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Research Article

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The Antifungal Activities of Rosemary against Trichophyton Tonsurans and Microsporum Canis

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ABSTRACT

Dermatophytosis (ringworm) is a contagious fungal infection of skin caused by dermatophytes and is threat to human health. In recent years, the infections caused by these fungi have been developing. Trichophyton Tonsurans is a anthropophilic dermatophyte and which causes Tinea Endothrix (hair), Tinea Corporis (body) and Tinea Unguium (nails). Microsporm Canis is a zoophilic (animal-friendly) dermatophyte which is considered as the main causes of dermatophyte infections such as Tinea Capitis, Tinea Corporis (Body) and Tinea Barbae (Bread). Rosemary is a plant whose antifungal effects have been proven by several studies. The current study intended to investigate the antifungal effects of rosemary on Trichophyton Tonsurans and Microsporum Canis. To this end, the antifungal activities of aqueous and hydro-alcoholic extracts of rosemary leaves against the intended fungi were, first, examined based on agar dilution method. Next, the most effective extract was fractioned based on decantation method. The antifungal effects of the observed fractions on the intended fungi was assessed. Afterwards, the most effective fraction was fractionated in Chloroform and pure ethyl stat based on solid phase extraction (SPE) using pure chloroform solvents and ethyl stat mixtures %1, %3 and %5, and their antifungal activities fungi were examined against the intended fungi. Finally, the most effective SPE fraction was measured in terms of Keratinase inhibitory activity. Furthermore, Terbinafine was used as a positive control. According to the results, both extracts showed antifungal effects. The hydro-alcoholic extract was more effective and M. Canis was more sensitive. It was indicated that N-hexane fraction of decantation, with a minimum inhibitory concentration (MIC) and a minimum fungicidal concentration (MFC) less than 1.25 mg/ml, had the maximum antifungal effects on the intended fungi. Amongst the SPE obtained fractions, Chloroform %100 with a MIC of 1.25mg/ml and a MIC of 0.625 mg/ml had anti-dermatophyte effects respectively on Trichophyton Tonsurans and Microsporum Canis. Consequently, it was found that Keratinase inhibition is not the most effective fraction mechanism. To conclude, hydro-alcoholic extract, N-hexane fraction of decantation, and Chloroform %100 fraction of SPE had anti-dermatophyte index; however, their potency was not comparable with Terbinafine.

Keywords: *Rosemary Leaves; Anti-dermatophyte; Trichophyton Tonsurans; Microsporm Canis; Mechanism.*

INTRODUCTION

Dermatophytes are a set of fungi which cause superficial infections, called dermatophytosis, by invading the keratinase tissues like hair, skin and nails in human and other vertebrates. Dermatophytes consists of three genera including Microsporum, Trichophyton and Epidermophyton. In recent decades, infections caused by these fungi

have been developing which is a considerably important in patients with immune deficiency and severe diseases. Dermatophytosis is very widespread around the world and M. Canis is considered as one of the main factors of dermatophyte infections all over the world. That dermatophyte i.e. M. Canis, mainly causes Tinea capitis in not only human beings but also animals such as cat and dog is of great importance because the infection can spread from animals to human. T. Tonsurans is a anthropophilic fungus that is recently prevalent in Iran with an increased cases of disease. The treatment of dermatophyte is a combination of tropical and systematic treatments. In Iran, the preferred treatments of dermatophytosis for human are Terbinafine and Griseofulvin. However, recent studies have attempted to find natural alternatives with less complications to the aforementioned treatments due to their multiple side effects, high costs, low efficiency and drug resistance properties.

