Identification and evaluation of potential biological activities of some secondary metabolites produced by local isolates of *Trichophyton rubrum*

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Abstract:

Secondary metabolites products permanently played an important role in medicine, fungi metabolites have increasingly become major players in recent pharmaceutical discovery in particular antimicrobial agents .The aim of study was evaluate of potential biological activities of the antioxidant activity and anticytotoxicity of secondary metabolites (SMs) extracted from *Trichophyton rubrum*. Eleven isolates of dermatophytes were examined for production of Secondary metabolites, Extracts were checked for their cytotoxicity using a sensitive *in vitro* Brine shrimp lethality bioassay. The results showed different mortality rate at different concentration acetone SMs extracts. The results were showed different mortality rate at different concentration acetone SMs Extracts. Acetone extracts of SMs from *T.rubrum* showed better antioxidant potential by DPPH radical scavenging method when compared to standard ascorbic acid ..

Keywords : *Trichophyton rubrum,* Secondary metabolites , antimicrobial activity .

Introduction

Secondary metabolites have been used as drugs or inspiring structural leads in the pursuit for new potent drugs. The main sources for secondary metabolites were filamentous fungi, marine, terrestrial plants and bacteria, (Klejnstrup *et al.*, 2016).

Secondary Metabolites consistently played an important role in medicine, in particular dermatophytes metabolites possess progressively become major participant in recent pharmaceutical discovery much of the medicaments**.** Fungi that classified pathogenic have been obvious great potential as a major source of pharmacologicall active metabolites while the biological activity of their metabolites was mainly of interest because of their antibiotic production (Swathi *et al.*, 2013**).**

Secondary Metabolites production by Dermatophytes exhibit different types of biological activities, and these metabolites have incredible array of chemical structures results in the many-sides biological activities. On the other side of their environmental ubiquity and hypercritical roles in nearly every ecological niche as primary decomposers, *Trichophyton spp* well known for producing a wealth of low molecular weight molecules of secondary metabolites, although the true ecological function of most secondary metabolites (SMs) was still unknown, their role as biotic and abiotic protectants or defensive metabolites was emerging (Benjamin *et al.*, 2015).

Trichophyton rubrum as a source of biologically active metabolites this implicates fungi to represent an enormous source for natural products with diverse chemical structures and activities. Of special interest were creative fungal strains. Creativity in this sense defined as the ability to produce compounds of interest for human activities. The *Trichophyton rubrum* were one of the major promise antibiotic-producing organisms

and one of the most diverse groups of fungi, so the search for novel antibiotics from *Trichophyton rubrum* were promising (Kjer *et al.*, 2010). So , the present study aimed to identify the secondary metabolites and evaluate the antibacterial activity produced by *T.rubrum*.

Materials and methods

Eight strains isolate were selected and brought from patients attended to Dermatology Department in Diwaniya Teaching Hospital were obtained during the period beginning the December 2014 till end of May 2015 were collected 30 samples different from hair, nail, and skin scratching and these samples were collected by using sterilized forceps, sterile fine scissors, sterile blunt scalpel, so that, the scraping skin was collected by sterilized the container, while infected hair was removed by a sterile forceps, but the infected nail was collect by used sterile fine scissors. Samples were treated, divided and processed by a potassium hydroxide (KOH) solution smears prepared and examined microscopically for the purpose of identification and characterization samples. The standard medium used for isolation of dermatophytes from clinical materials is Sabouraud's dextrose agar containing cycloheximide (0.5gm).

Preparation Production media

T. rubrum was grown on modification medium of Horse Hair Dextrose liquid medium **(**HHD) (Colin *et al*., 1981)**.** The *Trichophyton rubrum* fungus was grown on SDA for 7-10 days. inoculated into each 1-L flask containing 200 mL fermentation broth was inoculated with mycelium incubated for 10th days on a orbital incubator (150 rpm and a 5cm eccentric throw) at 28°C to obtain good growth for the Dermatophytes (Hammadi *et al.*, 2007).

Isolation and purification of crude extract secondary metabolite

After 10th days of incubation in fermentation broth and after end the incubation time centrifugation on the 6000 rpm for 20 min to the obtained mycelia were separated the mass of mycelia filtered with Whatman filter paper No.1 , the extracts of the ferment mediums then transferred into a vial and kept at -20°C until use. The extracts of the ferment mediums extract culture media was extracted with an equal volume of acetone the mixture was shaken in a separating funnel. The organic layer was separated and collected from the aqueous part and then divided into two part one that extracted was concentrated using partial evaporations were carried out a rotary evaporator under vacuum at 40°C, the solvent was removed in using a rotary evaporator and collected extract and weighed (Hammadi *et al*., 2007, Al-Shaibani *et al*., 2013)

Antimicrobial activity :

Crude extracts were screened for microbial properties using a modified Oxford disc diffusion assay against *Streptococcus pyogene:* ATCC49619,

Pseudomonas aeruginosa ATCC27853, *Staphylococcus aureus* : ATCC25923, *Staphylococcus epidermidis* ATCC25985 and *Escherichia coli* :ATCC25922.

