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Identification and Evaluation of Potential Biological Activities of Some Secondary Metabolites Produced by Local Isolates of *Trichophyton rubrum*

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By
Haider Abed Ali Abdalrahman Alshawi

B.Sc. Medical Laboratory Science Technology / 2007

M.Sc. Medical Laboratory Science Technology / 2012

Supervised by

Professor
Dr. Adnan Hamad Al- Hamadani
College of Medicine/ University of Al-Qadisiyah

Asst. Professor
Dr. Nawfal Hussein Al- Dujaili
Faculty of Sciences/ University of Kufa

October- 2016 A.D.

Muharrm-1437 A.H.

Certification

We, the members of the examining committee, certify after reading this thesis (**Identification and Evaluation of Potential Biological Activities of Some Secondary Metabolites Produced by Local Isolates of *Trichophyton rubrum***) and after examining the student **Haider Abed Ali Abdalrahman Alshawi** in its contents, we found it is adequate for the award of the Degree Doctorate of Philosophy in Medical Microbiology with Excellent Degree.

Signature
Professor
Dr. Jassim Mohammad
College of Medicine/ University of Baghdad
Chairman

Signature
Assistant Professor
Dr. Bassim I Mohammad
College of Pharmacy/ University of Al-Qadisiyah
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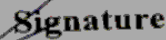

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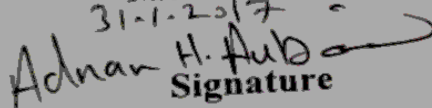


Signature
Professor
Dr. Adnan Hamad Al- Hamadani
College of Medicine/ University of Al-Qadisiyah
Member & Supervisor:

Signature
Assistant Professor
Dr. Nawfal Hussein Al- Dujaili
Faculty of Sciences/ University of Kufa
Member & Supervisor

Approval for college on Graduate Studies

Signature
Assistant Professor
Dr. Aqil Rahim Hassan
Dean
College of Medicine/ University of Al-Qadisiyah

Supervisor' s Certificate

We certify that this thesis: **Identification and evaluation of potential biological activities of some secondary metabolites produced by local isolates of *Trichophyton rubrum*** was prepared under our supervision at the Department of Microbiology, College of Medicine/ University of Al-Qadisiyah, in a partial fulfillment of the requirements of the degree of Doctorate of Philosophy of science in Medical Microbiology.

Signature

Professor

Dr. Adnan H.Al-Hamadani

Department of Microbiology

College of Medicine

University of Al-Qadisiyah

Date 21/10/2016

Signature

Asst. Prof.

Dr. Nawfal H. Al- Dujaili

Department of Biology

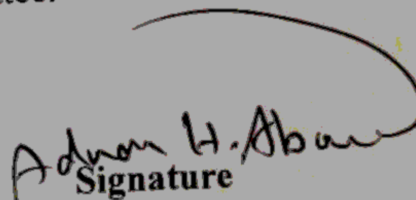
Faculty of Science

University of Kufa

Date: / /2016

Recommendation of Head of the Department of Microbiology

In view of the available recommendation, I forward this thesis for debate by the examining committee.



Signature

Professor

Dr. Adnan H.Al-Hamadani

Head of the Department of Microbiology

College of Medicine / University of Al-Qadisiyah

Date: 21/10/2016

بسم الله الرحمن الرحيم

قَالُوا سُبْحَانَكَ لا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَا إِنَّكَ أَنتَ الْعَلِيمُ الْحَكِيمُ

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Summary

In this study, one hundred patients were clinically diagnosed with dermatophytosis, which admit to Dermatology and Venereal Diseases at Diwaniyah Teaching Hospital during period December 2014 to May, 2015 in order to isolate a dermatophytes *Trichophyton rubrum* and evaluate its ability to produce bioactive metabolites that tested as antibacterial agent, anti-oxidant activity and cytotoxicity activity, samples of skin scraping, hair fragments and nail clipping were collected for direct and culture examination.

Out of eleven isolates of *Trichophyton rubrum* were identified using conventional and confirmed by molecular methods (PCR). The optimization of growth conditions and production of secondary metabolites by the isolated dermatophyte were studied it is revealed that optimum pH= 5, Temperature 28ºC and the Hores Hair Dextrose liquid media (HHD) and Keratinized Skin Dextrose liquid media (KSD) as substrate at 28ºC to promote the production of secondary metabolites .

In other hand, TLC showed the relative flow (R_f) of standards compounds was compared with extracts, also it is appearance as purple color spot under UV light. High performance liquid chromatography analysis of crude acetone of secondary metabolites of *T.rubrum* showed that each of HHD and KSD extracts given three peaks with different retention time, Benzylpenicillin G(BPG), fusidic acid(FA) and kojic acid were used as standard compounds.

Fourier Transform Infrared spectrophotometer (FTIR) analysis results revealed that the crude extract contains numerous functional groups The aromatic group was appeared in just the samples extract as well as the Sulfones groups in acetone extracts.

The results of crude acetone extract (100 µg/disc) exhibit wed antibacterial activity against several bacterial isolates, the crude acetone extracts of both media were showed largest growth inhibition zone (23 mm) against *Staphylococcus epidermidis* and the lowest was (10.5 mm) against; *Escherichia coli* and (7 mm) against *Pseudomonas aeruginosa*.

Acetone extracts of Secondary metabolites from *T.rubrum* showed better antioxidant potential on 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method when compared to standard ascorbic acid where the absorbance was at 517 nm, the antioxidant activity expressed as IC50 values were as 24.8 and 21.3 µg/ml while the standard Ascorbic acid was 9.3 µg/ml .

The crude extracts were checked for their cytotoxicity using a sensitive, *in vitro*, Brine shrimp lethality bioassay. The results were showed different mortality rate at different concentration of acetone Secondary metabolites extracts of HHD and KSD of *T. rubrum* the median lethal concentration (LC50) was 16µg/ml.

In conclusion, the present study established the ability of a dermatophyte , *T. rubrum* to produce antibiotic-like substances, especially the Sulfones which it is extracted and diagnosis at the first time from *T. rubrum* .

List of contents

Titles	Page
Summary	I-II
List of contents	8-VI
List of tables	VII-VIII
List of figures	IX-XI
List of Abbreviations	XII-XIII
Chapter one: Introduction and Literatures Review	
1.1 Introduction	1-4
1.2. Literatures review	5
1.2.1. The Dermatophytes	5
1.2.2. Etiologic Agent	5
1.2.3. Ecology of dermatophytes	7
1.2.4. Clinical manifestation of dermatophytes:	10
1.2.5. The dermatophyte:	13
1.2.5.1. Conventional method:	14
1.2.5.2. Polymerase Chain Reaction (PCR):	14
1.2.6. Natural Products	16
1.2.7. Production of Secondary Metabolites from Fungi	19
1.2.8. Classification of secondary metabolites	19
1.2.9. Isolation and Characterization of Secondary Metabolites	21
1.2.9.1. Isolation of metabolites	21
1.2.9.2.Characterization of metabolites	22
1.2.10. Applications of Secondary Metabolites	24
2.2.11. The secondary metabolites of *T.rubrum* Products:	26

1.2.10. Trichophyton rubrum:	30
1.2.12. Biological activity of Secondary metabolites of *T.rubrum*	32
Chapter Two: Materials and Methods	
2.1 Materials	35
2.1.1. Patients:	35
2.1.2 Apparatus	35
2.1.3 Chemicals and Biological Materials	36
2.1.4.Study Design	38
2.1.5. Culture media	38
2.1.6. Reagents:	40
2.1.7. PCR Kits	40
2.1.8. Primers	41
2.1.9.Molecular DNA markers	41
2.2.Methods	42
2.2.1.Collection of specimen:	42
2.2.1.1.Patients	42
2.2.1.2. Mycological Examination	42
2.2.1.3. Cultivation of dermatophytes:	43
2.2.1.4. Selection of *T.rubrum* isolates	43
2.2.1.5.Activation of fungal isolates	44
2.2.1.6.Identification of dermatophytes	44
2.2.2. Molecular methods	45
2.2.3 Preparation of Production secondary metabolites media	49
2.2.4. Isolation and purification secondary metabolite from *T. rubrum*	51
2.2.5.Antimicrobial activity of crud extract of *T. rubrum*	51
2.2.6. Thin layer chromatography (TLC)	52
2.2.7. Evaluation of Radical Scavenging Effects of *Trichophyton rubrum* Acetone secondary metabolites Extracts	54

2.2.8. Cytotoxic Activity	56
2.2.9. High Performance Liquid Chromatography (HPLC.)	60
2.2.10. Fourier Transform Infrared spectrophotometer (FTIR)	63
Chapter Three: Results & discussion	
3.1. Isolation and identification of *T. rubrum*	64
3.2. Confirmative identification of T. rubrum by PCR	66
3.2.1. DNA Quality and purity	66
3.2.2. amplification of extracted DNA with primers (IST1):	66
3.3. Determination the optimal growth conditions of *T. rubrum*:	67
3.4. Preparation of Crude extract from *T.rubrum:*	68
3.5. Antimicrobial activity of crude extracts of *T. rubrum*	69
3.6. Separation of *T.rubrum* crude extract by Thin Layer Chromatography	71
3.7. Evaluation of Radical Scavenging activity of crude Acetone secondary metabolites *T.rubrum* Extracts	75
3.8. Cytotoxic Activity of *T.rubrum* crude extract	77
3.9. Separation of crude extract by HPLC	82
3.10. Fourier Transform Infrared spectrophotometer (FTIR) analysis of the crude Acetone of *T.rubrum* extracts	90
Conclusions and Recommendations	
Conclusions	98
Recommendations	99
References	100-114
Arabic summary	أ-ب

List of Tables

Table No.	Title	Page
2-1	The apparatus used in this study	35
2-2	The Chemicals and Biological Materials used in this study	36
2-3	The read-mead media with remarked	39
2-4	Illustrated the contents of PCR kits	40
2-5	The primers used and their sequences	41
2-6	Molecular markers sizes as DNA marker for electrophoresis	42
2-7	The PCR master mix reaction preparation	47
2-8	The PCR Thermocycler Condition	48
2-9	bacterial strain that used in the study	51
2-10	Specifications of HPLC Model LC-2010HT	62
3-1	Value of DNA concentration and purity of selected sample of Extracted DNA	66
3-2	The Antibacterial activity in vitro of Acetone extract of *T. rubrum* and standard Chloramphenicol.	69
3-3	Determination of the Rf value of thin layer chromatography TLC of the crude acetone extracts of *T. rubrum* with compared with standard	75
3-4	Absorbance of different extract secondary metabolites of *T.rubrum* with standard ascorbic acid at 517 nm by UV visible spectrophotometer	76
3-5	inhibition percent of different extract of different extract secondary metabolites of T.rubrum. With standard ascorbic acid	77
3-6	Results of the bioassay of Vincristine sulphate	78

	Positive control	
3-7	Results of the cytotoxicity assay of Acetone secondary metabolites Extracts of HHDof *T. rubrum*	79
3-8	Results of the cytotoxicity assay of Acetone secondary metabolites Extracts of KSD	80
3-9	the peaks of HHD separation in HPLC and R. time	85
3-10	the peaks of KSD separation in HPLC and R. time	86
3-11	The peaks of Benzylpenicillin G separation in HPLC and R. time.	87
3-12	The peaks of Fusidic acid separation in HPLC and R. time.	88
3-13	The peaks of Fusidic acid separation in HPLC and R. time	89
3-14	FTIR spectral peak values and functional groups obtained for the crude Acetone secondary metabolites of *T.rubrum* extracts and Standard compound	91

List of Abbreviations

BPG	Benzylpenicillin G
CLSI	Clinical and Laboratory Standards Institute
ESI	electrospray ionization
FA	Fusidic acid
FDA	US Food and Drug Administration
GC	Gas chromatography
HBV	hepatitis B virus
HETCOR	Heteronuclear chemical shift correlation
HMG-CoA	3-hydroxymethyl glutaryl-CoA
HPLC	High Performance Liquid Chromatography
HSQC	heteronuclear single-quantum correlation
IR	infrared region
ITS1	internal transcribed spacer 1
KA	Kojic acid
LC	liquid chromatography
LDL	low-density lipoprotein
MRSA	methicillin- resistant *Staphylococous aureus*
MS	mass spectrometry
NCCLS	National Committee for Clinical Laboratory Standards
nm	Nanometer
NMR	Nuclear Magnetic Resonance
rpm	Round per minute
UV-VIS	Ultraviolet and visible spectroscopy

1. Introduction and Literatures Review

1.1 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,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 pharmacological active metabolites while the biological activity of their metabolites was mainly of interest because of their antibiotic production (Swathi *et al.,* 2013**).**

Fungi were of major interest because only a small percentage of them have been investigated for their role in producing novel bioactive compounds and hence offer huge potential. To date, soil fungi have yielded most of the compounds presently in commercial use. These bioactive compounds were mostly derived from fungal secondary metabolism. The secondary metabolism was required product usually was not extracted from the primary growth substrate, but rather a product formed from the primary growth substrate acts as a substrate for the production of a secondary metabolite and usually was suppressed by high specific growth rates of the secondary metabolites producing cultures (Batt *et al.,* 2014).

The products of fungal metabolism have a wide range of organic compounds including small primary metabolites, amino and fatty acids, peptides, and secondary metabolites such as penicillin, zearalenone, and statins. Fungal secondary metabolites account for many of the lifesaving

antibiotics, 3-hydroxy-3-methylglutaryl-coenzyme (HMG-CoA) reductase inhibitors and have bioactivities that make them of high value, including in cancer treatment. Primary metabolites were those metabolites necessary for the growth of an organism, such as polysaccharides, proteins, fats and nucleic acids. The primary metabolism process was similar among all organisms (Manitto, 1981).

Those metabolites that utilize primary metabolites as building blocks, and were not necessary to the overall function of the organism were referred to as secondary metabolites (Benjamin *et al.,* 2015).

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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 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 *T.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).

In spite of the fact that the production of antibiotics by dermatophytic fungi has long been known the earliest reports of the production of antibacterial substances by dermatophytes appear to representative of a previous study conducted proved that all species studied produced benzyl penicillin-like substances and some *Trichophyton* isolates also produced streptomycin-like antibiotics, a characteristic previously unrecorded for eukaryotic organism. Other antibiotics, which apart from azalomycin F could not properly classified, produced by *Epidermophyton floccosum* (Youssef *et al.,* 1978).

The natural function of secondary metabolites often was unknown, it was assumed that they play an important role in chemical defence and communication. Many of them have been suggested to act as pheromones, antifeedants or repellents, and as regulators in the development of organism

Although *T. rubrum* have proven to be prolific producers of diverse bioactive secondary metabolites, these observations argue strongly for continued exploration of fungal chemistry in order to help meet the increasing demand for new medicinally beneficial agents there have been few systematic studies of either production or the ecological effects of production (Scott, 2011).

Based on the previous literatures published in Iraq, there is no available studies were done concerning the approach of the present study. Last decades, the treatment of microbial infections has become increasing complicated due to their resistance mechanisms. Thus, this situation is driving the search for novel antibacterial agents that extracted from microorganism, especially the fungi because of increasing the antibiotic resistance by several microbes at their time. Accordingly, the present study was designed.

Aim of the study:

The aim of present study was to prepare, characterize and evaluate of secondary metabolites produced from *Trichophyton rubrum* as a bioactive materials against pathogens, *in vitro*.

To achieve this goal, the following objective were conducted.

1. Isolation and identification of *T. rubrum* using the conventional PCR
2. Determination the optimal conditions for the *T. rubrum* obtaining maximum metabolite.
3. Separation of the secondary metabolites using different solvent sequentially based on their ascending order of polarity using TLC HPLC.
4. Qualitative analysis of different chemical groups in the secondary metabolites to get preliminary idea about the compounds present in the extracts using FTIR.
5. Screening of various pharmacological activities of secondary metabolites.

1.2. Literatures review

1.2.1. The Dermatophytes

Dermatophytes which comprise a group of closely related fungimade up of three genera; *Trichophyton*, *Microsprum*, and *Epidermatophytes*, they have the ability to invade the stratum corenum of the epidermis and keratinized tissues derived from it, such as skin,hair,and nail of humans and other animals (Weitzman *et al.,*1995).

It was one of the most common infections all over the world cause superficial fungal infections that pose public health problems to man and animals). Dermatophytes infections can be disfiguring and recurrent and generally need long-term treatment with antifungal agents ((Havliekova *et al.,* 2008; Ameen, 2010)

Responses to a dermatophyte infection may extent from mild to severe because of the host's reactions to the metabolic products of the fungus, the virulence of the infecting strain or species, the anatomic location of the infection, and local environmental factors (Ameen, 2010).

1.2.2. Etiologic Agent

The etiologic agents of the dermatophytoses were classified in three anamorphic (asexual or imperfect) genera, *Epidermophyton*, *Microsporum*, and *Trichophyton*, of anamorphic class Hyphomycetes of the Deuteromycota (Fungi Imperfecti). The descriptions of the genera essentially follow the classification scheme of Emmons on the bases of conidial morphology and formation of conidia and were updated following the discovery of new species (Ajello 2012).

The genera and their descriptions were as follows; *Epidermophyton spp,. Trichophyton* and *Microsporum.*

***Trichophyton*:**

The genus *Trichophyton* included 24 species. The colonies on agar media SDA were powdery, velvety or waxy. The predominant spore type was microconidia with sparse macroconidia. Reverse side pigmentation was characteristics of the species and was used for the identification of the species within genus (Larone, 1995 ; Jagdish, 1995). The macroconidia were thin-walled with smooth surface and variable shape, some of the *Trichophyton spp* were fastidious in their requirement for amino acid as nitrogen source; *T. tonsurans* require ornithine, citrul-lime and arginine whereas *T. mentagrophytes* require methionine (Jagdish, 1995).

***Microsporum*:**

The genus *Microsporum* includes 16 species. The colony morphology on agar plate surface was either velvety or powdery with white to brown pigmentation (Jagdish, 1995). Both macro and microconidia were produced but the predominant conidial structures were macroconidia and microconidia were less abundant. The macroconidia were multiseptate with thick wall and rough surface. Rarely some species produce neither micron nor macroconidia and they do not have any special nutritional requirement the macroconidia may have thin, moderately thick-to-thick walls and 1 to 15 septa and range in size from 6 to 160 by 6 to 25 mm. Microconidia were sessile or stalked and clavate and usually arranged singly along the hyphae or in racemes as in *Microsporum racemosum*, a rare pathogen (Chen and Friedlander, 2001).

Macroconidia were characterized by the presence of rough walls which may be asperulate, echinulate, or verrucose. Originally, the macroconidia were described by Emmons as spindle shaped or fusiform, but the discovery of new species extended the range from obovate (egg shaped) as in *Microsporum nanum* to cylinder or fusiform as in *M. vanbreuseghemii* (Chen and Friedlander, 2001).

Epidermatophyton:

The genus *Epidermatophyton* includes only two species. The colonies were slow growing, powdery and unique brownish yellow in color. This genus was devoid of microconidia, macroconidia were abundant and produced in clusters these macroconidia were thin-walled with smooth surface. The macroconidia were broadly clavate with typically smooth, thin to moderately thick walls and 1 - 9 septa with size 20 to 60 by 4 to 13 µm in size. They were usually abundant and borne singly or in clusters. Microconidia were absent, this genus has only two known species to date, and only *E. floccosum* was pathogenic (Jagdish, 1995).

1.2.3. Ecology of dermatophytes

Anthropophiles were predominant in the central and northern European countries Examples include: T.rubrum, T. tonsurans (Havillickova et al., 2008).The primary hosts of anthropophilic species were human being but they may also cause infection in animals. Transmission of infection was from human to human (Jagdish, 1995) .

Zoophiles were pathogens with only one animal host and grow as saprophytes on animals materials. Zoophiles were also reported to infect human beings. Human beings acquire the infection from infected

animals; *T. simi* (monkey), *M. canis* (cats and dogs), *M. nanum* (pigs), *T. mentagrophyte* (bovine and sheep) (Schiavoni *et al.,* 2010).

Geophilic species were generally saprophytes and drive nutrients from keratinous substrates. Rarely these pathogens cause infection in animals and human, examples include *T. ajello, M. cookie* and *M. gypsum* (Connole, 1990).

The dermatophyte species within the three genera *Epidermophyton*, *Microsporum* and *Trichophyton* differ in their pathogenicity *in vivo*. While all species invade the stratum corneum of the epidermis and the follicular ostium of hairs, different species vary widely in their capacity to invade hair and nail. The reasons for this observed tissue specificity were unknown, but were thought to be related to specific nutritional requirements or the enzyme production of individual organisms (Alexopoulos *et al.,*1996).

Self-synthesised enzymes serve fungi in a number of ways. They enhance survival in tissues by chemically or physically altering the immediate environment and they act directly by digesting host proteins, thus providing a source of nutrition. Therefore the pathogenic potential of a fungal agent depends on its ability to produce enzymes. In turn variations in enzymatic potential of a fungus may be responsible for differences in the pathogenic effects of various strains. Although all dermatophytes were botanically closely related, each species has certain characteristics in its geographic distribution (Kwon-Chung and Bennett, 1992).

The most prevalent species of dermatophytes may vary strikingly from one geographic locality to another. While many species were cosmopolitan, others have very limited geographic ranges. The reason for these differences were unknown; however, it was clear that changing

patterns of prevalence occur, and were caused by several factors. Which including migration of labor, troop movement, emigration and other travel, changing world patterns of animals husbandry, evolution of new genotypes and the transfer or adaptation of species indigenous to wild animal populations to parasitism in man, and recent therapeutic advances, played important roles in speeding these fungi (Mackenzie *et al.,* .,1986).

The prevalence of the cosmopolitan anthropophilic species in each country was influenced by the constant change of the environment (Kwon-Chung and Bennett, 1992). The rapid transit from continent to continent, and the increasing mobility of people; agents of disease were no longer geographically restricted. Disease contracted half way across the world may become manifest in a country in which the pathogen was not normally found (Philpot, 1978).

Dermatophytosis of scalp (tinea capitis) due to *M. audounii, T. tonsurans, T. violaceum,* and *T. schoenleinii* was highly prevalent in Western Europe during the early 1900s, and then tinea capitis due to the four species has been disappearing since World War II from that region, excluding the Mediterranean area. Sporadic out breaks of tinea capitis still occur but they were usually due to *M. canis* (Kwon-Chung and Bennett, 1992).

Geographic dermatophytes were more significant as agents of ringworm in hotter ,drier climates, while zoophilic fungi usually were more important in colder climates where they may represent over 80% of human infections and this directly related to techniques in farming and animals husbandry in colder were as the high humidity, warmth, and low standard of living, poor hygienic conditions of living as well as customs and traditions were etiological factors causing the high incidence of

dermatophytes in a tropical country (Kamalom *et al.,* 1981). The incidence and type of mycoses vary according to age, geographical distribution of the organism, and the epidemicity of the prevalent species (Ali, 1990).

1.2.4. Clinical manifestation of dermatophytes:

Dermatophytes typically affect the keratinized tissues. They grow on nails, hairs and the outer layer of the skin of both human and other animals. Although, the clinical signs of dermatophytoses may vary depending on the affected region of the body, purities were the most common symptom in humans. The lesions on the skin were often characterized by inflammation severity of the lesions was often obvious at the edges. Scaling, erythematic and sometimes, blister formation were evident. This results in clinical ringworm formation as seen in tinea corporis often resembling a central clearance. Hair loss often results, especially on the facial hair and the scalp (Weitzman and Summerbell, 1995). The infections caused by dermatophytes were commonly referred to as "tinea" or "ringworm" due to the characteristics ringed lesions (Theodore *et al.,* 2008). Clinical types were:

1. Tinea capitis (ringworm of scalp):

It was a dermatophytic infection of the hair and the scalp and begins with a small papule, which spreads to form irregular and scaly forms of alopecia. Typical cases mostly result in the enlargement of cervical and occipital lymph nodes. Sometimes, a boggy inflammatory mass known as a kerion formed. This was common in children worldwide especially in African countries (Macura, 1993).