Dermatophytosis or ringworm (Tinea) is a contagious superficial fungal infection caused by dermatophytes which raises concerns about the health of a large number of mammals like humans (1-7). They are a group of fungi invading the Keratinase tissues of human and other vertebrates, including the skin, and causes superficial infections known as *Dermatophytosis*. So far, more than 40 species of dermatophyte have been identified which are mainly categorized into three genera namely Microsporum, Trichophyton and Epidermophyton. In recent years, the infections caused by these fungi have been developing to the extent that they cause advanced severe atypical lesions in patients with immune deficiency and severe diseases. Dermatophytosis infection is widespread throughout the worlds so that no geographical area can be found without ringworm. Today, zoophilic dermatophytes like Microsporum Canis are considered as the main causes of dermatophyte infections (Tinea Capitis, Tinea Corporis and Tinea Barbae) in human in different countries. Besides, Trichophyton Tonsurans is a anthropophilic dermatophyte that causes Tinea Endothrix (hair), Tinea Corporis (body) and Tinea Unguium (nails) (2, 3, 8-12). Nowadays, herbal extracts are being used to treat a variety of infection diseases caused by bacteria, fungi, protozoa and viruses; thus, considerations have been given to the application of natural herbal compounds against dermatophytosis-generating organisms (4, 5, 13-19). Rosemary is a perennial, aromatic and beautiful plant with fragrant evergreen leaves; the leaves and flowering branches of rosemary are used for medicinal applications. It is an herb native to Mediterranean Sea which is currently being cultivated all over the world including Iran. Rosemary contains phenolic compounds (Carnosol, Rosmarinic acid and Caffeic acid), Flavonoids (Diosmin and Luteolin) and Menotropins (Camphor, Cineol and Borneol) (6, 20-25).

Rosemary is active against the fungi including Candida Albicans, Aspergillus Flavus, Aspergillus Ochraceus, Microsporum Canis, Trichophyton Mentagrophytes, Trichophyton Rubrum, Epidermophyton Floccosum and Microsporum Gypseum (7, 8, 26-29). Nevertheless, the antifungal effects of this herb on other dermatophytes have not yet been investigated. with respect to the importance of dermatophytes infections and the need for new anti-dermatophyte medications, the current study intended to investigate the antifungal effects of Rosemary essence-free extracts on two common dermatophytes namely Trichophyton Tonsurans and Microsporum Canis in order to invent a natural anti-dermatophyte alternative.

1. Literature Review

The prevalence of dermatophytosis and its different causes, including Microsporum Canis, has been considered throughout the world due to the significance of this infection. Mugnaini et al. found that M. Canis is the most prevalent cause of dermatophytosis in head and body (Tinea Capitis and Corposris) in New Zealand (8, 30-33). Omidinia et al. (9, 34-39) studied the prevalence of dermatophytosis for 9 months (from Oct. 1991 to Jun. 1992) in Hamedan; they reported that the prevalence of M. Canis has got the third rank amongst other causes (%5.5).

Dermatophytosis has been always associated with pain, disability, loss of time and high treatment costs for human. According to the U.S. reports, millions of children and adults are suffering from one or more dermatophyte infections annually so that the sales of Griseofulvin have been over one hundred million dollars in only the United States within the last 16 years. This important issue is indicative of the prevalence of dermatophyte infections not only in the U.S. but also in other countries. Furthermore, it is an important health problem amongst livestock which leads to considerable economic losses (2, 40-43).

Materials and Methods

1.1. Plant Collection and Identification

The Rosemary plant was collected from an area located in between Bisoton and Songhor cities in Iran in May. Then, it was identified under the binomial name *Rosmarinus officinalis. L.*, using Flora of Iran, in the school of Pharmacy of the Kermanshah University of Medical Sciences.

1.2. Preparation of Extracts from Rosemary Leaves

After being collected, the plant leaves were dried in the shadow and pulverized by the mill. The dried powder of leaves was used to prepare extracts.

1.2.1. Preparation of Aqueous Extract by Brewing

In order to prepare an aqueous extract, the plant powder was mixed with boiling water in proportion of 1:10 (gram powder per ml solvent) and heated at 80-90˚C for 1 h according to Bain-Marie method. The obtained extract was dried at 40˚C by rotary after being filtered (by means of filter papers).