The in *vitro* antibacterial activity of the isolated secondary metabolites was determined by agar diffusion assay according to the standard method as described by the Clinical and Laboratory Standards Institute (CLSI), 2013 bacterial pathogens were prepared in the ready-made Mueller Hinton agar (MH) medium.

A 100µg sample of each crude extracts were prepared in chloroform (for complete evaporation of the solvent). Using a micropipette 100 µL of each crude extracts were loaded onto three

separate 5 mm diameter, Whatman No. 1 filter papers and allowed to dry in a sterile environment overnight. The plates were incubated at 37°C for overnight and the zone of inhibition measured (Swathi. *et al*.,2013).

Evaluation of Radical Scavenging Effects of *Trichophyton rubrum* Acetone SMs Extracts

1. Preparation of DPPH Solution:

Add 0.004 g of DPPH to 12 ml of 95% methanol measured with a graduated cylinder into a small flask wrapped in foil to protect the solution from light .The mixture was shaken vigorously and allowed to stand at refrigerator for 30 min extracts (Patel *et al*., 2011)

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2. Preparation of standard solutions

Prepare 0.004 gm of ascorbic acid was dissolved in 40 ml distilled water to prepare the standard solution for the experiments. The concentration of standard solution was 100µg/ml. From this solution, different required concentrations for different tests were prepared by serial dilution; the mixture was shaken vigorously and allowed to stand at room temperature for 30 min extracts (Patel *et al*., 2011)

3. Preparation of Extracts Solution

Prepare 0.004gm of both fungi extract (*T. rubrum*) extract (HHD) crude extracts were dissolved into 40ml of Methanol. The concentration was 100µg/ml, the mixture was shaken vigorously and allowed to stand at room temperature for 30 min. This was called the stock solution. From this solution, different required concentrations were prepared by serial dilution. Different Concentration of Extracts and Ascorbic acid: (200, 400, 600, 800, 100 µg/ml) extracts (Patel *et al*., 2011)**.**

Cytotoxic Activity

1. Preparation of the simulated seawater

Thirty seven grams sea salt (nonionized NaCl) was weighted accurately, dissolved in 1 liter of sterilized distilled water and then filtered to get clear solution. The pH of the sea water was maintained between 8.5 using 1N NaOH solution (Krishanaraju *et al*., 2005).

2. Hatching of brine shrimp eggs

Artemia salina Leach (brine shrimp eggs) was collected from the pet shop was used as the test organism. Simulated sea water was taken in the small tank and the shrimp eggs (1.5 gm /L) were added to one side of the tank and this side was covered(Krishanaraju *et al*., 2005).

The shrimp were allowed for two days to hatch and mature as nauplii (larvae). Constant oxygen supply was carried out during the hatching time. The hatched shrimps were attracted to the lamp on the other side of the divided tank through the perforated dam. These nauplii were taken for this bioassay .

3. Preparation of the sample of fungal Extracts solution:

Clean test tubes were taken different concentration (one test tube for each concentration) of test samples. 5 mg of methanol extracts of both types of broth extract was coded as Ext 1and Ext 2 of secondary metabolites of *T.rubrum* were accurately weighed and dissolved in 1000µl or 1 ml DMSO (dimethyl sulfoxide) in different beaker. Thus, a concentration of 5 mg/ml was obtained which used as a stock solution.

From this stock solution 400, 200,100, 50 and 25 µg/ml were taken in five test tubes respectively each containing 5ml sea water and 10 nauplii.

4. Preparation of control group:

Control groups were used in cytotoxicity study to validate the test method and ensure that the results obtained were only due to the activity of the test agent and the effects of the other possible factors were nullified. Usually two types of control groups were used positive control and negative control (Krishanaraju *et al*., 2005).

5. Preparation of the positive control group

Positive control in cytotoxicity study was a widely accepted cytotoxic agent and the result of the test agent was compared with the result obtained for the positive control. In the present study, Vincristine sulphate (VS) was used as the positive control 3 mg of vincristine sulphate was dissolved in 1.8 ml of distilled water to get a concentration of 5 mg/ml.

