1.1 Introduction

The use of systemic drugs is limited to treat man or animal due to their high toxicity and problems of residues in products intended for human consumption (Araujo *et al*,2009).Different treatments have been recommended to control dermatophytes in general pharmacological treatment option include antifungal agents(Aly,1997;Agwa *et al*,2000).Recently the use of some natural plant products has been emerged to inhibit the causative organisms .

The antimicrobial and antitoxin properties of some plants ,herbs and their components have been documented since the late 19th century (Saadabi, 200 6).These natural plants involve garlic ,lemon grass, datura, acacia. A triplex ginger, black seed, neem, basil, eucalyptus and alfalfa(Omer and Abd-El-Hal- im,1992; Aly *et al*,2000 and Aly and Dafedar, 2008).They are safe to human and the ecosystem than the chemical fungicides for ailments and [illegible] used them for thousands of years to [illegible] foods as well as its economic value (Shelf *et al* ,1980; Shelf *et al* , 1984). Early cultures also recognized the value of these plant materials in medicine .Plant extract has been used traditionally to treat a number of infectious dise- ases including those caused by bacteria ,fungi, protozoa and viruses (Soylu *et a*l,2005;Yoshida *et al*,2005; Nejad and Deokule,2009).

The activity of plant extract against dermatophytosis i.e. the superficial infections of skin or keratinized tissue of man and animals can be very well visualized from the reports of Venugopal and Venugopal (1995). They reported the activity of plant extracts against 88 clinical isolates of dermatophytes which includes *Microsporum canis, Microsporum audouinii ,Trichophyton rubrum , Trichophyton*

mentagrophytes, Trichophyton violaceum, Trichophyton simii, Trichophyton verrucosum, Trichophyton erinacci ,Epedermophyton flocosum by agar dilution technique. While (Vlietinck *et al*,1995)reported clinical findings of Rawandese medicinal plants (267)plant extracts used by traditional healers to treat microbial infections and found 60% of these extracts were active against dermatophytes species.

Recently more than 200 different biologically active substances have been isolated from plant extract ,among them organosulphur compounds such as all- icin ,azoenes and diallyl trisulfide .Eugenol, phenolic compounds the most imp- ortant biologically active compound found in many plant extract (Aly and Bafiel,2008).

efficient non-toxic antifungal s and increasing number of treatment failures have motivated current searches for therapeutic alternatives to include the testing of medicinal plant and essential oils as potential antimicrobial agents (Mondello *et al*,2003; Pina *et al*,2004).

People used many plants and plant preparations to cure various skin infections and other disease .Though the recovery is slow ,the therapeutic use of medicinal plant is becoming popular because of less side effects. Unlike the synthetic drugs medicinal plants have the ability to control antibiotic resistant micro organism (Rawat *et al*,2003). Dermatophytoses are one of the most frequent skin diseases of human

pets and livestock .It's widely distributed all over the world with various degrees and more common in man than in women(Brook *et al*,1998).

Prangos platychlaena is used as a folk medicine in eastern Turkey to treat under tail wounds of cattle and to stop bleeding in man by application on wounds. The antifungal activity of 30 aqueous medicinal plant extract which distributed in Jordanian environment ,especially *Mentha viridis* and *Rosmarinus officinalis* aquous extract were studied.In Malaysia the antifungal activity of Gignoniaceae family was studied and it revealed that the extracts which obtained by dichloromethane solvent of the following plant species *Tacaranda filiciefolia, Tabeduia chrysantha,Oroxylum indicum and Fernadoa adenophyla* are most effective against dermatophytes (AL-Suhaili,2001).

Aloe vera 5%gel applied topically 3 times daily to the patient ... area of glabrous skin ... cure rates were noted from the first week of treatment. Moham... *officinale* and *Pimpella anisum* were examined against bacteria as well as two molds *Trichophyton mentagrophytes* and *Microsporum gypseum* and one yeast species *Candida albicans* and they revealed a good results especially *Thymus vulgaris* oil secondly the oil of Eucalyptus and zengiber becoming in 3rd degree while *mentha piperita* and *Pimpella anisum* oil had no inactivation effect(Al-Jubori,2005) .

The alcoholic extract of *Allium cepa* high antifungal activity rate against dermatophytes species *Trichophyton mentagrophytes* (Mohammed ,2009). *Paliurus spina-christi* belonging to Rhamnaceae family commonly known as Jersalem thorn ,Garland thorn or crown of thorns, (Rushforth,1999),Its used as diuretics, against diarrhea and

rheumatism in traditional Croatian herbal med- icine (Grlic,1986)Antibacterial activity mainly on G+ve bacteria (Brantner *et a*l,1996).

Paliurus spina-christi rich source of flavonoids isoquercitin ,rutin ,hyperoside (Dalakishvili *et al*,1986; Kustark *et al* ,1990).A quercetin-3-O-triglycoside (Brantner and Males,1990).This plant found in the north of Iraq ,Al-Sulaimanya governorate ,Rania village ,kaladizah ,Beramagron mountains it doesn't studied in Iraqi universities or by any researchers. For above reasons its interesting to study the chemical and biological properties of this plant.

The aims of the study were proposed for:

1. Isolation of dermatophytes species from patient infected with
2. In vitro and in vivo (histopathological examination)study of the antidermatophyte activity of the alcoholic *Paliurus spina-christi* extract .using light and electron microscope.
3. Molecular assessment of Tri m4 gene.

1.2-Literature Review:

1.2.1 History of medicinal plants

Historically, therapeutic results have been mixed, quite often cures or symptom relief resulted. Poisonings occurred at a high rate also. Clinical microbiologists have two reasons to be interested in the topic of antimicrobial plant extracts.

First its very likely that these phytochemicals will find their way into the arsenal of antimicrobial drugs prescribed by physicians ,several are already being tested in humans ,its reported that on average ,two or three antibiotics derived from microorganisms are lunched each year (Clark,1996).Plants play an important role in the medicinal practices of many ,if not all peoples .Thus plants are used not only in the diagnostic process ,but also in the physical and ritual purification procedures that commonly precede ceremonies and in the act of healing itself (Moerman,1996). After a downturn in the use in recent decades, the pace is a gain quickening as scientists seek to the effective life span of any [illegible]

Second, the public is becoming increasingly aware of problems with the prescription and misuse of traditional antibiotics .In addition ,many people are interested in having more autonomy over their medicinal care . Although several synthetic antimycotic drugs are available in the market, at present the use of these drugs has minimized because of a number of factors which includes low potency, poor solubility, development of resistant strains, drug toxicity and side effects, like gastrointestinal disturbances, cutaneous reaction, hepatotoxicity, leucopenia …etc (Brownlee *et al,*1990). The poor availability of efficient non toxic antifungals and increasing number of treatment failures have motivated current searches for therapeutic alternatives to include the testing of

Medicinal Plant and essential oils as potential antimicrobial agents (Butler,1988; Cardellina *et al*,1993).

The uses of traditional medicinal plants for primary health care have steadily increased worldwide in recent years. The goals of using plants as sources of therapeutic agents are

a) To isolate bioactive compounds for direct use as drugs, e.g., digoxin, digitoxin, morphine, vinblastine, vincristine.

b) To produce bioactive compounds of novel or known structures as lead compounds for semisynthesis to produce patentable entities of higher activity and/or lower toxicity, e.g., metformin,nabilone, oxycodon (and other narcotic analgesics), taxotere, teniposide,verapamil, and amiodarone, which

taxol, podophyllotoxin,khellin, and khellin.

c) To use agents as pharmacologic tools, e.g., lysergic acid diethylamide, [illegible]

echinacea, feverfew, garlic, ginkgo biloba, St. John's wort, saw palmetto.

Scientists are in search to discover a new bioactive compounds Scientists are in search of new phytochemicals that could be developed as useful anti-microbials for treatment of infectious diseases. Currently, out of 80% of pharmaceuticals derived from plants, very few are now being used as anti-microbials. Plants are rich in a wide variety of secondary metabolites that have found anti-microbial properties (Samy and Gopalakrishnakone,2010).

1.2.2 Scientific Classification of plant used in this study:-

Kingdome :Plantae

Division :magnoliopsida

subclass:Rosidae

Order:Roales

Family:Rhamnaceae

Genus and species: Paliurus spina-christi

Trees or shrubs .Leaves simple ,usually stipulate .Inflorescence ... Flowers perigynous .Calyx 4- to 5-lobed, lobes valvate in bud ... Petals 4-5, often small and sometimes absent ... hooded over the stamens ... with the calyx-lobes; anthers versatile. ovary superior, 2 to 4-locular ;ovules solitary .Fruit often fleshy(Heywood *et al*,1968) .

Rhamnaceae, the Buckthorn family, is a large family of flowering plants, mostly trees, shrubs and some vines. The family contains 50-60 genera and approximately 870-900 species. The Rhamnaceae have a worldwide distribution, but are more common in the subtropical and tropical regions. The earliest fossil evidence of Rhamnaceae is from the Eocene.

The simple leaves can be either alternate and spiraling, or opposite. Stipules are present. These leaves are modified into spines in many genera, in some (e.g. *Paliurus spina-christi* and *Colletia* cruciata)

spectacularly so. Colletia stands out by having two *axillary* buds instead of one, one developing into a thorn, the other one into a shoot(Rushforth,1999).

1.2.3Description of *Paliurus spina-christi*

Paliurus spina-christi Miller. Nearly glabrous ,much –branched shrub up to 3cm.Twigs flexuous ,puberulent when young .Leaves 2-4 cm, alternate and distichous ,ovate crenate –serrate ,shortly petiolate. Flowers in small, axillary,hortly peduculate cymes. Fruit 18-30 mm in diameter; wing undulate. Dry slopes ;also often used for hedges. Miditerranean region. Balkan peninsula and black sea coast(Heywood *et al*.1968)

It is a deciduous shrub or small tree growing to 3-4 m tall. The shoots are zig-zagged, with a leaf and two stipule spines (one straight, one curved) on the outside of each kink. The leaves are oval, 2-5 cm long and 1-4 cm broad, glossy green, with an entire margin. The fruit is a dry woody nutlet centered in a circular wing 2-3.5 cm diameter, The name reflects an old legend that the spiny branches were used to make the crown of thorns placed on Christ's head before his crucifixion (Rushforth,1999).

1.2.4 Distribution of *Paliurus spina-christi*

Paliurus spina-christi. is a traditional Mediterranean and Asiatic medicinal plant (Polunin and Huxley,1981). widely distributed in dry and rocky places in the Mediterranean region and Asia (Mosaddegh *et al*, 2004)

Cyclocarya paliurus (Batal.) Iljinskaja (*C. paliurus*) is a Juglandaceae plant, an endemic tree growing on cloudy and foggy highlands in the south of China.(Kurihara *et al*,2003) .*Cyclocarya paliurus* (Batal.) Iljinskaja, grown on cloudy and foggy highlands in southern China, is a well-known edible and medicinal plant and is commonly called the 'sweet tea tree' because of the flavour of its leaves (Liu *et al*, 2009).