2. Tinea corporis (glaborous region of the body):

It was often referred to as ringworm, was characterized by single or sometimes multiple scaly lesions, and occurs on the trunk, extremities and face of human. It was more common in children than in adults and occurs most frequently was hot climates similar to that found in many African countries (Macura, 1993).

3. Tinea pedis (athlete's foot):

It was an infection of the foot, characterized by fissures, scales and maceration in the toe web, or scaling of the soles and lateral surface of the feet. It was more common in those who wear occlusive shoes. In majority of cases, vesicles, erytherma, pustules and bullaen may also be present. Anthropophilic dermatophytes were the major cause of tinea pedis. Most common agents were T. rubrum, *T. mentagrophytes var interdigitale* and *E. floccosum* (Macura, 1993).

4. Tinea unguium (onychomycosis, nail):

Thickened; broken and discolored nails usually characterize tinea ungium, a dermatophyte infection of the nail. It was often referred to as onychomycosis and may result in the separation of the nail plate from the nail bed. Both anthropophilic and zoophilic dermatophytes can cause tinea ungium. *T. rubrum* and *T. mentasgrophytes var mentagrophytes* were the most common agents (Nenoff *et al.,* 2007).

5. Tinea manuum (hands infection):

Tinea manuum was a fungal infection of one or occasionally, both hands.It often occurs in patients with tinea pedis. The palmar surface was diffusely dry and hyperkeratosis. When the fingernails were involved vesicles and scant scaling may be present (Goldstein *et al.,* 2000).

6. Tinea barbae (barbaesitch; bearded region of face and nick):

It was an infection of the skin and hair in the beard and mustache area.It was more common in adult men and hirsute women. Because the usual cause was zoophilic organism, farm workers were most often affected. The lesions may include erythematic, scaling and follicular pustules (Elewski, 1999).

7. Tinea cruris (ringworm of the groin):

Tinea cruris, an infection of the groin, this was occurs when ambient temperature and humidity were high. Occlusion from wet or tight-fitting clothing provides an optimal environment for infection. The dermatophytosis was more common in men than in women due to spread of the fungus from feet. Tinea cruris affects the proximal medial thighs and may extend to the buttocks and abdomen thus; the usual causative agents were *T. rubrum* and *T. interdigitale* (Hainer, 2003).

8.Tinea faciei (ringworm of the face):

Tinea faciei seen on the face especially on the non– breaded area the lesions were mostly pruitic with itching and with burning sensation exposure to sunlight could make infected cases worse due to itching and burning. However, red area may be indistinct, especially on pigmented skin, and lesions may have little or no scaling or raised edges. Because of the subtle appearance, this dermatophytosis was sometimes known as tinea "incognito"(Zuber and Baddam, 2001).

1.2.5. *Trichophyton rubrum*:

Trichophyton rubrum was anthropophilic dermatophytes. It was most common agents of dermatophytes, primarily causing tinea pedis,

onychmycosis, tinea corporis and tinea capitis. This fungus was first described by Malmsten in 1845(Hainer, 2003).

Two types of *Trichophyton rubrum* may be distinguished down type and granular type. The downy type has become the most widely distributed dermatophytes of human. It frequently causes chronic infection of skin,nail and rarely scalp. The granular type was frequent cause of tinea corporis in South East Asia (Rebell and Taplin, 1974).

The growth rate of *T. rubrum* in the laboratory can be slow to rather quick. Their texture was waxy, smooth and even to cottony. From the top, the color was white to bright yellowish beige or red violet. Reverse was pale yellowish, brown, or reddish–brown. Microscopically, the downy type was characterized by the production of scanty to moderate numbers of slender cleavage microconidia and no macroconidia. The granular type was characterized by the production of moderate to abundant numbers of cavetto pyriform microconidia and moderate to abundant numbers of thin–walled, cigar-shaped macroconidia. The macroconidia may or may not have terminal appendages. This agent may remain viable in the environment for over six months, thus accounting for widespread infection. Transmission occurs most often from person to person (Rippon, 1988).

There were two methods to identification of *T. rubrum*, the first one, which depended on the phenotype differences (conventional method), and the second method which depended on the molecular differences like PCR method.

1.2.5.1. Identify of *T. rubrum* by Conventional method:

These methods were considered the routine way to characterize *T. rubrum,* the conventional laboratory technique based on detection of

phenotypic characteristic such as (microscopy and *in-vitro* culture) and physiologic properties, which played an essential role in dermatophytes identification (Rippon, 1988).

Conventional method was divided into two ways, the first way direct microscopic examination, which based on detect septate of hyphae and shape of conidia. The advantage of this method was rapid and inexpensive but did not provide genus or species identification and results were negative in 5% to 15% (Mohanty *et al.,* 1999).

Microscopic examination was not species-specific, therefore they were used the second way *in-vitro* culture for identification of species but this way need long time to give result about 3-4 weeks and require a range of culture media such as Urea test medium, Dermatophyte test medium, Sabourauds dextrose agar, Corn meal agar which used to stimulate condition or pigment production, Phenotypic identification can be difficult because intra species morphological polymorphism and phenotypic pleomorphism (Robert and Pihet, 2008).

1.2.5.2. identify of *T.rubrum* by molecular methods:

Polymerase chain reaction was biochemical technology in molecular biology to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating thousand to millions of copies of a particular DNA sequence. Which was developing in 1983 by KaryMullis (Bartlett and Stirrling, 2003).

PCR was common and often indispensable technique used in medical and biological research labs for a variety of applications, These include DNA cloning for sequencing; DNA-based phylogeny or functional analysis of genes; the diagnosis of hereditary disease; the identification of genetic fingerprints (used in forensic sciences and

paternity testing); and the detection and diagnosis of infectious diseases. consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase were key components to enable selective and repeated amplification. As PCR progresses, the DNA generated was itself used as a template for replication, setting in motion a chain reaction in which the DNA template was exponentially amplified PCR can be extensively modified to perform a wide array of genetic manipulations (Saiki *et al.,* 1988).

The ability to accurately identify microorganisms was fundamental to all aspects of fungal epidemiology and diagnosis. In the last ten years, advancements have been made in the molecular diagnosis of fungi through PCR technology. Unlike conventional methods, samples can be tested directly through PCR and isolated without the need for cultures. The technique was fast and highly specific. It can be used to detect trace amounts of fungal DNA from environment samples before symptoms occur. It therefore allows the implementation of early disease control methods (Atkins *et al.,* 2004).

PCR performed routinely and does not require specialized skill to interpret the results. The technology can also offer more accurate quantitative data, providing additional information necessary for decision making and the assessment of how effective fungal agents were in biological control. Since its introduction in the mid-1980s, PCR has become the cornerstone of DNA technology and has cleared the path for the creation of innumerous associated technologies (Dahllof, 2002).

It was remarkable for its ability to detect amounts of DNA amplified from one or few original sequences. Conventional PCR was not

quantitative, but rather qualitative. It was using to detecting, monitor and identify fungi from an entire set of environmental samples and was the core of molecular fungal diagnostics (Atkins *et al.,* 2004).

Real-Time PCR has been successfully used to quantify the number of pathogens, thereby assisting in decisions regarding how to treat fungal diseases and assess the effects of fungi (Mauchline *et al.,* 2002).

1.2.6. Natural Products

The term "natural product" was any biological molecule, but the term usually for secondary metabolites, small molecules (M. wt < 1500 amu). That was produced by an organism but that were not strictly necessary for the survival of the organism, unlike the more prevalent macromolecules such as proteins, nucleic acids, and polysaccharides that make up the basic machinery for the more fundamental processes of life (Richard, 2012).

Concepts of secondary metabolism include products of overflow metabolism because of nutrient limitation, shunt metabolism produced during idiophase, defense mechanism regulator molecules (Satyajit *et al.,* 2006).

Fungal secondary metabolites (figure 1.1) were mostly derived from acetate and shikimic acid while the production of a required secondary metabolite, it was vital to make suitable conditions for metabolic pathways with providing throughout the trophophase to maximize growth of the microbial species. They were important that the conditions were altering properly at the suitable time of fermentation to get the best product yield (Jacob *et al.,* 2011).

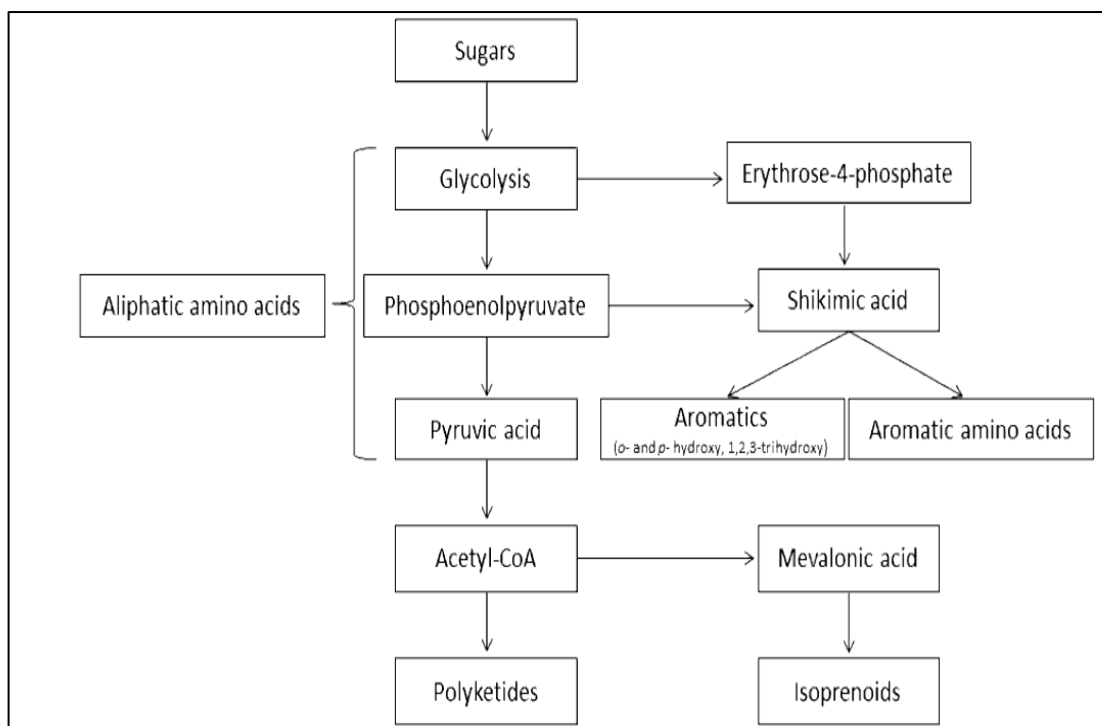


Figure 1.1.Scheme of enzymatic pathways for secondary metabolite production in fungi (Jacob *et al*., 2011)

Besides its fundamental role in fatty acid synthesis (FAS), the primary metabolite acetate was the most common carbon source for secondary metabolites of the polyketide and terpene classes. Polyketides were derived from the activated forms of acetic acid; acetyl CoA and malonyl CoA (Manitto, 1981).

This biosynthetic pathway was responsible for the wide variety of secondary metabolites produced by fungi and bacteria. Highly reactive poly-β-ketoacids were formed by the sequential condensation and addition of C2 units. This reaction was similar to FAS and was catalyzed by polyketide synthases (PKS). Unlike in FAS, the growth of the polyketide chain does not require reduction of the previous carbonyl unit to a methylene group. The first C2unit was derived from acetyl CoA while the subsequent C2 units were derived from malonyl-CoA (Manitto 1981).

Microorganisms were cultured under perfect conditions for primary metabolism without environmental limitations effort to make as large or great as possible the microbial biomass formation. Under conditions of balanced growth, however, the fungi cell minimizes the accumulation of any particular cellular building blocks in amounts beyond those required for growth. Consequently, the metabolic pathway of a particular microorganism can be manipulated for the production of a large surplus of the desired metabolite. The production of secondary metabolites starts as growth was limited due to the unavailability of one of the key nutrients for example, nitrogen, carbon, phosphorus, and so on (Vaishnav *et al.,,.* 2011).

Most Secondary metabolites were complex organic molecules that need a large number of specific enzymatic reactions for synthesis. The enzymes involved in the making of the secondary metabolites were controlled independently from the enzymes of primary metabolism. In some cases, specific inducers of secondary metabolite production have been identified. The metabolic pathways of these secondary metabolites start from primary metabolism, because the starting materials for the secondary metabolism come from the major biosynthetic pathways. Many structurally complex secondary metabolites arise from structurally quite similar precursors. Thus, the secondary metabolite generally produced from several intermediate products that accumulate in the fermentation medium or in microbial cells during primary metabolism (Arnold, 2014).

1.2.7. Production of Secondary Metabolites production from Fungi

The Secondary metabolites usually accumulate during the later stage of fermentation, known as the idiophase, which follows the active growth phase called the trophophase. Compounds produced in the

idiophase have no direct relationship to the synthesis of cell material and normal growth of the microorganisms. Secondary metabolites were forming in a fermentation medium after the microbial growth was completed. Comparatively, a few fungi produce the majority of secondary metabolites, a single microbial type has the capacity to produce very different metabolites, for example, *Streptomyces griseus*, and *Bacillus subtilis* each can produce more than 50 different secondary metabolites. The most common secondary metabolites were antibiotics; others include mycotoxins, ergot alkaloids, the widely used immunosuppressant cyclosporin, and fumagillin, an inhibitor of angiogenesis and a suppressor of tumor growth (Batt *et al.,* 2014).

The *T. rubrum* usually produce secondary metabolites in only tiny amounts due to the evolution of regulatory mechanisms that limit production to a low level. Such a level was probably enough to allow the organism to compete with other organisms and/or coexist with other living species in nature (Arnold, 2014).

1.2.8. Classification of secondary metabolites

Secondary metabolites can be divided into several chemical classes. Most fungal secondary metabolites fall into four main chemical classes; peptides, alkaloids, terpenes and polyketides (Arnold, 2014).

A. Peptide metabolites can be either ribosomal peptides or nonribosomal peptides. Ribosomal peptides synthesized by translation of mRNA. These kinds of peptides usually undergo a post-translational modification after its being translated. Fungal nonribosomal peptides, were synthesized by specific enzymes rather than the ribosomes fungal peptides include penicillin, which

has anti-bacterial properties and destruxin A, which has insecticidal as well as anti-viral properties (Keller *et al.,* 2005)

B. Alkaloids were derivatives of amino acids. Many alkaloids possess pharmacological effects on humans and animals. They can be further divided into 10 group (pyridine, pyrrolydine, tropane, quinoline, isoquinoline, phenetylamine, indole, purine, terpenoid and vinca alkaloids) based on the metabolic pathway. Examples of fungal alkaloids were ergopeptides, used to treat migraine as well as Parkinson's disease and fumitremorgen C, a mycotoxins (Hoffmeister *et al.,*2007)

C. Terpenes were a large and varied class of hydrocarbons. They were derived from polymerization of isoprene subunits. The basic molecular formula for terpenes was $(C_5H_8)_n$, where n was the number of linked isoprene units. The terpene metabolites can be further divided into hemiterpenes, monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, triterpenes and tetraterpenes, depending on their size. Examples of fungal terpenes were vomitoxin. Vomitoxin possesses mycotoxin properties while (Karioti *et al.,* 2006).

D. Polyketides were the most abundant fungal secondary metabolites and have an extremely broad range of biological activities and pharmacological properties. Polyketides was derived from the condensation of acetyl-SCoA with malonyl-ScoA units. For fungal polyketides, the enzymes responsible for their synthesis were type I polyketide synthases, examples of fungal polyketides include the mycotoxic aflatoxins and lovastatin, a cholesterol-lowering agent (Keller *et al.,* 2005).

1.2.9. Isolation and Characterization of Secondary Metabolites

1.2.9.1. Isolation of metabolites

Chromatography was a useful technique for the separation of compounds from a complex mixture, such as a fungal extract. Based on the physical and chemical properties of compounds and their affinities for certain solid phase materials (e.g., silica), a mixture can be separated into its individual compounds, or at least into mixtures containing fewer compounds with similar characteristics by selecting the appropriate elution solvent or solvent system (Harris 2003).

Often, when working with complex mixtures such as biological it was necessary to repeat the technique, varying the parameters until compounds of sufficient purity for structural characterization can be obtained or even employ many chromatographic techniques in succession. The most common technique employed in the separation and analysis of low molecular weight secondary metabolites was reverse-phase High Performance Liquid Chromatography (HPLC) (Frisvard, 1987). Many robust reverse-phase HPLC methods were described in the literature for the identification and analysis of various mycotoxins (Frisvard and Thrane 1987).

High Performance Liquid Chromatography (HPLC) system with a larger column, semi-preparative or preparative HPLC can be used to isolate and purify individual compounds from a mixture. The larger sized column allows for the introduction of larger samples into the column. Individual peaks can then be collected once they exit the detector. Isolation of pure metabolites was essential for structural elucidation. Once a pure metabolite has been isolated, mass spectrometry along with

1D and 2D Nucleic magnetic Resonance (NMR) experiments were used to unambiguously elucidate the chemical structure.

1.2.9.2.Characterization of metabolites

1. Mass spectrometry

The mass of a pure compound was determined using mass spectrometry (MS). Often a gas or liquid chromatography (GC or LC respectively) system was coupled to a mass spectrometer. Coupling an LC system to an electrospray ionization (ESI) MS system allows for the sample to be dissolved in a solvent, introduced into the LC system, separated into its individual components and then transferred to the gas phase (without decomposition) and ionized before entering the mass analyzer (Pavia *et al.,*. 2009; Kebarle and Verker, 2010).

Electrospray ionization -mass spectrometry (ESI-MS) was a useful technique for analyzing high molecular weight biomolecules as well as small non-volatile compounds (Pavia *et al., 2009*). It was especially useful when trying to characterize unknown compounds since it may not be known if the compounds were thermally stable (Pavia *et al.,* 2009). ESI was a soft ionization technique that, when operated in positive mode, often produces the protonated molecular species ([M+H]+) for a variety of different types of compounds (Smedsgaard and Nielsen 2005).

The mass spectrum analysis can also provide other useful information about the compound being studied. For example, molecules containing chlorine or bromine atoms will display two molecular ion peaks; one for each of its commonly occurring isotopes (Pavia *et al.,* 2009).

2. Nuclear Magnetic Resonance NMR

In conjunction with the mass of the compound, both 13C and 1H NMR data were necessary to properly characterize a metabolite. Obtaining a 135-DEPT spectrum can also be useful since it can help to assign carbons as quaternary, methylene, methine or methyl. To be able to correlate proton signals with their corresponding carbon signals, or to determine whether they belong to an amine or hydroxyl group, heteronuclear correlation spectroscopy was employed (Balci, 2005).

Heteronuclear chemical shift correlation (HETCOR), or heteronuclear single-quantum correlation (HSQC) reveal the correlation between protons and carbons with a two dimensional plot. HSQC was a 1H-detected experiment whereas HETCOR was an X-detected experiment (here 13C). Since 1H protons were more abundant than 13C atoms, and have a higher magnetogyric ratio they were easier to detect and therefore result in a more resolved spectrum obtained over a shorter period of time, HETCOR would be a more useful technique when the carbon spectrum was crowded and better resolution of that parameter was required (Balci, 2005; Pavia *et al.,* 2009).

3. Ultraviolet and visible spectroscopy UV-VIS

Ultraviolet and visible spectroscopy were of limited use in characterizing compounds, however, along with infrared spectroscopy and NMR can provide valuable structural information to support a potential structure. For example, it was possible to correlate certain absorptions in the UV-VIS wavelengths range with features such as the presence of alkenes, and carbonyls (Pavia *et al.,* 2009).

4. Infrared region IR

Each type of bond in a molecule absorbs a different frequency of energy. Therefore, certain characteristic absorptions in the infrared region (IR) can give valuable information on the structure of the compound being analyzed. For example, the presence of a broad O-H stretch in the range of 3400-2400 cm-1 and a carbonyl stretch between 1730 and 1700 cm-1 indicates the presence of a carboxylic acid. Unless two compounds were identical it was not possible to use an IR spectrum to identify an unknown metabolite, however it can be used to confirm the presence of certain chemical features (Pavia *et al.,* 2009).

1.2.10. Applications of Secondary Metabolites

Through the years, the pharmaceutical manufacturing increased their biological effective screening programs secondary metabolites Fungi were such a prolific origin of structurally various bioactive metabolites; the industry extended their screening programs order to look for fungi with activity in other disease areas. As a result of this move, some of the most important products of the pharmaceutical industry were obtained for example, the antimicrobial have uprising medicine by facilitating treatment of infectious disease (Kremer *et al.,* 2000).

Other products include antitumor drugs, hypocholesterolemic drugs, enzyme inhibitors, gastrointestinal motor stimulator agents, ruminant growth stimulants, insecticides, herbicides, antiparasitics versus coccidia and helminths, and other pharmacological activities. Catalyzed by the use of simple enzyme assays for screening prior to testing in intact animals or in the field, further applications were emerging in various areas of pharmacology and agriculture (Verdine, 2015).

1. Antibiotics

The 12,000 antibiotics known in 1955, filamentous fungi produced 22 %. The beta-lactams were the most important class of antibiotics in terms of use. They constitute a major part of the antibiotic market. Including penicillins, cephalosporins, clavulanic acid, Kojic acid, Fusidic acid and the carbapenems. Fungi were responsible for production of penicillins and cephalosporins (Berdy, 1995 ,Brown, 1996 and Yang *et al.,* 2012).

Despite these impressive figures, more antibiotics were needed to struggle evolving pathogens, indeed resistant microbes, bacteria and fungi that have developed resistance to current antibiotics. A new and approved cephalosporin was ceftobiprole, which was active against methicillin-resistant *Staphylococous aureus* (MRSA) and was not hydrolyzed by a number of beta-lactamases from Gram-positive bacteria. Another antibiotic was cerulenin, an antifungal agent produced by *Acremonium caerelens*. It was the first inhibitor of fatty acid biosynthesis discovered. It alkylates and inactivates the active-site nucleophylic cysteine of the ketosynthase enzyme of fatty acid synthetase by epoxide ring opening. Other properties that were desired in new antibiotics were improved pharmacological properties, ability to combat viruses and parasites, and improved potency and safety (Shang, 2010).

2. Pharmacological Agents

Noninfectious diseases were mainly treated with synthetic compounds.Despite testing thousands of synthetic chemicals; only a handful of promising structures was obtained. As new synthetic lead compounds became extremely difficult to find, microbial products came into play. Poor or toxic antibiotics produced by fungi such as cyclosporin A or mycotoxins such as ergot alkaloids, gibberellins, zearelanone were

then successfully applied in medicine and agriculture. This led to make fungal products as immunosuppressive agents, hypocholesterolemic drugs, antitumor agents, and for other applications (Shang, 2010).

A. Hypocholesterolemic Agents

Only about 30 % of cholesterol in humans comes from the diet. The body, predominantly in the liver, synthesizes the rest. Many people cannot control their level of cholesterol at a healthy level by diet al.,one and require hypocholesterolemic agents. High blood cholesterol leads to atherosclerosis, which was a chronic, progressive disease characterized by continuous accumulation of atheromatous plaque within the arterial wall, causing stenosis and ischemia. Atherosclerosis was a leading cause of human death (Nicholls, 2007).