1.2.2. Preparation of Hydro-Alcoholic Extract

In order to prepare a hydro-alcoholic extract, the plant powder was mixed with methanol %80 in proportion of 1:10 (gram powder per ml solvent) and extracted by Magnet Stirrer at room temperature for 12-24 hrs. The obtained extract was dried at 40˚C by rotary after being filtered (by means of filter papers).

1.3. Preparation of Fungi

The Microdporum Canis (PTCC 5069) was provided by Persian Type Culture Collection (PTCC) affiliated with IROST. The other fungus i.e. Trichophyton Tonsurans was isolated from the patients referring to Mythology Laboratory of Mahdieh Clinic affiliated with Kermanshah University of Medical Sciences. Dextrose Agar Culture Medium containing Sabouraud Chloramphenicol and Cycloheximide (SCC) was prepared according to the recipe. The molten agar was placed in plates and, then, refrigerated, after being stored under in-vitro conditions about 30 minutes, to transfer the fungi on them. Each of the intended fungi was cultivated on separate plates of Dextrose Agar Culture Medium containing Sabouraud Chloramphenicol and Cycloheximide (SCC) for 14 days.

1.4. Determination of MIC and MFC based on Agar Dilution Method

The plates were inspected for four weeks in terms of the growth of fungi to record the results of growth or non-growth. The minimum concentration of the extract which inhibited fungal growth till the end of the second week, was considered as the minimum inhibitory concentration (MIC) of the intended fungus (10, 11). The MIC is reported as the amount of extract per unit volume of Dextrose Agar Culture medium containing SCC. If the fungus grew within the two weeks after recording the MIC of growth, the extract could inhibit the fungal growth but not destroy it. On the other hand, if the fungus did not grow until the end of the fourth week since the cultivation, the fungs would destroy; then, the minimum concentration of the extract which had this property would be considered as the Minimum Fungicidal Concentration (MFC) (10, 13).

1.5. Preparation of Fractions from the Most Effective Extracts based on Liquid-Liquid Extraction (Decantation)

The most effective extract, identified in the preceding stage, was sufficiently prepared as described in the section of ‘’ Preparation of Extracts from Rosemary Leaves.’’ Next, the extract was mixed with distilled water in ratio of 1:5, and the mixture was decanted with N-Hexane, Chloroform, Ethyl Stat and N-Butanol solvents. The decantation process of each solvent was continued until the extracting solvent was decolored. Finally, all the aqueous and organic phases extracted from decantation were dried at less than 50˚C by rotary. The dried fractions were stored at -20˚C in capped containers until the time of inspecting their fungal effects.

1.6. Investigation of Antifungal Effects of Decanted Fractions on Sensitive Fungus

The procedure of this stage was similar to the investigation of antifungal effects of extracts whereas they were different in that the former examined the antifungal effects of fractions, prepared by decantation, on the sensitive fungus, identified earlier, instead of the antifungal effects of extracts. At the end of this stage, the most effective fraction on the most sensitive fungus was determined and used for the next stage. The procedure of this stage was similar to the ''Agar Dilution of Extracts''; however, the difference was that the former examined the dilution of antifungal effects of fractions, prepared by decantation, on fungi sensitive to extracts instead of the dilution of antifungal effects of extracts. To this end, some solutions with different concentrations were obtained from fractions. That is, solutions at %2, %1, %0.5, %0.25 and %0.125 concentrations were obtained in culture medium from all fractions including N-Hexane, Chloroform, Ethyl Stat, N-Butanol and aqueous fraction. Moreover, the plates containing extract blanks, positive control and Terbinafine blank were prepared. The rest of the procedure followed the stage related to the antifungal effects of extracts. At the end of this stage, the most effective fraction on the fungi sensitive to extracts was determined and used for the next stage.