1.2.5 Chemical constituents

Chemical investigation of *Paliurus spina-christi* fruits and leaves indicated the presence of the flavonoides isoquercitin,rutin, hyperoside (Dalakishvili *et al*, 1986, Kustark *et al*,1990) .Sterols (Dalakishvili *et al*,1985) .In addition, the fruits contain alkaloids (Velcheva,1993). Generally plant parts contain flavonoids and tannins (Males,1995).

1.2.6 Biological potentials

Paliurus spina-christi commonly used as a diuretic and against diarrhea and rheumatism (Grlic, 1986). A remarkable antibacterial activity, mainly on Gram-positive bacteria, of the ethanolic extracts from different plant parts was found ,*Paliurus spina-christi* demonstrate a clear inhibition zone against gram positive and negative bacteria (*Staph*

aureus, Streptococcus faecalis, Micrococcus luteus, Shigella sonnei, Proteus mirabilis, E.coli) (Brantner *et al*., 1996) .Also used against rheumatism in ethnomedicine (Wichtl,1997). *Paliurus spina-christi* fruit could be a good candidate for clinical investigation towards the treatment of hyperlipidemia. This can explain the reason for its use in traditional medicine as a lipid-lowering plant. (Mosaddegh *et al*,2004) The leaves of *Cyclocarya paliurus* have been a food source for maritime people for a long time, and are known to have beneficial effects on health and used as a traditional remedy for ailments, the enhancement of mental efficiency, and recovery from mental fatigue (Kurihara *et al*,2003).

1.2.7 High performance liquid Chromatography

The newest addition to the chromatographic family of analytical procedures, most commonly known as HPLC. This form of chromatography resembles column chromatography except that the process involves pumping liquid eluant under pressure instead of depending on gravity for the eluant to pass through a number of fractions to detect separated products ,The experimenter runs the eluant through a detector ,most commonly an ultraviolet detector or a refractive index detector. The detector is connected to a chart recorder that gives a record of U.V. absorbance or refractive index versus ml of eluant –a chromatogram .The U.V. detector can detect as little as 10^{-10} g of solute ,while the refractive index detector can detect 10^{-6} g of solute .

1.2.7.1 Types of High performance liquid chromatography

- Exclusion chromatography
- Ion exchange chromatography
- adsorption chromatography

- ❖ Partition chromatography
- ❖ Reversed phase chromatography

In general each type of separation uses a different kind of packing material, and since each type of separation exploits a different property of the molecules, the choice of packing really comes down to which property of the molecules would be most useful in achieving the separation (Rossomando,1987)

1.2.8Gas chromatography

Gas chromatography (GC) ,also called vapor phase chromatography is a means of separating volatile mixture ,the components of which may

Williamson,1983). The Gas chromatograph was introduced by James and Martin in 1952(Skoog *et al*,2004). The GC process is similar to fractional distillation and used a glass column or a coiled stainless steel sponge the GC column used which is a3-10m long coiled metal tube (diameter 6 mm)packed with ground firebrick .

The firebrick serves as an inert support for a very high boiling liquid is essentially non volatile such as silicon oil and low molecular weight polymers like carbo wax these are the liquids of gas liquid chromatography and are referred to us the stationary phase the sample (1-52 µl) which is injected through silicon rubber septum into the column ,which is being swept with a current of helium(ca,200ml/min). Gas chromatography determines the number of components and their relative amounts in a very small sample. The small sample size is an advantage in many cases, but it precludes isolating the separated components .Some specialized chromatographs can separate samples as large as 0.5 ml per injection and automatically collect each fraction in a separate container .

At the other extreme gas chromatographs equipped with flame ionization detectors can detect micrograms of sample and are used to analyze for traces of drugs in blood and urine .Clearly a gas chromatograph gives little information about the chemical nature of the sample being detected. However ,its sometimes possible to collect enough sample at the exit port of the chromatograph to obtain an infrared spectrum .The sample ,if its not too volatile ,will condense in the gold glass tube. Subsequently the sample is washed out with a drop or two of solvent and an infrared spectrum obtained (Fieser and Williamson, 1983**).**

Gas chromatography (G.C.) is one of the most widely used techniques for qualitative and quantitative analysis. In(G.C.), the components of a vaporized sample are separated as a consequence of being partitioned between a mobile gaseous phase and a liquid or a solid stationary phase held in a column. An important fact of the G.C is the use of a carrier gas such as hydrogen or helium, to transfer the sample from the injector through the column and [illegible] to most other types of chromatography, the mobile phase does not interact with molecules of the analyte; its only function is to transport the analyte through the column.

1.2.8.1Two types of gas chromatography are encountered

1.2.8.1.1 Gas-liquid chromatography (GLC) and

1.2.8.1.2 Gas-solid chromatography (GSC)

Gas-liquid chromatography finds widespread use in all fields of science; in 1955, the first commercial apparatus for gas-liquid. Chromatography appeared on the market. Since that time, the growth in applications of this technique has been phenomenal. Currently, several

hundred thousand gas chromatographs are in use throughout the world (Skoog *et al*,2004).

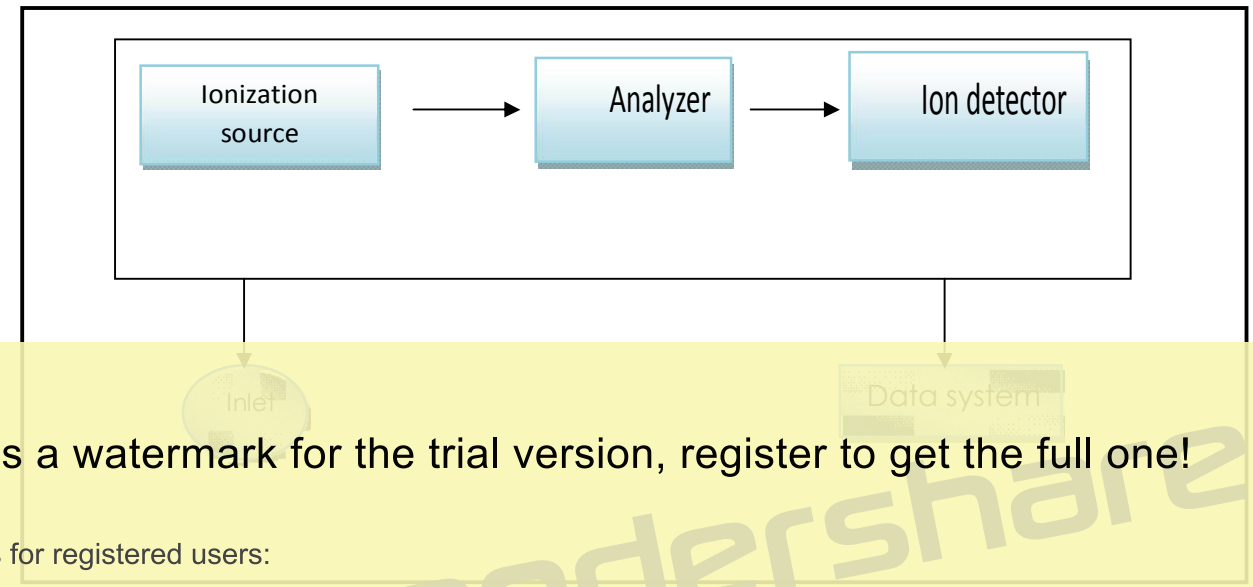
1.2.9 Mass Spectrometry

One of the most powerful detectors for gas chromatography is the mass spectrometer. The combination of gas chromatography and mass spectrometry is known as GC/MS. A mass spectrometer measures the mass-to charge ratio (m/z) of ions that have been produced from the sample. Most of the ions produced are singly charged (z = 1), so that mass spectrometer is often speak of measuring the mass of ions when mass-to-charge ratio is actually measured. A block diagram of a typical molecular-mass spectrometer is shown in Figure(1-1).

Sample molecules enter the mass spectrometer through an inlet system. In the case of GC, the sample is in the form of a vapor, and the inlet must interface between the atmospheric pressure GC system and the low-pressure (10-4 to 10-6 torr) mass spectrometer. In the ion source of the mass spectrometer, sample molecules enter an ionization source, which ionizes the sample. The ionization sources for molecular mass spectrometry are energetic enough to break chemical bonds in the sample molecules but not so energetic as to decompose the sample molecules into their constituent atoms, as is done in atomic mass spectrometry. The ionization sources in GC/MS produce fragments, which can also be ionized. Hence, leaving the ion source are ions of the sample molecules, called molecular ions, fragment ions, and un-ionized molecules. The uncharged molecules and fragments are normally

pumped out of the ion source by the vacuum pumps used to produce the low pressure environment. The next part of the mass spectrometer is an analyzer stage. The analyzer serves to sort the ions according to their

m/Z; values, just as in atomic mass spectrometry. The separated ions are then detected, and a plot of the ion intensity versus mi; value is produced by the data system (Skoog, 2004).



ions strike an ion detector, where they produce an electrical signal that is recorded and plotted by the data system.

1.2.10 Dermatophyte infections

Defenition. Cutaneous lesions due to dermatophytes ,presenting most commonly as athlete's foot(tinea mannum) ,nail infections(tinea unguium) ,body infection (tinea corporis) ,and scalp ringworm(tinea capitis). These pathogenic fungi inhabit the keratinized tissue of skin ,hair and nails, and generally do not invade living tissue ,in active

infections they are seen as branched hyphae .They grow down the pilosebaceous follicles towards the hair bulb ,but are arrested before reaching the bulb itself and fan out to form multiple hyphal fronds known as Adamson's fringe(Mackie,1983).

Dermatomycoses due to *Trichophyton mentagrophytes Trichophyton.rubrum ,Microsporum gypseum* and *Epidermophyton floccosum* occur commonly world wide and these are one of the most common skin infections in north east region of India. Unlike other superficial fungal infections ,the incidence of dermatophytoses ,commonly called ring worm or tinea , (Weitzman and Summerbell,1995; Jessup *et al*,2000)

The prevalence of different types of superficial fungal infections of the skin is variable among different age group ,gender ,socio-economic levels and countries . In Iraq ,tinea pedis infection age group 15-45 years showed the highest frequency ,male :female ratio was 1.37:1(Habbu and industrial workers formed the largest group (42.4%) of the patients and the rate of ability was seems USA is 8.1%and in general it is more prevalent in male's children (Al-Kayali *et al*,2011).

Three genera of dermatophyte fungi cause tinea infections (tinea worm):

- Trichophyton (skin, hair and nail infections)
- Microsporum (skin and hair)
- Epidermophyton (skin and nails)

Dermatophytes invade keratin only, and the inflammation they cause is due to metabolic products of the fungus or to delayed hypersensitivity. In general ,zoophilic fungi (those transmitted to humen by animals)are responsible for the majority of the cases (Hasegawa,2000).

1.2.10.1 Tinea types

1.10.1.1 Tinea pedis (athlete's foot). This is the commonest type of fungal infection in man (Hunter *et al*,1995). Ring worm of the feet it is the most prevalent of all dermatophytoses .It occurs as a chronic infection of the toe webs. other varieties are the vesicular ,ulcerative and moccasin types ,with hyper keratosis of the sole ,initially there is itching between the toe webs becomes chronic ,peeling and cracking of the skin are the principle manifestations ,accompanied by pain and pruritus (Brook *et al*,1998).

