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Study of Antifungal Activity against Trichophyton Rubrum ATCC 28188 of Lemon Seed Extract

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ABSTRACT

In this study, the antifungal activity of lemon seed extract on Trichophyton rubrum growth and development of Trichophyton rubrum was thoroughly investigated .Different ratios and concentrations of lemon seed extract were cultured in two separated characteristics of medium (agar and broth)for testing the colony formation and minimum inhibitory concentration (MIC)) against T. rubrum. The results of this study have indicated that the extract of Lemon seed only the statistically significant reduction of T.rubrum colony formation at 8% in agar test and 9% at broth test with the ratio of 1:4 v/v but also the improved herbal advantages of aqueous extracts of lemon seed usage against the growth and development of T.rubrum. In conclusion, the lemon seed extract could effectively inhibit the growth of T.rubrum at the selectively applied concentration.

Keywords: *Lemon seed extract, Trichophyton rubrum, antifungal activity.*

INTRODUCTION

Dermatophyte infections are known as the one of the earliest fungal infections of mankind and they are very common throughout the world. Dermatophytosis do not cause the mortality, it can cause the morbidity and poses a major public health problem [1], it occurs especially in tropical countries (such as Viet Nam, India, Indonesia…) because of the hot and humid climate. Belong to the Indonesia Health Profile Data the number of outpatients have medical diagnoses of "skin diseases and subcutaneous tissue" visited in 2008 was 15,100 visits. In 2010 the number of patients increases to 192,414, of which 122.076 were new cases.

Dermatophytes are a group of filamentous fungi that cause infections of the skin (see Figure 1 for diseases, Figure 2 for typical dermatophyte species). Diseases caused by dermatophytes include athlete's foot, ringworm, jock itch, and nail infections (onychomycosis). The medical terminology for dermatophyte infections is to use the word tinea (to denote a fungal infection of the skin) followed by a word that describes the site of infection. Tinea pedis which is called Athlete's foot or "Ring worm of the foot" is the most common dermatophyte infection. The most common cause is Trichophyton rubrum *(T. rubrum).* [2]

Trichophyton is keratophakic that digest skin and anthropophilic keratin which selects humans as their permanent hosts. This kind of fungi can live in air, land, water, clothing, or even human body. This fungus group can cause chronic and residual disease due to the body's very mild rejection reaction, on the human body of this fungus concerning the skin of ankles, soles of the feet and the sidelines of the toes. Singapore's National Skin Care Hospital in 1999-2003 showed a percentage of Tinea pedis reaching 27.3% while Chumitshu Chuo Hospital Tokyo Japan showed a percentage of Tinea Pedis reaching 64.2%. Approximately 15% of the population has a podiatric fungal infection at any given time and it is estimated that over 70% of the population have suffered at some point in their lives from tinea pedis [3]

Lemon is an important medicinal plant of the family Rutaceae, it is used mainly for its alkaloids, which have anticancer activities and the antibacterial potential in crude extract of different parts (leaves, stem, root, juice, peel, seed, and flower) of lemon. Lemon (Citrus limon) juice contains many bioactive compounds such as flavonoids, carotenoids, limonoid, tannin, and terpenoids. The bioactive compounds contained in lemon (Citrus limon) each have an antibacterial [4]. The main content of lemon (Citrus limon) juice is vitamin C and

citric acid. The content of vitamin C and citric acid makes the degree of acidity (pH) the lemon (Citrus limon) juice become acidic [5].Also, the citrus secondary metabolites contributed to antimicrobial, antifungal [6], antiviral, and other beneficial activity [7]. Furthermore, lemon is one of the major plants in Citrus groups which contains many biological active compounds such as polyphenolic compounds of flavonoids and a high amount of limonoids[8]. So, it is scientifically and economically important to know whether the extracts from lemon seeds can be used as antifungal activity against *T. rubrum*. Also, consequence of choosing a suitable concentration of the lemon seeds extracts to meet the proposed works need to be determined.

In summary, this study has been conducted to determine as well as evaluate the possible and highly suitable concentration of lemon seeds extracts against the growth of *T. rubrum*. The aim of this study was to evaluate the effect of the lemon seed extracts against *T. rubrum*.

MATERIALS AND METHODS

Materials

Lemon fruits were collected from the Southwestern province of Ben Tre province, Vietnam. The lemon seeds were dried in drying oven at 105^0C to get the consistent moisture content of 5% and then store in Desiccator ready for further use and analysis.

