrogen/Korea for Sanger sequencing. After trimming of each sequence, the result of the trimmed sequence was blasted in NCBI to check the similarities and differences with the database. Mega 6 Soft was used to check the similarities and differences among ITS products of different *M. canis* samples. The primers were extracted according to the nucleotide sequences of the SUB1-3 genes in the GenBank database for *M. canis* [Table 2]. All PCR amplification was conducted, as shown in Tables 3-5.

PCR

Target DNA was amplified through conventional PCR with the help of specific primer pairs. PCR comprises three main steps through which the PCR product (amplicon) is obtained. These are denaturation, annealing, and elongation. Table 3 provides the PCR thermal cycling conditions. Electrophoresis helped visualize the PCR products (5 µL) to determine their size. This was achieved using 1.5% (w/v) agarose gel, a ×1 TBE buffer, a safe dye for staining purposes. To determine the size, the PCR products were compared with Sizer100 bp DNA ladder (Intronbio/Korea). The following [Tables 3-5] shows the PCR conditions.

Table 1: Contents of the reaction mixture

No.	Contents of the reaction mixture	Volume (µl)
1	Master mix	5
2	Template DNA	4
3	Forward primer (10 pmol/µl)	2
4	Reverse primer (10 pmol/µl)	2
5	Nuclease free water	7
Total volume		20

Table 2: Primers used to detect subtilisin genes (SUB1-3)

Primer name	Sequence 5′-3′
SUB1 F	AAGGTTTGTGACCAGTGCCA
SUB1 R	AGCGGAACCAGAGATACCCT
SUB2 F	CTCGGCCTGCTTCTCCTAC
SUB2 R	TGCGTCTTTCTATTGGGAGCAT
SUB3 F	GCGCTTTCTTTCACAACCGT
SUB3 R	AGAGGAGCTTGTTGGTGGTG

Agarose Gel Electrophoresis

This technique is effectively used to separate DNA fragments. The sizes of the fragments determine the agarose concentrations incorporated into the gel. Usually, the agarose concentration lies between 0.5% and 2%. For the current study, 0.7% gel was utilized to separate genomic DNA (5–10 kb) and another 1.5%–2% utilized to obtain a better resolution for smaller fragments of the PCR product (0.2–1 kb). The first step of gel preparation was adding the required amount of agarose to 100 ml of ×1 TBE buffer, and then melting it in the microwave until it becomes a clear solution. The next step was cooling the agarose to 50–55°C. Further, 100 ml of the melted agarose gel was mixed with 5µl of simply safe dye (10 mg/ml) to achieve the required 0.5 µg/ml concentration. The agarose was then dried on a gel tray sealed at the ends. Afterward, the samples were placed in a different well of gel, with one well containing a marker. This was followed by an accurate connection of electrodes and application of the run according to size and concentration of the gel (agarose gel electrophoresis took 45 min for the genomic DNA and 90 min for the PCR product).

Statistical Analysis

Statistical analysis included ANOVA performed on SPSS software, version 23. To distinguish between the different treatments, the means obtained from the analysis were compared using the 5% least significant difference LSD test.

RESULTS

Effect of *Moringa oleifera* on Growth of *M. canis*

Based on the results, *M. oleifera* extract inhibited the growth of *M. canis* isolate mainly after 6 and 8 days of inoculation [Figure 1a]. After 4 days of inoculation, *M. oleifera* inhibitory effect was evident in the radial growth of *M. canis*, the treated one reached 3.8, 3.3, and 3.1 cm of growth at concentrations of 10, 20, and 40 mg/ml, whereas the untreated one shown 4.3 cm of growth. However, there was a significant inhibition of fungal growth after 6 and 8 days of inoculation for all the concentrations. For 10 mg/ml, fungal growth was 5.1 and 6.5 cm; for 20 mg/ml, the fungal growth was 4.5 and 5.4 cm, and finally for a 40 mg/ml concentration, the fungal growth was 3.8 and 4.3 cm. For all three, the growth was lower than that in the control treatment which exhibited 5.9 and 7.8 cm. The interaction among extracts, concentration