Infections are most common during the warm ,humid months, *T.mentagrophytes,T.rubrum and E.floccosum* are the dermatophyte species most commonly recovered .The increased amount of keratin on the soles of the feet and the palms of the hands makes these two sites selectively vulnerable to infection with *T.mentagrophytes* and other dermatophytes (Washington *et al*,2006).

1.2.10.1.2Tinea cruris : When the infection occurs in the groin area, Its called tinea cruris, or jock itch. frequ[illegible] [illegible] m[illegible] *rubrum,T.mentagrophytes,E.floccosum* (Al-Ani,2006).The theory is that the feet get infected first from contact with the ground. The fungus spores are carried to the groin from scratching (Dermatophytes , 2008).

1.2.10.1.3Tinea Corporis: Typically appears as single or multiple annular ,scaly lesions with clearing ,a slightly elevated ,reddened edge and sharp margin on the trunk, extremities or face(Hubbard,1999). *Tinea corporis* was a major problem for U.S. troops during the Vietnam conflict, particularly those assigned to combat units exposed to wet terrain. In the United States, the most common organism recovered is *T. rubrum*; however, during the Vietnam conflict, the most common isolated species recovered from

lesions of tinea corporis and tinea cruris was a zoophilic strain of *T. mentagrophytes* that accounted for 73% of fungal infections in combat servicemen (Mondello *et al*,2003) .

1.2.10.1.4 Tinea capitis: Hair infectied with one of the following fungi *Microsporum ferrugineum, Microsporum audouinii and cause tinea capitis* . *Microsporum canis* var. *distortum* is a zoophilic fungus known to cause infections in cats, dogs and other animals. It is a rare cause of tinea capitis in New Zealand, Australia and North America. Clinical disease is similar to *M. canis* . Invaded hairs show an ectothrix infection and fluoresce a bright greenish-yellow under Wood's ultraviolet light (Ellis *et al*,2007).

which becomes yellow and crumbly .Subungual hyperkeratosis ,separation of the nail from its bed ,and thickening may then follow .Usually only a few nails are infected but rarely all are .finger nail lesions are similar ,but less common ,and are seldom seen without a chronic *T.rubrum* infection of the skin of the hands(Hunter,1995) . Onychomycosis , its caused by members of the fungal triad, namely dermatophytes, yeasts and other molds. It is estimated that fungi cause 50% of all nail disorders, and onychomycosis accounts for 30% of all superficial fungal infections (Ercan *et al*, 2004).

1.2.10.1.6 Tinea mannum: it's the infection of the hands this is usually asymmetrical and associated with tinea pedis .*T.rubrum* may cause a barely perceptible erythema of one palm with characteristic powdery scales in the creases (Hunter,1995).

1.2.11 Diagnoses using molecular and Immunological techniques

1.2.11.1 Immunological tests

Trichophyton is a crude Anti gene preparation that can be used to detect immediate or delayed type hypersensitivity to dermatophytic anti gene. Many patients who develop chronic ,non inflammatory dermatophyte infections have poor-cell mediated immune response to dermatophyte Anti gene in the normal host immunity to dermatophyte infection a[illegible] Serologic tests for Anti body detection are generally considered to be in adequate for use in diagnoses .In only 25%of known cases are immunodiffusion and complement fixation assays +ve .Enzyme immunoassays (EIAs)are more sensitive than immunodiffusion (ID),but less specific (Washington *et al*,2006).

1.2.11.2 Molecular techniques

The application of nucleic acid amplification technology has made rapid and precise identification of dermatophytes possible. Morphological and physiological characteristic can frequently vary in fact, the phenotypic feature can be easily influenced by outside factor such as temperature variations medium and chemotherapy

(Mondello,2003). Molecular approaches have been developed to provide more rapid and accurate alternatives for dermatophyte identification. These methods include restriction fragment length polymorphism (RFLP)analysis (Kawasaki *et al* ,1996; Kanbe *et al* ,2003) sequencing of protein encoding genes (Kano *et al* ,2000) random amplification of poly morphic DNA (RAPD) (Mochizuki *et al*,1997). PCR finger printing and amplified fragment length polymorphism analysis (Graser *et al*,2000),and dot blot hybridization (El Fari *et al*,1999). Internal transcribed spacer (ITS) region of the RNA gene(Graser *et al*,1999). Faggi *et al* (2001)reported that the application of PCR finger printing for the identification of species and verities of common dermatophytes and related fungi utilizing arbitrary single primer the simple repetitive oligonucleotide (GACA)4,the primer was able to amplify all the strains ,producing species –specific profiles for *M.canis,M.gypseum T.mentagrophytes,T.rubrum,E. floccosum* (dpsD1)for these species and species –specific primer sets(PsT-PsME)for each species were designed based on the genomic sequences of the DNA topoisomerase II genes of the dermatophytes. Using dPsD1,a DNA fragment of 3390pb was amplified from the genomic DNA of all the dermatophyte species .second subsequent nested PCR using species-specific primer sets (psT-PsME),both sets amplified unique sizes of PCR products ,all of which corresponded to a species of the dermatophytes even in the presence of other fungal DNA.PCR-based identification targeting the DNA topoiomerase II gene is rapid and simple and is a available as a tool for the identification of the major dermatophyte species .

Developing of light cycler PCR method for the rapid detection and differentiation of fungal DNA in dermatological specimens such as skin scales and skin swabs light cycler melting points were defined by amplification of DNA from 21 fungi and sensitivity was determined by amplification of serial dilutions of fungal DNA .Two subsequent light cycler PCR reactions and one RFLP reaction allowed the differentiation of dermatophytes and non-dermatophytes molds and subclassification of yeasts .analysis of clinical samples from 38 patients with fungal skin diseases provide conclusive new diagnostic information in 9/38 cases (23.7%)by this PCR protocol that was not equally provided by direct microscopy and mycological culture(Gutzmer, 2004).

and invasive infections. Of 397 fungal strains (290 target and107non-target strains)tested ,the sensitivity and specificity of the array was 98.3% and 98.1%respectively.

When one of the 4 random primers (OPAA11, OPD18, OPAA17 and OPU15) was used to arbitrarily primed polymerase chain reaction (AP-PCR)up to 20 of the 25 of dermatophyte species or subspecies under investigation could be distinguished on the basis of characteristic band patterns detected in agarose gel electrophoresis (Liu *et al,* 2000).

1.2.12 Treatment of Tinea by herbs

Cure ringworm with Garlic capsules:Garlic has been used since Biblical times for treating infections. Garlic has both antiseptic and immune stimulant properties. It is most effective if crushed and eaten raw also Cure for ringworm with Grapefruit Seed Extract.

Grapefruit (*Citrus paradisi*) has strong antimicrobial properties as anti-bacterial, anti-viral and anti-fungal activity. It has been often used in the topical and internal treatment of different fungal infections. Therefore grapefruit seed extract is a powerful ringworm remedy both orally and topically(Capek *et al*,2009).

1.2.13 Treatment of Ringworm: Anti-fungal Ginger Paste

1.2.14 In vitro Antifungal activity evaluations

Plant extract has been used traditionally to treat a number of infectious diseases including those caused by bacteria ,fungi, protozoa and viruses (Soylu *et al*,2005)A number Of reports are available *invitro* and *invivo* efficacy of plant extract against plant and human pathogens causing fungal infections (Natarajan *et al*,2003). The antifungal activity of methanolic extract of different types of zygophyllum plant species (venugopal and venugopal, 1995) reported the activity of plant extracts against 88 clinical isolates of dermatophytes which includes *Microsporum canis*, *M. audouinii*,*Trichophyton rubrum* , *T.*

mentagrophytes , *T. violaccum* , *T. simii*, *T. verrucosum*, *Trichophyton erinacci* and *Epidermophyton floccosum*.While Vlietinck *et al*(1995)revealed clinical findings of Rwandese medicinal plants (267)plant extracts used by traditional healers to treat microbial infections and found 60% of these extracts were active against dermatophytes.Inhibition effect of 30 aqueous extract of medicinal plants were studied against *Alternaria solani* and *Fusarium* oxysporum especially *Menta viridis* and *Rosmarinus* officinalis are the highest inhibition rate. (AL-Suhaili,2002)Found that the extracts of 9 plant species in Jordan extracted with different solvents have antifungal activity against 3 types of fungi, especially *Bryonia syriaca* and *Onion spinosa*. Al-Taie (2001)studied the antifungal activity of water and alcoholic extracts of (*Mentha longifolia*, *Matricarica chemomilla*, *Albezia lebbeck* and *Citrus sinensis*)the alcoholic extract showed the highest antifungal activity against (*Microsporum gypseum* and *Trichophyton mentagrophytes*. (Mohammed ,2004,repo[illegible] [illegible]tophytes while the pruritus and s[illegible] after 4 weeks treatment of tinea corporis patients with *Aloe vera* 5%gel.

Ethanolic extract of *Fumaria officinalis* showed a good therapeutic when used to treat mouse skin infected with *Trichophyton rubrum* ,after 11 days of treatment the incision was completely covered with newly formed epithelial and hair seen in comparison with fugidin ointment (Al-Ani,2006).

The antidermatophytic efficacy of other plant extracts also studied such as *Nerium oleander* extract against Tinea corporis and tinea capitis (Heba,2007) Acetone extract of leaves *combretum nelsonii*, *combretum albopunctatum*, *combretum imberbe*and *Terminalia sericea* possessed remarkable growth inhibitory activities against fungal pathogens(Masoko,2007) for example

Garlic sativum extract (Mustafa,2009).Methanolic extract of lemon grass,lanta,nerium,basil,olive oil(Bokhari,2009) .

Chloroform ,methanol and aqueous extracts of *Solanum melongena*, *Lawsonia inermis*, and *Justicia gendarussa* were evaluated against four common dermatophytic species (T. *metagrophytes*, *T. rubrum*, *M. gypseum and M. fulvum*(Sharma *et al* , 2011).

1.2.15 Gene expression of dermatophytes virulence factors

In host pathogen interactions the gene expression of the pathogen is modulated by signals from the host, and understanding the expression patterns may provide insight into the mechanisms of disease .Little or no information is available on the transcription patterns of dermatophyte [illegible] *Cryptococcus neoformans*_and pathogens of plant (Hube *et al*,1994 and (Rhodes *et al* 2001) Kaufman characterized the expression of an [illegible] *mentagrophyte* is closely related to TRI 14. Trim4 resembles other protease other protease encoding genes though to be virulence factors ,Expression of Trim4 gene was induced several fold when *T.mentagrophyte* was grown on keratin and elastin. Exvivo ,strong induction was observed after culture on blood plasma ,differential cDNA screening was performed to identify additional gene encoding putative virulence factors .By this method a fungal thioredoxin and a cellulase homolog were identified and both genes were found to be strongly induced by skin extracellular matrix proteins .Induction by superficial (keratin)and deep (elastin)skin elements suggest that the products of these genes may be important in both superficial and deep dermatophytosi and models for their function are proposed .(Kaufman *et al*, 2005)Secreted proteolytic activity is considered a virulence trait of these fungi .In a

medium containing protein as a sole N.A. and C source *T.rubrum* secretes a m*etal*locarboxy peptidase (TruMcpA)of the M14 family according to the MEROPS proteolytic enzyme database. TruMcpA is homologus to human pancreatic carboxy peptidase A, and is synthesized as a precursor in a preproprotein form In addition *T.rubrum* was shown to possess 2 genes (TruSCP A and TruSCP B)encoding serine carboxypeptidases of the S10 family while are homologues of the previously characterized *Aspergillus* and *Penecillium* secreted acid carboxypeptidases (Zaugg *et al*, 2008).