The last two decades have witnessed the introduction of a variety of anti-atherosclerotic therapies. The statins form a class of hypolipidemic drugs, formed as secondary metabolites by fungi, and used to lower cholesterol by inhibiting the rate-limiting enzyme of the mevalonate pathway of cholesterol biosynthesis; i.e., 3-hydroxymethyl glutaryl-CoA (HMG-CoA) reductase. Inhibition of this enzyme in the liver stimulates low-density lipoprotein (LDL) receptors, resulting in an increased clearance of LDL from the bloodstream and a decrease in blood cholesterol levels (Shang, 2010).

This first member of the group was isolated as an antibiotic product of *Penicillium brevicompactum* at about the same time, it was found by Endo as a cholesterolemic product of *Penicillium citrinum,* although compactin was not of commercial importance, its derivatives achieved strong medical and commercial success (Endo, 2010).

B. Anticancer Drugs

More than 12 million new cases of cancer were diagnosed in the world in 2015; 6.6 million cases were in men and 6.0 million in women, resulting in 7.6 million cancer related deaths. The tumor types with the highest incidence were lung (12.7 %), breast (10.9 %), and colorectal (9.8 %). Some of the anticancer drugs in clinical use were secondary metabolites derived from plants and fungi. Among the approved products were taxol and camptothecin. Taxol (paclitaxel) was first isolated from the Pacific yew tree, *Taxus brevifolia* (Wall *et al.,* 1996).

It was a steroidal alkaloid diterpene alkaloid that has a characteristic *N* -benzoylphenyl isoserine side chain and a tetracycline ring the chemical structure which The benzoyl group was located in the left side of the structure that shown in Figure (1.2) (Newman *et al.,* 2007).

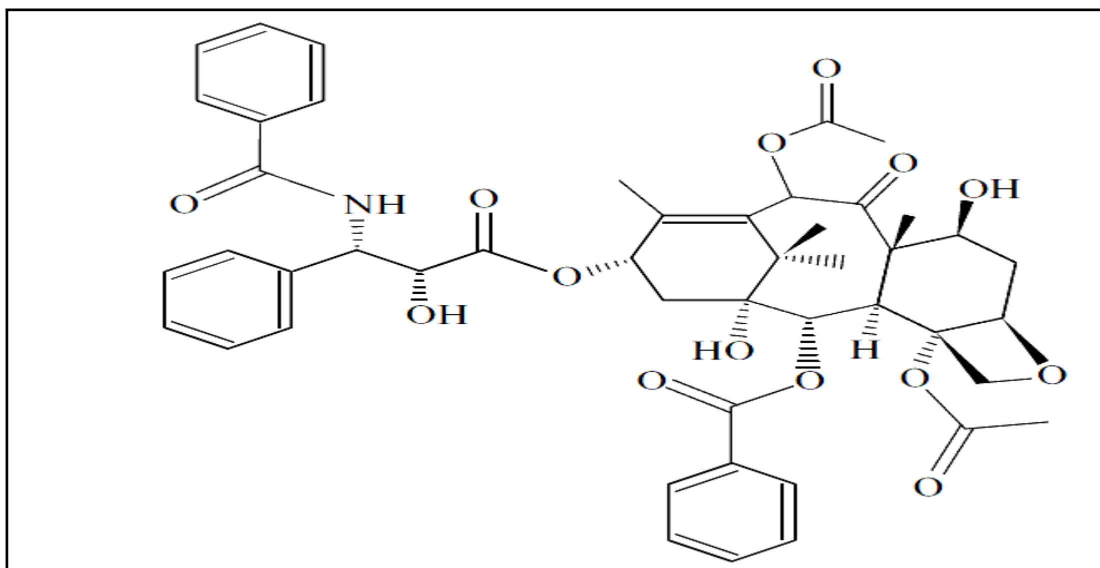


Figure 1.2 Chemical structure of Taxol. The benzoyl group was located in the left side of the structure (Newman *et al.,* 2007).

It inhibits rapidly dividing mammalian cancer cells by promoting tubulin polymerization and interfering with normal microtubule

breakdown during cell division. The benzoyl group of the molecule was particularly crucial for maintaining the strong bioactivity of taxol. The drug also inhibits several fungi (species of *Pythium, Phytophthora, Aphanomyces*) by the same mechanism. In 1992, taxol was approved for refractory ovarian cancer and used against breast cancer and advanced forms of Kaposi's sarcoma (Newman *et al.,* 2007).

Another important antitumor agent was camptothecin, a modifi ed monoterpene indole alkaloid produced by certain plants (angiosperms) and by the endophytic fungus, *Entrophospora infrequens.* The fungus was isolated from the plant *Nathapodytes foetida* (Wall *et al.,* 1996).

In view of the low concentration of camptothecin in tree roots and poor yield from chemical synthesis, the fungal fermentation was very promising for industrial production of camptothecin. It was used for recurrent colon cancer and has unusual activity against lung, ovarian, and uterine cancer (Amna *et al.,* 2006).

Colon cancer was the second-leading cause of cancer fatalities in the USA and the third most common cancer among US citizens. Camptothecin was known commercially as Camptosar (Lorence *et al.,* 2004). Camptothecin's watersoluble derivatives irinotecan and topotecan have been approved and were used clinically. Metastatic colorectal cancer was treated by irinotecan whereas topotecan has use for ovarian cancer, cervical cancer, and small-cell lung cancer. A review of the activities of camptothecin and its many small and macromolecular derivatives has been published by Venditto and Simanek (Venditto *et al.,* 2010).

3. Immunosuppressant Drugs

An individual's immune system was capable of distinguish between native and foreign antigens and to mount a response only against the latter. Suppressor cells were critical in the regulation of the normal immune response. The suppression of the immune response, either by drugs or by radiation, in order to prevent the rejection of grafts or transplants or to control autoimmune diseases, called immunosuppression (Borel,2002).

Microbial compounds capable of suppressing the immune response have been discovered as fungal secondary metabolites. Cyclosporin A was originally discovered in the 1970s as a narrow-spectrum antifungal peptide produced by the mold, *Tolypocladium nivenum* (previously *Tolupocladium infl atum*) in an aerobic fermentation (Borel,2002).

Cyclosporins were a family of neutral, highly lipophilic, cyclic undecapeptides containing some unusual amino acids, synthesized by a nonribosomal peptide synthetase, cyclosporin synthetase. Discovery of the immunosuppressive activity of this secondary metabolite led to use in heart, liver, and kidney transplants and to the overwhelming success of the organ transplant field (Borel 2002).

Cyclosporin was approved for use in 1983. It was thought to bind to the cytosolic protein cyclophilin (immunophilin) of immunocompetent lymphocytes, especially *T* -lymphocytes. This complex of cyclosporin and cyclophilin inhibits calcineurin, which under normal circumstances was responsible for activating the transcription of interleukin-2. It also inhibits lymphokine production and interleukin release and therefore leads to a reduced function of effector *T* -cells. Cyclosporin A also has activity against corona viruses (DeWilde *et al.,* 2011).

2.2.11. The secondary metabolites of *T.rubrum* Products:

The production of antibiotics by dermatophytes fungi has long been known to produce antibiotics in vitro and in vivo, showed that of *T.rubrum* and *T. mentagrophytes* strains tested would produce penicillin and other antibiotics whilst of *Epidermophyton floccosum* could produce penicillins, fusidanes and a variety of other compounds (Youssef *et al*.,1979).

Using HPLC showed that penicillins X and G were produced whilst found that the fusidanes included fusidic acid, 3-ketofusidic acid and the diketo- compound. The diversity of antibiotics produced suggested to us that antibiotic production might be used as a tool for typing the dermatophytes (Lappin *et al*.,1985; Perry *et al*.,1983). Four types of antibiotics have been revealed: a penicillin-like substance produced by the strains of *Trichophyton* rubrum and two different types of unknown substances obtained from *Trichophyton mentagrophytes* var. *interdigitales* and the Kojic acid-like antibiotics substance which has been given by Trichophyton *verruccosum* strain(Hammadi *et al*.,2007).

1.Benzyl penicillin G

Benzylpenicillin also known as penicillin G, Benzylpenicillin (figure 1.3) was a β-lactam antibiotic produced by *Penicillinum spp*. It was bactericidal against streptococci, neisseriae, many anaerobes and spirochaetes. Penicillin G was typically given by injection parenterally, bypassing the intestines, because it was unstable in the highly acidic stomach. Because the drug was given parenterally, higher tissue concentrations of penicillin G can be achieved than was possible with phenoxymethylpenicillin. These higher concentrations translate to increased antibacterial activity (Rossi,2013).

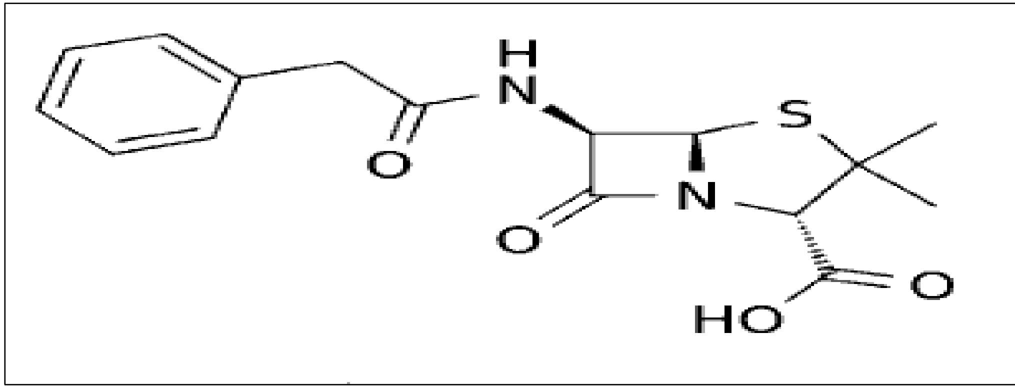


Figure: (1-3) chemical structure of penicillin G (Rossi,2013).

2. Fusidic acid

Fusidic acid was a therapeutically useful antibiotic, being particularly active against penicillin resistant staphylococcal infections, and has been isolated from Fusidium coccineuml', Mucor ramannianus Cephalosporiurn lamellaecula and Paecilomyces fiusidioides). The structure of fusidic acid was characterised by the presence of the cyclopentanoperhydrophenan threne ring system show in figure (1-4) (Perry *et al.,*1983). Although certain strains of *M. gypseum*, *Trichophyton longifusum* and *E. floccosum* have previously been reported to produce an antibiotic with TLC mobility similar to fusidic (Elander *et al*.,1969).

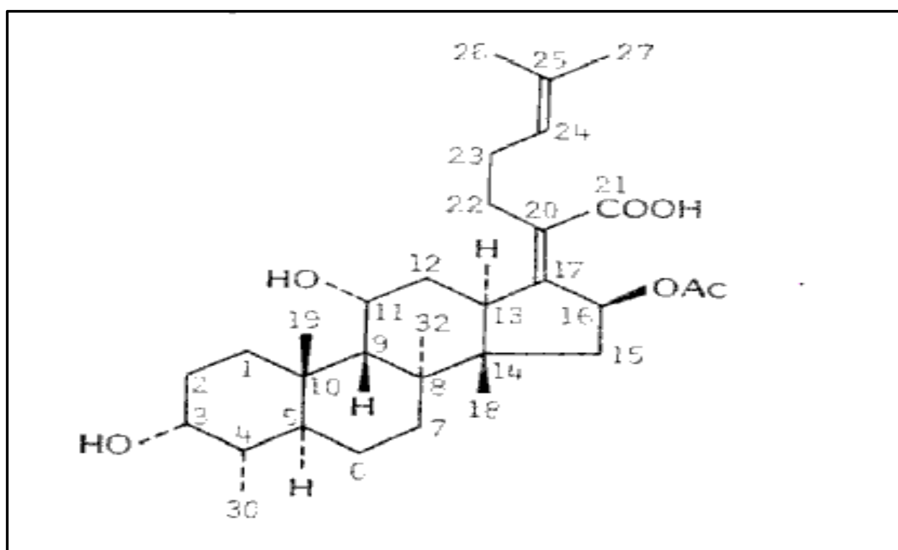


Figure: (1-4) chemical structure of fusidic acid (Elander *et al.,*1969).

3. kojic acid

Kojic acid (5-hydroxy-2-(hydroxymethyl)-4-pyrone; KA) was a major secondary metabolite produced by a limited range of microorganisms, including *A. oryzae, Aspergillus flavus*, and *Aspergillus tamarii*, as well as *Penicillium* species and certain bacteria in the stationary phase of growth (Bentley, 2006; Yasunobu *et al*., 2010) show structure Kojic acid (figure 1-5).

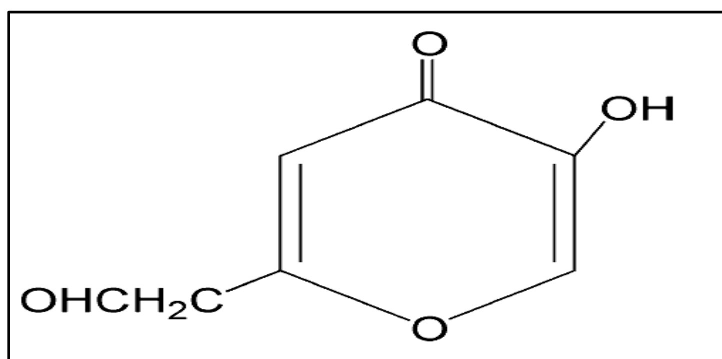


Figure: (1-5) chemical structure of the kojic acid structure (Yasunobu *et al.,* 2010)

1.2.12. Biological activity of Secondary metabolites of *T.rubrum*

1. Antimicrobial Screening:

The main objective of performing the antibacterial screening was to determine the susceptibility of the pathogenic microorganisms to test secondary metabolites that, in turn was used to selection of the compound as a therapeutic agent, Aantimicrobial assay was essentially a qualitative or semi qualitative test that indicates the sensitivity or resistance of microorganisms to the secondary metabolites. However, this technique cannot be used to distinguish between bacteriostatic and bactericidal agents. The primary assay can be performed in vitro by disk diffusion assay method, which includes (Reiner *et al*., 1982).

a) Plate diffusion test

b) Streak test

The plate diffusion test utilizes different concentrations of a test compound absorbed on sterile filter paper disks on the same plate whereas the streak test permits the determination of the antibacterial effect of a test compound on several microorganisms simultaneously and was suitable for the estimation of the spectrum of the activity. However, the plate diffusion test was commonly used (Reiner *et al*., 1982).

2. Cytotoxicity Study

Cytotoxicity was the quality of being toxic to cells. One biological approach involves monitoring the cytotoxicity of the extracts of subfractions against the napulii, *Artemia salina*. The susceptibility *of Artemia salina* (*Artemiidae*), or brine shrimp larvae to treatment with secondary metabolites extracts can be used as a measure of toxicity of chemicals as well as natural products (Logarta *et al*., 2001).

The Brine Shrimp lethality test was a simple bench top bioassay used to screen secondary metabolites extracts of fungi for biological activity and has yielded good results (Ajaiyeoba *et al*., 2006).A wide variety of chemicals as well as natural products were toxic towards brine shrimp nauplii; the death of this organism when exposed to the various secondary metabolites extracts of fungi concentrations forms the basis of the toxicity test the assay was capable of detecting a broad spectrum of bioactivity present in crude extracts (Reiner *et al*., 1982).

3.Antioxidant activity

An antioxidant was a molecule that inhibits the oxidation of other molecules. Oxidation was a chemical reaction that transfers electrons or

hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants were often reducing agents such as thiols, ascorbic acid, or polyphenols (Krishnaiah *et al.,* 2010).

Free Radical Scavenging Activity (DPPH Assay Method) was first described by Blois in 1985 and was later modified slightly by numerous researchers. A rapid, simple and inexpensive method to measure antioxidant capacity involves the use of the free radical, 2,2-Diphenyl-1-picrylhydrazyl (DPPH). DPPH was widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and to evaluate antioxidant activity (Krishnaiah *et al.,* 2010). Where the DPPH was a dark-colored crystalline powder composed of stable free-radical with red color(absorbed at 517nm. DPPH was a well-known radical and a trap ("scavenger") for other radicals. Therefore, rate reduction of a chemical reaction upon addition of DPPH was used as an indicator of the radical nature of that reaction (Krishnaiah *et al.,* 2010).

MATERIALS AND METHODS

2.1 Materials

2.1.1. Patients:

One hundreds of patients from both sexes and with different ages were clinically diagnosed as cases of dermatophytoses by clinician after attended the outpatient clinic of the Department of Dermatology at AL-Diwaniya Teaching Hospital during period from the December 2014 till to of May 2015 . a questionnaire forma was done for each patient to record the patient' s information.

2.1.2 Apparatus:

Table (2-1) shows the apparatus and their remarks that used in this study.

Table (2-1): The apparatus used in this study.

Apparatus Names	Company and Origin
Autoclave	Express -Japan
Deep freeze	Sanyo-Japan
Distiller	Controls-England
Electrophoresis unit	Consort-Belgium
Eppendorf tubes	Sigma/England
Filter paper	Biobasic inc./korea
Gel documentation system	Consort-Belgium
Hot plate magnetic stirrer	IKA-USA
HPLC- Shimadzu model LC-2010HT	Shimadzu- Japan
Incubator	Memmert – Germany
IR Prestige-21 (Fourier Transform	SHIMADZU , Japan

InfraredSpectrophotometer)	
Laminar air flow hood	Techne- UK
Magnetic stirrer	Scientific Industries-USA
Nanodrop	Thermo Scientific/ UK
pH-Meter	Bio Red – Italy
Power supply	Consort- German
Refrigerator	Ishtar-Iraq
Rotary evaporator	Buchii -Switzerland
Scanning electron microscope	Inspect S50/FEI/Netherland
Sensitive electronic Balance	Sortorius-Germany
Spectrophotometer	Shimadzu- Japan
Thermo cycler	Bioneer/ Korea
Ultra violet transilluminator	Consort-Germany
Ultraviolet Spectrophotometer	Agilent 8453 , Agilent Technologist , USA
Vortex Stuart	Scientific-UK
Water bath	Memmert – Germany

2.1.3 Chemicals and Biological Materials:

Table (2-2) shows the chemicals and Biological Materials used in this Study.

Table (2-2): The Chemicals and Biological Materials used in this study.

Materials	Company and origin
1,1-Diphenyl-2-picryl hydrazyl (DPPH)	MP Biomedicals Ltd., USA
Absolute ethyl alcohol	BDH-England
Acetone	BDH-England
Acetonitrile	BDH -England
Agarose	Promega-USA
Ammonium Acetate (CH3COONH4)	Thomas Bakar-India
Ascorbic acid	SD fine chemicals Ltd.,India

Boric acid	Fluka – Switzerland
Bromophenol Blue	Sigma-USA
Chloramphenicol	SDI-Iraq
Chloroform	BDH-England
CTAB (cetyltrimethyl ammonium bromide)	Riedel-de Haën –Germany
Cycloheximide	Fluka-Germany
DEPC water	Bioneer (Korea)
EDTA(ethylene diamintetra acetate)	BDH-England
Ethanol Alcohol 70%	Hazard-UK
Ethidium bromide	Promega- USA
Glutaraldehyde	BDH –Netherland
Glycerol	Fluka – Switzerland
HCl	BDH – England
Hexan	BDH – England
Isoamyl Alcohol	Thomas Bakar-India
Isopropanol	BDH (England)
lactophenol blue stain	BDH-England
Peptone	Difco-USA
Phenocrystals	Fluka-Germany
Potassium dihydrogen phosphate	Hazard-UK
Sodium chloride (NaCl2)	BDH-England
Sodium hydroxide (NaOH)	Fluka – Switzerland
Sodium sacodylate buffer(PH 7.4)	BDH -Netherland
TBE-buffer (10x)	Promega – USA
Tris-Base	Thomas Bakar-India
Vincristine sulphate	Ibn sina industrial company-Iran
Yeast extract	Fluka – Germany

2.1.4. Study Design

The present study was designed according to the following steps (figure 2.1)

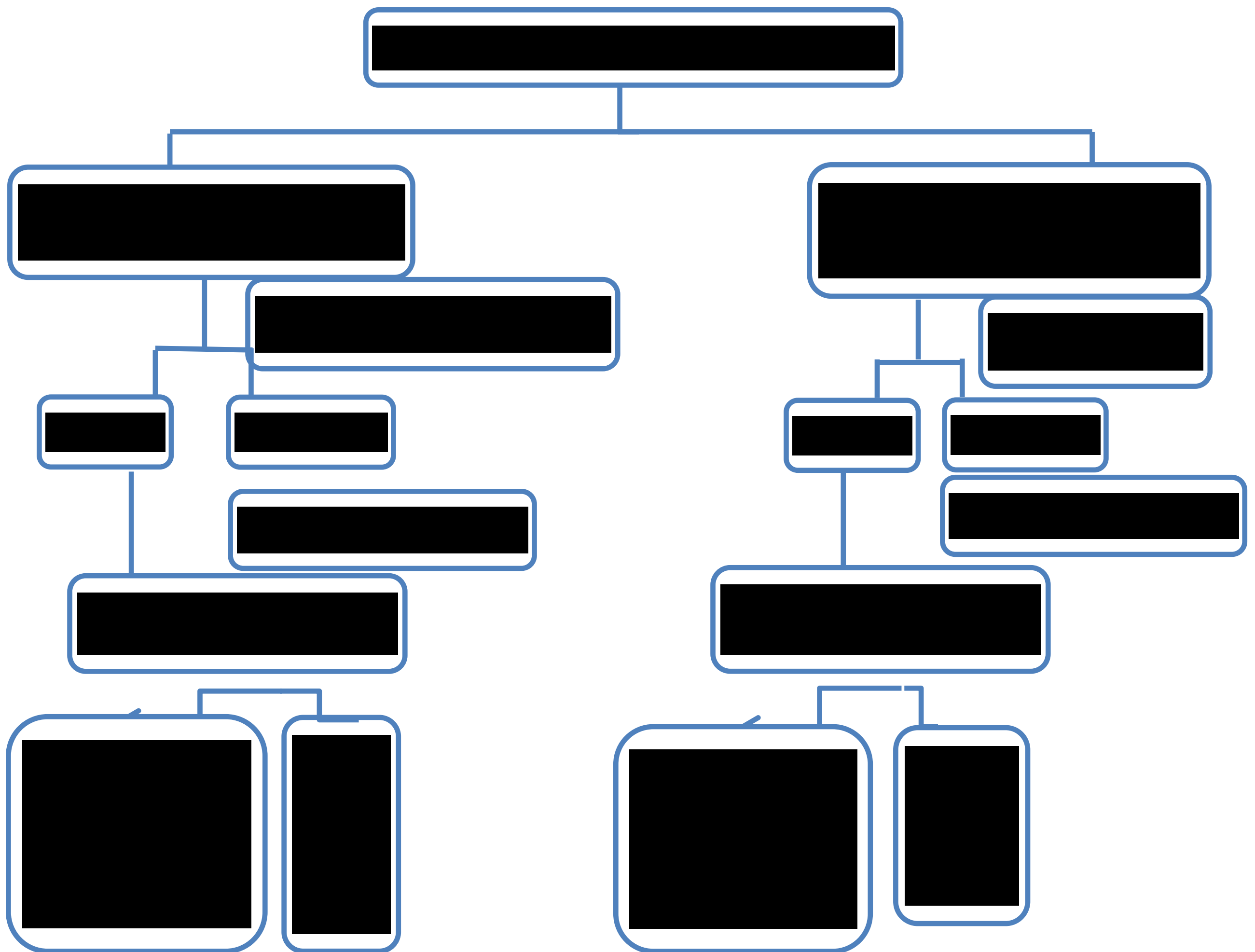


Figure: 2.1 Scheme of study design of Acetone Secondary metabolites of *T. rubrum* extraction

2.1.5. Culture Media:

The media which were used in the present study divided as ready-made media and prepared media.