1.7. Antifungal Mechanism of the Most Effective SPE Fraction

1.7.1. Keratinase Examination

The cultivation method for enzyme examination was submerged cultivation method. The mineral liquid medium containing all nutrients of solid medium except for Agar including MgSO4.7H2O (0.5 g), KH2PO4 (0.1 g), FeSO4. 7H2O (0.01 g), ZnSO4.7H2O (0.005g), NaH2PO4 (3.86 g), Na2HPO4 (3.97 g), Cycloheximide (0.5 g) and Chloramphenicol (0.05 g) were prepared in a liter of distilled water and autoclaved (sterilized) for 15 minutes. An amount of 10 ml of culture medium was transferred to test tubes to which different concentrations of intended fraction (%0.5, %0.25, %0.125, %0.0625 and %0.03125) were added separately. Then, a minimum fungal suspension of 10^6 cells/mL was added to the culture medium. The inoculated tubes were incubated at 28˚C for 15 minutes. Phosphate-Buffered Saline (PBS) was considered as a control solution. The experimentation was replicated for three times (14) and the kinetic activity of refined culture keratinase was measures based on spectrophotometric method (15). That is, 2 grams of human hair in sizes of 1 to 2 millimeters were cut by a scissor, washed in 70 ml of Chloroform %50 in methanol, treated with soap particles at 42˚C for 12 hours in a shaking incubator, washed with water for several times and dried after being filtered. To prepare phosphate buffer (pH 7.8, 28mM), an amount of 0.945 grams of KH2PO4 was dissolved in 250 ml of water using a magnet stirrer and the pH was adjusted to 7.8 in pH-meter using concentrated soda solution. The enzyme solution was prepared by mixing 3 ml of buffer to 3 ml of fractions. Afterwards, 50 mg of dried hair was added to 6 ml of the enzyme solution, diluted in phosphate buffer (pH 7.8, 28mM) and the reaction mixture was incubated at 37˚C in a shaking incubator or warm water bath. After incubation, the remaining hair were removed by filtering and the absorbance was measured at the wavelength of 280 nm by means of UV-Visible Spectrophotometer. The boiled enzyme solution was considered as a control solution (for denaturation and deactivation). The increase of a 0.1 of absorbance is equal to one unit of enzyme activity; thus, the special enzyme activity was calculated as enzyme unit per mg protein. The mg of sample protein was measured based on Bradford assay (16). The enzyme activity was assessed at the presence of fractions. The Bradford assay is one of most sensitive and accurate protein measurement assays in the range of microgram sensitivity. This assay based on an absorbance shift of the dye Coomassie Brilliant Blue G-250 from purple to blue under acidic conditions and at the presence of protein. This dye has a negative charge and ionize with Amino proteins, which have positive charges. The binding form of protein and dye has the most absorbance at the wavelength of 595 nm. Therefore, the more protein the sample has, the more intense the blue dye and the more the absorbance of the sample will be. It is worth noting that Bovine Serum Albumin (BSA) was use to plot a standard curve (17). Also, Sigma Bradford reagent was used in this project. The Bradford reagent %20 was prepared using distilled water and BSA solution at 1 mg/ml concentration in ration of 1:4. The method presented in Table 1 was used to measure the protein.

The concentration of protein was determined at the wavelength of 595 nm. The calibration was done with Blank solution (Albumin-free). It took almost 5 minutes to have the purple dye of Bradford solution changed to blue after adding BSA to the Bradford solution. In addition, the absorbance of each standard sample along with the unknown (unidentified) sample was read. The standard curve was drawn by plotting the values of optical absorption versus

their identified concentrations. In finale, the concentration of protein was determined in the intended samples after identifying the final linear equation.

Results and Discussion

1. Data Analysis and Results

Data analysis included the estimation of means, SEM and Univariate test to compare the differences between different extracts under in-vitro conditions. ANOVA was done based on a randomized complete block design and the mean comparison was made based on Tukey method at %5 probability level for the data of under in-vitro conditions by $SPSS_{18}$.