Chen *et al*,(2010)reported the secreted proteases were considered to be the most important virulence factors .first time they used the keratins from human nail and skin stratum corneum as the growth medium to investigate the different expression patterns of *T.rubrum* secreted endoprotease genes .so they found that SUB7 and MEP2 were the highest expressed gene in each family during growth in both keratin containing media. Also they found that [illegible] structures of SUB1,SUB4 and MEP4 may be more suitable for degradation of nail original keratins.

Chapter Two

2 Materials and Methods

2.1 Materials

2.1.1 Equipments and Apparatus

The following equipments and apparatus were used through out this study:

Apparatus	Company
Autoclave	**SES Little Sister(England)**
Centrifuge	**Beckman(England)**
Incubator	**Memmert (Germany)**
Microscope	**Olympus(Japan)**
Oven	**Jan rkamp**
Rotary Evaporator	**Buchi (Switzerland)**
Spectrophotometer	**Cecil(France)**
Thermal regulator	**Eppendorf**
Water bath	**Gallen kamp(England)**

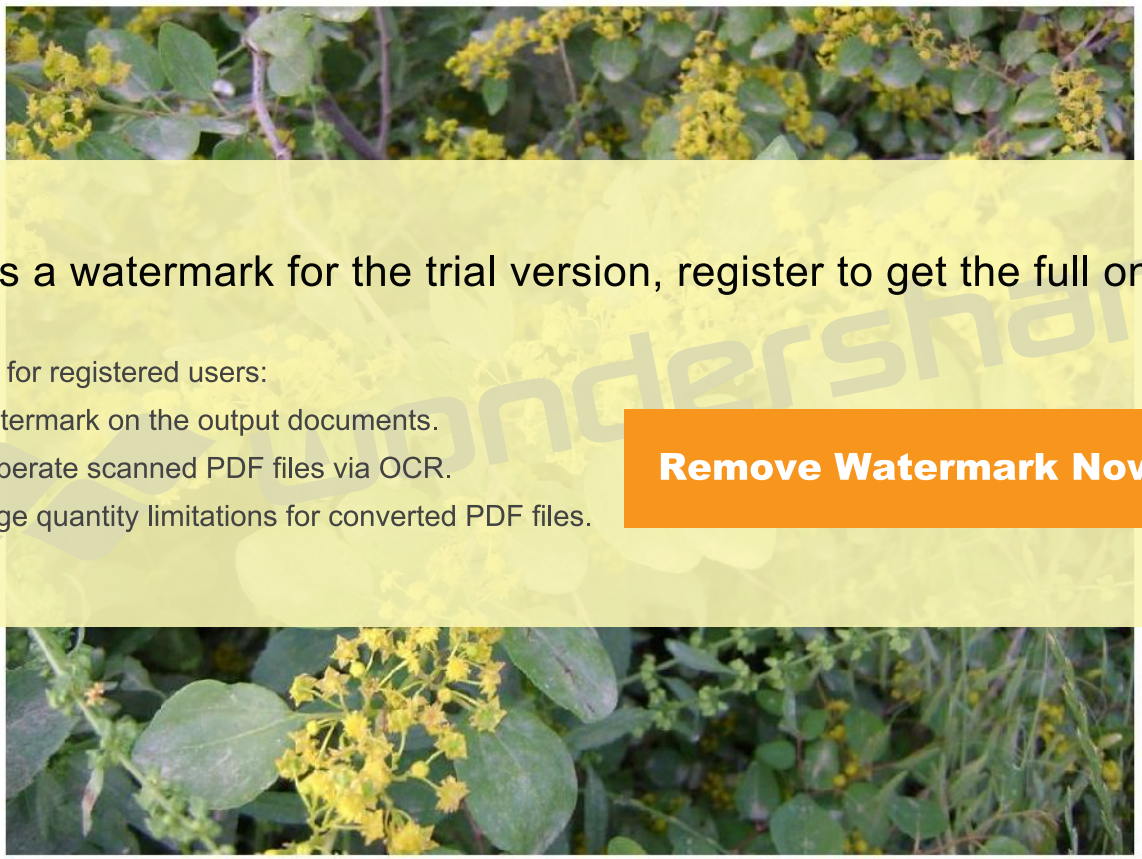
2.1.2.Chemical reagents

MATERIALS	COMPANY
Acetone	**BDH(England)**
Benzen	**BDH**
Cephalexin	**BDH**
Chloroform	**BDH**
Dermatophyte test media	**Conda, Spain**
Diethyl ether	**BDH**
Ethanol	**BDH**
Ethyl acetate	**BDH**
Formaline	**BDH**
Glacial acetyle	**BDH**
HCl	**Fluka**
Methanol	**BDH**
Saboraoud dextrose agar media	**Oxoid(England)**
Standard of phenolic compounds	**Sigma(England)**
Vaseline	**Kawa-Erbil**

2.2 .Methods

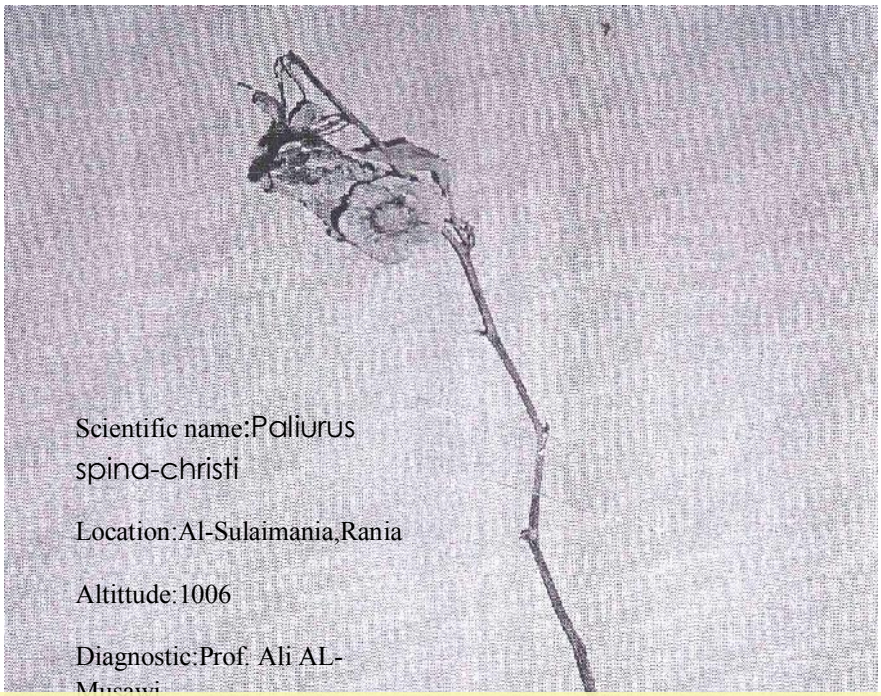
2.2.1.Collection of *Paliurus spina-christi*

The plant *Paliurus spina- cheristi*, was collected from Al-Sulaimanyia the mountain regions(Rania Village) in may 2010,and was identified by Professor Dr.Ali AL-Mosawy, Biology Department, College of science ,Baghdad University .Aerial parts of the plant were air dried at room temperature and grinded into powder form as shown in figures (2-1)and (2-2).



(Figure 2-1) *Paliurus spina-christi* during may in flowering period **.**

The photo captured by author



Figure(2- 2):Dried flowers of *Paliurus spina-christi* captured by

2.2.2. Plant Extraction

Solutions

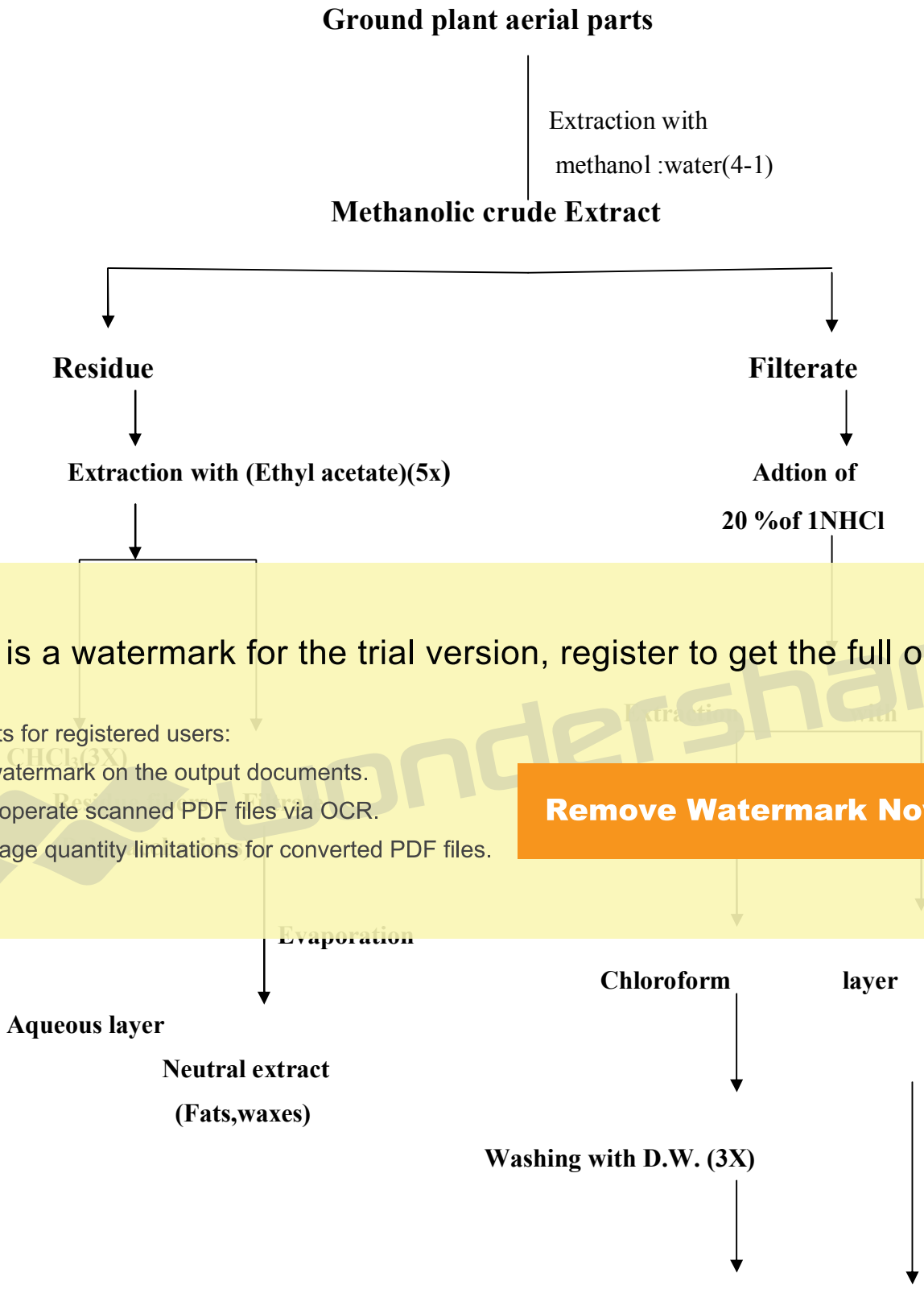
i. Ethanol 80%
ii. Methanol 80%

Method

The dried aerial parts of the plant (stem) were powdered using a seeds grinder for 5 minutes, and two extract were obtained using two types of solvents (Ethanol and methanol). In both cases, 50 grams of the processed plant were extracted in 250 ml of the solvent apparatus and the source of heating was a hot plate (70°C). The obtained extract was then evaporated at 40°C using a rotary evaporator, and the resultant crude extract was

lyophilized then used to prepare the required doses and concentrations (Arokiyaraj *et al*, 2007).