This was used as stock solution of vincristine sulphate using micropipette 25, 50, 100, 150 and 200 µl of the stock solution were transferred in 5 different vials. NaCl solution (brine water) was added to each vial to making the volume up to 5 ml. The final concentration of vincristine sulphate in the vials became 25, 50, 100, 150 and 200 µg/ml respectively.

6. Preparation of the negative control group

One hundred µl of distilled water was added to each of the three remarked glass vials containing 5 ml of simulated seawater and 10 shrimp nauplii to use as control groups. If the brine shrimp nauplii in these vials show a rapid mortality rate, then the test considered as in valid as the

nauplii died due to some reason other than the cytotoxicity of the samples.

Result :

The crude acetone extract of *T. rubrum* showed antibacterial activity,100 µg/disc of each crude extracts (HHD,KSD) compared with Chloramphenicol antibiotic as the control. The results of the antibacterial effect of the acetone extracts of both media (HHD,KSD) against several bacterial isolates showed growth inhibition effect for both types of bacterial isolate as showed in tables (1) and figure (1). The highest inhibition zone diameter was 23 mm on *S.epidermidis* and the lowest inhibition zone diameter was 9 mm *S. typhi coli* and 7mm *P. aeruginosa* .The Reference drug Chloramphenicol showed highest zone of inhibition of 30 mm on *S. typhi* and lowest zone of inhibition on *S. pyogenes*.

The absorbance at 517 nm by UV visible spectrophotometer were found to standard ascorbic acid and Acetone extracts of SMs of *T.rubrum* that show in the table (2) and IC 50 value obtained were as 9.3 , 24.8 and 21.3µg/ml table (3). for same ascorbic acid and Acetone extracts respectively. It means Acetone extracts of SMs of *T.rubrum* at higher concentration So, comparison with the ascorbic acid, it is clear that *T.rubrum* extracts possess moderate antiradical activity. The antioxidant power of Acetone extracts of SMs of *T.rubrum* was less than the antioxidant power of Ascorbic acid.

The results of brine shrimp lethality bioassay were shown in the table (4) and figure (2), test samples showed different mortality rate at different concentration. The mortality rate of brine shrimp napulii was found to be increased with the increase with the concentration of the sample. The effectiveness of the concentration and mortality percent

relationship of Acetone extraction of SMs of *T.rubrum* was expressed as a median Lethal Concentration (LC50) value.

Vincristine sulphate (VS) was used as reference standard, which chemotherapy used to treatment of acute lymphoblastic leukemia in children that show in the table (5) and mortality curve of Vincristine sulphate Positive control revealed in the figure (3) that was explain the mortality. The results of brine shrimp cytotoxicity bioassay are shown in the table (5) and mortality carve in figure (3) test acetone SMs extract of HHD broth showed different mortality rate at different concentration

Table (1): The Antibacterial activity *in vitro* of Acetone extract of *T. rubrum* and standard Chloramphenicol.

Standard strains	Mean Diameter of inhibition zone (mm)	
	Acetone extract of HHD	Acetone extract of KSD
S.aureus	20	18
S.epidermidis	*23*	*20*
S. pyogenes	*12*	*10*
E. coli	*10.5*	*9*
S. typhi	*9*	*8*
P. aeruginosa	*7*	*7*

Table (2): Absorbance of different extract SMs of *T.rubrum* with standard ascorbic acid at 517 nm by UV visible spectrophotometer

Concentration (µg/ml)	**HHD extract SMs**	**KSD Extract SMs**	**control Scorbic Acid**	
200	0.2421	0.244	0.238	Control: 0.2444
400	0.2428	0.242	0.1719	
600	0.244	0.218	0.0469	
800	0.244	0.1619	0.0415	
1000	0.2379	0.142	0.041	

Table (3): inhibition percent of different extract of different extract SMs of *T.rubrum*. With standard ascorbic acid

Conc.(µg/ml)	Ascorbic acid (% Inhibition)	IC50 Value (µg/ml)	HHD extract SMs (% Inhibition)	IC50 Value (µg/ml)
200	20.61%	9.3	0.94%	24.8
400	29.66%		0.65%	
600	80.80%		0.16%	
800	83.01%		2.65%	
1000	83.22%		9.24%	

Table 4: Results of the cytotoxicity assay of Acetone SMs Extracts of HHD.