1.1. Results of Anti-Dermatophyte Effects of Extracts

Table 1 presents the results of antifungal effects of extracts on the intended dermatophytes. The final results of MIC (Table 1) and MFC of each extract and Terbinafine against each fungus is shown in Table 2.

Trichophyton Tonsurans

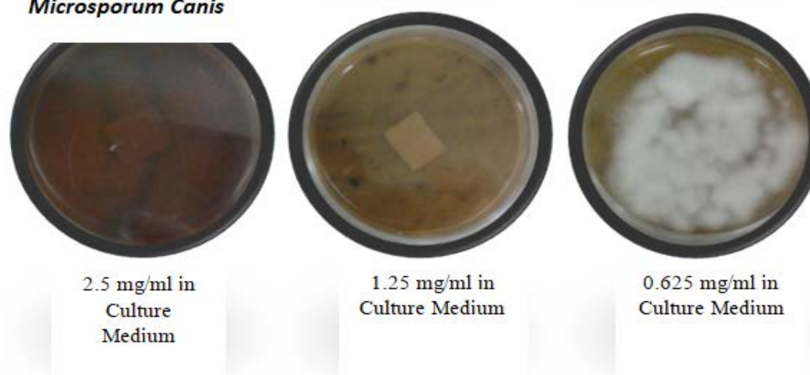

Figure 1: The MIC of hydro-alcoholic extract on M. Canis and T. Tonsurans after two weeks since cultivation

Table 1: The results of antifungal effects of extracts based on Agar dilution method.

Extracts \ Fungi		T. Tonsurans		M. Canis	
	Concentration(mg/ml)	End of 2nd Week	End of 4th Week	End of 2nd Week	End of 4th Week
Aqueous Extract	10	-	-	-	-
	5	+	+	-	-
	2.5	+	+	+	+
	1.25	+	+	+	+
	0.625	+	+	+	+
Hydro-Alcoholic	10	-	-	-	-
	5	-	-	-	-

	2.5	-	-	-	-
	1.25	-	-	-	-
	0.625	+	+	+	+
Blank		+	+	+	+

(+) refers to the growth & (-) refers to the non-growth of fungus at different concentrations (The mean of three plates)

Table 2: Determination of MIC and MFC of extracts against the intended fungi

Fungi / Extracts	T. Tonsurans		M. Canis	
	MIC	MFC	MIC	MFC
Aqueous	10	10	5	5
Hydro-Alcoholic	1.25	1.25	1.25	1.25
Blank	0.05	0.05	0.05	0.05

Note: The concentration of extracts was obtained as (mg/ml) i.e. extract mg per culture medium ml. The concentration of Terbinafine was obtained as (μg/ml) i.e. Terbinafine μg per culture medium ml.

1.2. Preparation of Fractions from Hydro-alcoholic Extract based on Liquid-Liquid Method (Decantation)

Table 3 below presents certain fractions with specific yields was obtained by the decantation of hydro-alcoholic extract with different organic solvents from non-polar to polar solvents.

Table 3: Fraction yields obtained by decanting hydro-alcoholic extract

Fraction	Yield (%)
N-Hexane	18.97
Chloroform	12.34
Ethyl Stat	9.95
N-Butanol	26.29
Aqueous	31.07

Note: The decantation was obtained as fraction gram per hydro-alcoholic extract 100 grams

1.2.1. Results of Antifungal Effects of Fractions obtained from Hydro-alcoholic Decantation

The final results for the MIC and MFC of each fraction and Terbinafine against the intended fungi is presented in Table 4.