The ethanol or methanol crude extract was dissolved in sterile distilled water to prepare the dose by which the infected mice were treated.



Scheme(2-1):Extraction of phenols from *Paliurus spina-christi* (Harborne,1973)

2.3. Extracts preparation

Stock solution was prepared by (1mg) of plants extract powder in 0.1ml ethanol and then the volume was completed to 10ml sterile distilled water .The plant extract was prepared at different concentration started with (25, 50, 75, 100, 125) µg/ml. These concentrations were prepared according to the equation (Al-Ani,2006).

$$C_1V_1 = C_2V_2$$

Plant Extracts was sterilized by filtration using Millipore filter 0.22µm size .Then each concentration was poured into plates in addition to the control plates.

2.4. *In vitro* antifungal evaluation

Plant extracts were added to modified sabouraud dextrose agar [illegible] petridishes were inoculated with spore and incubated at 30 C for 3-7 days (Al-Sammarei *et al* ,2001). The diameter of fungal colonies was [illegible] incubation.

2.5.Detection of Some active compounds:

2.5.1 Detection of tannins:

Solutions:

lead acetate

Method:

(10)g of the plant powder was mixed with 50 ml distilled water in a magnetic stirrer .The mixture was boiled in a boiling water bath for few minutes .Then filtered ,and the filtrate was treated with few drops of 1% lead acetate solution .The development of greenish –blue precipitate is an indicator for the presence of tannins(Shihata,1951).

2.5.2 Detection of Saponins :

This method was done according to method described by Stahl,(1969).

Saponines were detected by two methods :

i. Aqueous extract of plants powder was shaken vigorously with D.W. in a test tube .The formation of foam standing for a time indicate a positive result.

ii. Five milli liters of aqueous extract of the plant was added to 1-3ml of 3%ferric chloride solution ,a white precipitate was developed which indicates a positive result.

2.5.3 Detection of terpenes and steroids :

Solutions:

a) chloroform

b) acetic anhydride

c) sulphuric acid

Method

(1ml)of ethanolic extract was mixed with few drops of chloroform, then a drop of acetic anhydride and drop of concentrated sulphuric acid were added , brown precipitate appeared which presenting the presence of terpene., whith the appearance of dark blue color after few minutes would represent the presence of steroids(Al-Abid,1985).

2.5.4 Detection of flavonoids:

Solutions

a) petroleum ether
b) ammonia solution

Method

Ethanolic extract of the plant was partitioned with petroleum ether ,the aqueous layer was mixed with ammonia solution. The appearance of dark color is an evidence for the presence of flavonoids (Harborn,1973).

2.5.5 Detection of Alkaloids:

Solutions

a) Mayer's reagent: Two solutions were firstly prepared; the first one was prepared by dissolving 1.58 g of mercuric chloride (HgCl2) in 60 ml of distilled water, while the second solution was prepared by dissolving 5 grams of potassium iodide (KI) in 10 ml of distilled water. Both solutions were mixed and the volume was made-up to 100ml with distilled water (Smolenst *et al*, 1972).

2.5.6 Detection of Flavonoid Compounds by TLC

The procedure of Harborne (1984) was followed, in which a silica gel TLC plate (200×200×0.25mm) was activated in the oven at 105°C for one hour, and after cooling the plate at room temperature (around 30 minutes), a marginal line was drawn on the upper and lower side of the plate with a distance of 2 centimeters from the margins. On the lower line of the plate, 0.25 ml of concentrated sample (methanol extract) and a flavonoid standard (rutin) was applied. After around 5-7 minutes, the plate was placed in a jar-closed-system containing a solvent (n-Butanol:

Acetic Acid :H2O at a ratio 4:1:5), and the system was left for two hours at room temperature.

Then plate was examined under UV light to detect the separated flavonoid compounds in the methanol extract samples as compared to the flavonoid standard and the RF value .The RF value is the distance compound moves in the chromatography relative to the solvent front. It is obtained by measuring the distance from the origin to the center of the spot produced by the substance, and this is divided by the distance between the origin and the solvent front (i.e. the distance the solvent travels). This always appears as a fraction and lies between 0.01 and 0.99. It is convenient to multiply this by 100 and RF is quoted as RF (×100) (Harborne, 1984). Such value was used to assess the presence of flavonoid compounds in the plant extracts.

2.6. High Performance Liquid Chromatography

The chemical analysis of methanolic extract of *Paliurus spina-christi* was carried out in the laboratories of science [illegible] technology center,the equipment type is Agilent 1100/1200 Binary pump column ,flow 0.750ml/min and stop time 60 min. solvents A:95%(0.2%TFA),solvent B:5%(MEOH), For pressure limits; minimum pressure :0 bar, maximum pressure:400 bar. Column diameter(0.2-0.17)mm And the flow of solvent B for different concentration(5,80,5)at different time (0,50,60) was 0.750. The sample injection volume was 50 µL.

Peak purity detection at wave length 280nm , signal polarity +ve and the scan from190nm to 400nm in 2 step scan was accomplished using an Agilent 1100 .

2.7. Gas chromatography mass spectrometry

Sample preparation

Weight about three of the dry sample was dissolved in 1 ml dichloromethane, then sonicate for 5 min, centrifuge at 4000 rpm for 3 min, the upper layer was transfer to test tube and evaporated at 35C° under N_2 gas then 100µl of BSTFA was added and put on oven for 20 min at 70C°finally 1 µl was injected on GC/MS.

Method

Chemical separation was in the same above place .Gas chromatography Ajilent type of Varian 450, composed of two parts A-Seperator and B-Detector (MS model Varian320), injected volume: 1µl,splitless injection type ,250C° injection temperature ,helium gas was

2.8 Isolation of the Dermatophytes species

2.8.1 Sampling of the dermatophytes species

i. Sabouraud dextrose agar media.

ii. Dermatophytes test media.

iii. Potassium hydroxide solution: It was prepared by dissolving 10 grams of potassium hydroxide in 100 ml of distilled water (Collee *et al*, 1996).

Method

Skin scales ,nail clippings , and hairs were examined after KOH preparation is made by emulsifying the specimen in a drop of 10 %KOH on a microscope slide .Lactophenol was added to stain the specimen after that the specimen was examined under light microscope at (10,40)X powers respectively (Washington *et al*,2006).

Isolation of dermatophytes were done by culturing above specimen(skin scales ,nail scrapings , and hairs directly on the media and incubated at 30C° for 7 days (Washington,2006) after that sub culturing of resulted growth into selective media (dermatophytes test media).

2.9 Laboratory Animals

Balb/C male mice were the laboratory animals that were employed in carrying out the experiments of the study. They were supplied by the Drug surveillance centre /Ministry of Health .Their age at the start of experiments was 8-10 weeks, and their weight was 25-27grams. They were divided into groups, and each group was kept in a separate plastic cage (details of these groups are given in the section of experimental design). The animals were maintained at a temperature of 20-25°C, and

experimental work.

The experiments were designed to evaluate antidermatophytic effects of *Paliurus spina-christi* (methanol)

2.9.1 Experiment

The experiment was designed to assess the histopathological effects of *Paliurus spina -christi* extracts (methanol)on skin. The animals were divided into four groups each group consist of six animals in comparison with negative and positive composed.

- Group I (negative controls): The animals without any treatment.
- Group II (positive control): The animals were treated with clotrinazol-p powder + thin layer of vaselin. (0.23 mg/ml) .
- Group III: The animals treated with dose (0.23 mg/ml) of methanolic extract .
- Group IV: The animals were treated with vaselin.

The animals were treated with the plant extract topically by applying the extract topically to the infected area twice daily then the insicion biopsy was taken from the mouse each week for histopathological manifestation .

2.9.2 Spore suspension preparation

Spore suspensions were prepared according to Faraj method (Faraj,1990),spores were harvested by adding 5ml/slant of sterilized water containing 0.1 % tween 80 to aid wetting and separation of spores ,The suspension was filtered through sterile cotton wool ,the filtrate was centrifuged at 3000 r.p.m for 5 minutes. The supernatant was removed and the spores were washed twice by resuspending in sterile distilled water and further centrifuged .Then 5 ml of sterile distilled water was added to precipitate and mixed vigorously by the vortex for [illegible] drop of the suspension was added to the haemocytometer by Pasteur pipette, spores were calculated under high [illegible] of light microscope using the following equation:

Concentration of spores $=(Z\times4\times10^{6})/n$ spores/ml

Where n: total No. of small squares.

Z: total No. of spores. (Faraj,1990).

2.10. Histopathological Study

2.10.1 light microscopic examination

Solutions

i. Phosphate Buffer Saline (PBS): The solution was prepared by dissolving the following chemicals in 950 ml of distilled water and then the volume was made-up to 1000 ml:

- Sodium chloride (NaCl): 8.0 g
- Potassium chloride (KCl): 0.2 g

- Di-sodium hydrogen phosphate (Na2HPO4): 1.15 g
- Potassium dihydrogen phosphate (KH2 PO4): 0.2 g

The pH was adjusted to 7.4, and the solution was completed to 1000ml with D.W, autoclaved and stored at 4 C (Hudson and Hay,1989).

ii. Potassium chloride (KCl) hypotonic solution (0.075M): was prepared by dissolving (5.75g) in 1000 ml of distilled water, and then the solution was autoclaved and stored at 4°C (Allen *et al*, 1977).

iii. Haematoxylin stain: The stain solution was ready supplied by the Histo- pathology Department at Educational Laboratories of Medical Baghdad City.