Test tube no.	**Concentration (µg/ml)**	**LogC**	**Number of nauplii alive**	**Number of nauplii dead**	**Mortality %**	**LC50 (µg/ml)**
1	25	1.398	4	6	60	16
2	50	1.699	4	6	60	
3	100	2.000	3	7	70	
4	200	2.301	3	7	70	
5	400	2.602	2	8	80	

Table 5: Results of the bioassay of Vincristine sulphate Positive control

Test tube no.	**Concentration (µg/ml)**	**LogC**	**Number of nauplii alive**	**Number of nauplii dead**	**Mortality %**	**LC50 (µg/ml)**
1	25	1.398	5	5	50	13.38
2	50	1.699	3	7	70	
3	100	2.000	2	8	80	
4	200	2.301	1	9	90	
5	400	2.602	0	10	100	

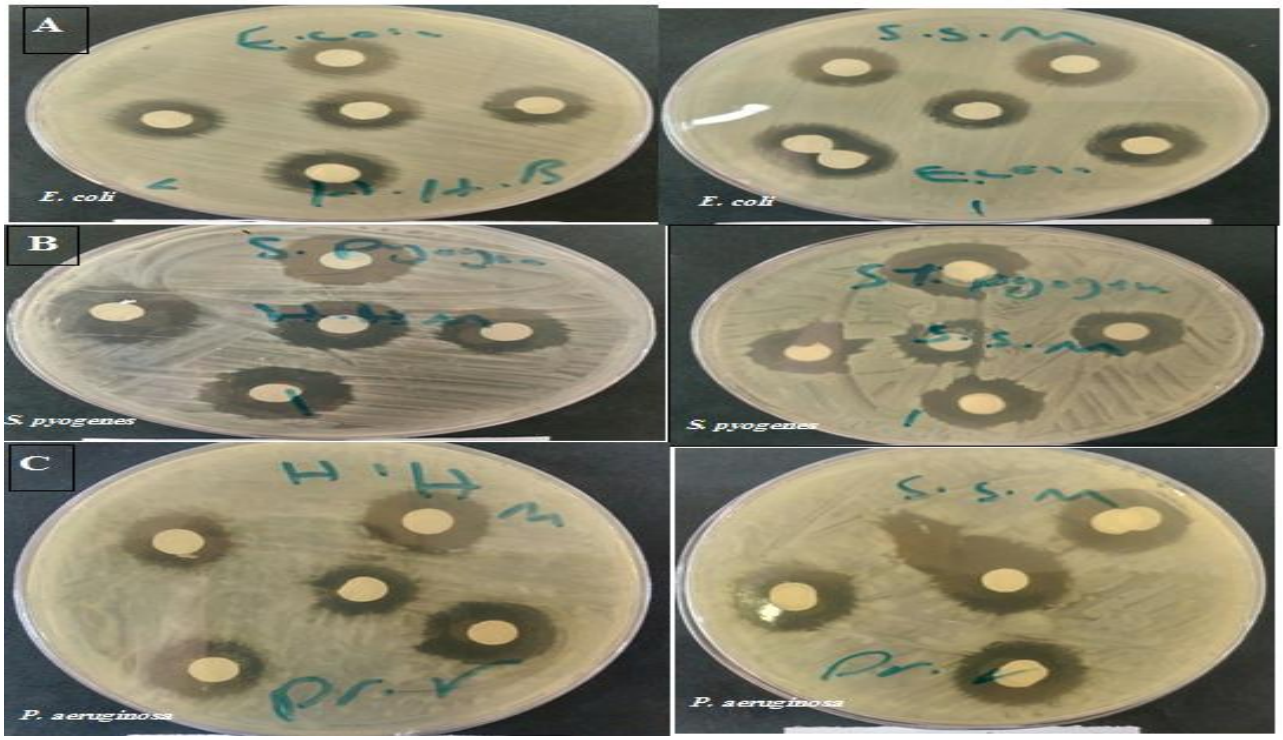

Figure (1): GrowthInhibitor zone (A)*E. coli,* *(B) S. pyogenes* and (C) *P. aeruginosa* by using *T. rubrum* secondary metabolites extract in both media, HHD, KSD