Table 4: The MIC and MFC of Fractions obtained from decantation of hydro-alcoholic extract against the intended fungi

Fungus / Fraction	T. Tonsurans		M. Canis	
	MIC	**MFC**	**MIC**	**MFC**
N-Hexane	<1.25	<1.25	<1.25	<1.25
Chloroform	20>	20>	10	10
Ethyl Stat	10	10	5	5
N-Butanol	20>	20>	5	5
Aqueous	20	20	20	20
Terbinafine	0.05	0.05	0.05	0.05

Note: The concentration of extracts was obtained as (mg/ml) i.e. extract mg per culture medium ml. The concentration of Terbinafine was obtained as (μg/ml) i.e. Terbinafine μg per culture medium ml.

1.3. Preparation of Fractions from N-hexane based on SPE on Silica Gel Absorbent

By fractionating the N-hexane based SPE on Silica Gel absorbent, their corresponding dried fractions were obtained. Table 5 below presents the fraction yields.

Table 5: The yields of fractionating N-hexane on SPE on silica gel based

Fraction	Yield (%)
Chloroform %100	18
Ethyl Stat %1 in Chloroform	24.5
Ethyl Stat %3 in Chloroform	23.5
Ethyl Stat %5 in Chloroform	16
Ethyl Stat %100	13.5

Note: The fraction was obtained as Fraction gram per initial fraction 100 grams.

1.3.1. Results of Antifungal Effects of Fractions Extracted from N-Hexane

The final results of MIC and MFC for each fraction and Terbinafine against the intended fungi is shown in Table 6.

Table 6: The MIC and MFC of Fractions obtained from SPE N-hexane resulted from decantation of hydro-alcoholic extract

Fungus	T. Tonsurans		M. Canis	
Fraction	MIC	MFC	MIC	MFC
Chloroform %100	1.25	1.25	0.625	0.625
Ethyl Stat %1 in Chloroform	5	5	2.5	2.5
Ethyl Stat %3 in Chloroform	>5	>5	5	5
Ethyl Stat %5 in Chloroform	>5	>5	5	5
Ethyl Stat %100	>5	>5	>5	>5
Terbinafine	0.05	0.05	0.05	0.05

Note: The concentration of extracts was obtained as (mg/ml) i.e. extract mg per culture medium ml. The concentration of Terbinafine was obtained as (µg/ml) i.e. Terbinafine µg per culture medium ml.

1.4. Mechanism of Antifungal effects of Chloroform Fraction based on SPE

1.4.1. Results of Keratinase Activity Assay

The results of keratinase activity assay for Mi. Canis and T. Tonsurans fungi is displayed in Figure 2.