- The ready-made media are illustrated in table (2-3).

Table 2-3: The ready- made media with their remarks.

Media	**Company /origin**	**Purpose of used**
Sabouraod s dextrose agar	Difco-USA	Routine culture of dermatophytes
Mueller Hinton Agar	Himedia-India	Antibiotics sensitivity test

- The prepared media included the following:-

1. Sabouraud's Dextrose agar with chloramphenicol and Cyclohexamide modified

It was prepared from Dextrose 40 g, Peptone 10 g, Agar 20 g, Chloramphenicol 0.05 g, Cyclohexamide 0.5 g then dissolved in 1000 ml distal water. (Mackenzie *et al*., 1986) , it used isolation and purification of isolate *T.rubrum*.

2. The Modified Horse Hair dextrose medium (HHD):

It was prepared from Sabouraud dextrose broth 35g in 1000ml D.W, tryptone 5g and horse hair 2g, the medium was adjusted in accordance with pH 4.8 and was administered in 250 ml volumes in 500 ml cone like flagons (Erlenmeyer flask) closed with cotton fleece plugs (Hammadi *et al.,*2007), it used for production secondary metabolites *T.rubrum* .

3. The modified keratinized skin dextrose medium (KSD):

It was prepare from Human callus stratum corneum (3g) as sole supplements with Sabouraud dextrose broth 35g in 1L D.W, media will be adjusted to pH 4.8 and was apportioned in 250 ml volumes in 500 ml funnel shaped cups (Erlenmeyer flagon) closed with cotton fleece plugs (Colin *et al.,*1981) it used for production secondary metabolites *T.rubrum* .

All the used media were sterilized by autoclaved at 15 Ibs/inch ^{2} , 121 ^oC and 15 min.

2.1.6. Reagents:

1.Potassium hydroxide (KOH)

It was prepared KOH crystals (10 g), Glycerol (10 g) and Distilled water (80ml) used for direct detection of fungal elements in Clinical specimens by microscopically examination (Suhnen *et al*.,1999).

2. Lacto phenol cotton blue stain:

It was used for staining and microscopic identification of fungi Cotton blue (0.05 g), Phenol crystals (20 g), Glycerol (40 ml) and Lactic acid (20 ml) and Distilled water (20 ml). The ingredients were dissolved by heating in a water bath. Then (0.05 g) of Cotton Blue added (Ellis, 1994).

2.1.7. PCR diagnostic Kits:

Table (2-4) shows the contents of PCR kit that used for extraction of genomic DNA of the fungus *T. rubrum*.

Table (2-4): Illustrated the contents of PCR kits

No.	Kit	Company	Country
3	**EZ-10 Spin Column Fungal Genomic DNA Mini-Preps Kit**	**BioBasic**	**Canada**
	EZ-10 Column 50		

	2.0 ml Collection Tube 50		
	Universal Digestion Buffer 12 ml		
	Universal Buffer PF 6 ml		
	Universal Buffer BD 12 ml		
	Universal PW Solution (concentrate) 18 ml		
	Universal Wash Solution (concentrate) 7.5 ml		
	TE Buffer 10 ml		
	Proteinase K (10mg/ml) 1.2 ml		
4	AccuPower^{TM} PCR PreMix	Bioneer	South Korea
	Taq DNA polymerase		
	dNTPs (dATP, dCTP, dGTP, dTTP)		
	Tris-HCl pH 9.0		
	KCl		
	$MgCl_2$		
	Stabilizer and Tracking dye		

2.1.8. Primers

The PCR primers that used in the present study were designed by using the complete sequence of *T.rubrum* 5.8S rRNA gene, internal transcribed spacer 1(ITS1) Gene Bank: (AJ270806.1) by using NCBI Gene Bank data base and Primer 3 plus online, and provided by Bonier company, south Korea) company as shown in table (2-5) (Brasch et al.,2010).

Table (2-5): The primers used and their sequences for detection of *T.rubrum*

Primer name	**Sequence(5'-----3')**	**Amplicon size**
ITS1	**F-5'ACGATAGGGACCGACGTTCC-3'**	601bp

	R-5'-CCCTACCTGATCCGAGGTCA-3'	

F: Forward , R:Reversed

2.1.9. Molecular DNA markers

The molecular markers that used in this work was listed in table (2-6)

Table (2-6): Molecular markers sizes as DNA marker for electrophoresis

DNA markers	Description	Manufacture company/state
100 bp ladder	100-200 base pairs. The ladder consist of 20 double strand DNA fragment with size of 100-200 bp present at triple the intensity of other fragment and serve as reference all other fragments appear with equal intensity on gel.	US Biological USA .

2.2. Methods

2.2.1. Collection of specimens:

One hundreds patients were clinically diagnosed as cases of dermatophytoses after attended the consultant clinic of the Department of Dermatology and venereal diseases at AL-Diwaniya Teaching Hospital during period from the December 2014 till to of May 2015. Duplicate specimens(skin scrapings, hair fragments, nail clippings) from each lesion (tinea) were collected using standard procedures then transferred to

laboratory for processing , examination and culturing (Suhnen *et al.*,1999).

2.2.1.2. Mycological Examination

The confirmation of disease in view of show of contagious components (stretching septate hyphae and spore (Arthroconidia) by the direct microscopically examination where tests treated and separated and handled by a potassium hydroxide (KOH) arrangement and get ready smears inspected minutely with the end goal of distinguishing proof and portrayal tests examination (Suhnen *et al.*,
1999 ; Ellis, 1994).

2.2.1.3. Cultivation of dermatophytes:

The standard medium for isolation of dermatophytes from clinical materials was Sabouraud's dextrose agar containing cyclohexamide (0.5gm) to suppress the development of saprophytic parasites and chloramphenicol (0.05gm) to stifle the development of quickly developing microorganisms. Moreover, another altered Sabouraud's dextrose agar containing penicillin and streptomycin utilized for segregation of organisms from clinical materials. Plates were inoculated with skin scratching by help of sterile scalped. Cultures were brooded at 28°C. Cultures were firstly inspected following 7-10 days. The recognizing components of dermatophytes were normally created inside of 10-20 days. Most dermatophytes lose their particular Culture and microscopically includes when kept for quite a while in Cultures. Recognizable proof of the development relies on upon the accompanying (Rippon, 1988):

Microscopic examination was made by examination of many preparations from different areas of fungal growth mounted with lacto phenol cotton

blue stain to reveal spores, which include large septate macroconidia, and small, single-celled microconidia (Rippon, 1988).

2.2.1.4. Selection of *T.rubrum* isolates

Eleven isolates of *T. rubrum* were identified according to the morphological qualities of the fungus and confirmed by Polymerase chain reaction (PCR) , and selected then for further studies . Subculture media were done for storage and maintains the isolates using saborouad broth media supplemented with 15% glycerol under 2-8 ^{o} C.

2.2.1.5. Activation of fungal isolates

Sabouraud Dextrose agar was used for activation of Dermatophytes the was prepared the medium by Suspended 40 grams in 1000 ml distilled water, sterilize by autoclaving at 15 lbs /inech pressure 121°C for 15 minutes and pH was adjusted to 5.6±0.2, then, pouring the medium in the sterile Petri dishes and incubation in incubator in 28 °C for 7-10 days, the formulation described by Michel for the cultivation of fungi, particularly those associated with skin infections (Michel *et al.*, 2005)

2.2.1.6. Identification of *T.rubrum*

The identification of the fungal species was depended on the following criteria:

1- Colony characteristics (Color, Consistency and Topography).

2- Colony Reverses (Color, Significant pigment).

3- Microscopic Morphology (Microconidia and Macroconidia: their Size, Shape, an Arrangement, and hyphal Structures).

4-Resistance to cycloheximide.

identification was built on colonial and microscopic features, and was certain media according to Rippon.

Microscopic examination was made by examination of many preparations from different areas of fungal growth mounted with lacto phenol cotton blue stain to reveal spores which include large septate macroconidia and small, single-celled micro conidia (Rippon *et.,al.,* 1988).

2.2.2. Molecular methods

1. Culture and harvest the isolates

A portion of colonies was cultured on SDA by spot inoculation technique .The cultures were incubated at 29 ^{0}C until visible fungal growth or (5-10) day-old for early PCR analysis.

2. Fungal genomic DNA Extraction

Fungal genomic DNA from of fungal isolates were extracted by using EZ-10 Spin Column Fungal Genomic DNA Mini-Preps Kit, and done according to company instructions as following steps:

- A100mg of fungal culture was Grinded in liquid nitrogen by using a pestle. Transfer the sample was transferred to a clean 1.5 ml microcentrifuge tube.
- A180 µl of Universal Digestion Buffer and 20 µl Proteinase K was added to the sample, and mix thoroughly by vortexing. Then incubated at 56°C for 60 min.
- A100 µl of Universal Buffer PF was added, and mixed by inverting and incubated at -20°C for 5 minutes. Then centrifuged at 1000 rpm for 5 minutes at room temp4 ^{o}C . After that the supernatant was transferred in to a new 1.5 ml microcentrifuge tube.

- A200 Universal Buffer BD was added and mixed thoroughly by vortexing.
- A200 µl ethanol (96%) was added and mixed thoroughly by vortexing.
- EZ-10 column was placed in a 2 ml collection tube and transferred all of the mixture (including any precipitate) to column. Then centrifuged at 12000rpm for 1 minute. And the 2 ml collection tube containing the flow-through was discarded and placed the column in a new 2 ml collection tube.
- A 500 µl of Universal PW Solution were added to the DNA filter column, then centrifuge at 10000rpm for 1 minute. The flow-through was discarded and placed the column back in the 2 ml collection tube.
- A 500 µl of Universal Wash Solution was added to each column. Then centrifuged at 12000 rpm for 1 minute. The flow-through was discarded and placed the column back in the 2 ml collection tube.
- All the tubes were centrifuged again for 3 minutes at 12000 rpm to dry the column matrix.
- The dried DNA filter column was transferred to a clean 1.5 ml microcentrifuge tube and 50µl of pre-heated 60 ^{o}C TE elution buffer were added to the center of the column matrix.

The tubes were let stand for at least 5 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 30 seconds to elute the purified DNA.

3. Estimation of DNA yield and quality

The extracted genomic DNA was checked by using Nanodrop spectrophotometer (THERMO. USA) that check DNA concentration at

260 nm and estimation of DNA purity through reading the absorbance at (260 /280 nm) (Sambrook and Russel 2001).

4. Preparation of primers

The primers were prepared depending on the manufacturing instructions by dissolving the lyophilized primers with TE buffer to stock solution with counteraction of 100 pmole/ul , after spinning-down and stay overnight at 4c,primers working solution with TE buffer , using the equationC1V1=C2V2 (concentration versus volume) to get final working solution (10 p mole /ul) for each primer(Maniatis *et al.,* 1982 ; Brasch *et al.,*2010).

5. Polymerase chain reaction (PCR)

PCR master mix reaction was prepared by using AccuPower PCR PreMix Kit and this master mix done according to company instructions as shown in table (2-7):

Table (2-7):The PCR master mix reaction preparation

PCR Master mix	**Volume**
DNA template	5µL
Promegagreen mix(2x)	1.5µl
ITS1 Rverese primer (10 pmol)	1.5µL
PCR water	12 µL
Total volume	20µL

PCR reaction components were placed in standard PCR tubes containing the PCR PreMix as lyophilized materials containing all other components

needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, MgCl2,stabilizer, and tracking dye). Then the tube placed in centrifuge for 5 sec . Then transferred in Mygene PCR thermocycler (Rabbani *et al*., 2008; Brasch *et al.*,2010).

.2. PCR Thermo cycler Conditions

PCR thermocycler conditions for each gene fragments were done using convential PCR thermocycler system as following tables (2-8):

Table (2-8) :The PCR Thermocycler Condition

PCR step	Repeat (cycle)	Temp.	Time
Initial denaturation	1	95 ^{0}C	5min
Denaturation	30	95^{0}C	30sec.
Annealing		60^{0}C	30sec
Extension		42^{0}C	45sec
Final extension	1	72^{0}C	7min
Storage	-	4^{0}C	For long time

3. The protocol of PCR

PCR was performed with a protocol includes the following:

-PCR primer: the random PCR primer as indicated in table (2 -4)

-PCRmix: about 12.5µl of PCR ready mix(Go Taq Green master mix)was added when the final reaction volume was 25µl to obtain a final concentration (1x)as recommended by provider and sterile distilled water

was used to achieve a total volume of 25µl after added each of primers and DNA template.

3. PCR product analysis:

The PCR products of ITS1gene were analyzed by agarose gel electrophoresis by following steps:

- A 1% Agarose gel was prepared in using 1X TBE dissolving in water bath at 100 ^{o}C for 15 min., after that left to cool 50 ^{o}C.
- Then 3ml of ethidium bromide stain were added into agarose gel solution.
- Agarose gel solution was poured in tray after fixed the comb in proper position, after that, left to solidified for 15 min at room temperature, then the comb was removed gently from the tray and 10 ml of PCR product were added into each comb well and 5ml of 100bp ladder in one well.
- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volt and 80 AM for 1hour.
- PCR products were visualized by using ultraviolet transilluminator.

2.2.3 Preparation of Production secondary metabolites media

Optimal growth and secondary metabolites production condition by *T. rubrum* was grown on Sabouraud's broth as the control and two different broth modification medium were used; Horse Hair Dextrose liquid medium (HHD) and keratinized skin dextrose modified liquid medium (KSD) to promote production of secondary metabolites product. The effect of some environmental factors in the growth of *T. rubrum*.

The fungus development on three substrate; HHD used the Horse hair as the Nitrogen source , KSD used the keratinized skin as the Nitrogen source and Sabouraud's broth as the control and taken from SDA disc diameter of 10 milliliters of the edge of the colony fungal aged 7-10 days .

Temperature was estimated the optimal growth temperature of fungus and ability to produce secondary metabolites in varying degrees temperature (25, 28,30 and 35)^{o} C (Hammadi *et al*., 2007).

The optimal pH growth was estimated of *T. rubrum* by different pH 4,5,6 and 7, while were excluded pH 6 and 7, to a complete inhibition of the growth of fungal isolated when this pH.

Incubation period was estimated the optimal growth incubation of fungus after different incubation periods by transferring a disk diameter of 10 mm from the edge of the colony fungal in SDA to the broth media after incubation periods 5-14 Day (Colin *et al*., 1981; Hammadi *et al*., 2007).

1. Preparation media inoculation

The *T. rubrum* fungus was grown on SDA for 7-10 days. After that, agar blocks of 5 x 5 mm dimension were cut off using a sterile sharp blade. The modification medium Horse Hair Dextrose liquid medium (HHD) so distinct from the other and keratinized skin dextrose modified liquid medium (KSD) was used as the culture medium of the Dermatophytes *T. rubrum* for its secondary metabolite productions. Five to seven pieces of agar blocks were inoculated into each 1L flask containing 250 mL fermentation broth was inoculated with mycelium incubated for 10 days on an orbital incubator 150 rpm at 28°C to obtain good growth for the Dermatophytes (Hammadi *et al*., 2007)

2. Selection of an Extraction Method and Solvents

The selection of a suitable extraction method depends mainly on the work to be carried out, and whether or not the metabolites of interest were known. Extraction processes employed water-miscible and water-immiscible solvents. The selected solvent selected have a low potential for artifact formation, a low toxicity, a low flammability, and a low risk of explosion. The main solvents used for extraction of secondary metabolites *T. rubrum* Acetone (Hamide *et. al.,* 2007).

2.2.4. Isolation and purification secondary metabolite from *T. rubrum*

1.Isolation

After incubation, the maximum production of secondary metabolites from the strains were obtained at the 10th days of incubation in fermentation media and after end the incubation time, centrifugation on the 6000 rpm for 20 min was done to obtain mycelia were separated the mass of mycelia filtered with Whatman filter paper No.1 , the filtrate was collected and the volume and pH measured then transferred into a vial and kept at -20°C until use.

2. Liquid-liquid extraction

Using a 2 L separatory funnel, the filtrate from each 1 L flask was extracted with 1 L Acetone .The organic phase was collected in 2 L Erlenmeyer flasks, and filtered through a Whatman No. 1 filter paper. The solvent was then evaporated under vacuum using a rotary evaporator (Hammadi *et al*., 2007, Abdulwahid *et al*., 2013). This crude extracts were dissolved when required in 5ml of aqueous acetone (10%) and stored at 4°C for future use.

2.2.5. Antimicrobial activity of crude extract of *T. rubrum*

Test Strains:

Table: 2-9 bacterial strain that used in the study

Bacterial strain	Code No.	Reference
Streptococcus pyogenes	ATCC49619	public Health laboratories / Ministry of Health –Baghdad
Pseudomonas aeruginosa	ATCC27853	
Staphylococcus aureus	ATCC25923	
Staphylococcus epidermidis	ATCC25985	
Escherichia coli	ATCC25922	

Crude extracts were screened for microbial properties using a modified Oxford disc diffusion assay. 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).

2.2.6. Thin layer chromatography (TLC)

A TLC plate was a sheet of glass, metal, or plastic, which it coated with a thin layer of a solid adsorbent (usually silica or alumina). A small amount of mixture was spotted near the bottom of plate. The TLC plate that was

placed in a shallow pool of a solvent in a developing chamber so that only the very bottom of the plate was in the liquid. This liquid was the (mobile phase) slowly rises up the TLC plate by capillary action. As the solvent moves past the spot that was applied, equilibrium was established for each component of the mixture between the molecules of that component, which were adsorbed on the solid, and the molecules which were in solution. In principle, the components will differ in solubility and in the strength of their adsorption to the adsorbent and some components will be carried farther up the plate than others (Randerath *et al*., 1968).

Analytical thin layer chromatography was performed on aluminium pre-coated plates of silica gel 60 F254 with a 0.2 mm layer thickness. Visualization of the TLC spots was achieved under UV light at 254nm and by spraying with the *p*-anisaldehyde reagent (prepared by mixing 0.5ml an aldehyde mixed with 10ml glacial acetic acid, followed by 85ml of chilled methanol and 5ml of 98% sulphuric acid) (Randerath *et al*., 1968).

1. Mobile phase of TLC

A comparison of TLC solvent systems was performed on the both standards and sample extracts. A wide variety of solvent mixtures from literature were tested which included : Methanol (70%) + Chloroform (30%), DW(10%)+Acetone(30%) + Ethyle acetate(60%) , Hexane (80%)+Ethyl acetate(20%) and chloroform (90%) + ethanol (10%) (Hammadi *et al*., 1988)

Comparisons of the different solvent systems were performed using identical standard s and samples, where it was determined that chloroform: ethanol 90:10 provided the best separation and results.

2. Standard materials preparations

Benzyl penicillin G, fusidic acid and Kojic acid were used as standard compounds for comparison in chromatography

Procedure of TLC:

1- Development tank was prepared using a clean dry jar and allow it to equilibrate for 10 min.

2- The plastic –backed TLC plate was prepared by drawing a fine line (the base line) in pencil about 2cm above the bottom with carful to scrape off any of the stationary phase. Three pencil dots were put on the base line- one in the center and the other equidistant on the either side, but no closer than 3mm to the edges of the plate.

3- The crude mixture (1mg) in 150µl of suitable volatile solvent Acetone in Eppendorf tube.

4- Using micropipette the crude extracts was poured onto one of the pencil spots.

5- Compared sample (reference compound) was poured using micropipettes onto other spots as an appropriate.

6- The solvent from the plate was evaporated.

7- The plate was lowered into the developing tank, holding it by the jar cover and the eluent did not cover the base line .

8- The lid was put on the jar and allowed the eluent to rise up the plate to about 2 cm from the top of the plate.

9- The plate was removed from the tank and quickly marked the height of the eluent ,using a pencil.

10- The chromatogram was evaporated by the air

11- The chromatogram was visualized by placing the dry chromatoplate in the UV cabinet and using a pencil to draw round the spots.

The R_f values of the spots were calculated by the formula) (Randerath *et al*., 1968),

$$R_f = \frac{\text{Distance travelled by the sample}}{\text{Distance travelled by the solvent}}$$

2.2.7. Evaluation of Radical Scavenging Effects of *Trichophyton rubrum* Acetone secondary metabolites Extracts

One of the quick methods to evaluate antioxidant activity was the scavenging activity on 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), a stable free radical and widely used index. Screening method adopted in accordance with the method described by Patel in the detection of antioxidants activity of fungal extracts (Patel *et al*., 2011)**,** DPPH was found as dark-colored crystalline powder composed of stable free-radical molecules and forms deep violet color in solution. The scavenging of DPPH free radical (neutralization) was indicated by the deep violet color being turned into pale yellow or colorless. This can be quantified spectrophotometrically at 517 nm to indicate the extent of DPPH scavenging activity by the *T. rubrum* extracts ((Brand *et al.,* 1995;Patel *et al*., 2011)

1. Preparation of DPPH Solution:

A 0.004 g of DPPH was added 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.

2. Preparation of standard solutions

A 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.

3. Preparation of Extracts Solution

A 0.004gm of both fungi extract (*T. rubrum*) extract (HHD and KSD) 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).

5. Measurement of the Absorbance

1. The absorbance of the solution was measured at 517nm using a spectrophotometer
2. A typical blank solution contains 3ml reagent solution and the appropriate volume 300µl of the same solvent used for the sample and it was incubated under the same conditions as the rest of the sample solution.

Formula of the calculation the percentage scavenging activity of the extract was calculated using the formula:

% scavenging activity = $\{(A_0 - A_1)/ A_0\} * 100$

Where, A_0 was the absorbance of the control and A_1 was the absorbance of the extract.

%inhibition =(1- absorbance of sample/ absorbance of control) * 100

The IC 50 value of the sample, was the concentration of sample required to inhibit 50% of the DPPH free radical, and was calculated using Log dose inhibition curve.

2.2.8. Cytotoxic Activity

Brine shrimp lethality bioassay

Screening method adopted in accordance with the method described by Farhana in the screening cytotoxicity activity of fungal secondary metabolites extracts (Farhana *et al.*, 2010) . The test achieved in laboratories of the pharmacologic college /Tehran University

Principle

Test samples were prepared by the addition of calculated amount of DMSO (dimethyl sulfoxide) for obtaining desired concentration of test sample. The nauplii were counted by visual inspection and were taken in vials containing 5 ml of seawater. The samples of different concentrations were added to pre-marked vials with a micropipette. The vials were left for 24 hours and then nauplii were counted again to find out the cytotoxicity of the test agents.

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.

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.

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.

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.

7. Application of brine shrimp nauplii

Ten living nauplii were added to each of the vials containing 5 ml of simulated seawater. A magnifying glass was used for convenient count of nauplii. If the counting of the 10 nauplii was not being possible accurately, then a variation in counting from9-11.

8. Counting of the nauplii

After 24 hours, the vials were observed using a magnifying glass and the number of survival nauplii in each vial were counted and recorded. From

this data, the percentage of mortality of nauplii was calculated for each concentration of the sample. The median lethal concentration (LC50) of the test samples was obtained by a plot of percentage of the killed shrimps against the logarithm of the sample concentration.Cytotoxicity as LC50 was calculated using the formula:

$$LC50 = \frac{\text{Log LC50} - \text{Log Concentration A}}{\text{Log Concentration B}}$$

Log Concentration A = 50% - M / N – M, Where concentration A was the concentration before LC50, Concentration B was the concentration after LC50, M was the % killed before LC50, and N was the % killed after LC50.