Method

The tissue was fixed in 10% formalin for 48 hours and then prepared as of Bancroft and Stevens (1982) was followed to prepare sections for histopathological examinations. The procedure is outlined as the following:

i. Fixation: The sample was fixed in 10% formalin for 48 hours.

ii. Dehydration: The sample was dehydrated with ascending concentrations (50, 70, 90 and 99)% of ethanol (two hours for each concentration).

iii. Clearing: The sample was placed in xylene for two hours.

iv. Infiltration: The sample was first placed in paraffin-xylene (1:1) for 30 minutes at (57-58)ºC, and then in paraffin alone for 2 hours at (60-70)ºC.

v. Embedding: The sample was embedded in pure paraffin wax (melting temperature: (60-70)ºC and left to solidified at room temperature.

vi. Sectioning: The paraffin block was sectioned (rotary microtome) at a thickness of 5 microns, and then the sections were transferred to a

slide with Mayer's albumin. The section of tissue was placed in a water bath (35-40)ºC for few seconds.

vii. Staining: The slide was first placed in xylene for 15-20 minutes, descending concentrations (90, 80 and 70)% of ethanol (two minutes for each concentration) and finally distilled water. After that, the slide was stained with haematoxylin for 10-20 minutes and then washed with distilled water for 5 minutes. Then, the slide was placed in acidic alcohol for one minutes and washed with distilled water. After washing, the slide was placed in eosin stain for 10-15 seconds, and then in ascending concentrations (70, 80, 90 and 99)% of ethanol (two minutes for each concentration). Finally, the slide was cleared with xylene for 10 minute.

viii. Mounting: The slide was mounted with Canada balsam and was covered with a cover slip. Then, the slide was examined microscopically to inspect the histopathological changes.

i. Cut paraffin sections at 5 µm.

ii.Deparffinzed by xylene.

iii.Absolute alcohol.

iv.Add alcohol 95%.

v. Rinsed in distilled water.

vi. Periodic acid solution for 5minutes (oxidizer).

vii. Rinsed in distilled water.

viii. placed in schiff's leuco. Fuchsin for 15 minutes for pink color to develop.

ix. Stained in Harries's Hematoxyline for 6 minutes ,or light green counter stain for few seconds .Light green recommended for counter staining sections in which fungi are to be demonstrated .

x. Rinsed in tap water .

xi. Differentiated in acid alcohol- quick dips.

xii. Washed in tap water .

xiii. Dip in ammonia water to blue sections.

xiv. Wash in running tap water 10 minutes.

xv.Alcohol,95%.

xvi. Absolute alcohol,2 changes .

xvii.Xylene,2 changes.

Mountin in permount(Ambrogi,1960).

2.10.2 .Preperation for transmission electron microscope (T.E.M)

A-Fixation ,dehydration and embedding

The fixation, dehydration and embedding were performed by using the method of Glauret(1975) and AL-Kaisy,(1988).

1-Samples were obtained from skin and cut into small pieces (0.1-0.2) cm fixed in 2.5% glute[illegible] (pH 7.4),the buffer was rinsed for several times and left in the PBS for 12 hours.

2-The specimens were post fixed in 1% osmium tetraoxide for 1 hour.

3- The specimens were dehydrated through a series of ethanol concentrations (30,50,70,80,90 and 100)%.

4-The specimens were cleared in propylene oxide for 15 minutes (two times).

5-The specimens were placed in mixture of propylene oxide and embedding materials (araldite) for 1 hour, then left in araldite for 12 hours at room temperature.

6-Each specimen was cleaned from adherent araldite by filter papers then placed in plastic capsule and filled with araldite and left in oven for 48 hours at 60 °C, left at room temperature for sectioning and staining .

B-Sectioning and staining

The glass knives prepared by cutting the sheet of glass plate by the knife maker .The specimen blocks were cut electrically by ultra microtome. Semi-thin sections of (0.5-1)Mm were collected on a dry glass slide to which a drop of water was added and placed on a hot plate at 60°C to dry out for few minutes ,then stained with 1 % methylene blue.

The ultra –thin sections that have silver color (60-90mmthickness) with uranyl acetate that saturated in 70% ethanol then stained with lead citrate for 20 minutes and washed again in distilled water then dried by special filter paper.

The sections examined by electron microscope(Boch,1981) using special films which photographed and printed in electron microscope unit/Al-Yarmouk university ,Jordan.

2.11.Molecular identification

2.11.1.DNA Extraction

The genomic DNA was extracted according to the method of intron biotechnology company kit**.** Mycelial samples of *T. mentagrophytes* were grounded in liquid N_2 and transferred into eppendorf tubes.

I. Pre- lyses step

1. 100 µl of MP buffer and 3µl lysozyme solution were added into sample tube ,and mix well by vortex for 30 sec or pipetting vigorously .

2.Incubation of the lysate for 15 min. at 37 °C.

during this time the tubes inverted 5-6 times .

II. Lyses step

3. Aquantity of 200µl buffer MG ,10µL proteinase K, and 5µl Rnase A solution were added into sample tube, and mixed by vortexing vigorously.

4. Lysis sample was incubated for (30-45)min. at 65C° ,during this period the tube inverted 5-6 times .

III. Binding step

5. Add 250 µl ethanol (96-100%) to the lysate ,and mix by pipetting or gently inverting 5~6 times. After mixing the tube spin down to remove drops from inside the lid.

6. 250 µl of 80% EtOH were added gently and mixed or inverted **.**

7. 750 µl of the mixture was transferred to the spin column .then centrifuged at 13.000 rpm for 1 minute.

8. The collection tube was Discarded then replaced by new one ,after that 700 µl of buffer MW was added to the spin column.

9. The spin column was centrifuged at 13,000 rpm for 1 minute.

10.The collection tube was replaced with new eppendorf tube, then 50µl of buffer ME was added followed by incubation for one minute at room temperature.

11.The spin column was centrifuged at 13,000 rpm for 1 min. then the resulted precipitant is DNA template can be use in the new eppendorff tube.

2.11.2.Spectrophotometric analysis and electrophoresis

The extracted DNA was quantified using spectrophotometer .To do that, extracted DNA was diluted 100X using nuclease free water then DNA was analyzed spectrophoto metrically at λ=260 nm. The formula used to calculate DNA concentration is as follows:OD_{260}=50 µg/ml(Faggi

purity of DNA preparation .DNA quality was tested by running

flame ,the flask was shaken repeatedly to ensure complete dissolution of agarose .The molten agarose was cooled to about 60°C and 2.5µl of red safe solution was added ,mixed thoroughly. The warmed agarose was carefully poured into a previously prepared horizontal casting tray .After the gel was completely set (40 minutes at room temperature),the comb was carefully removed .The agarose tray was submerged in 1X TBE running buffer such that a level of around 0.5 cm above the gel. The voltage was set for 120 volts and timed for 1 hour. The gel was visualized under UV light and photographed using gel documentation system.

2.11.3 PCR of Template DNA

PCR was performed according to (chung *et al*,1999).Ready mix Taq PCR was carried out in reaction volume of 25 µl of ready mix Kapa(2 G fast ready PCR) and 1.25 of 10µM of each specific *(T.menragrophytes)* primers (Table2-1) and 2µl(0.1 µg/µl)template DNA .Blank which contained only water instead of DNA ,was used in each PCR run to check for DNA contamination.

DNA amplification was carried out by thermal cycler .the sample was denatured at 95 °C for 3 minutes ,followed by 25 cycles of 95°C for 10 seconds (denaturation step),55°C for 30 seconds (annealing step),72°C

Following amplification, PCR product was kept at -20 °C until tested by 1% agarose gel electrophoresis (as described in section 3.12.2).

Table 2-1) Primers used for *T.mentagrophytes*

Primer name	Sequences (5' to 3')	Target gene	PCR product/bp	Aim and References
T.mentagrophytes	F)5'-CGA GCG TGG CTA CAG CTT CT-3' R)5'-CTCCTTGAT ACG GAC GAT-3'		<120	Kaufman *etal*.,2005
M4#804	f)5'-CAG	Tri	<120	Kaufman

	GACTTCAAC GGA ACC TTC T-3'	m4		*etal.*,2005
M4#1859	R)5'-CAA TCC CAG CGG TCA TAG TTC T-3'	Tri m4	120	=
#219	F)5'-CGA GCG TGG CTA CAG CTT CT-3'	Actin	120	=
#279	R)5'-CTC CTT GAT GTC ACG-3'	Actin	120	=

transferred to eppendorf tubes and was centrifuged at 20,000 g for one minute. Then the supernatant was discarded and pelleted cells were frozen in liquid nitrogen and stored at -80°C until used.Frozen cells were resuspended in 0.5 ml of lysis buffer TES (10 mM Tris-HCl (pH8.0),1 mM EDTA (pH8),SDS2%)and incubated at 65 °C for 2 minutes. Then 5µl of 1 M sodium acetate(Ph5.0) and 0.5 ml of acid phenol (Ph5.2)was added and vortexed vigorously for 30 seconds. The mixture was incubated at 65°C for 6 minutes followed by centrifugation at 20,000 g for 10 minutes. The aqueous layer was aspirated which was approximately 0.5 ml, then transferred to a new eppendorf tube. Equal volume of chloroform was added and vortexed for 30 seconeds and

centrifuged at 20,000 g for 10 minutes. The upper layer was aspirated and transferred to a new eppendorf tube,RNA was precipitated by addition of 50 µl of 3 M sodium acetate (pH5) and 1 mM EDTA (pH8.0). Isopropanol (1ml)was added and mixed gently by inverting the tube several times. To precipitate nucleic acid, the mixture was incubated at -80 °C for 20 minutes or 1 hr at -20°C.After that ,the tubes were centrifuged at 12,000 g for 20 minutes and the supernatant was discarded .Nucleic acid pellet was washed with 1 ml of cold 70% ethanol and centrifuged again at 12,000g for 5 minutes.RNA pellet was air-dried for 10 minutes then dissolved by addition of 30-50µl nuclease –free water and stored at -20°C until use.

2.11.5 Power cDNA Synthesis

cDNA synthesis was according to the kit protocol of Intron Biotechnology ,Inc.and as follwing:

briefly in order to collect the solution at the bottom of the tube. and then

the plates were placed on ice for at least 1 min..A group of reagents were added in the order and mixed gently they are (RNase inhibitor(1µl),5Xrt buffer(4µl),dntP 2µl,DTT 2µl and AMV RT 0.5µl. followed by incubation at 42°C for 60 min. and heating to 70°C.Finally the above reactant diluted by adding 50-80 µl sterile water .

The resulted product electrophoresis according to the method (2.11.2) and examined under U.V.

2.11.6 Statistical analysis:

The Statistical Analysis System- SAS (2004) was used to find the effect of the different concentrations in study percentage. Least significant difference –LSD test was estimated to compare between means.

<u>3.1 Detection of Some Active compounds in *Paliurus spina-christi* extract:</u>

In this study the active constituents of alcoholic extract of *Palliurus spina-christi* were determined .Depending on results there were classes of active compounds using different specific reagents they were(Alkaloids, Tannins, Flavonoids, Saponin, Terpenes and Steroids) were detected (Table 3-1). Previous work on the same plant indicated the presence of the same classes of compounds (Lee *et al*,2001) found that 13-memberd cyclopeptide alkaloids ,paliurines G,H and I ,together with six known alkaloids, were isolated from the stem of *Paliurus* species .

Another study indicated that *Paliurus spina- christi* alcoholic extract was rich of flavonids contents of different plant parts and the different composition of the flavonoid pattern , rutin are the main flavonoids compounds present in all parts (leaves, flowers, fruits) investigated (Brantner and Males,1999).

phytochemical screening of compoun[illegible] flavonoids, tannins in all plant parts of *P. s*[illegible] 1999).

Table (3-1): Some active compounds in *Paliurus spina- christi* methanolic extract.