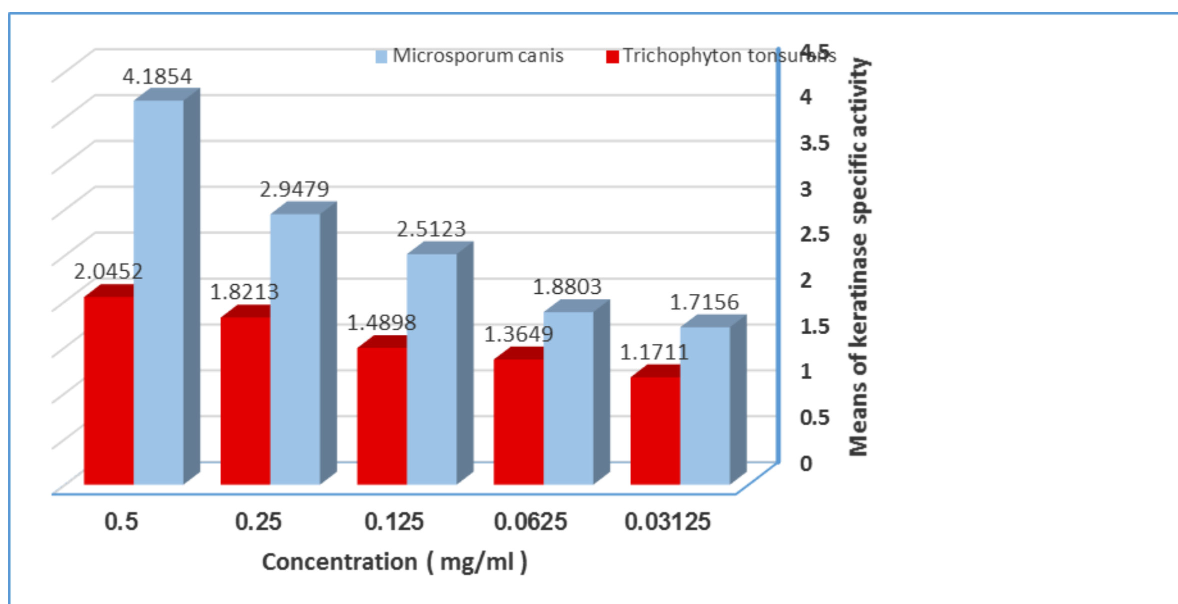

Table 2: The comparison results of Keratinase activity assay for M. Canis and T. Tonsurans at the presence of Chloroform fraction resulted from SPE

Discussion and Conclusion

1.8. Yield of Extracts

With respect to the appropriateness of the extract's yield, it was indicated that the yield of aqueous extract was higher than hydro-alcoholic yield.

1.9. Results of Anti-Dermatophyte Effects of Extracts

According to the results, both extracts have antifungal property which was proved by the growth of fungus at lower concentrations of the intended extracts in an essence-free culture medium. Furthermore, the hydro-alcoholic extract has more effect on the tested fungi (Trichophyton Tonsurans and Microsporum Canis) than aqueous extract since the MIC of hydro-alcoholic extract is lower than the MIC of Aqueous extract against the fungi. It was concluded, then, that rosemary inhibits the growth of fungi due its concentration-dependent antifungal activity. Comparing the results between the MIC and MFC of rosemary extracts and Terbinafine drug, there was observed a significant difference in their antifungal effects. As a consequence, the aqueous and hydro-alcoholic extracts of rosemary do not have the ability to compete with or be substituted for Terbinafine under in-vitro conditions. Regarding the proven antifungal and antibacterial effects of rosemary, it is expected that, by fractionating the most effective extract (hydro-alcoholic), the obtained fractions have more antifungal effects than the aforementioned extract. The results of the antifungal effects of rosemary's hydro-alcoholic extract on dermatophytes in the present study are in line with another similar study. According to study on microbial activity of rosemary's essence and methanol extract based on Agar dilution method, it was found that the bacteria including Staphylococcus Aureus, Proteus Vulgaris, Pseudomonas Aeruginosa, Klebsiella Pneumoniae, Enterococcus Faecalis, Escherichia Coli, Staphylococcus Epidermidis, Bacillus Subtilis and Candida Albicans were sensitive to rosemary essence while they showed a relatively sensitivity to Methanol extract (10).

1.10. Preparation of Fractions from Hydro-alcoholic Extract based on Liquid-Liquid Method (Decantation)

As shown in Table 3, the highest yield of extracted fraction belonged to the more polar solvent in liquid-liquid extraction; therefore, the fraction yields from the highest to the lowest values would be in the order of Aqueous Fraction → N-Butanol → N-Hexane → Chloroform → Ethyl Stat. The yield of each solvent is dependent on the type of plant and the type of extract (18, 19, 44-46).

1.11. Results of Antifungal Effects of Fractions Obtained from Hydro-alcoholic Decantation

Although different research has been conducted on the anti-dermatophyte effects of rosemary's extracts, the present study not only examined the antifungal effects of this plant cultivar on the dermatophytes which had not already been experimented but also set its objectives beyond investigating the anti-dermatophyte effects of extract to segment the hydro-alcoholic extract based on polarity for observing its anti-dermatophyte effects.