Table 3: Polymerase chain reaction conditioned for amplification of SUB1 gene

Step	Temperature (°C)	Time (second)	No. of cycles
1	95	2	1
2	95	30	29
3	58.3	30	
4	72	100.0	
5	72	5	1
6	4	Forever	-

Table 4: Polymerase chain reaction conditioned for amplification of SUB2 gene

Step	Temperature (°C)	Time (second)	No. of cycles
1	95	2	1
2	95	30	29
3	59.6	30	
4	72	120.0	
5	72	5	1
6	4	Forever	-

Table 5: Polymerase chain reaction conditioned for amplification of SUB3 gene

Step	Temperature (°C)	Time (second)	No. of cycles
1	95	2	1
2	95	30	29
3	58.3	30	
4	72	140.0	
5	72	5	1
6	4	Forever	-

Table 6: Minimum inhibitory concentration

Fungal species	*Moringa oleifera*	*Pimpinella anisum*	Terbinafine
Microsporum canis	80 mg/ml	60 mg/ml	6 µg/ml

and periods was crucial for the assessment of fungal growth.

Effect of *P. anisum* Extracts on Growth of *M. canis*

Figure 1b shows how different concentrations of *anisum* extract (10, 20, and 40 mg/ml) affect the growth of *M. canis*. After 6 and 8 days of inoculation, the growth inhabitation power of *P. anisum* differs across the different concentrations: 4 and 5 cm for 10 mg/ml, 3.2 and 3.9 cm for 20 mg/ml, and 2.8 cm and 3.1 cm, respectively, for 4mg/ml. Increasing the concentration of the extract increased the inhibition effect on fungal growth. The interaction between extracts, concentration, and periods also plays a significant role in this process.

Effect of Terbinafine on Growth of *M. canis*

The results of this assessment are shown in Figure 1c. It is evident that the growth of *M. canis* differs across different concentrations (2 µg/ml, 4 µg/ml, and 6 µg/ml). Throughout the experiment, terbinafine was seen to inhibit the growth of *M. canis* at concentrations of 4 and 6 µg/ml. The growth was as follows: 2.2 and 1.8 cm after 2 days, 3.6 and 2.7 cm after 4 days, 3.9 and 3.2 after 6 days, finally 4.1 and 3.5 cm after 8 days, respectively. On the other hand, the growth exhibited in the control experiment 2, 4, 6, and 8 days post-inoculation was 3.3, 4.6, 5.2, and 6.5 cm, respectively. For a 0.2 µg/ml concentration, the effect of terbinafine was only felt 6 and 8 days after inoculation.

Effect of Combination between Plant Extract of *M. oleifera, P. anisum,* and Terbinafine on Growth of *M. canis*

The experiment results, as shown in Figure 2, indicate that different combinations of extracts had a considerable effect on the growth of *M. canis*. A combination of terbinafine, *M. oleifera* and *P. anisum* had the greatest effect in inhibiting the development of pathogenic fungi.

In comparison to the control experiment, all combinations (P+T, M+T, P+M, M+P+T, and T) were found to have a considerable impact on the growth of *M. canis* throughout the experiment. In addition, all the treatments comprising terbinafine and a plant extract has a higher inhibitory effect compared to P+M treatments and the control experiment. This implies that the terbinafine was more effective in inhibiting fungal growth when used in a combination treatment.

MIC of Aqueous Plant Extracts and Antibiotic

After a 7 days incubation period, the MIC of aqueous plant extract (*M. oleifera*+*P. anisum*) and antibiotic against *M. canis* was determined, as shown in Table 6. The results summarize the MIC of terbinafine, *M. oleifera*, and *P. anisum* as follows: Terbinafine has a MIC of 6 µg/ml, whereas *M. oleifera* and *P. anisum* have a MIC of 80 mg/ml and 60 mg/ml, respectively. This implies that *P. anisum* has a higher effect compared to *M. oleifera*.