Trichophyton Rubrum ATCC 28188 with freeze-dried status was purchased from Lan Oanh Company, Ho Chi Minh city.

Brain Heart Infusion Broth (BHI broth) and Brain Heart Infusion Agar (BHI agar) were purchased from Ensure company, Ho Chi Minh City.

Methods

Preparation of Lemon Seed Extract

The well-prepared lemon seeds were well ground into fine powder by using electric grinder.

The powder was homogenized to deionized water with the ratio 1:10 (weight / volume), then the mixture was put into sonicator to agitate particle of powder from 2 – 3 hours at 90oC and the mixture was cooled down to 50 – 55oC. Then the mixture was filtered with standard funnel and filtered paper to collect the filtrate. The filtrate was centrifuged at 2000 rpm at 4oC in 15 minutes to remove the insoluble debris. The insoluble debris is dried and weighed to know the amount of lemon seed powder dissolve inthe solution after subtracted by the initial biomass. The calculation of the obtained yield usingthe formula:

$$\textbf{Yield of extraction} = \frac{Dissolved\ powder\ in\ solution}{initial\ biomass\ of\ powder}\ x\ 100$$

Different concentrations were diluted by using deionized water. Then each concentration was label and stored at 4^oC. Before using the lemon seed extract, the solution should be autoclaved at 121oC for effective antifungal test. [9]

Preparation of the Testing Microorganisms

The commercial *Trichophyton rubrum* ATCC 28188 is in freeze-dried form, which needed to be cultured on cultured on Sabouraud Dextrose Agar and incubated at 25^0C before culturing in Brain-Heart infusion broth (BHI). The inoculation *Trichophyton rubrum*was performed at 35^oC for 24-36h. After checking without contamination on Agar Base, one colony was randomly taken for culturing on BHI broth medium for 24-36 hours at 37^oC and used for testing the antifungal activity.

Before testing anti-fungal activity, concentration of *Trichophyton rubrum*needed to be known prior to counting colony on the agar plate by making serial dilution method ,then making sure that the number of colonies on agar plate was not too many or few. A plate having of 30-300 colonies was chosen due to this range is considered statistically significant.

Testing Antibacterial Activity

This testing was performed by culturing Trichophyton Rubrum on medium which contained several concentrations they are described in following steps:

Broth Test

Broth medium was autoclaved at 121oC for 15 minutes then cooled down to 60oC. Trichophyton Rubrum is cultured in BHI broth medium containing specific lemon seed extract concentration, for each treatment the number of replicated is five times. Three different ratio 1:4 v/v, 1:2 v/v, 1:1 v/v (lemon seed solution/ medium) is performed to determine the minimal inhibitory concentration of lemon seed extract against T.rubrum

Table1. *Experimental design for culturing the Trichophyton rubrum for each treatment.*

	No.of replicated	Added materials		
		Medium	Inoculum	Lemon seed extract
Negative control	1	√	X	x
Negative control 2	1	√	X	√
Positive control1	1	√	√	x
Sample	5	√	√	√

(√: with; x: without)

Table2. *Experimental design for culturing Trichophyton rubrum in broth*

Treatment No.	Concentrate of sample	No. of replicates
1	4%	5
2	5%	5
3	6%	5
4	7%	5
5	8%	5
6	9%	5

The results of this test were used for quantitative determination of CFU. CFU was used for the reduction's proportion to determine the antifungal activity. This method was used to determine MIC (minimum inhibitory concentration) as the minimum concentration of the extract solution that completely inhibited the growth of visible bacteria. CFU was calculated by the following formula:

$$\text{CFU/ml} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volume plated}}$$

, and the effect of the lemon seed was decided by fungal counting and it was calculated by the following formula:

$$\textbf{\% Reduction (D-value)} = \frac{[(Mean\ CFU)control - (Mean\ CFU)sample]x\ 100}{(Mean\ CFU)control}$$

$$\textbf{Log (Reduction)} = \textbf{Log10}(Mean\ CFU)control - \textbf{Log10}(Mean\ CFU)sample$$

Agar plate test

This test was performed by culturing the inoculum on BHI agar containing specific concentration of lemon seed extract to determine the concentration of lemon seed extract that inhibit T.rubrum. There were three ratios of 1:4 v/v, 1:2 v/v, 1:1 v/v and repeated 5 times for each ratio with each concentration.