Chemical compounds of alcoholic extract	**Reagents used for detection**	**Result**
Alkaloids	Mayer's reagent	White PPt.
Tannins	FeCl2	Greenish blue
Flavonoids	Ammonia	Yellow color
Saponin	Ferric chloride	White PPt.
Terpenes and steroids	H2SO4	Brown PPt.

3.2. HPLC and TLC Analysis of *Paliurus spina-christi* Extract

HPLC analysis was done to detect the most important active compounds phenols found in the plant ,using phenols as a standard reference .

Table (3-2) revealed a major peak with different retention times of phenolic compounds which detected in our results including :(Gallic acid, caffiec acid, syringic acid and epecotechin) .

Gallic acid is the basic unit of tannines (Al-Rawi and sulaiman,1984).Caffeic acid is a simplest bioactive phytochemical consist of a single substituted phenolic ring(Cowan,1999). Gallic acid and epicotechin are the major phenolic compounds (Terashima *et al*,1999; Kakuda *et al*,2000).

spina-christi. This may be due to different plant parts used. While other study revealed the presence of amino acids (Bogdanova,1963);alkaloids (Velcheva ,1993)and (Lin *et al*,2000). Methanolic extract of leaves of another species of *Paliurus* resulted in caffeic acid with 10 known phenolic compounds(Zhang *et al*,2009).

Table (3-2) phenolic compounds assessed by HPLC in *Paliurus spina-christi* extract.

Compounds	Retaintion Time	Area	Area%
Gallic acid	4.632	7411.067	82.3599
Caffeic acid	19.418	278.787	3.0982
Syringic acid	20.408	217.197	2.4137
Epichotichen	21.871	1090.168	12.115

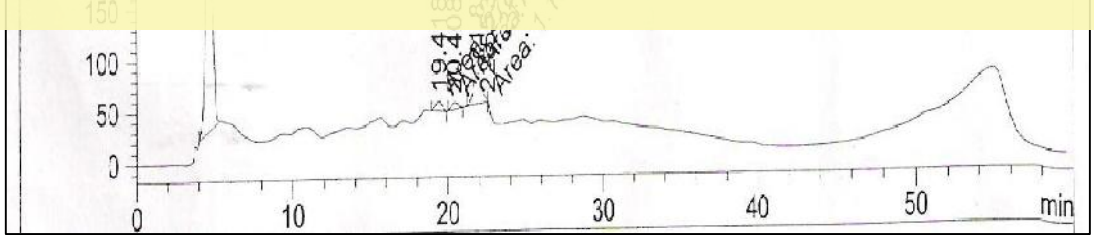


Figure (3-1)HPLC Analysis Of *Paliurus spina -christi* extract

3.3. Gas chromatograph mass spectrometry analysis of the *Paliurus spina-christi* extract

Using gas chromatograph revealed a numbers of chemicals such as fatty acids (Myristic acid), sugars (arabinofuranose,xylose and glucose), amino acids (1-aminocyclopentancarboxylic acid), phenols (benzoic acid) were identified as shown in table(3-3).

In general there are similarities in some classes of active compounds between genera belonging to the same family , Soluble sugars ,non-volatile acids and phenolic compounds were identified in *Ziziphus mauritiana* fruits by using TLC and HPLC techniques, sugars were galactose ,fructose and glucose whereas phenolic compounds were ,caffeic acids, p-hydroxy benzoic acid and coumaric acid in addition organic acids were identified malonic acid, malic acid and citric acid(Muchuweti *et al*,2005).Polysaccharides were extracted from Cyclocarya paliurus by ultrasonic wave technique (Ke-yue,2011).

Table (3-3): Biologically active constituents of *Paliurus spina-christi* extract assessed by GC/MS chromatogram.

No	Retention time/min.	Name
1	6.181	Propanoic acid, 2-[(trimethyls[illegible]
2	[illegible]	Trisiloxane, octamathyl
3	[illegible]	Acetic acid, [(-[(trimethylsilyl)oxy], trimethylsilyl ester
4	7.832	Silane, trimethyl (4-methylphenoxy)
5	8.953	3,7-Dioxa-2,8-disilanonan-5-one, 2,2,8,8-tetramethyl
6	9.408	Arabinofurannose,1,2,3,5-tetrakis-o-(trimethyl)
7	9.467	Benzoic acid trimethyl ester
8	9.729	Trimethylsilyl ether of glycerol
9	10.048	Acetoacetic acid, bis(trimethylsilyl)
10	10.209	1-Aminocyclopentancarboxylic acid, N-methoxycarbonyl-, heptyl
11	10.488	Butanedioic acid, bis(trimethylsilyl)ester
12	10.682	Propanoic acid, 2,3 – bis[(trimethylsilyl)oxy], trimethyl ester
13	11.286	Nonanoic acid. trimethyl ester
14	11.988	Hexadecane
15	12.640	2-Isopropyl-3-ketobulyrate, bis(O-trimethylsilyl)
16	12.858	Decanoic acid, trimethyl ester
17	13.244	Malic acid, (O-trimethylsilyl), bis(trimethylsilyl)ester
18	13.640	Benzoic acid,2-[(trimethylsillyl)oxy], trimethylester
19	13.767	n-octanoic acid, 2-[(trimethylsillyl) amino], trimethylsilyl ester

20	**13.844**	**N,O-Bis(trimethylsilyl)-2-pyrrolidone carboxylic acid**
21	**14.288**	**L-threonic acid, trimethylsilyl)ether, tnmethylsilyl ester**
22	**15.174**	**D-Xylofuranose, 1,2,3,5-tetrakis-O-(trimethylsilyl)**
23	**15.784**	**Lauric acid**
24	**15.893**	**Mannoic acid, 2,3,5,6-tetrakis-O-(trimethylsilyl), lactone**
25	**17.132**	**D-Glycero-L-manno-Heptonic acid 2,3,5,6,7-pentakis-O-(trimethylsilyl), gamma-lactone**
26	**17.680**	**Arabinofurannose,1,2,3,5-tetrakis-(trimethylsilyl)**
27	**17.782**	**D-Glycero-L-manno-Heptonic acid 2,3,5,6,7-pentakis-O-(trimethylsilyl), gamma-lactone**
28	**17.857**	**Mannoic acid, 2,3,5,6-tetrakis-O-(trimethylsilyl), lactone**
29	**17.932**	**1,2,3,-Propanetricarboxylic acid, 2-[(tetrakis-(trimethylsilyl)oxy], tris(tetrakis-(trimethylsilyl) ester**
30	**18.268**	**Glucofurancoside, methyl 2,3,5,6-tetrakis-O-(trimethylislyl)**
31	**18.319**	**2-Doxy-galactopyranose,tetrakis-(trimethylsilyl)**
32	**18.445**	**Myristic acid**
33	18.778	Xylulose tetraki tetrakis-(trimethylsilyl)
34	18.846	Glucose
35	18.959	Beta-D-Galactopyranoside, methyl 2,3,4- tris-O-(trimethylsilyl)
36	18.972	2,3,4,6-tetra-Otrimethylsilyl-1-t1,7-dicarbacloso dodecaborane-yl]-D-glucopyranose

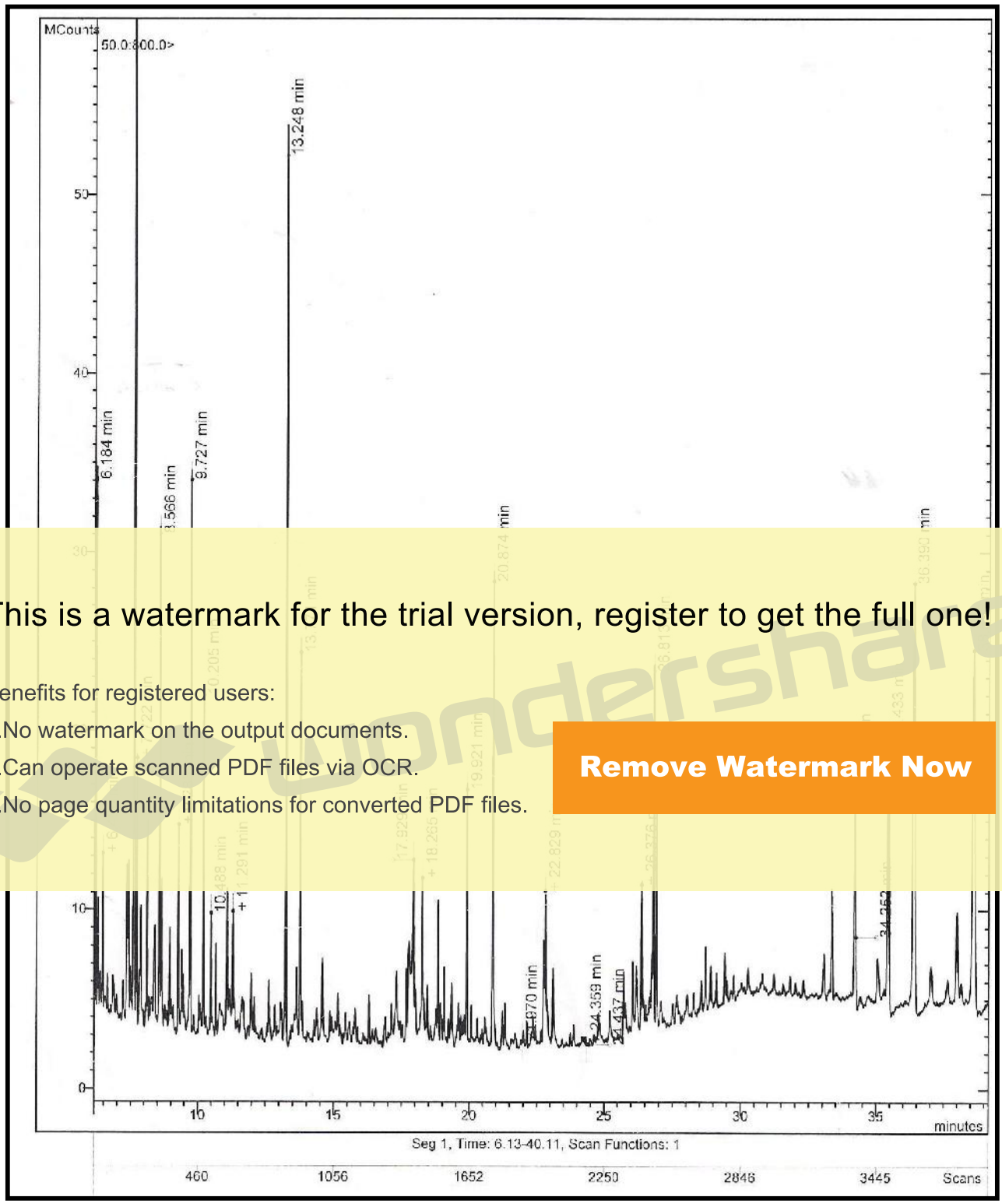


Figure 3-3 :Gas-mass spectrometer chromatograph analysis of *Paliurus spina -christi* methanolic extract

3.4. Diagnosis of Dermatophytes species

A total of fifty five samples (male and female) were collected from the tinea diagnosed patients who submitted to the consultant of Baquba Teaching Hospital of Diyala City from the period June 2010 to September 2010 .

All cases were successfully assessed regarding its clinical types of tinea. The direct KOH mount smear showed the presence of fungal elements in all of the cases while the growth of dermatophytes was positive in 18(32.27%) of these 55 patients .