According to the results of Table 4, N-hexane fraction had the lowest MIC against the intended fungi to the extent that the MIC and MFC of this fraction was lower than the intended MIC; thus, it did not result in any growth within the concentration limit. It was also indicated that with the polarization of fractions, their antifungal potency decreases (the values of MIC increases). With regard to point that the MIC of N-hexane, in this stage, was lower than the MIC of hydro-alcoholic extracts, in the first stage, the potency of antifungal effect is mainly due to the concentration of active components in fraction. Since all the fractions had antifungal effects on M. Canis and T. Tonsurans, it can be concluded that the active components had already been added to the fractions. That the non-polar fraction (N-hexane) had more effects than polar fraction contributes to the fact that the active components had been mainly added to non-polar phases; that is the active components may have a non-polar nature. Additionally, N-hexane fraction resulted from Liquid-Liquid extraction was selected for the process of fractionation at the next stage since it showed higher antifungal effects on M. Canis and T. Tonsurans compared with other fractions.

1.12. Preparation of Fractions from N-hexane based on SPE on Silica Gel Absorbent

According to the fraction yields in Table 5, a highest amount of N-hexane fraction, obtained from the preceding stage, entered Ethyl Stat %1 in Chloroform in this stage. Amongst the fractions containing the mixture of Chloroform and Ethyl Stat, the ones with higher chloroform showed the highest yield than the fractions with lower chloroform. Also, the pure Ethyl Stat fraction had the lowest yield. SPE has many advantages than Liquid-Liquid method. The demerits of liquid-liquid method are incomplete isolation of phases, less solvent recycling and the use of expensive fragile glass tubes. The SPE is more efficient, more facile and faster than Liquid-Liquid method.

1.13. Results of Antifungal Effects of Fractions Extracted from N-Hexane

The aforementioned fractions did not have antifungal effects on M. Canis and T. Tonsurans within the intended concentration range in the present study. The results indicated that three fractions including Ethyl Stat %3 and %5 in chloroform as well as pure ethyl stat had less antifungal effects than Ethyl Stat %1 in chloroform and pure chloroform. According to the comparison results between the antifungal effects of fractions based on SPE and the antifungal effects of fractions based on decantation, the SPE fractions had more antifungal effects. That is, fractionation based on SPE increased and improved the antifungal effects of fractionated fractions due to the increased concentrations of antifungal active components in these fractions. However, the antifungal effects of SPE fractions were more than the antifungal effects of hydro-alcoholic extract on M. Canis and T. Tonsurans.

The results of all three stages indicated that the extracts and fractions of rosemary leaves inhibits and destroys dermatophytes through dose-dependent activities. Therefore, based on the results of antifungal effects of hydro-alcoholic extract, the fractions obtained from the decantation of hydro-alcoholic extract as well as the fractions from decanted N-Hexane SPE, it was indicated that pure chloroform fractions resulted from SPE have the most anti-dermatophyte effects. Comparing these results with the results of Terbinafine, the replacement of this fraction with Terbinafine is not acceptable to this level of isolation of components.

1.14. Results of Keratinase Activity Assay

The results showed that the keratinase activity of both fungi increased with the increase of chloroform concentration; thus, there are a significant difference between different levels of concentrations for both fungi. In other words, there is an absolute dose-dependent effect. Consequently, the chloroform fraction resulted from SPE reinforce keratinase activity and is considered as an enzyme activator for keratinase.

To conclude, different extracts and fractions of rosemary leaves have anti-dermatophyte effects. With respect to the higher effect of N-hexane fraction in decantation, these fractions are recommended to be used in proper percentage to prepare appropriate topical formulations (Cream, Ointment or Losion) and to investigate their efficiency under in-vivo conditions which may result in better anti-dermatophyte effects. In fact, the ultimate objective of in-vitro tests is identifying antifungal factors and predicting their clinical outcome. Since treatments are getting more specialized every day and requires the strongest antifungal drugs to be identified for any fungal agent, it is necessary to identify the most effective drug against M. Canis and T. Tonsurans fungi which are the most common causes of ringworm (Tinea) by precise assay of the chemical components of rosemary and investigation of its antifungal effects.

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