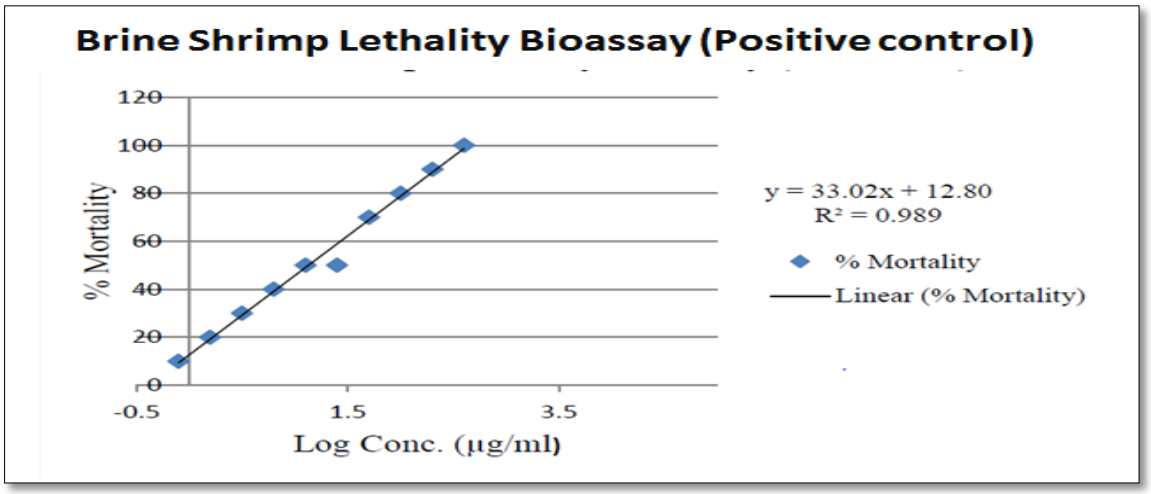

Figure (2): mortality percent curve of Vincristine sulphate Positive control

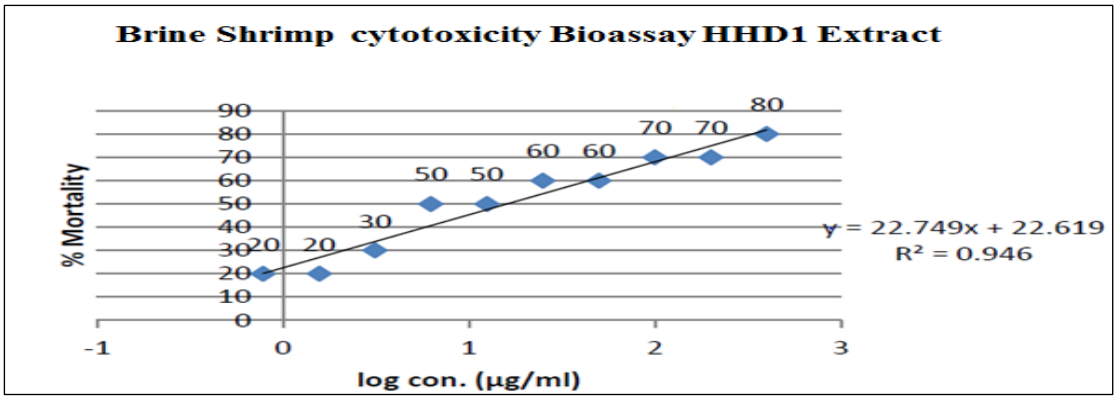

Figure 3: % mortality curve of Acetone SMs extract of HHD1 broth

Discussion:

The results of antimicrobial activity indicated that both the extracts had significant antimicrobial activity. this result were confirmed with other study Lennart and Goran (1970) which they carried out that dermatophytes have ability to inhibit bacterial growth, which showed that matches with current study, besides conform to study carried out by Youssef *et al*, (1979) Kheira *et al.* (1988) that show the ability of secondary metabolites extract by acetone from *T. rubrum* to inhibition the bacteria isolations.

The absorbance at 517 nm by UV visible spectrophotometer were found to standard ascorbic acid and Acetone extracts of SMs of *T.rubrum* that shown in the table (2) and IC 50 value obtained were as 9.3 , 24.8 and 21.3µg/ml table (3). for same ascorbic acid and Acetone extracts respectively. It means Acetone extracts of SMs of *T.rubrum* at higher concentration So, comparison with the ascorbic acid, it is clear that *T.rubrum* extracts possess moderate antiradical activity. The antioxidant power of Acetone extracts of SMs of *T.rubrum* was less than the antioxidant power of Ascorbic acid.

In Brine Shrimp cytotoxicity bioassay, varying degree of cytotoxic was observed with exposure to different concentrations of the test

samples extracts of *T.rubrum*. The degree of cytotoxic was found to be directly proportional to the concentration ranging from the lowest concentration to the highest concentration in both standard and Acetone SMs of *T.rubrum* extracts in HHD. Mortality increased gradually with an increase in concentration of the test samples. Maximum mortalities took place at the highest concentration of 400µg/ml, whereas the least mortalities at lowest concentration 1.398 µg/ml as shown in table (4) and (5).

In this investigation, positive control and extracts fraction exhibited cytotoxic activities with the LC50 values 13.38µg/ml, 16µg/ml and 1.428 respectively, which indicates that the extract has less potent activity than standard Vincristine sulphate (VS) against brine shrimp napulii.

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