BHI Agar was autoclaved at 121°C for 15 minutes and cooled down to about 60°C before transferring the lemon seed extract into the medium, vortex well and poured into the petri dish. A sterile cotton swab was used for spreading out 20µl inoculum on the surface plates and incubated at 37°C, 24 hours. There are several treatments which distinct in added the sample into medium. The lemon seed extract is transferred into medium with the specific concentration and poured into the petri dish. Colony which was taken from the source was spread on the surface of the agar with a sterile cotton swab.

Table 3. *Experimental design for culturing Trichophyton rubrum in broth for each treatment*

	No. of replicated	Added materials		
		BHI broth	Inoculum	Lemon seed extract
Negative control	1	√	X	x
Negative control 2	1	√	X	√
Positive control1	1	√	√	x
Sample	5	√	√	√

(√: with; x: without)

Table 4. *Experimental design for culturing Trichophyton rubrum in agar plate.*

Treatment No.	Concentration of sample	No.ofreplicates
1	4%	5
2	5%	5

3	6%	5
4	7%	5
5	8%	5
6	9%	5

Data analysis

All data were treated and presented as means ± standard deviation of representatives of similar test carried out in 5 times of replication. Statistical differences in colony forming unit (CFU) were determined by student's t-test in which, P-values less than 0.05 was considered statistically significant.

RESULTS

Preparation of Lemon Seed Extract

After the dried powder of lemon seed was added to deionized water, then sonicated in water batch at 90°C for 2-3 hrs. with the ratio of 1:10 w/v followed by filtering, using filter paper, the aqueous phase of lemon seed was obtained. It had a dark brown colored solution. The formula below was used to calculate the obtained yield:

$$\text{Yield of extraction(w/v \%)} = \frac{\text{dissolved powder in solutionx 100}}{\text{intitial biomass of powder}}$$

Theinitial biomass of lemon seed powder = 30 grams

The insoluble weight= 20.15 grams

The powder that dissolved in the solution = 9.85 grams

Figure1. *Different concentrations of lemon seed extract*

Consequently, the concentration of lemon seed extract was 9.85% (w/v) which was followed by adding deionized water to have it diluted into different concentrations of lemon seed solution (4%, 5%, 6%, 7%, 8%, 9%) by applying the formula C1V1= C2V2 (Where C1 and C2 are the concentration (%) of the initial and final solution, respectively, V1 and V2 are the volume (ml) of the initial and final solution).

Lemon seeds are commonly herbal medicine in traditional medicine systems which contain the high level of bioactive compounds, it used to be potent analgesic, anti-inflammatory, antibacterial, antifungal agent [10].

Preparation of Microorganisms: Identification of the cultured Trichophyton Rubrum

Trichophyton Rubrum is the most common causative agent of dermatophytosis worldwide, mainly occur in humans' feet, skin and between fingernails (Yang, J., Chen, L., Wang, L., et al 2007). Moreover T.rubrum is also known as the most prominent anthrophilic species [11]. This fungus appears in shades of white cotton, and the colony of *T.rubrum* are formed on Sabouraud dextrose agar and BHI agar after they are incubated at 25°C in 2 days.

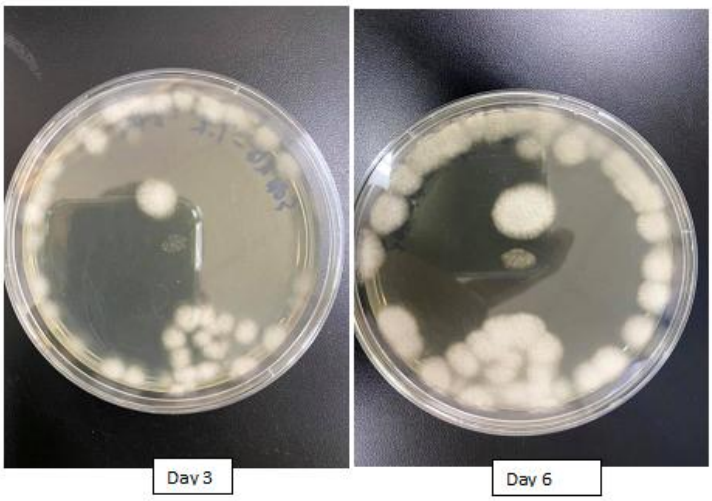

Figure2. *Trichophyton Rubrum colonies*

Figure3. *Structure of Trichophyton Rubrum*

Determination of antifungal activity

Broth test

The broth experiment was conducted with 3 control samples and 9 testing samples with a different concentration of lemon seed extract and was repeated five consecutive times. The turbidity was used to test the bacterial growth in the liquid medium. After 24 hours of incubation, the dilution method was used for counting colony from broth medium into agar plates. Taking 0.1 ml of the suspension was added to 0.9 ml of sterile distilled water. From this suspension, 8 dilution factors were done and 100 µl of lasted dilution is cultured on agar plated for 370C, 24hours.