Identification of SUB1-3 Genes

Different fragments of SUB3 (1324bp), SUB2 (1120bp), and UB1 (609bp) positively identified PCR products. They were found to contain subtilisin virulence genes in *M. canis* DNA, as shown in Figures 3-5, indicating some risk of developing infections related to the presence of SUBs. The SUB1 [Figure 3] SUB2 [Figure 4] and SUB3 genes [Figure 5] were found to be transcribed in fungal tissues of *M. canis*, strongly suggesting that the corresponding encoded proteases are produced in these tissues. SUB1 [Figure 4] was normally produced in control treatment (C), and in culture supplemented with a combination of M+P, P+T, T alone, and M extract alone. In contrast, the bands that appeared in M+T and P treatments were quite faint but completely disappeared in M+P+T.

The bands in SUB2 [Figure 4] were more obvious than that in SUB1, in particular, C treatment, M+P,

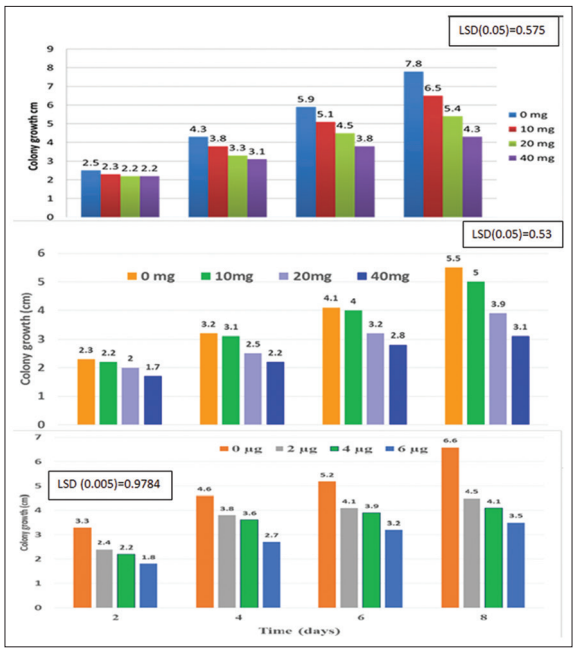

Figure 1: (a-c) Effect of (a) *Moringa oleifera* leaf extract (0.0, 10, 20, and 40 mg/ml), (b): *Pimpinella anisum* seeds extracts and (c) Terbinafine (0. 2 µg/ml, 4µg/ml, and 6 µg/ml) on the growth of *M. canis* on Sabouraud dextrose agar at 25±1°C during 8 days of incubation

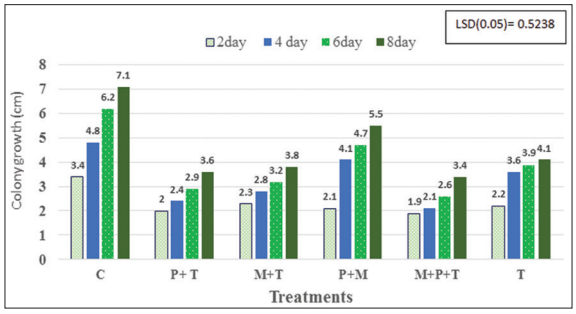

Figure 2: Effect of combination treatments on growth of *M. canis*: 0 (control), P+T (*Pimpinella anisum*+terbinafine), M+T (*Moringa oleifera*+terbinafine), P+M (*P. anisum*+*M. oleifera*), M+P+T (*M. oleifera*+*P. anisum*+terbinafine and T terbinafine alone) on Sabouraud dextrose agar at 25±1°C during 8 days of incubation

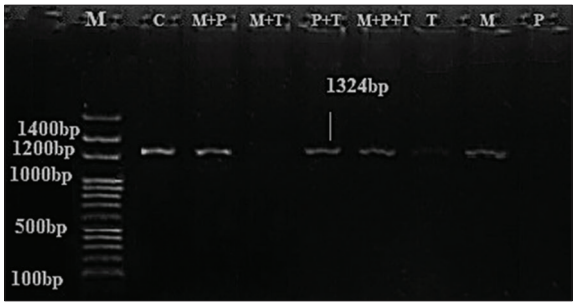

Figure 3: 1.5% agarose gel electrophoresis at 72 volt for 80 min of polymerase chain reaction to SUB1 amplicon (906 bp) M (DNA marker size [100bp]). 1-C, 2-M+P, 3-M+T, 4-P+T, 5-M+P+T, 6-T, 7-M, and 8-P

P+T, M+P+T, T, and P compared with that in, M which appeared less clear than other treatments but the bands completely disappeared in M+T.