The percentage mortality at each concentration was determined using the following formula:

$$\%\ \text{Mortality} = \frac{(\text{Number of dead nauplii}) \times 100}{\text{Total number of nauplii}}$$

The LC50 of the test samples was obtained by a plot of percentage of the shrimps died (% Mortality) against the logarithm of the sample concentration (Log C) and the best-fit line was obtained from the curve data by means of regression analysis.

2.2.9. High Performance Liquid Chromatography (HPLC.)

Analyses were performed in Ibin Sina in Laboratory – university of Baghdad using an HPLC system equipped with a model LC-10ATvp pump (Shimadzu), covering the 190–600 nm range with Shimadzu Client/Server software, plus auto-sampler, Separations of extracts and Standard were C18 analytical column performed on a 250 mm x 4. mm, 5 µ. In the table, 2-10 below shows the device details and specifications.

1.Preparation Acetone secondary metabolites Extracts of *T.rubrum* Sample for HPLC

The Acetone extract from *T.rubrum* was initially fractionated by Column C18 250*0.4 mm , 5 µ. Column was first flushed with 100% MeOH to activate the column packing and flush any column bleed through. The both extracts HHD1and KSD were mobile phase eluted with the Acetonitrile–Potassium dihydrogen phosphate ($H2KPO_4$) (30 % -70%) respectively, the Potassium dihydrogen phosphate have PH 3 , the wave length 240 nm with flow rate 1.5 ml/min , volume injection 20µ, the final concentration 250 ppm.

2.Parparation Banzilpancillin G Standard for HPLC

Benzylpenicillin G was initially fractionated by Column C18 250*0.4 mm , 5 µ. Column was first flushed with 100% MeOH to activate the column packing and flush any column bleed through, While the mobile phase eluted with the Acetonitrile–Potassium dihydrogen phosphate ($H2KPO_4$) (30 % -70%) respectively, the Potassium dihydrogen phosphate have pH 3 , the wave length 240nm with flow rate 1.5 ml/min , volume injection 20µ, the final concentration 250 ppm.

3.Parparation fusidic acid Standard for HPLC

Fusidic acid was initially fractionated by Column C18 250*0.4 mm , 5 µ. Column was first flushed with 100% MeOH to activate the column packing and flush any column bleeds through, While the mobile phase eluted with the Acetonitrile–Potassium dihydrogen phosphate (H2KPO4) (70 % -30%) respectively, the Potassium dihydrogen phosphate have pH

3 , the wave length 210nm with flow rate 1.5 ml/min , volume injection 20μ, the final concentration 250 ppm.

4.Parparation Kojic acid Standard for HPLC

Kojic acid was initially fractionated by Column C18 250*0.4 mm , 5 μ. Column was first flushed with 100% MeOH to activate the column packing and flush any column bleed through, While the mobile phase eluted with the water-Acetonitrile (92 % -8%) respectively, the Potassium dihydrogen phosphate have pH 3 , the wave length 210nm with flow rate 1.5 ml/min , volume injection 20μ, the final concentration 250 ppm.

Table (2-8): Specifications of HPLC Model LC-2010HT

Item			LC-2010CHT (228-45103-3X)	LC-2010AHT (228-45102-3X)
Pump	Pump type		Serial dual pluger, micro volume (10 µL on primary side, 5 µL on secondary)	
	Flow rate setting range		0.001–5 mL/min [1.0–35 MPa / 145–5076 psi]	
	Flow rate accuracy		±1% or ±2 µL/min, whichever is larger (1 mL/min, 3–10.0 MPa / 435–1450 psi, water, ambient temperature at 20°C, except in gradient solvent delivery), ±2% or ± 2 µL/min, whichever is larger (except above condition and in gradient solvent delivery)	
	Flow rate precision		±0.075% RSD or 0.02 min SD, whichever is larger (0.05–2 mL/min, 1.0–35 MPa / 145–5076 psi, methanol, ambient temperature at 20°C)	
	Pressure display accuracy		±2% or ±0.5 MPa, whichever is larger	
	Plunger cleaning		Automatic rinsing mechanism as standard equipment	
	LPGE	Number of solvents and setting range	Up to 4 solvents 0–100%, 0.1% step	
		Concentration accuracy	±0.5% (0.1–2 mL/min, 1.0–20.0 MPa / 145–2900 psi, aqueous acetone / water)	
		Concentration precision	±0.1% (0.1–2 mL/min, 1.0–20.0 MPa / 145–2900 psi, aqueous acetone / water)	
Degasser	Type		Membrane on-line degasser, 5 lines (4 × mobile phase, 1 × autosampler rinsing liquid)	
	Volume		4 mL/line for mobile phase, 2 mL/line for autosampler rinsing liquid	
Autosampler	Injection method		Full volume injection, variable injection possible (no sample loss)	
	Injection volume setting range		0.1–100 µL (standard), 1–2000 µL (optional) (0.1–0.9 µL in 0.1 µL step, 1–2000 µL in 1 µL step)	
	Sample capacity	1 mL vials	350	
		1.5 mL vials	140	210
		4 mL vials	100	
		Microtiter plates	Max. 4 plates (up to 384 samples with 96 well or 1536 samples with 384 well plates)	
		Deep well plates	Max. 4 plates (up to 384 samples with 96 plates)	
	Injection volume repeatability		RSD < 0.3% (10 µL injection)	
	Carryover		0.01% (under conditions determined by Shimadzu)	
	Injection volume accuracy		±1% (50 µL, n = 6)	
	Repetition injection frequency		1–99 / sample	
	Analytical run time setting		0.01–9999.9 min (0.01 min step)	
	Injection condition steps		Max. 202 steps	
	Operating pressure		35.0 MPa maximum	
	Rinsing liquid degassing		Standard	
	Sample cooler (LC-2010CHT only)	Type	Direct cooling type. (For setting temperature at 4°C, ambient temperature shall be 30°C or lower and humidity 70% or lower.) Dehumidifier incorporated.	———
		Temperature setting range	4–40°C (For setting temperature at 4°C, ambient temperature shall be 30°C or lower and humidity 70% or lower.)	———
Column Oven	Type		Block heating with preheating of the mobile phase	
	Temperature setting range		4–60°C, 1°C step	
	Temperature control precision		±0.1°C	
	Temperature control range		(Ambient temperature-15°C) –60°C	
	Columns accommodated		2 × 250 mm columns	
	Preheater volume		4 µL (for Semi-micro), and 9 µL, 2 channels	

2.2.10. Fourier Transform Infrared spectrophotometer (FTIR)

Analyses were performed in Ibin Sina in Laboratory –university of Baghdad using The FT-IR spectrometer used was a Shimadzu® (Kyoto, Japan), model IR Prestige-21, which allows spectral digitalization for obtaining electronic files of the analysis. The region comprised in the spectral analysis was from 500 to 4000 cm^{-1} at 2 cm^{-1} intervals. After obtaining the IR spectrum and with the assistance of the IR Solution software, quantitative analysis was carried out in the spectral region between 1800 and 1700 cm^{-1}. The concentrated partial purified sample was measured under FTIR the sample powder was run at infrared region between 400 nm and 4000 nm.

Preparation of samples RS Pellets

Amounts of powder equivalent to 2.0 mg of (HHD, KSD, Benzylpenicillin G, fusidic acid and kojic acid) (20.0 mg of the 1:10 dilution in potassium bromide) were taken and homogenized with 130 mg of potassium bromide, making the total pellet weight of 150 mg ,this mixture was compressed in a mechanical die press for 15 minutes to obtain translucent pellets, through which the beam of the spectrometer can pass.

Method Validation

The method was validated by determining the following parameters: linearity, precision, accuracy, robustness, and detection and quantification limits, according to the literature recommendation.

Statistical analysis

Statistical analysis was carried out with the SPSS software, version 17, after translated data into codes. Suitable statistical methods were used in order to analyze and assess the results.

3. Results and Discussion

3.1. Isolation and identification of *T. rubrum*

Out of 100 samples, 11 isolates of *T. rubrum* were identified based on morphology and culture characterizes, the colonies were slow-growing , while , and cottony to velvety appeared on the modified Sabouraud's dextrose agar and their diameter reaching 2.5cm as shown in (Figure 3.1). Also, the isolates of *T. rubrum* had ability to produced pink pigment on the reverse side when cultured on potato dextrose agar (Figure 3.2). Microscopically, this organism produced very small microconida, which oval and born along the sides of hyphae when Colony mounted with lacto phenol cotton blue stain (Figure 3.3). Aalso, macroconidia were rarely formed and they were cylindrical with thin, smooth wall, while the microconidia were spherical, abundant and born both in clusters along the hyphae when the colony mounted with 10 % KOH (Figure 3.4).

Figure 3.1: Morphology feature of *T. rubrum* on Sabouraud s dextrose agar at 28C for 3-4 weeks incubation.

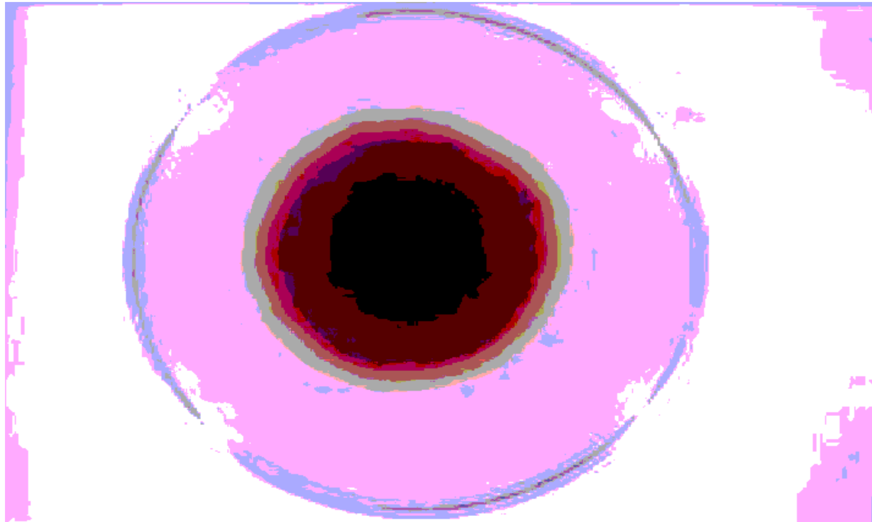


Figure (3.2): Morphology feature of *Trichophyton rubrum* on potato dextrose agar, (background color) at 28C for 3-4 weeks incubation.

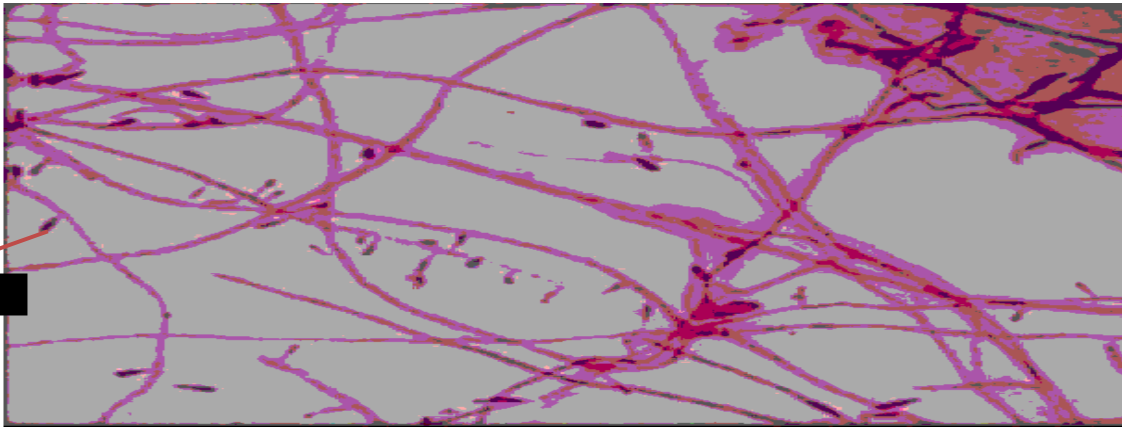


Figure (3.3): Microscopic morphology of *Trichophyton rubrum*, Colony mounted with lacto phenol cotton blue stain (40x).

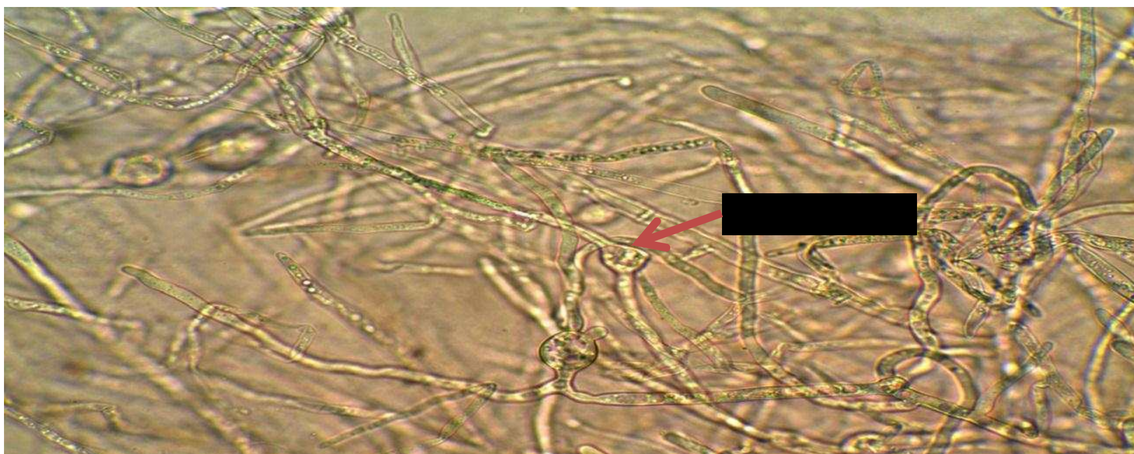


Figure (3.4): Microscopic morphology of *Trichophyton rubrum* mounted with 10 % KOH

3.2. Confirmative identification of *T. rubrum* by PCR

3.2.1. DNA Quality and purity

The results of evaluating and estimating the DNA extraction of *T.rubrum* was measured by Nano drop spectrophotometer at wave length (260-280)nm. It gave an optimal concentration of DNA for amplification range from 20.6 to 39.4 ng/µl and purity ranged from 1.8 to 2.17 (Table 3-1).

Table 3.1: Value of DNA concentration and purity of selected sample of Extracted DNA.

Sample no.	Concentration ng/µl	Purity 260/280 nm
1	35.1	2.03
2	23.5	2.05
3	39.4	2.17
4	26.7	1.87
5	33.7	1.88
6	27.1	1.89
7	23.8	2.00
8	21.5	1.97
9	30.2	2.03
10	38.1	2.00
11	22.9	2.11
12	72.4	1.4

Based on the standard values of DNA concentration for amplification, the values of the present study are considered an efficient values and amplification.[Applied Biosystems,2008].

3.2.2. Amplification of extracted DNA with primers (ITS1):

In the present study the confirmation process of eleven *T.rubrum* isolates was conducted by conventional PCR to detected the presence of specific 5.8S rRNA gene. The extracted genomic DNA of these isolates were used as template for amplification with primers of internal transcribed spacer (ITS1). The results of revealed that only 8/11 (33.33%)

isolates were classified as *T.rubrum* with amplicon size equal 601 bp after band electrophoresis and UV-transilluminated of product (Figure 3.5).

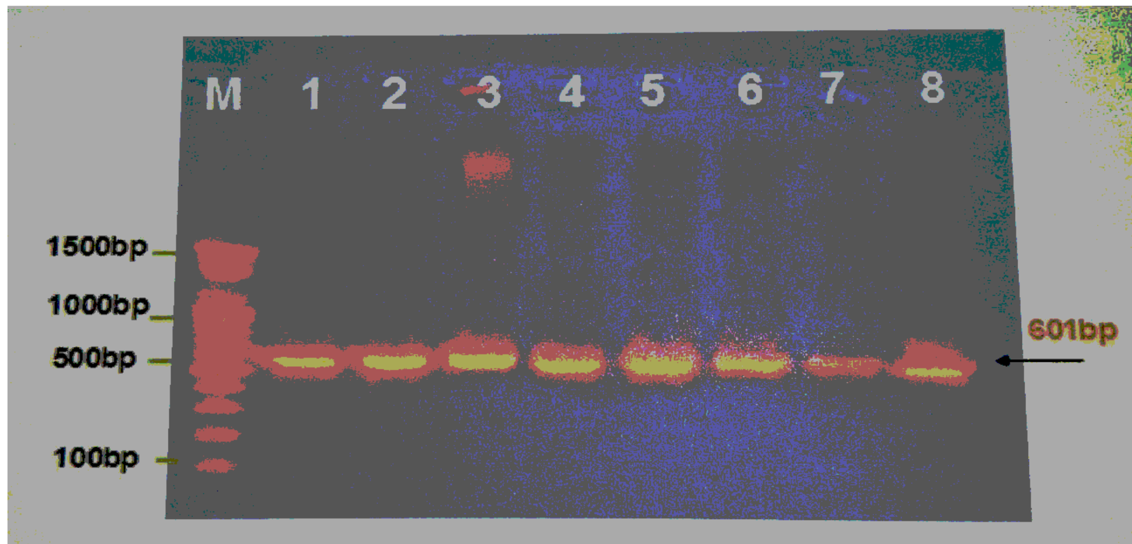


Figure (3.5): Agarose gel electrophoresis that shows the PCR product was 601 bp . Where M: marker (2000-100bp), lane (1-5) *Trichophyton rubrum* isolates

The internal transcribed spacer (ITS) region of the fungal ribosomal DNA (rDNA) has been used as one of the techniques for species identification because it is faster, accurate species determination , specific and are less feasible to affected by exterior effects such as temperature changes and chemotherapy (Girgis *et al.,* 2006).

3.3. Determination the optimal growth conditions of *T. rubrum*:

Studied the effect of substrate source in the production secondary metabolites the growth and production of secondary metabolites of *T. rubrum* was examining to determine the optimal growth conditions for the major factors affecting metabolite production. *T. rubrum* was grown on Sabouraud's broth as the control and there are used two different broth modification medium viz.,Horse Hair Dextrose liquid medium (HHD) and keratinized skin dextrose modified liquid medium (KSD) to promote production of secondary metabolites. The result obtained from this study

indicate the superiority of organic substrate used in the study as a source of carbon , and this result is agree with those obtained by Kheira *et al*. (1988) that used keratinized skin to promote fungus to produce the secondary metabolites product and Youssef *et al* (1979) where was used hair to promote growth *T.rubrum* as good stimulate to produced antibiotics.

Effect of different pH values results showed that the best growth of *T.rubrum* and production of secondary metabolites was at pH 5. These findings were compatible with what referred to Cole (1966) , where concentrated of pH in the production, through the influence on the characteristics of the broth media as the dissolved of ingredients media and its transition and concentration resulting from the melting of carbon oxide.

While the effect of temperature on the growth and production of secondary metabolites revealed that the best temperature was 28 ^{o}C, this is based on previous studies that showed that this class of optimal temperature to produce secondary metabolites of *T.rubrum* Youssef *et al* (1979), Kheira *et al*. (1988) and Hammadi *et al*., (2007).

3.4. Preparation of Crude extract from *T.rubrum*:

The *T.rubrum* was grown in the (HHD) and (KSD) after 10 days incubation the fermented broth (10 liters) was separated from mycelium by filtration and was then partitioned with equal volume of acetone for three times. The crude acetone/water mixture was filtered and reduced to dryness in a rotary evaporator pressure at 45°C to yield 0.640 gm of crude acetone extract from liquid media HHD as whitish to yellowish and 0.553 gm of crude acetone extract from liquid media KSD , which exhibit like extract HHD in color. The crude acetone extracts was further

examined for antimicrobial activity, antioxidant activity and anticytotoxicity activity also physical test TLC , HPLC and FT-IR.

3.5. Antimicrobial activity of crude extracts of *T. rubrum*

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 (3-3) and figure (3-6). The highest inhibition zone diameter was 23 mm on *S.epidermidis* and the lowest inhibition zone diameter was 9 mm *Escherichia coli* and 7mm *Pseudomonas aeruginosa* .

Table (3-2): 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
Staphylococcus aureus	20	18
Staphylococcus epidermidis	23	20
Streptococcus pyogenes	12	10
Escherichia coli	10.5	9
Pseudomonas aeruginosa	7	7

The results 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 .