Figure4. *Concentration broth with the ratio 1:4(v/v) in test 1*

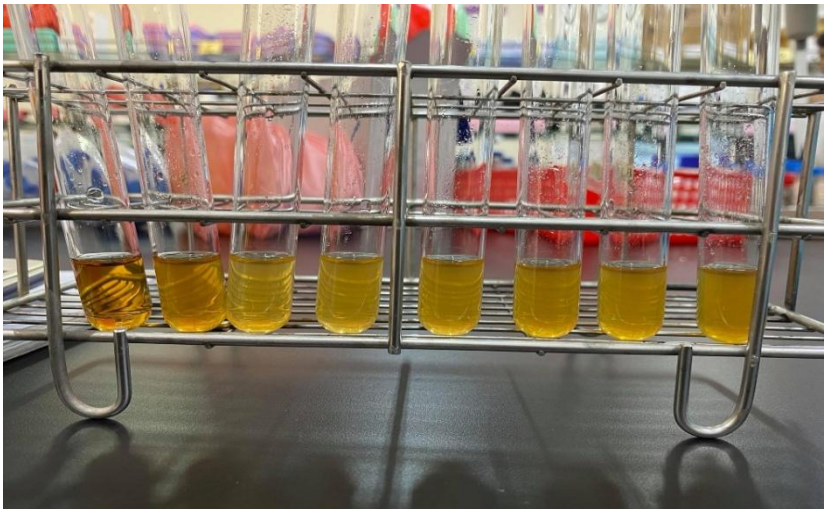

Figure5. *Concentration broth with the ratio 1:2 (v/v) in test 2*

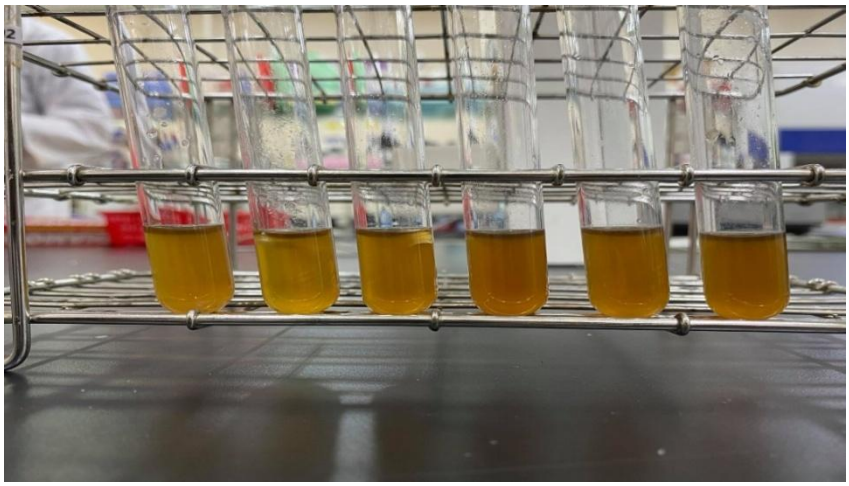

Figure6. *Concentration broth with the ratio 1:1(v/v) in test 3*

Table5. *The antifungal activity result of lemon seed extract testing after sub-culture and incubation at 37oC for 24 hours on BHI agar plate from broth piece*