The result of this experiment revealed that the bands of SUB3 [Figure 5] were present in C treatment, M+P, P+T, M+P+T, and M alone treatments were appeared clearly while the bands of SUB3 in both M+T and P treatments were disappeared completely.

DISCUSSION

Extracts from the *M. oleifera* family are usually used in ethnomedicine to manage a variety of ailments.[29,30] In addition, *P. anisum*, which is a member of the Umbelliferae family, is used in traditional medicine as a disinfectant, galactagogue carminative, and aromatic.[31] This work focused on the pharmacological properties of these plant extracts, particularly their ability to inhibit the growth of *M. canis* and reduce its virulence. Based on the results obtained, the two plant extracts (*P. anisum* and *M. oleifera*) showed high antifungal potencies against *M. canis*. While the two plant extracts had the power to fight the pathogen, a MIC analysis indicated that higher concentrations of *M. oleifera* aqueous extract are required to completely prevent the growth of *M. canis* (80 mg/ml) compared to *P. anisum* (60 mg.ml). These results were consistent with the findings of Chuang *et al*. 2007[32] that the extracts

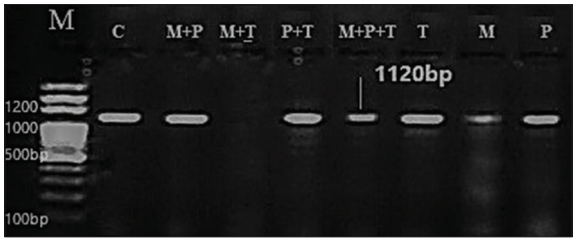

Figure 4: 1.5% agarose gel electrophoresis at 72 volt for 80 min of polymerase chain reaction to SUB2 amplicon (1120 bp), M (DNA marker size [100bp]). 1-C, 2-M+P, 3-M+T, 4-P+T, 5-M+P+T, 6-T, 7- M, and 8-P

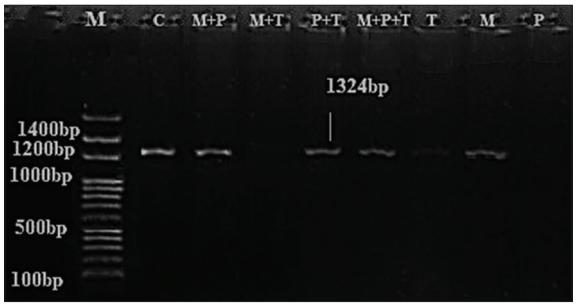

Figure 5: 1.5% Agarose gel electrophoresis at 72 volt for 80 min of polymerase chain reaction to SUB3 amplicon (1324 bp) M (DNA marker size [100bp]). 1-C, 2-M+P, 3-M+T, 4-P+T, 5-M+P+T, 6-T, 7-M, and 8-P

of *M. oleifera* seeds and leaves had antifungal activities *in vitro* against dermatophytes.[33] Both plants have antifungal activity against dermatophyte fungi (fungus), possibly related to the presence of small peptides which could have a crucial role in the plant's antimicrobial defense system.[34] The proteins/peptides are believed to participate in the defense mechanism against phytopathogenic fungi by preventing the growth of micro-organisms through different molecular modes. These include binding to chitin or making the fungal membranes or cell wall more permeable.[32]

The presence of chemical elements in *M. oleifera* is clear proof that the plant, if properly screened, could be used to produce drugs for pharmaceutical use. This is better supported by the fact that other members of the family of this plant have been used in ethnomedicine to manage a variety of ailments.[29,30]

The use of medicinal plants and artificial antibiotics is a multi-targeted line of single or multi-extracts combinations affecting not only on one target but also numerous, collaborating in an agonistic synergistic approach. The present results revealed a synergistic effect in P+T, M+T, and M+P+T treatments for increased inhibition effect to pathogen. It was observed by other researchers that *Rhamnus staddo*, *Aloe* sp., *Momordica foetida*, and the combined proportions against *C. albicans* had higher antifungal activity than Griseofulvin and Ketoconazole.[35] Our results showed that the mixture of *M. oleifera*, *P. anisum,* and terbinafine could be used effectively to control the growth of *M. canis*.