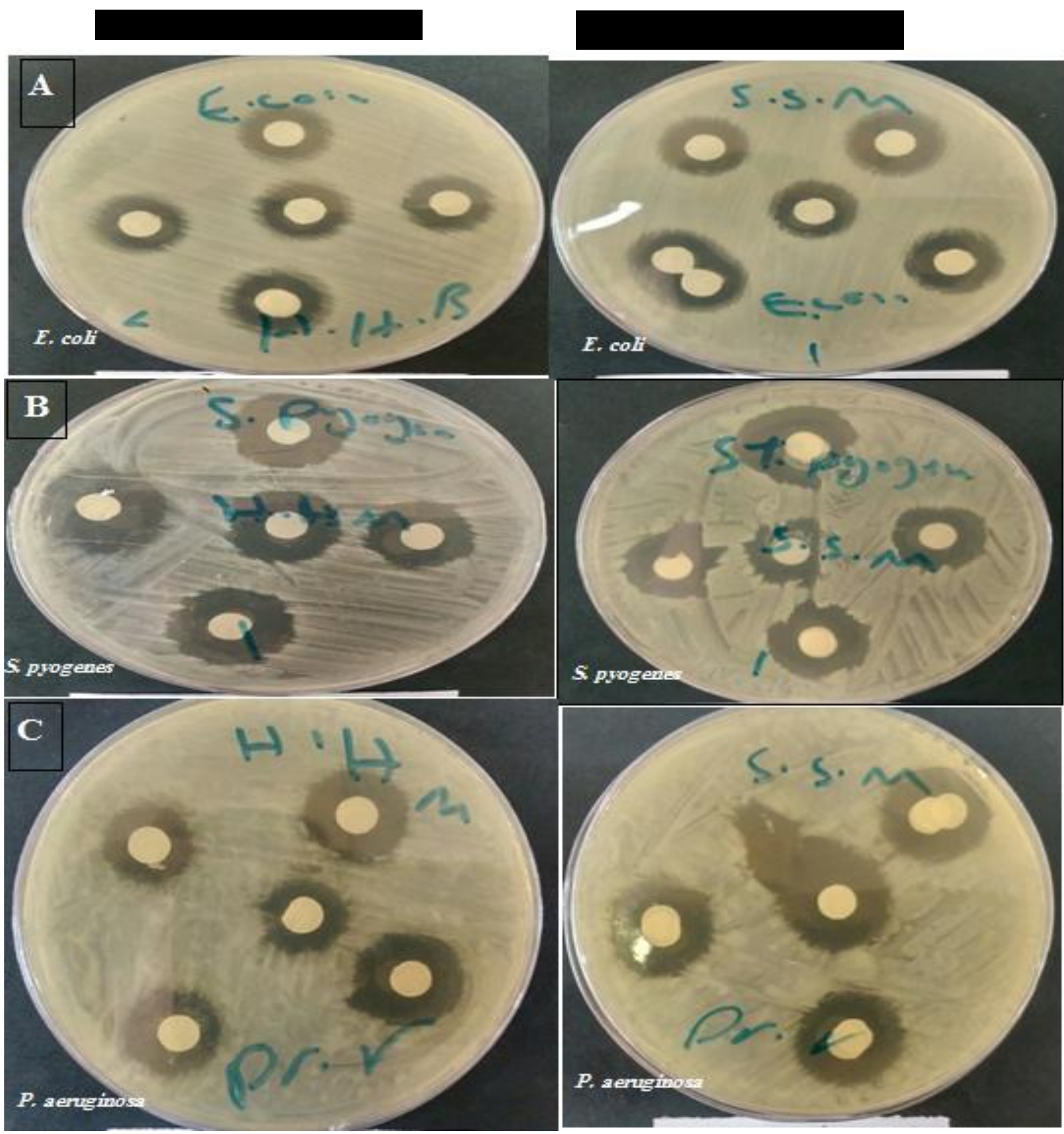


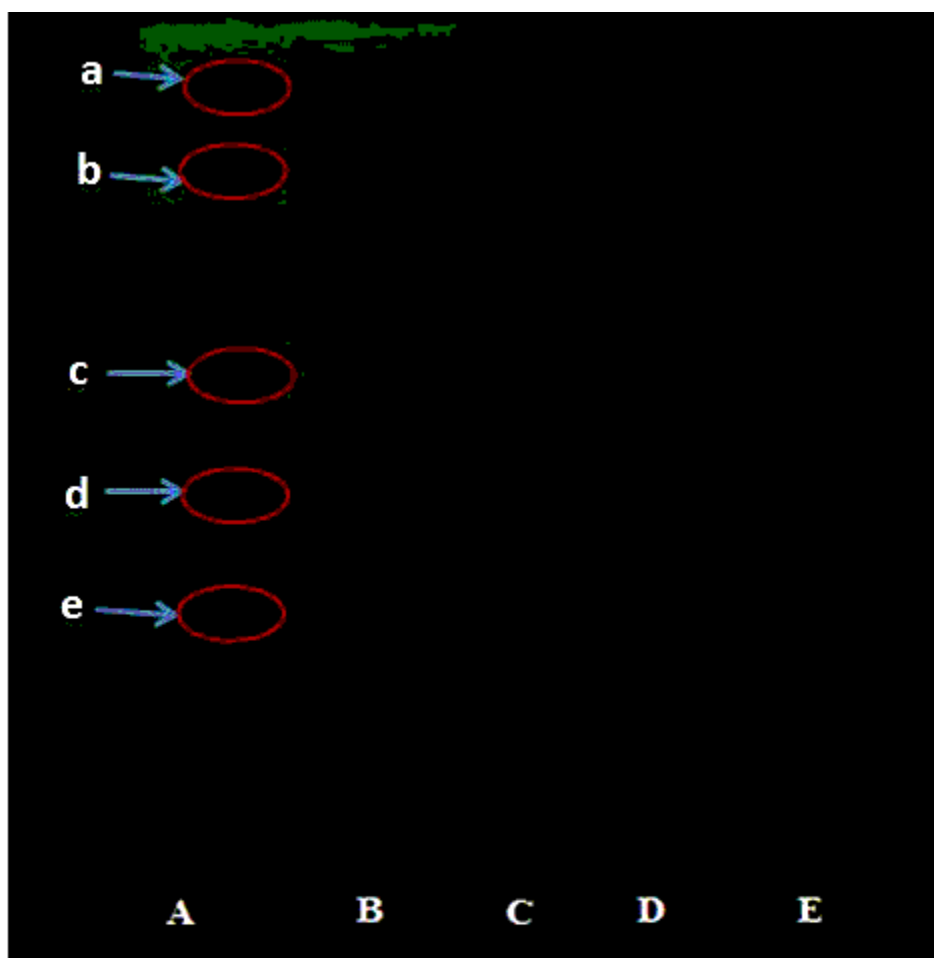
Figure (3-6): Growth Inhibitor zone (A)*E. coli,* *(B) S. pyogenes* and (C) *P. aeruginosa* by using *T. rubrum* secondary metabolites extract in both media, HHD, KSD

3.6. Separation of *T.rubrum* crude extract by Thin Layer Chromatography

A comparison of TLC solvent systems was performed of the both standards and secondary metabolites extracts. A wide variety of solvent mixtures from literature were tested which included : Ethanol (70%) + Chloroform (30%), DW(10%)+Acetone(30%) + Ethyle acetate(60%) , Hexane (80%)+Ethyl acetate(20%) and chloroform (90%) + Methanol (10%).

Comparisons of the different solvent systems were performed using identical standard s and samples, where it was determined that chloroform: ethanol 90:10 provided the best separation and results and this result agreed with Panchal *et al.*(2012), because chloroform is more efficient when diluted than concentrated one ,and the best extraction capacity ,due to its lowest boiling point (65) °C in comparisons with other solvents which were (79 , 69, 100) °C for ethanol, hexane and hot water respectively , moreover the increasing in the chloroform polarity, when diluted with water is acts as another reason for perfect extraction of this solvent ,despite the convergence polarity with that of methanol, while the polarity of water is high but not all the fungi material could be dissolved and extracted with high efficiency , this result confirmed with Hammadi *et al.*(1988) and Dawit (2009). Therefore the extraction by chloroform was selected for further separation and partial purification of acetone extracts of secondary metabolites of *T.rubrum*.

Active compound in the crude extracts of secondary metabolites from *T. rubrum* was determined using TLC separation and each of the fractions compared with standard compounds Benzyl penicillin G, fusidic acid and Kojic acid that were suspected according to the TLC test to determine the R_f value which was reached, the data revealed that TLC fractionated the crude extract into 5 components fluorescent spots on UV (366nm) as shown in figure (3-7).



Figure(3-7): The thin layer chromatography (TLC) separation of crude secodary metabolites of T.rubrum with standard compounds; A. secondary metabolites extracts from HHD, B. secondary metabolites extract from KSD ,C. Benzyl penicillin G, D. fusidic acid, E. Kojic acid and show the spots.

TLC is a simple, quick, and inexpensive procedure that gives the chemist a quick answer as to how many components are in a mixture. TLC is also used to support the identity of a compound in a mixture when

the relative flow (R_f) of a compound is compared with the (R_f) of a known compound (preferably both run on the same TLC plate), the detection of the standard compoundsin the partially purified extracts in both HHD and KSD after concentrated the collected fraction using TLC technique that show in the table (3-3)

Table (3-3): Determination of the R_f value of thin layer chromatography TLC of the crude acetone extracts of *T. rubrum* with compared with standard

Fraction	Total spot	Spot	R_f Value
HHD	5	A	0.85
		B	0.82
		C	0.72
		D	0.52
		E	0.35
KSD	5	A	0.85
		B	0.80
		C	0.72
		D	0.52
		E	0.35
Benzyl penicillin G	1	D	0.56
Fusidic acid	1	E	0.40
Kojic acid	1	C	0.64

The results showed a positive reaction for Benzyl penicillin G throughout the appearance of purple color under UV light , and also by calculating the R_F value which reached into 0.56 as noted in figure (3-7)

and(table 3-3). It showed convergence in the value of the extracts, which due to perhaps the presence of impurities in the standard or related with the degree of sample purity this result was identical to several recent researches that mention by Hammadi *et al*. (2007) that was revealed showed approximate in the value of the extracts and also instructed the cause of this difference to the extract and purity standard compounds used in comparative isolation conditions through TLC were claimed that this component to be like Benzyl penicillin G.

The present showed a match to a large extent with the study conducted by Peck *et al*.(1945) which showed the possibility of production of an antibiotic substance similar to penicillin by pathogenic fungi dermatophytes *T.rubrum*, and agreement with study of Cole (1965) have reported the presence of small amounts of a substance, closely resembling 6-Aminopenicillanic Acid (6-APA), in fermentations carried out with species of both *Epidermophyton floccosum*, and *Trichophyton rubrum*

The results presented in this paper represent the first conclusive evidence for the production of fusidane antibiotics by dermatophytes (*T.rubrum*) The results showed a positive reaction for throughout the appearance of purple color under UV light , and also by calculating the R_F value which reached into 0.40cm as noted in figure (3-7). It showed convergence in the value of the extracts, which due to perhaps the presence of impurities in the standard or related with the degree of sample purity this result was identical to several recent researches that mention by both Hammadi *et al*. (2007) and Perry *et al* (1983) . Nevertheless, not agreement with that reported by Youssef *et a*l., (1979) which did not mention in his search for existence of the proportion of this antibiotic

might be due to the lack of direction in the search for so antibiotic limited to beta-lactam antibiotics.

The detection of the kojic acid in the partially purified of crude extract of secondary metabolites from *T.rubrum* after concentrated the collected fraction using TLC technique, the results showed the appearance of purple color under UV light , and also by calculating the R_F value which reached into 0.64cm as noted in figure (3-7). This apparently compared with the nearest spot to the extracts (HHD and KSD) that were given the 0.72 to sign e in figure (3-7), which showed the presence of spacing between spots.

And that many of the studies done in the search for antibiotics produced by the Dermatophytes did not refer to the presence of a compound kojic acid in the secondary metabolism extracts of these dermatophytes, and this is evident as conducted by researchers Youssef *et al.,* (1979) ,Perry *et al*. (1983) , Elander *et al.*(1969), Cole (1965) and Peck *et al*.(1945).

Except study by Hammadi on the dermatophytes that may have the ability to extract kojic acid from the secondary metabolites of dermatophytes (Hammadi *et al*., 2007).

3.7. Evaluation of Radical Scavenging activity of crude Acetone secondary metabolites *T.rubrum* Extracts

This study determined that acetone extracts of secondary metabolites from *T.rubrum* showed better antioxidant potential by DPPH radical scavenging method when compared to standard ascorbic acid, DPPH is a reactive free radical that acts as an electron acceptor (oxidant/oxidizing agent) and causes oxidation other substances. On the other hand, antioxidant acts as electron donors (reluctant/reducing

agent).antioxidants neutralize DPPH by oxidized themselves; the odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm and is purple in color (Brand *et al.,* 1995).

The antioxidant effect of HHD acetone crude extract and KSD acetone crude extract of *T.rubrum* were significant in the DPPH method used. Free radical scavenging abilities of the test samples were determined by measuring the change in absorbance of DPPH at 517 nm by UV-Spectrophotometer. Concentration providing 50% inhibition (IC50) was calculated from the graph plotted inhibition percentage versus concentration of the extract. IC50 value of the standard and Acetone extracts secondary metabolites of *T.rubrum*.

The absorbance at 517 nm by UV visible spectrophotometer were found to standard ascorbic acid and Acetone extracts of secondary metabolites of *T.rubrum* (Table 3-4) and IC 50 value of ascorbic acid and acetone extracts were as 9.3 , 24.8 and 21.3μg/ml (Table 3-5). for same ascorbic acid and Acetone extracts respectively. It means Acetone extracts of secondary metabolites of *T.rubrum* at higher concentration, comparison with the ascorbic acid, it is clear that *T.rubrum* extracts possess moderate antiradical activity. The antioxidant power of Acetone extracts of secondary metabolites of *T.rubrum* was less than the antioxidant power of Ascorbic acid.

Table (3-4): Absorbance of different extract secondary metabolites of *T.rubrum* with standard ascorbic acid at 517 nm by UV visible spectrophotometer

Concentration (μg/ml)	**HHD extract secondary metabolites**	**KSD Extract secondary metabolites**	**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 (3-5): inhibition percent of different extract of different extract secondary metabolites of *T.rubrum*. With standard ascorbic acid

Conc.(µg/ml)	Ascorbic acid (% Inhibition)	IC50 Value (µg/ml)	HHD extract secondary metabolites (% Inhibition)	IC50 Value (µg/ml)	KSD extract secondary metabolites (% Inhibition)	IC50 Value (µg/ml)
200	20.61%	9.3	0.94%	24.8	0.16%	21.3
400	29.66%		0.65%		0.98%	
600	80.80%		0.16%		10.80%	
800	83.01%		2.65%		33.75%	
1000	83.22%		9.24%		41.89%	

3.8. Cytotoxic Activity of *T.rubrum* crude extract

The results of brine shrimp lethality bioassay were shown in the table (3.6) figure (3-8), Test samples showed different mortality rate at different concentration. The mortality rate of brine shrimp napulii was found to be increased with the increase of samples concenteration. The effectiveness of the concentration and % mortality relationship of Acetone extraction of secondary metabolites of *T.rubrum* was expressed as a median Lethal Concentration (LC50) value.

Figure 3-8: Brine shrimp lethality bioassay.(A) hatched eggs,(B) shrimp nauplii

1. Preparation of Curve for Positive control Vincristine sulphate (VS)

Vincristine sulphate (VS) was used as reference standard, which chemotherapy used to treatment of acute lymphoblastic leukemia in children that show in the table (3-6) and mortality curve of Vincristine sulphate Positive control revealed in the figure (3.9) that was explain the mortality.

Table 3-6: 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	

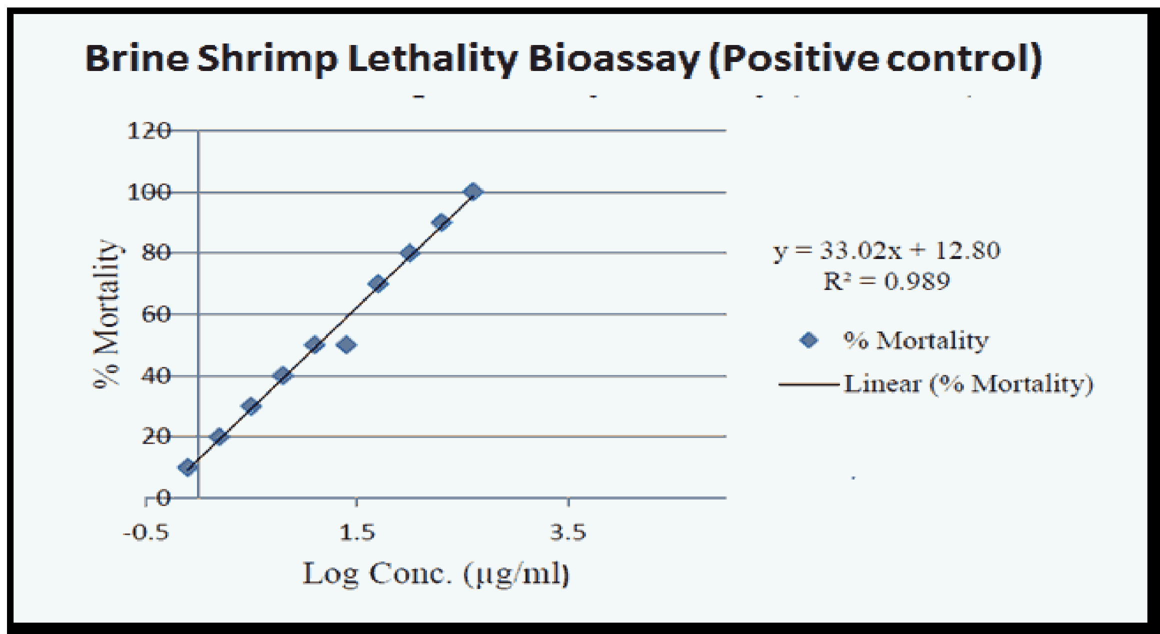


Figure 3.9: % mortality curve of Vincristine sulphate Positive control

2. Preparation of Acetone secondary metabolites Extracts of *T. rubrum* cytotoxicity assay

Acetone secondary metabolites of *T. rubrum* in HHD Extracts of cytotoxicity assay

The results of brine shrimp cytotoxicity bioassay are shown in the table (3-7) and mortality carve in figure (3-10) test acetone secondary metabolites extract of HHD broth 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 extract. The median lethal concentration (LC50) was calculated 16µg/ml.

Table 3-7: the cytotoxicity assay of Acetone secondary metabolites Extracts of HHD produce by *T. rubrum*.

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	

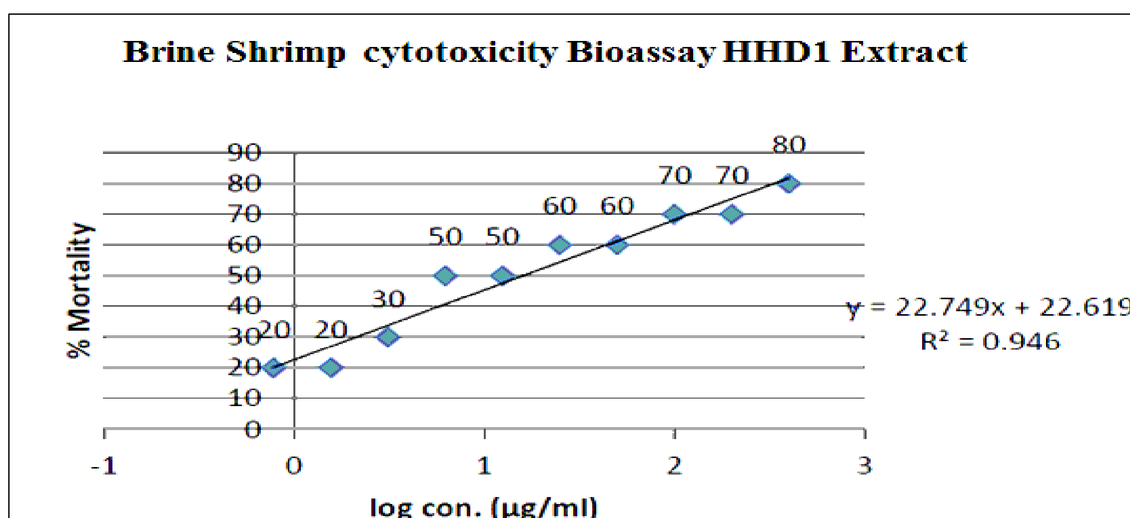


Figure 3.10: mortality percentage curve of Acetone secondary metabolites extract of HHD broth

3. Acetone secondary metabolites of *T. rubrum* in KSD Extracts of cytotoxicity assay

The results of brine shrimp cytotoxicity bioassay are shown in the table (3-8) and mortality carve in figure (3-11) test acetone secondary metabolites extract of KSD broth 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 extract. The median lethal concentration (LC50) was calculated 16µg/ml.

Table 3-8: Results of the cytotoxicity assay of Acetone secondary metabolites Extracts of KSD

Test tube no.	Concentration (µg/ml)	LogC	Number of nauplii alive	Number of nauplii dead	Mortality %	LC50 (µg/ml)
1	25	1.398	7	3	30	1.428
2	50	1.699	6	4	40	
3	100	2.000	2	8	80	
4	200	2.301	0	10	100	
5	400	2.602	0	10	100	

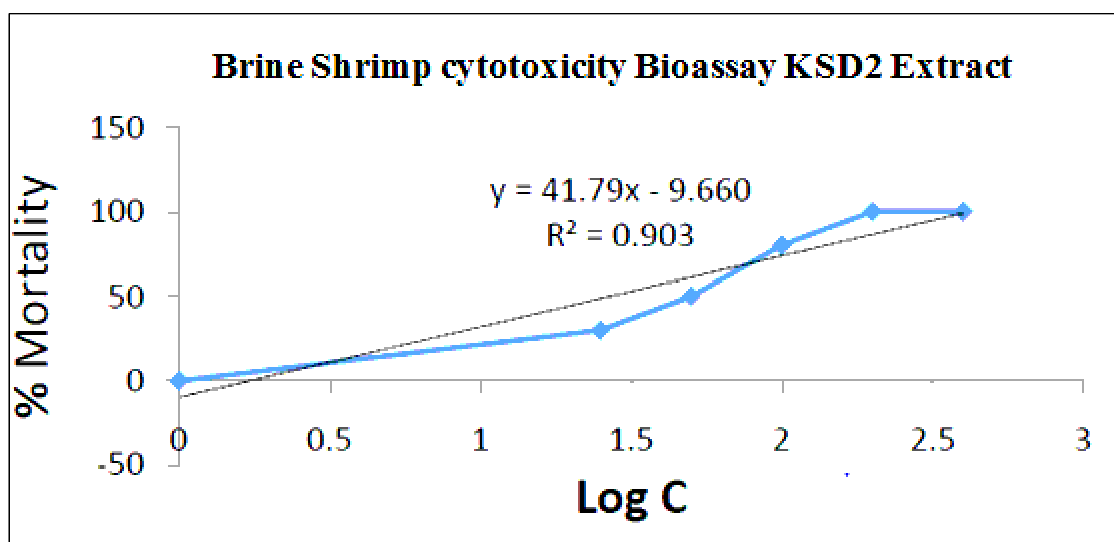


Figure 3.11: mortality percent curve of Acetone secondary metabolites extract of KSD broth

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 secondary metabolites of *T.rubrum* extracts in HHD, KSD samples.

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 3-6.3-7 and 3-8. 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.

Nevertheless, the found a difference between both extracts where extracted had the HHD high-level of toxicity compared to the KSD extracted Display level of toxicity less at lowest concentration with mortality 30%.

In terms of previous studies, this study showed affinity between them and those that have adopted screening toxicity secondary metabolism-fungal extracts and this seems clear in the study that was conducted to test the toxicity of extract dermatophytes by Hammadi *et al*., 2007and Perry *et al*. (1983) .

3.9. Separation of crude extract by HPLC

To determine of the purity the HPLC technique were used of both samples extracts and standards, as well as the expense of both samples extracts and standards retention time as evident from (Figures 3-12, 3-13,3-14,3-15 and 3-16).

The emergence of peaks absorption and this is evident in the table accompanying figures attached table formats, which explains all the peaks area and time of retenation. The result in Table (3-9) and Figure (3-12) were revealed HPLC analysis of crude acetone secondary metabolites of *T.rubrum* extract of HHD extract chart given the three retention time peak with measure 2.831,9.440 and 12.894 respectively and excluded the other may be turn to the purity of extracts process and have the more purity extract broth.

In the other hand figure (3-13) and table (3-10) were shown HPLC analysis of crude acetone secondary metabolites of *T.rubrum* extract of KSD which is exhibited also three retention time peak with measure 4.999,8.486 and 9.313 respectively , overall look the similarity separation between both fermentation media extract that was corresponded with TLC separation which were shown the identify separation.

The figure (3-14) and table (3-11) were represented the Benzylpenicillin G HPLC result as standard which was revealed three obvious retention time peaks with measure 4.503,6.321and 2.105 respectively, When corresponding with the chart HPLC results of crud acetone secondary metabolites extracts observe convergence in the level of the time of retenation for penicillin, which can be regarded as having similar to this antibiotic as a secondary metabolism products , which gave top 6.321 and the estimated area of the peak within the time of the retenation of about 212 062 and this seemed to clear through the figure and table accompanying.

This was corresponded to previous studies which illustrated the potential for isolating penicillin G or semi-penicillin G and particularly referred to Hammadi *et al.*(2007) in this study, which stated that the products of metabolism secondary *T.rubrum* contain a semi-penicillin G, as well as a reference study, which was completed by Youssef *et al.* (1979) in this regard, which isolate penicillin G from the Dermatophytes ,

But were found of impurities extracts and requests for additional processes to purify we could obtain high purity ratio extracts and our dependence on the pharmaceutical compounds standardized as materials

may container on extra material given the volatility and homosexuality in the classroom high efficiency.

The figure (3-15)and table (3-12) were represented the Fusidic acid result as standard, which was revealed six obvious retention time peaks with measure 0.728,1.768,2.765,6.038,8.395 and 10.547, When corresponding with the diagram extracts observe convergence in the level of the time of retenation Fusidic acid When matching these results with the results of extracts find When matching these results with the results of extracts, we find convergence in the summit area curve chart, which was 8.395 given a clear approach with a the chart of the extracts HHD and KSD in areas 9.440 and 9.313 respectively, when corresponded with TLC result .

We was found and to some extent the existence of convergence in the matching results obtained from HPLC and this indicates the possibility of the presence of Fusidic acid in the secondary metabolism products of *T.rubrum* and this corresponds to the study Perry *et al*. (1983) Isolates of *Microsporum canis, Microsporum gypseum* and *Epidermmphyton floccosumwere* observed to produce antibacterial activities of fusidic acid.