	Concentration (%)	Mean (CFU/ml)	Standard deviation	Reduction (%)	Log Reduction
Test 1 (1:4)	Control	**1128.2**	**135.08**		
	4%	168.2	38.67	85.09	0.034
	5%	165.4	11.05	85.34	0.036
	6%	68.6	31.01	93.92	1.216
	7%	35.8	24.2	96.83	1.498
	8%	15.6	4.72	98.62	1.859
	9%	12	1.5	98.93	1.973
Test 2 (1:2)	Control	1168.2	105.5		
	4%	75	3.16	93.58	1.19
	5%	66.2	2.77	94.33	1.24
	6%	54.2	6.76	95.36	1.33
	7%	35.8	3.83	96.94	1.51
	8%	25.2	3.42	97.84	1.66
	9%	10.4	2.3	98.83	1.93
Test 3 (1:1)	Control	1238.2	214.2		
	4%	1043.6	89.1	15.7	0.07
	5%	654.6	140.4	47.13	0.27
	6%	567.4	46.8	54.18	0.33
	7%	326	24.76	73.67	0.57
	8%	26.2	7.19	97.88	1.67
	9%	12.8	5.26	99	1.98

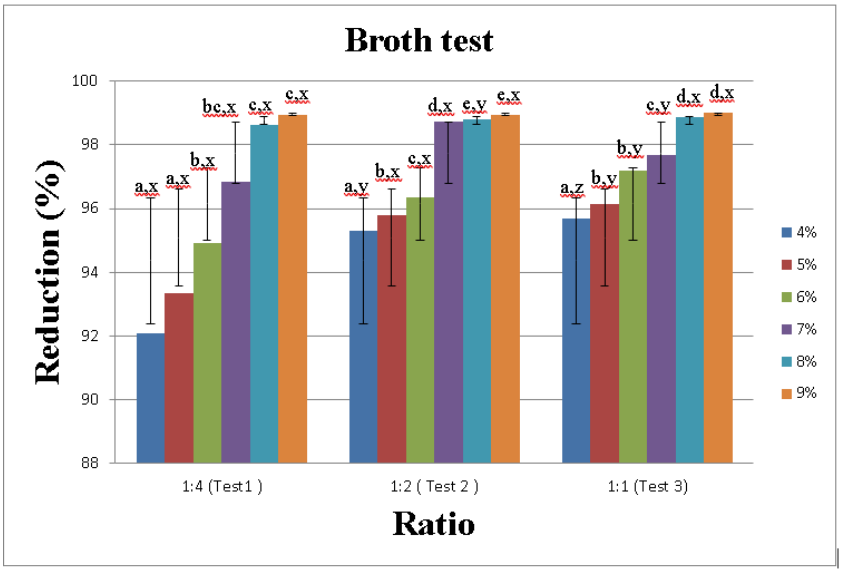

Figure7. *The proportion of fungus reduction (%) at different concentration lemon seed extract with different ratios in broth media (a,b,c,d,e,f,x,y,z: same letters in the same row express those values are not significantly different)*

Agar Plate Test

This type of experiment was done with three ratios of 1:4, 1:2, 1:1 v/v (ml medium/ ml of lemon seed extract) and every ratio was named with test 1, test 2, test 3, with different concentrations of lemon seed extract (4%, 5%, 6%, 7%, 8%, 9%)

This experiment was repeated five times. After that, adding lemon seed extraction and incubate it in 37°C, collect the data after 2 days.

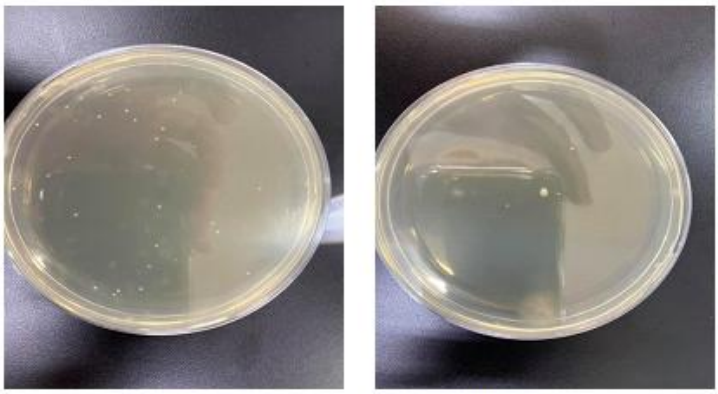

Figure8. *Sample concentrations testing on agar plate with ratio 1:4 (v/v) with 8% concentration (on the left) in comparison with 9% of sample (on the right).*