This research showed that combining more than one medicinal plant enhanced their efficacy against disease.[36] Plant extracts and drugs can be used to achieve multiple targets in an agonistic synergistic way. However, this method is not limited to extract combinations, since combinations of single extracts with antibiotics could be more efficient.[37,38]

Dermatophytic fungi are controlled by synthetic antibiotics, but the subsequent use of antibiotics is increasingly restricted due to its negative effects on human health.[39] There has been a constant increase in alternative search for efficient compounds for the treatment of fungal diseases, aiming at partial or total replacement of antimicrobial chemicals.

Thus, plant products that prevent fungal growth without harming the host represent a potential therapeutic agent. As earlier mentioned, two types of plants belonging to different families were used against *M. canis* in this research. These medicinal plants were selected due to their suggested antimicrobial ability.[40] The results showed statistically significant differences between the antimicrobial activities of the two plants' extracts, at a 0.05 level of probability of most significance were plant type and the concentration of fungi and extracts.

Dermatophytes are pathogenic fungi that infect healthy people, and the production of subtilisin proteases *in vivo* infection is known as an indicator of their importance in virulence of dermatophytes.[7,41] Aserine protease (SUBs) inhibitor plays a major role in inhibiting the adherence of *M. canis* arthroconidia to stratum corneum.[10] The presence of subtilisin genes (SUBs) coding for serine proteases in *M. canis* DNA contributes to the adherence of fungi to keratinized tissues. This study showed that high percentage of SUB1, SUB2, and SUB3 virulence genes for *M. canis* isolate is crucial for preventing initial contact.[10] Positive results of PCR products were observed in different fragments of SUB1 (906bp), SUB2 (1120bp), and SUB3 (1324bp), indicating the presence of subtilisin virulence genes.

CONCLUSION

The results obtained in this investigation suggest that both *M. oleifera* and *P. anisum* could be a source of metabolites with terbinafine to be used against *M. canis*. Our findings demonstrated that extracts from *M. oleifera* (leaves) and *P. anisum* (seeds) have strong fungicidal activity against *M. canis*. This good antifungal performance suggests that plant extracts may be useful as an alternative treatment for this fungus.

This study is, to the best of our knowledge, the first to reveal the expression of the keratinolytic protease Sub3 produced by *M. canis* under the combined effect of medicinal plants and Terbinafine. These data support previous hypotheses concerning the major role of SUBS in the earliest events of the infectious process, i.e., adherence to corneocytes. We believe that our findings provide a positive catalyst to prompt further experiments devoted to developing an intimate understanding of cellular and molecular mechanisms involving SUBS in dermatophyte pathogenesis. The study is a novel which highlights the combinatorial use of plant extracts of well-known medicinal plant extracts with a known antifungal agent, terbinafine, for the treatment of acute infections associated with *M. canis*. The study could be considerable, useful in improving new, novel and more efficient medication regimens for *M. canis* based on infections particularly for Babylon where the frequency of such infections is more distinguished.[42]

ACKNOWLEDGMENT

Authors sincerely thank assistant Prof. Dr. Husain Al-Dehmushi, Department of Biology University

of Babylon, Iraq, for technical assistance and Prof. Dr. Ali Abdul- Hussain Al-Janabi, College of Medicine, Karbala University, Iraq, for providing us with *M. canis* isolate.

FUNDING

This work was financially supported by the Department of Biology, College of Science, University of Babylon, Iraq.

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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.

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Source of support: Nil; Conflict of interest: None Declared