Observed that the results we had obtained in our research is not consistent with studies that have implemented by researchers Hammadi *et al*.(2007) Youssef *et al*. (1979) the two made no mention of the presence fusidic acid in the secondary metabolism of *T.rubrum* extract. Our research first to emphasize the possibility of extracting Fusidic acid.

The figure (3-16) and table (3-13) were represented the Kojic acid HPLC result as standard, which was revealed seven obvious retention time peaks with measure 4.281, 5.502, 9.358, 9.983, 11.902, 19.300 and

14.775 respectively. When corresponding with the chart HPLC results of crud acetone secondary metabolites extracts observe , where Display the chart with high level of multiple peaks and when matched with the results extracts showed lack of convergence between them and that match the results obtained from separation through the TLC This shows that the lack of the product acid kojic acid in secondary metabolism of the *T.rubrum* , this result were with those studies carried out by both Youssef *et al*. (1979) and Perry *et al*. (1983) .

However, it does not match with the study conducted by Hammadi *et al*. (2007) and those who were claimed the possibility of producing Kojak acid and extracted from the secondary metabolism of *T.rubrum*.

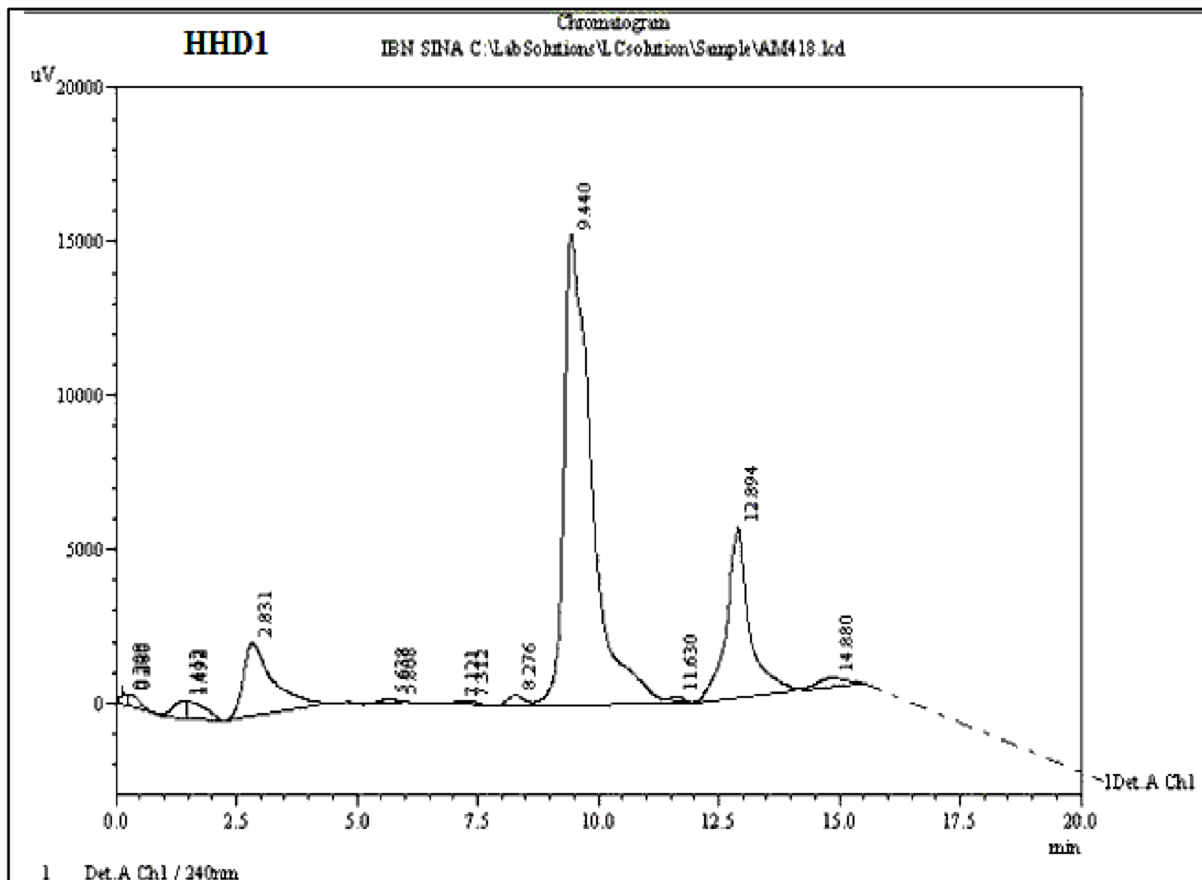


Figure (3-12): HPLC chromatogram of the HHD extract

Table (3-9): the peaks of HHD separation in HPLC and R. time

Peak	Ret. Time	Area	Height	Area %	Height T

1	2.831	101235	2374	10.479	9.023
2	9.440	617973	15205	63.967	58.167
3	12.894	182293	55334	18.869	21.034

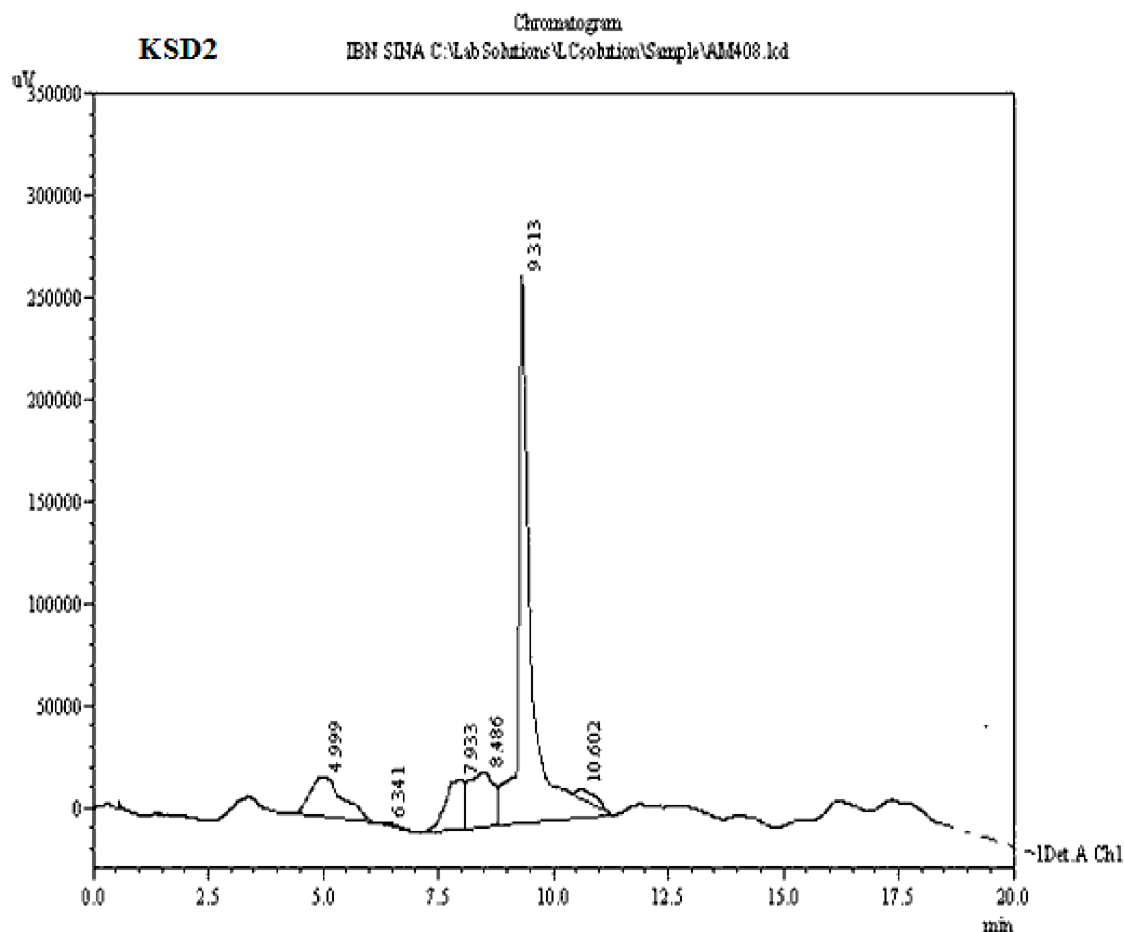


Figure (3-13): HPLC chromatogram of the KSD extract

Table (3-10): the peaks of KSD separation in HPLC and R. time

Peak	Ret. Time	Area	Height	Area %	Height %
1	4.999	1008043	19954	12.370	5.789
2	8.486	1024408	27128	12.571	7.871
3	9.313	5211970	267996	63.957	77.753

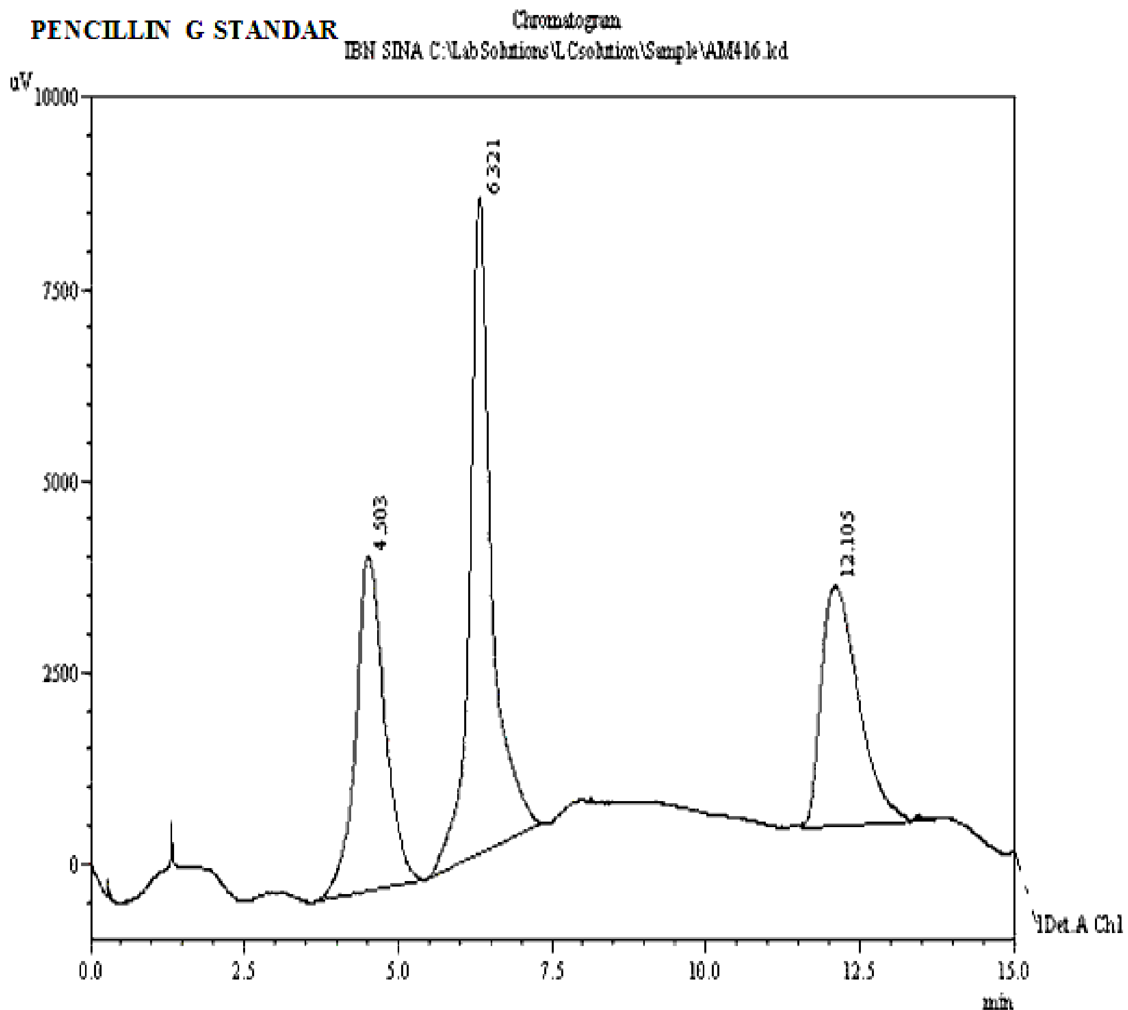


Figure (3-13): HPLC chromatogram of standard The Benzylpenicillin G

Table (3-11): the peaks of Benzylpenicillin G separation in HPLC and R. time.

Peak	Ret. Time	Area	Height	Area %	Height %
1	4.503	143297	3461	29.440	27.187
2	6.321	212062	8558	43.568	53.353
3	12.105	131383	3122	26.992	19.640

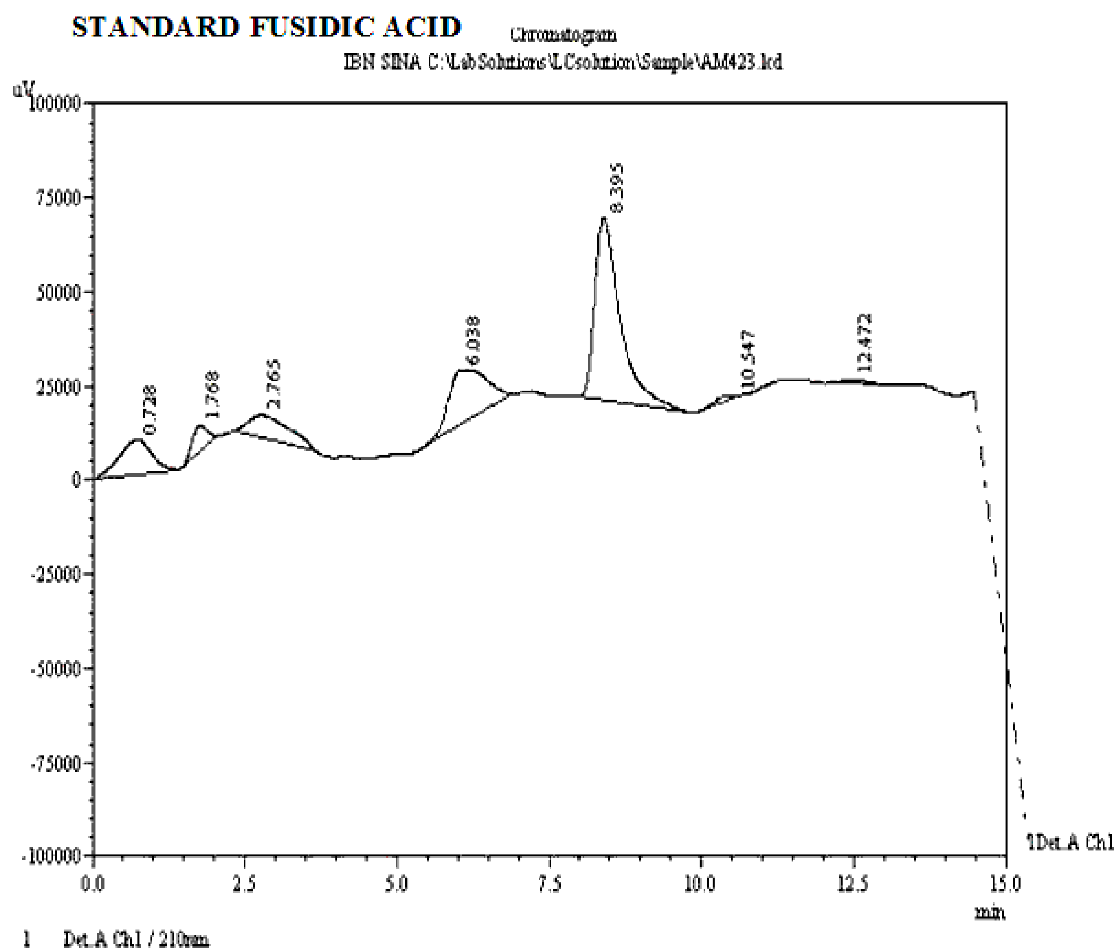


Figure (3-14): HPLC chromatogram of standard Fusidic acid

Table (3-12): the peaks of Fusidic acid separation in HPLC and R. time.

Peak	Ret. Time	Area	Height	Area %	Height %
1	0.728	349910	9261	12.533	10.816
2	1.768	124593	6702	4.463	7.826
3	2.765	278501	5557	9.975	6.489
4	6.038	584855	14322	20.948	16.726
5	8.395	1398551	48260	50.093	56.359
6	10.547	25857	390	0.926	0.456

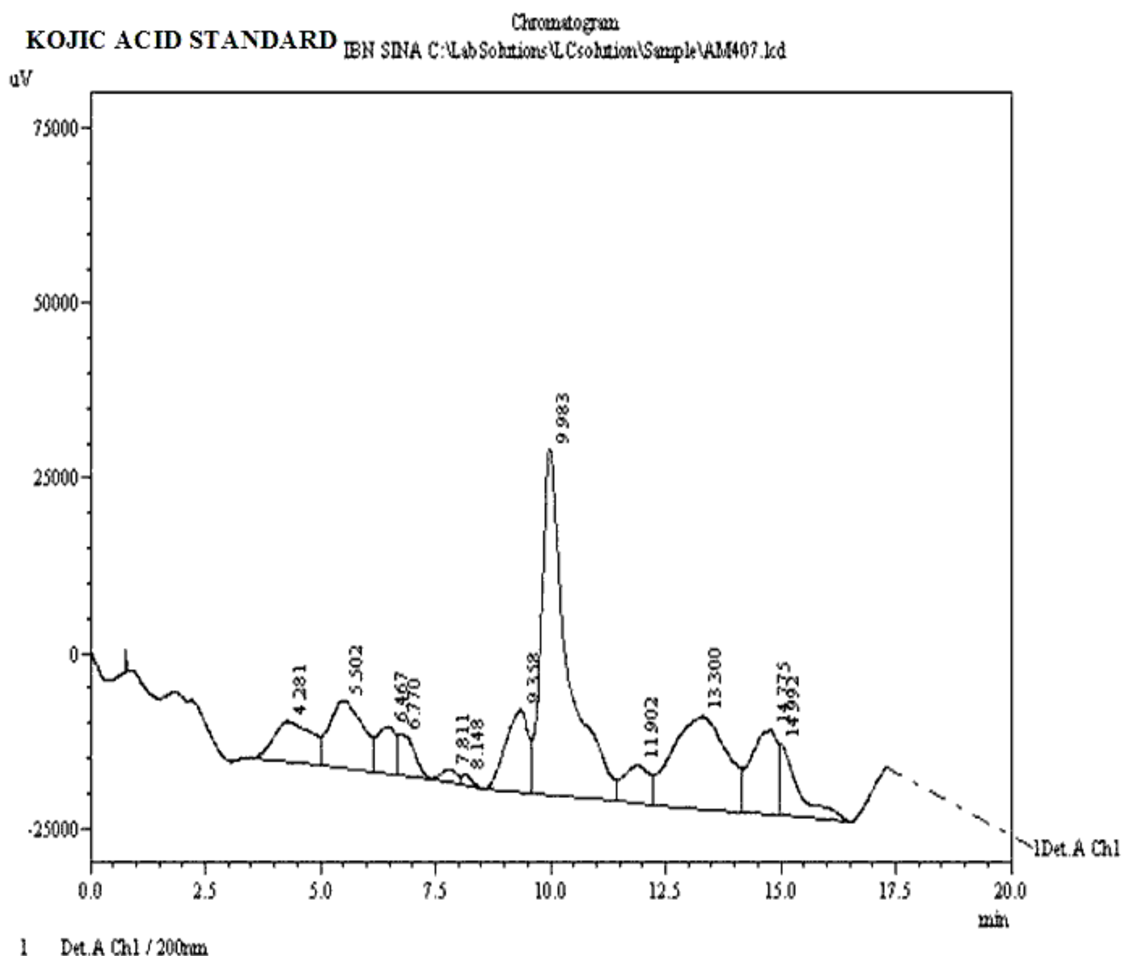


Figure (3-16):HPLC chromatogram of standard Kojic acid

Table (3-13): the peaks of Fusidic acid separation in HPLC and R. time.

Peak	Ret. Time	Area	Height	Area %	Height %
1	4.281	337140	5832	5.959	4.352
2	5.502	490484	9707	8.670	7.243
3	9.358	411679	11884	7.277	8.868
4	9.983	1943006	49418	34.344	36874
5	11.902	213982	5426	3.782	4.048
6	13.300	1089337	13191	19.255	9.842
7	14.775	476263	12257	8.418	9.146

3.10. Fourier Transform Infrared spectrophotometer (FTIR) analysis of the crude Acetone of *T.rubrum* extracts

FTIR used to characterize the organic groups of the wanted active material in crude Acetone secondary metabolites of *T.rubrum* extract and compared with standard compound by documented spectrum in the range of wave (400 nm and 4000 nm) Figures (3-17,3-18,3-19,3-20,3-21) revealed the results obtained from the FTIR analysis charts to detect functional groups in both standards compound and crude acetone extracts shown in the following table(3-14), and the presence of these functional groups and their frequencies throughout the peaks and, this shifting may be due to the few impurities were found in the sample.

Depending on the frequency found that the crude extract contains numerous compounds, where the following frequencies in HHD , KSD extracts, Benzylpenicillin G and fusidic acid the absorption package appearance at frequencies (3444.87,3410.15,3510.5,3444.87cm^{-1}) respectively indicated to poly phenol.

The appearance of the package at the frequency (1735-1686 cm^{-1}) indicate the presence of carboxylic acid. which were the package frequencies of the group amides in both the samples extracts and standard BPG, FA (3444.87, 3410.15, 3444.87, 3444.87cm^{-1}) respectively, also the package frequencies of the hydroxyl group were (3170.9-3263.56cm^{-1}) emerged only with BPG and FA.

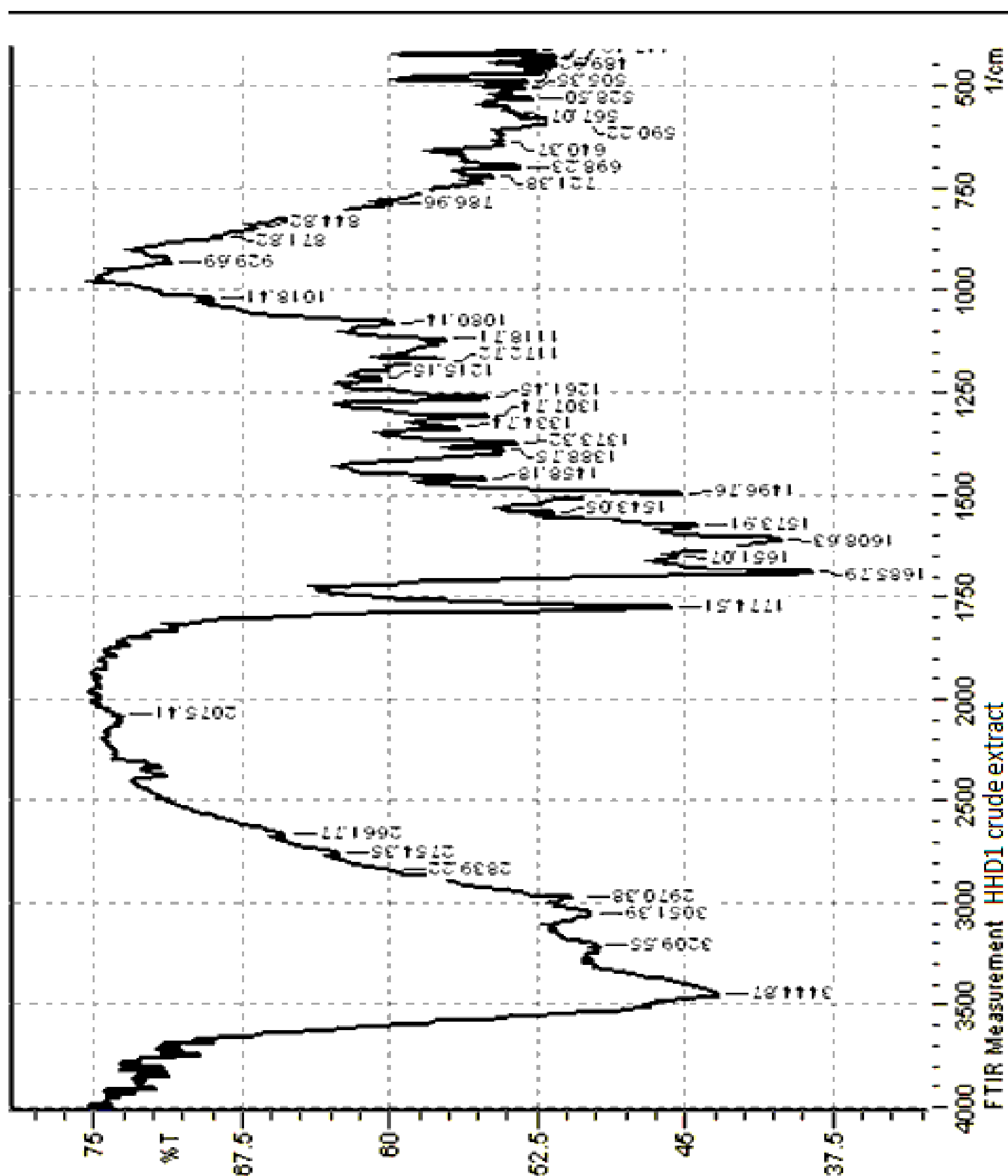
In addition, package frequencies of the Aromatic group (3150-3050cm^{-1}) in just the samples extracts as well as the Sulfones groups (1375-1360 cm^{-1}) in just the samples extracts, which was proved for the first time about the possibility of isolated and extracted from secondary metabolism of *T. rubrum*, which was the organic compounds that have a

high affinity to inhibit pathogenic bacteria, which enters in the pharmaceutical compositions and especially the antibiotics as Sulfonamides. The frequencies that were indicating to Sulfates (1200-1100 cm^{-1}) appeared in both extracts and standards compound except KA.