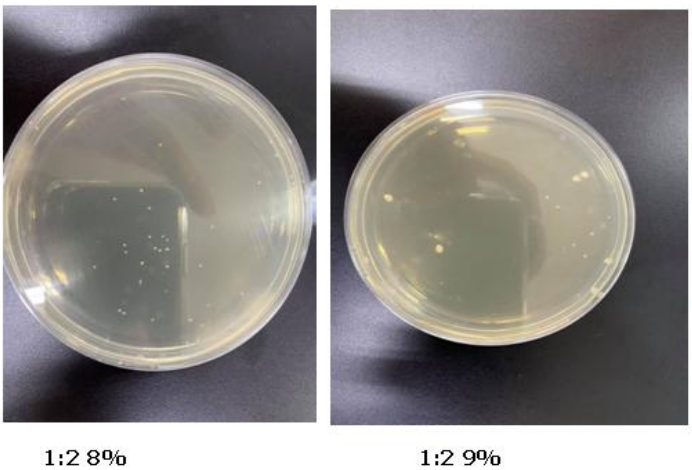

Figure9. *Sample concentrations testing on agar plate with ratio 1:2 (v/v) with 8% concentration (on the left) in comparison with 9% of sample (on the right).*

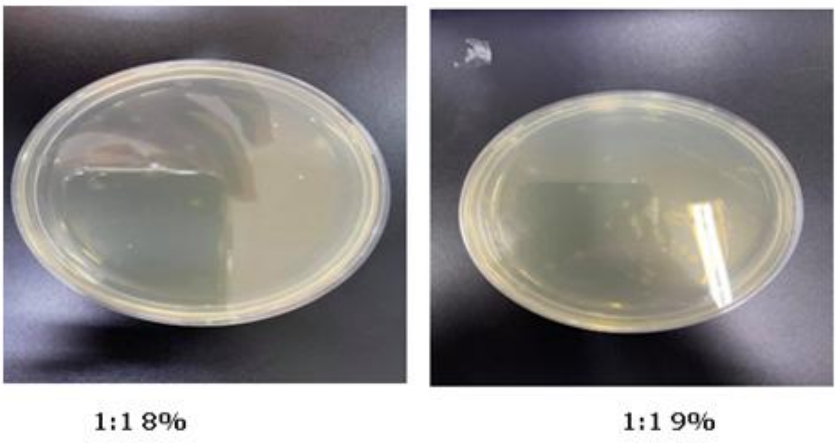

Figure10. *Sample concentrations testing on agar plate with ratio 1:1 (v/v) with 8% concentration (on the left) in comparison with 9% of sample (on the right).*

Table6. *The antifungal activity result of lemon seed extract testing after sub-culture and incubation at 37°C for 24 hours on BHI agar plate*

	Concentration (%)	Mean (CFU/ml)	Standard deviation	Reduction (%)	Log Reduction
Test 1 (1:4)	Control	1129.6	137.4		
	4%	674.6	73.01	40.27	0.22
	5%	540.4	42.07	52.16	0.32
	6%	234.4	121.54	79.24	0.68
	7%	166	18.8	85.3	0.83
	8%	35.8	4.3	96.8	1.49
	9%	14.8	5.2	98.7	1.88
Test 2 (1:2)	Control	1015.4	113.5		
	4%	704	47.5	30.66	0.16
	5%	718	61.8	29.23	0.15
	6%	339.8	93.7	66.53	0.47
	7%	55.2	4.1	94.56	1.26
	8%	25.8	4.3	97.45	1.59
	9%	10.4	2.3	98.83	1.93
Test 3 (1:1)	Control	1006.2	104.3		
	4%	30.2	2.4	96.98	1.52
	5%	17.8	4.6	98.23	1.75
	6%	13	2.7	98.70	1.88
	7%	4.8	2.8	99.52	2.32
	8%	4	1.5	99.60	2.4

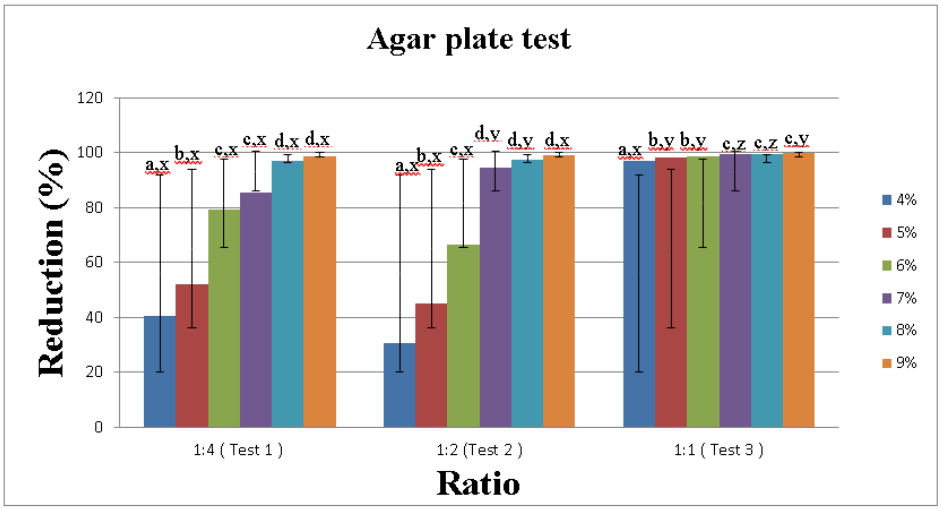

Figure11. *The percentage of fungal reduction (%) at different concentration lemon seed extract* ***with different ratios in agar media.*** *(a,b,c,d,e,f,x,y,z: same letters in the same row express those values are not significantly different)*

DISCUSSION

A lemon seed extract is a crucially traditional herbal medicine which has been used for the treatment of inflammatory conditions of the respiratory system [12. Analytical differences provoke the difficulty in comparison in-between studies' results. Such factors would alter the obtained results include the type of the material used, manufacturers, and the extraction technique implied during the experiment. Additionally, during the extraction preparation processes, some or all the active extraction components might be inactivated, or the active ingredients' concentrations might be different in terms of the geographical location, seasonal, and cultivation processes and all these factors affect the efficacy of lemon seed extract [13].

Trichophyton rubrum (T. rubrum) is a dermatophyte responsible for causing most superficial fungal infections worldwide [14]. Dermatophytes are a subset of fungi that can invade keratinized tissues, such as skin, hair, and nails. This group of fungi can cause infection anywhere on the skin, however, they most commonly affect the feet, inguinal region, axillae, scalp, and nails [15]. The infection results in mild to moderate dermatological symptoms, with a range of severity of infection([14],[15]. the formation of colony of *T.rubrum*on Sabouraud dextrose agar and BHI agar after incubated at 25^{o}C in 2 days as opposed to the negative control is shown in the figure 2.

From the figure 2 and 3, it could be clearly indicated that the identification tests were conducted in appropriate ways since *T.rubrum*is the primary component of gram-positive bacterium, and the color of gram stain was paired in cocci shape.

From the Table 5, it can easily be seen that the percentage of reduction of *Trichophyton Rubrum* which was performed in broth medium and cultured in the agar plate. Then incubated it in 2 days at 25^{o}C for counting the colony formation. CFU was calculated for the reduction to detect the antifungal activity and it also used for minimum inhibitory concentration (MIC). As presented in the Table 5, the comparison between the fungal reduction of different concentrations have shown the significant difference among 3 tests.

In general, we can see that there has no significant different at 9% concentration between test 1, test 2, test 3, the lemon seed extract has high antifungal activity with high percentage of *T.rubrum* reduction which was higher than 90% due to the low CFU. In test 1 and test 2 there are no significant difference at 5%, 6%, 7% compared to the test 3, of which there was a significant difference to the test 1 and the test 2 at 5%, 6%, 7%. There was a significant difference at 8% concentration in the test 2 compared to the test 1 and the test 3. So, the antifungal ability of lemon seed extraction increase proportion to increasing of the ratio and concentration.

In the agar plate test, there were significant differences in reduction at concentrations of 4%, 5%,6% of test 3 in comparison to the concentration of 4%, 5%, 6% of test 1 and test 2. At concentration of 9% there were significant differences among the test , the test1 and the test 2.

In general, the higher concentration of lemon seed extract, the higher antifungal activity against *T.rubrum* were seen.

CONCLUSIONS

In this study, the effects of the lemon seeds extract against the growth and development of Trichophyton Rubrum was successfully and thoroughly investigated. It was clearly found that the lemon seed concentration at least 8%(w/v) accounted for a quarter per total medium affected on the reduction percentage of Trichophyton Rubrum over 95%.The most suitable concentration of the extract at 9% showed the strongest effect on the inhibition of *Trichophyton Rubrum*.Moreover, it could be stated that the inhibition capacity of the extracted Limonoid from lemon seeds showed the highly potential for the mentioned matter of concern.

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