As well as Benzene ring (939.33-765.74 cm^{-1}) were appeared in both extracts and standards compound except FA the emergence of the absorption packages when preparing wavelengths (939.33 and 862.18 and 765.74) cm -1 is attributable to compensation duo to the benzene ring at the site 1,4 (1,4-α-disubtituted of ring) (Saleh *et al*., 2011). Moreover, it should be mentioning that IR spectroscopy was used to detect vibrations of molecules and properly diagnosed and private vibrational distinctive double and triple bonds in many effective units (Vijayakumar *et al.*, 2008).

Function groups	**Symbol**	**Frequency range(cm^{-1})**	**Crude Acetone secondary metabolites extract**		**Std.**		
			HHD	**KSD**	**BPG**	**FA**	**KA**
Poly phenol	OH	3410-3320	+	+	-	+	
Carboxylic acid	O-H	1735-1686	+	+	+	+	
amides	N-H	3590-3440	+	+	+	+	-
hydroxy group	-OH	3170.9-3263.56	-	-	+		+
Aromatis	C-H	3150-3050	+	+			
Alkanes	C-H	3000-2800	+	+	+	+	+
Aldehyde	C-H	2800-2700	+	+	+	+	
anhydride	C=O	1810-1760	+	+	+	-	-
Aromatic	C=C	1600-1475	+	+	-	-	+

Sulfones	R–S(=O)2–R	1375-1360	+	+	-	-	-
Sulfates	S=O	1200-1100	+	+	+	+	-
Benzene ring	C6H6	939.33-765.74	+	+	+		+



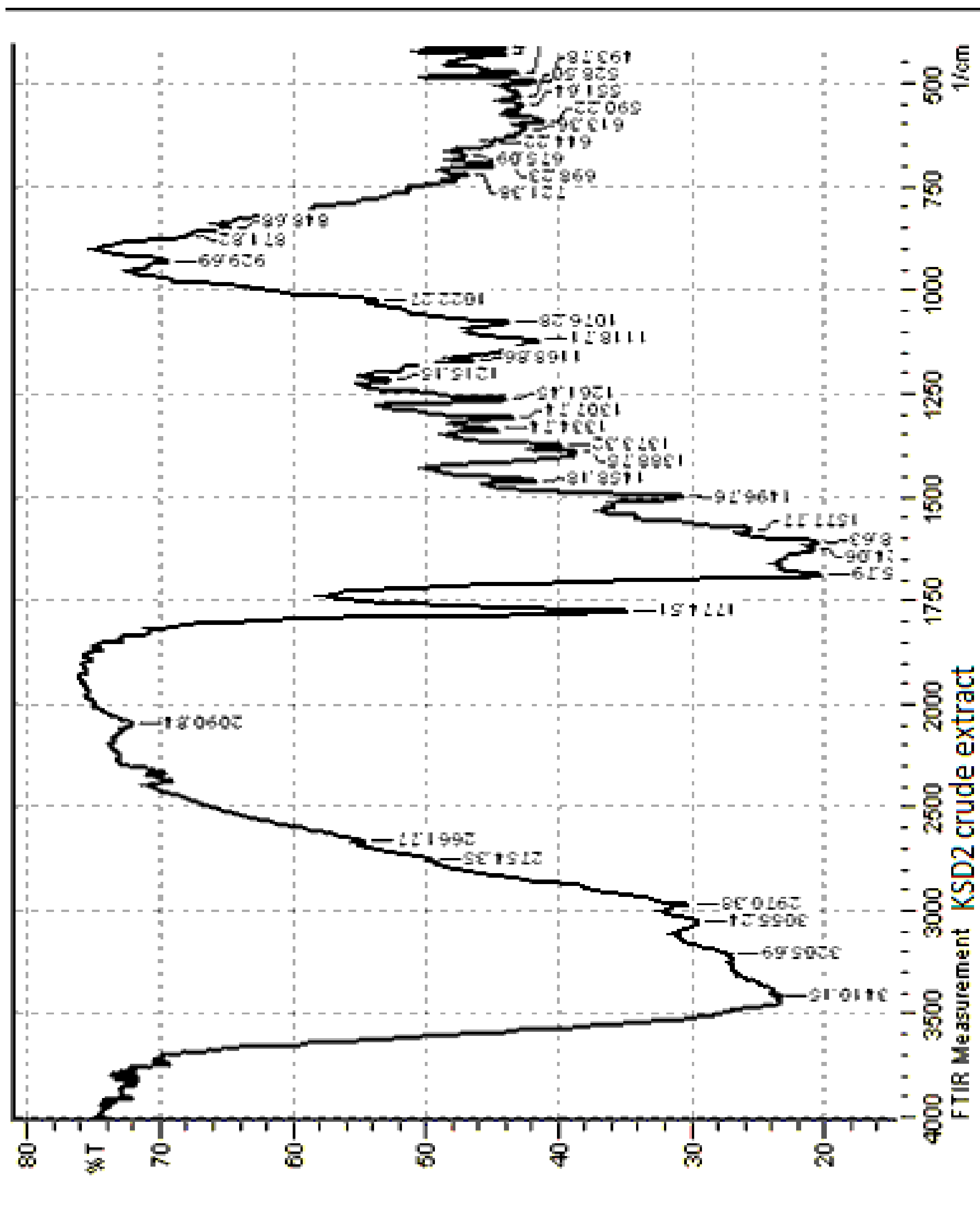
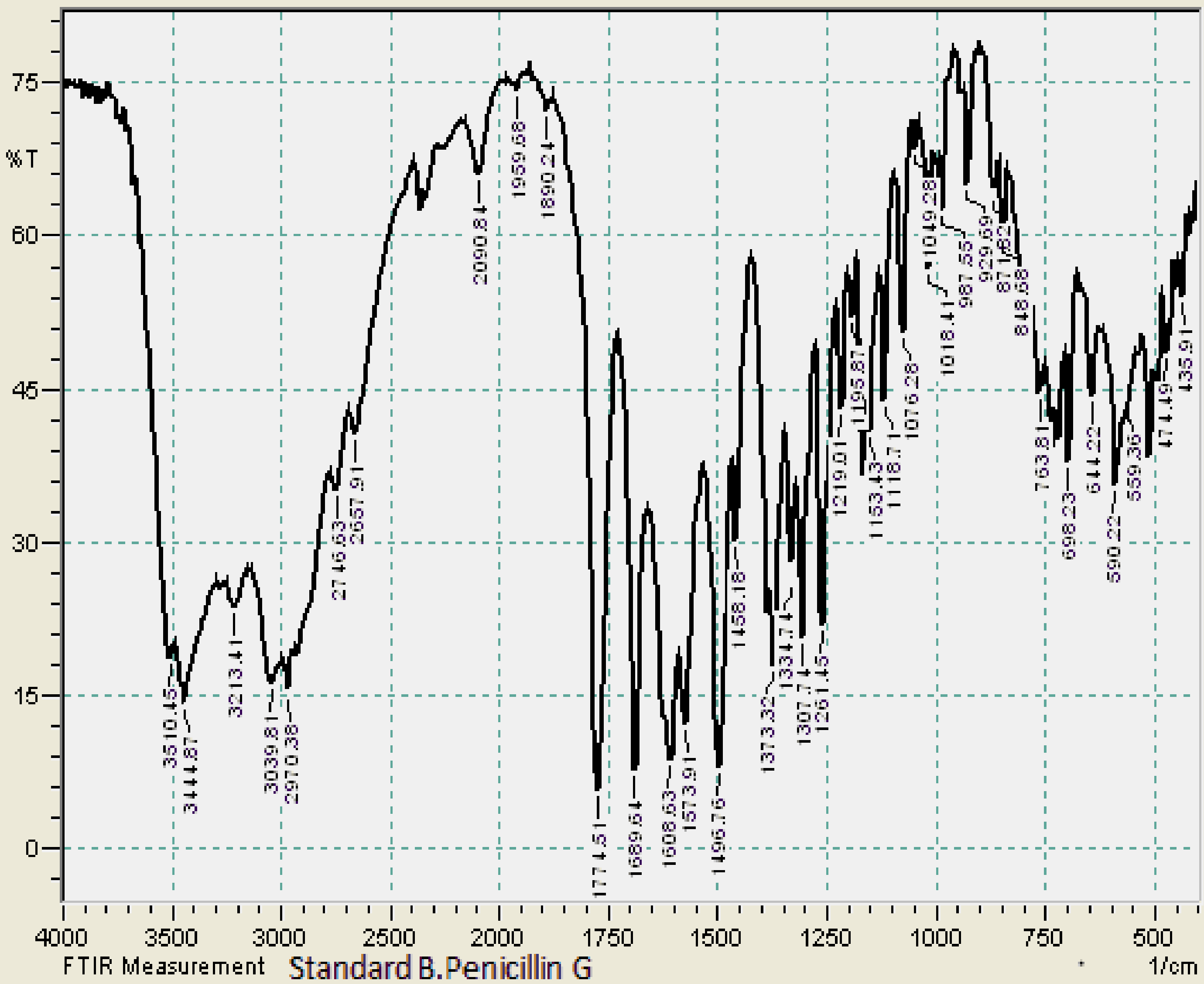
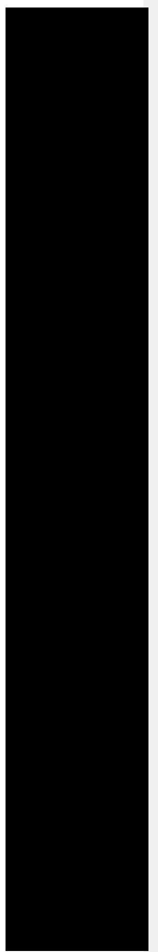


Figure (3-18): FTIR spectrum of KSD2crude Acetone of secondary metabolites of *T.rubrum* extract in the collected fractions by Fourier Transform Infrared spectrophotometer (FTIR).



%T
75
60
45
30
15
0
4000
3500
3000
2500
2000
1750
1500
1250
1000
750
500
1/cm
3510.45
3444.87
3213.41
3039.81
2970.38
2746.63
2657.91
2090.84
1959.58
1890.24
1774.51
1689.64
1608.63
1573.91
1496.76
1458.18
1373.32
1334.74
1307.74
1261.45
1219.01
1195.87
1153.43
1118.71
1076.28
1049.28
1018.41
987.55
929.69
871.82
848.68
763.81
698.23
644.22
590.22
559.36
474.49
435.91
FTIR Measurement
Standard B.Penicillin G

%T
75
60
45
30
15
0
3444.87
3236.55
3047.53
2970.38
2924.09
2746.63
2657.91
2090.84
1959.68
1890.24
1774.51
1689.64
1608.63
1573.91
1496.76
1458.18
1373.32
1334.74
1307.74
1265.30
1215.15
1153.43
1118.71
1076.28
1022.27
991.41
929.69
871.82
848.68
736.81
721.38
698.23
667.37
644.22
586.36
570.93
501.49
466.77
4000
3500
3000
2500
2000
1750
1500
1250
1000
750
500
1/cm
FTIR Measurement
Standard Fusidic acid


%T
80
60
40
20
0
4000
3500
3000
2500
2000
1750
1500
1250
1000
750
500
1/cm
FTIR Measurement
Standard Kojic Acid
3271.27
3174.83
2924.09
2854.65
2665.62
2522.89
2222.00
2013.68
1924.96
1766.80
1697.36
1658.78
1627.92
1608.63
1581.63
1523.76
1469.76
1438.90
1392.61
1350.17
1280.73
1226.73
1141.86
1072.42
987.55
941.26
864.11
775.38
640.37
609.51
551.64
513.07
470.63
439.77

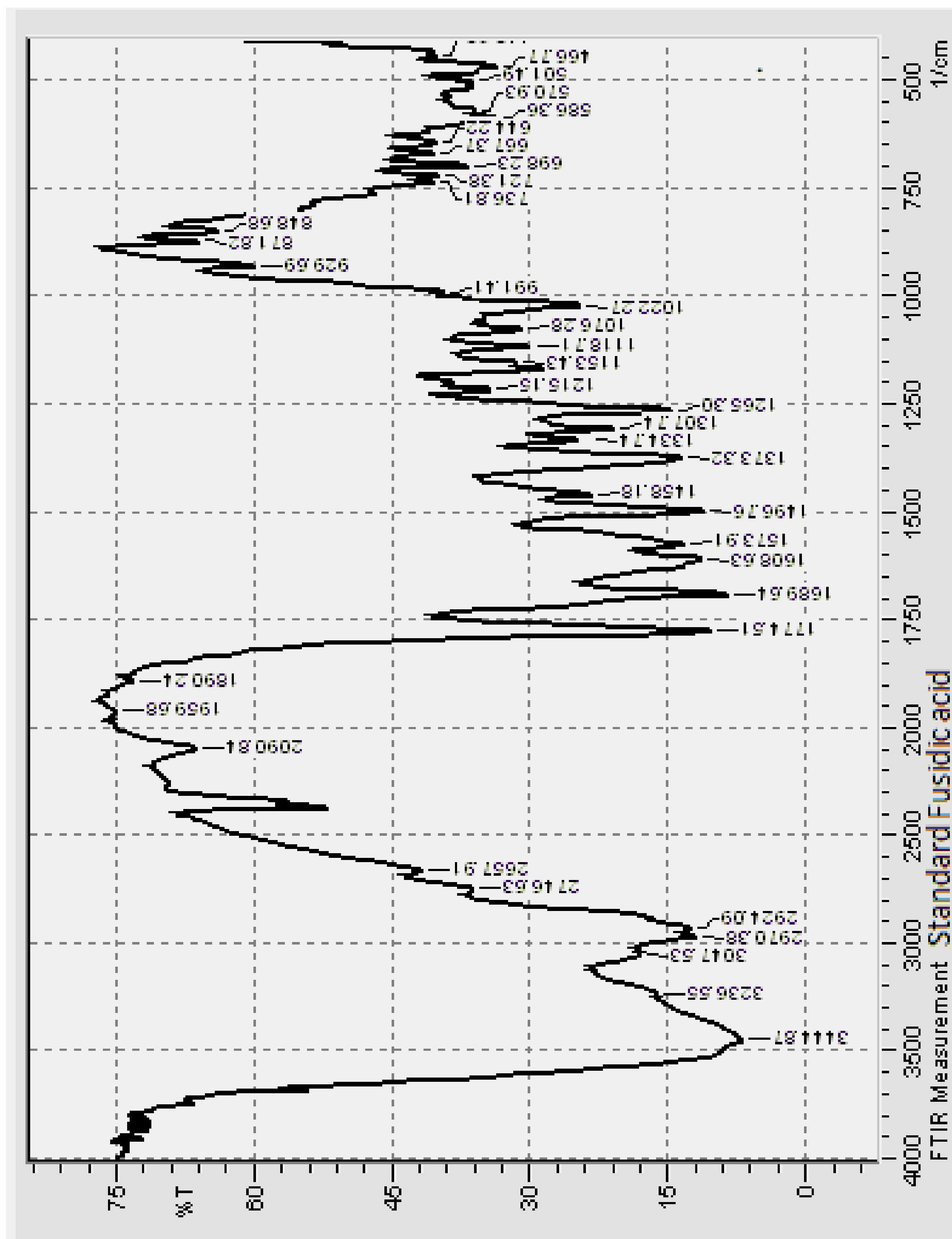


Figure (3-22): The IR value for Standard Kojic Acid in the collected fractions by Fourier Transform Infrared spectrophotometer (FTIR).

CONCLUSIONS

1. Horse Hair Dextrose liquid medium (HHD) and keratinized skin dextrose liquid medium (KSD) revealed promotion to produced secondary metabolites from *T. rubrum.*
2. The crude acetone were exhibited antibacterial activity against some pathogenic bacteria and showed antioxidant activity using DPPH radical scavenging method.
3. The mortality rate of brine shrimp napulii was increased with increasing the concentration of the 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.
4. Crude extract from HHD revealed high-level of toxicity compared with KSD extracted with mortality 30%.
5. The FTIR and HPLC tests provided a high purity level for the Crude acetone of secondary metabolites of *T.rubrum* when compared with standard compound, and lead to increase the installed fact that the compound likes antibiotic substances as semi-Benzyl pencilline and semi –fusidic acid.
6. Isolation and identification Sulfones for the first recorded in Iraq from SMs extracts of *T. rubrum*, which are potent antibacterial effective against many gram-positive and gram-negative organisms and are widely used in treatment and which enters in pharmaceutical compositions for the treatment of many diseases

RECOMMENDATIONS

1. Further experiments with multiple strains of other organisms to confirm the utility of the bioactive crude acetone SMs extracts.
2. Using different culture media of fungus to improve the production of bioactive compounds at industrial level.
3. Further investigation of this potential antimicrobial agent may help in developing new chemical classes of antibiotics.
4. Purification and identification the bioactive compounds using different advanced techniques like NMR, as well as chromatography.

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الخلاصة

شخص مئة مريض سريريا في هذه الدراسة يعانون من داء الفطار الجلدي والذين راجعوا استشارية الامراض الجلدية والزهرية في مستشفى الديوانية التعليمي خلال الفترة من كانون الاول/ 2014 لغاية ايار 2015 لغرض عزل الفطر الجلدي *Trichophyton rubrum* وتقييم قابليته على انتاج مواد حيوية فعالة واختبارها كعوامل بكتيرية مضادة. من هؤلاء المرضى ,جمعت عينات من قشطات جلدية و اجزاء شعر وقضمات اضافر لغرض الفحص المباشر والزرع المختبري.

شخصت 11 عزلة من الفطر *T.rubrum* باستخدام الطرق التقليدية وتوكيد تشخيصها بالطرق الجزيئية (تفاعل سلسلة البلمرة), اختبرت الظروف المثلى لنمو وانتاج المنتجات الثانوية للفطر*T.rubrum* تحت درجات حرارة ودالة حامضية واوقات ومواد تفاعل مختلفة .واظهرت النتائج ان الدالة الحامضية المثلى هي(5) ودرجة الحرارة (28ºC) ووقت (10) ايام ومواد تفاعل هي (وسط الدكستروز الحاوي على شعر الحصان و الوسط الدكستروز الحاوي على جلد بشري متقرن).

تم فصل بواسطة طبقة الكروماتوغرافيا الرقيقة(TLC) للمركبات القياسية ومقارنتها مع المستخلص والتي اظهرت بقع بنفسجية تحت الضوء الاشعة فوق البنفسجية.

ثم شخص بواسطة الكرماتوغرافيا السائل العالي الاداء (HPLC) و تحويل فورييه مطياف الأشعة تحت الحمراء(FTIR).

اظهرت نتائج الكرماتوغرافيا السائل العالي الاداء (HPLC) ان كل من المستخلص HHD وKSD اعطيا ثلاث قمم بوقت استرجاع مختلف عند استخدام Benzyl penicillin G وحامضfusidic وحامضkojic كمركبات قياسية حيث اظهرت نتائج HPLC عن وجود Benzyl penicillin G وحامضfusidic وحامضkojic في نواتج الايض الثانوي للفطر *T.rubrum* .

شخصت المجاميع الفعالة لمستخلص الايض الثانوي بواسطة طيف FTIR واظهرت النتائج ان المستخلص احتوى مجاميع فعالة متعددة ,حيث ان التكرارات العالية في المستخلص HHD وKSD :البنزيل بنسلينG وحامض الكوجيك ,ومن ناحية اخرى مجموعة الفعالة

السلفونات في مستخلص الايض الثانوية حيث ان مجموعة الكبريت ظهرت في كلاً المستخلصين.

اظهرت مستخلص الايض الثانوي لفطر *T.rubrum* في كلا الوسطين HHDو KSDتثبيطا نمو *Staphylococcus epidermidis*(32 مليميتر) و10.5مليميتر ضد كل من, و*Escherichia coli* و.*Pseudomonas aeruginosa* .

بينت النتائج ايضاً ان مواد الايض الثانوي المستخلصة بالاسيتون من الفطر *T.rubrum* ذات فعالية ضد الاكسدة باستخدام طريقة كاسح الجذور DPPH عند مقارنتها بالمادة القياسية Ascorbic acidحيث الامتصاصية كانت 517 نانوميتر ,وقد تم التعبير من هذه الفعالية بقيم IC50حيث كانت 14.8و21.3 مايكروغرام /مل بينما المادة Ascorbic acid القياسية كانت 9.3مايكروغرام/مل .

فحصت سمية المستخلصات باستخدام يرقات الروبيان ذو الحساسية العالية لمواد السمية في داخل الخلايا, اظهرت النتائج اختلاف في مستوى السمية بتغير التراكيز المستخدمة وكان متوسط تركيز المميت 16مايكروكرام/مل

وكانت الخلاصة الدراسة الحالية بينت القدرة الفطريات الجلدية على انتاج مواد ايض ثانوية كمضادات حياتية وخصوصا مركبات السلفونات والتي عزلت لأول مرة في العراق من هذا الفطر.

وزارة التعليم العالي والبحث العلمي
جامعة القادسية
كلية الطب

تشخيص وتقييم الفعاليات الحياتية لبعض المنتجات الايض الثانوية المنتجة من عزلات محلية من الفطر الشعروية الحمراء

اطروحة مقدمة
الى مجلس كلية الطب جامعة القادسية
وهي جزء من متطلبات نيل شهادة دكتوراه فلسفة في علم الأحياء المجهرية الطبية

من قبل

حيدر عبد علي عبدالرحمن الشاوي

بكالوريوس تقنيات التحليلات المرضية – 2007

ماجستير تقنيات التحليلات المرضية– 2012

بإشراف

ا. د. عدنان حمد الحمداني　　　　ا.م. د. نوفل حسين الدجيلي

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