Depending on species identification of the positive dermatophytes cultures, *Trichophyton mentagrophytes* percentage was (13.63%) for male and (22.72%)for female ,*Trichophyton rubrum* the percentage was (12.5%)for male only, followed by *Microsporum gypsum* (35.29%)detected in male and for female(23.52%). The detection was based on macroscopic colonial morphology and microscopic features as well . The age range was between (4 to 66)years. The age group (20-40)years old showed the highest frequency . Male : female ratio was 1:1, labor and industrial workers formed the largest group of the patients as well as house wives and the rate among military personnel seems to be high. Persons living in villages near by the animals , big families, public bathes attendants those with positive family history and persons with history of atopic dermatitis were at high risk of developing the disease.

These results agree with, (Abass,1995) who reported that the predominant fungal species were *T. mentagrophytes* (35.6%),of the positive dermatophytes culture followed by *Microsporum* canis species in (22.3%) . while disagree with(Ali,1990) and (Al-Janabi,1993) and (Al-Samarrai ,2008) who reported that *T. rubrum* were the predominant species(63.26%) followed by *T.*

mentagrophytes (19%)and *E. floccosum* (14.28%)this due to several factors such as climate differences, period of sampling, type of samples(hand, groins, foot skin scrub) (Ellis *et al*,2007).

The fungal species *Trichophyton mentagrophytes* is zoophilic dermatophytes which transmitted from the animal to human and cause a number of tinea types especially tinea capitis ,tinea unguium .The prevalence of different types of superficial fungal infections of the skin is variable among different age group ,gender, socio-economic levels and countries (Khalifa, 2011).

Table(3-4) Percentage of Dermatophytes species isolated from patient diagnosed with Tinea.

Fungal species	No. of samples	Male No.	Male %	Female No.	Female %
Trichophyton rubrum	16	2	12.5	-	-	٢
Microsporum gypseum	17	4	35,29	4	23.53	٨
Total	55	9	٦١.٤٢	9	٤٦.٢٥	١٨

Figure(3- 4)*Trichophyton mentagrophytes* growing on dermatophytes test media

Using the five different concentrations of methanolic extract maximum growth inhibition was at 125µg/ml while minimum inhibitor concentration observed at 25µg/ml. Table(3-5)shows the means of *T. mentagrophytes* growth diameter with percentage of inhibition ,the differences between means statistically non significant (P≥0.05) with the concentrations (25 and 50) µg/ml while the concentrations(75,100 and 125)µg/ml the effect of extract on growth diameter is significantly at level p≤0.05 .The differences between percentage of inhibition is statistically significant (P<0.05) for all concentrations of *P.spina-christi* extract. At the concentration 25µg/ml of methanolic plant extract *T. mentagrophytes* growth diameter after period of incubation was(6.90) cm and

the percentage of inhibition in contrast was (16%) .Using concentration 50 µg/ml of *P. spina- christi* methanolic extract the *T. mentagrophytes* growth diameter after incubation for seven days was (6.50)cm as demonstrated in Table 3-5.The inhibition percentage at this concentration was (21%) .

By increasing the concentration to 75 µg/ml of plant extract ,the mean diameter of *T. mentagrophytes* after seven days of incubation was (4.56)cm this indicated the ability of this concentration to inhibit the growth of *T. mentagrophytes* .The inhibition percentage was (45%) .

The *T. mentagrophytes* maximum growth diameter was (2.73)cm after incubation for seven days in media treated with 100 µg/ml of plant extract concentration. The inhibition percentage was statistically significant (67%).

While at the 125 µg/ml of plant methanolic extract concentration *T. mentagrophytes* growth diameter aft [illegible] ... [illegible]) cm. This effect was statistically [illegible] inhibition concentration was statistically significant(82%).

Table 3-5. The *In Vitro* antifungal activity of Methanolic extract (diameter of the growth, cm) of *Paliurus spina-christi* against *Trichophyton mentagrophytes* after seven days.

Concentration (µgl/ml)	Average of colonial diameter (cm) ± SE	Percentage of inhibition (%)
0	8.30 ± 0.87	0 ± 0.0
25	6.90 ± 0.62a	16 ± 0.94a
50	6.50 ± 0.49a	21 ± 1.02a
75	4.56 ± 0.31b	45 ± 2.37b
100	2.73 ± 0.25c	67 ± 2.93c
125	1.50 ± 0.03d	82 ± 3.77d
LSD value	2.508 *	9.366 *

A numbers of plant extract were tested for their antifungal activity against dermatophytes species *T.mentagrophytes* or indirectly treated dermatophytes caused tinea (Tinea corporis ,Tinea cruris and Tinea facie)(Sharma2011)This inhibition may be attributed to different compounds detected in methanolic extract such as flavonoids, phenols (including tannins, gallic acid and other) .

These ingredients were known to have antimicrobial activity and were found in many plant species and genera(Cown,1999).

Aloe vera gel acts as anti-inflammatory drug reducing the pruritus and the scales of the lesion caused by Tinea corporis and tinea cruris (Mohammed ,2004)*Aloe vera* is known to contain flavonoids, glycosides(Chithra *et al* ,19

98).while the methanolic extract of *Lemon grass* ,*Lanta* and *Nerium* followed by their ethyl acetate extracts showed the highest activities against *T.mentagrophytes* ,extract of lemon were the most effective followed by *Lanta* , *Nerium* and Basil showed moderate activities this affectivity may be due to free and bound flavonoid fractions (Bokhari,2009). In addition the presence of phenolic compounds which can be hold a good promise as a natural fungicide against common pathogens of crops (Nwachukwu and Umechuruba,2006). A wide variety of flavonoids, sesquiterpenoid alcohols, triterpenoids and quinic acid caffeates product from plants may also be useful as antimicrobials. The activity is probably due to their ability to form a complex with extra cellular and soluble proteins, which then binds to bacterial cell wall . More lipophilic flavonoids may also disrupt microbial membranes (Hu and Chen, 1997).

3.5.2 *In Vitro* Antifungal activity of *Paliurus spina-christi* Ethanolic extract

Different concentrations of ethanolic extract (25, 50, 75, 100)µg/ml are statistically significant (P≤0.05) as compared with control, while statistically non significant (P≥0.05) at concentration 25 µg/ml. The differences between percentage of inhibition is statistically significant (P≤0.05)for all concentrations after incubation period as shown in table(3-6).the *T. mentagrophytes* growth diameter after incubation for seven days in media non treated with *P. spina- christi* ethanolic extract the maximum growth diameter was at seventh day (8.3)cm which is consider control group for the growth of the fungal species.

By using the 25 µg/ml of plant ethanolic extract concentration *T. mentagrophytes* growth diameter after incubation for seven days was (7.30)cm table(3-6).The inhibition concentration was (12%).The *T. mentagrophytes*

maximum growth diameter was (5.2)cm after incubation for seven days in media treated with 50 µg/ml of ethanolic plant extract concentration. The inhibition percentage was (37%) which .

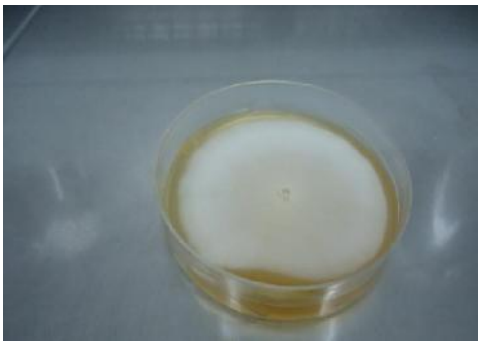
Using concentration *75* µg/ml of Plant ethanolic extract the *T.mentagrophytes* growth diameter after incubation for seven days was (4.6)cm as demonstrated in Table 3-6. The inhibition percentage was (44%).The *T. mentagrophytes* maximum growth diameter was (3.5)cm after incubation for seven days in media treated with 100 µg/ml of plant extract concentration. The inhibition percentage was (58%). The mean diameter of *T. mentagrophytes* growth after incubation for seven days in media treated with 125 µg/ml of *P. spina- christi* ethanolic extract concentration was(2.4) cm as seen in table(3-6). The inhibition percentage was (71%).

Table 3-6. The antifungal activity of *Paliurus spina-christi* Ethanolic extract (Mean of the growth, cm) against *Trichophyton mentagrophytes*

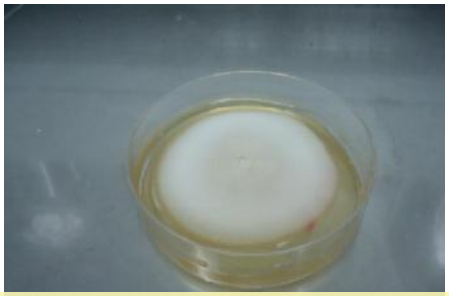
[illegible]	Average of colon diameter (cm) ± SE	inhibition (%)
0	8.30 ± 0.87	0 ± 0.0
25	**7.30 ± 0.65a**	**12 ± 0.67a**
50	**5.2 ± 0.49ab**	**37 ± 1.28b**
75	**4.6 ± 0.32c**	**44 ± 2.66c**
100	**3.5 ± 0.14d**	**58 ± 2.81d**
125	**2.4 ± 0.09e**	**71 ± 2.93e**
LSD value	**2.574 ***	**12.633 ***
***** **(P<0.05)**		

The inhibition may be attributed to different compounds found in the ethanolic extract in addition to the anti microbial activity of these compounds were found in many plant species and genera.

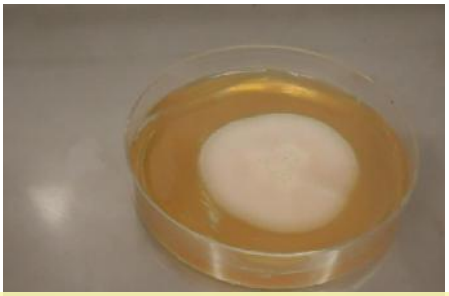
The ethanolic extract of Pomegranate peel at 5mg /ml concentration was the potent extract in complete inhibition the *T. mentagrophytes*(Al-Jumaily,2008)another study revealed that red and white onion extracts have highest inhibition rate on dermatophytes growth ,the alcoholic extracts were better than the aqueous extract in their effect against *T.mentagrophytes* at concentration 40mg/ml of alcoholic extract of red onion which give complete inhibition (Mustafa,2009).Of 22 plant extracts clove and ginger ethanolic extracts showed inhibitory activity against ten food associated fungal isolates by poisoned food technique. While garlic ethanolic extract also showed inhibitory activity against all the tested fungi except *pencilium expticum* (Ruqaia and Jalil,2010).



Control



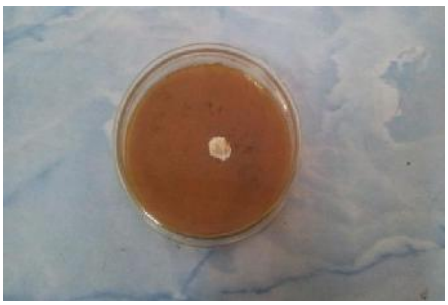
25µg/ml



50µg/ml

75µg/ml

100µg/ml



125 µg/ml

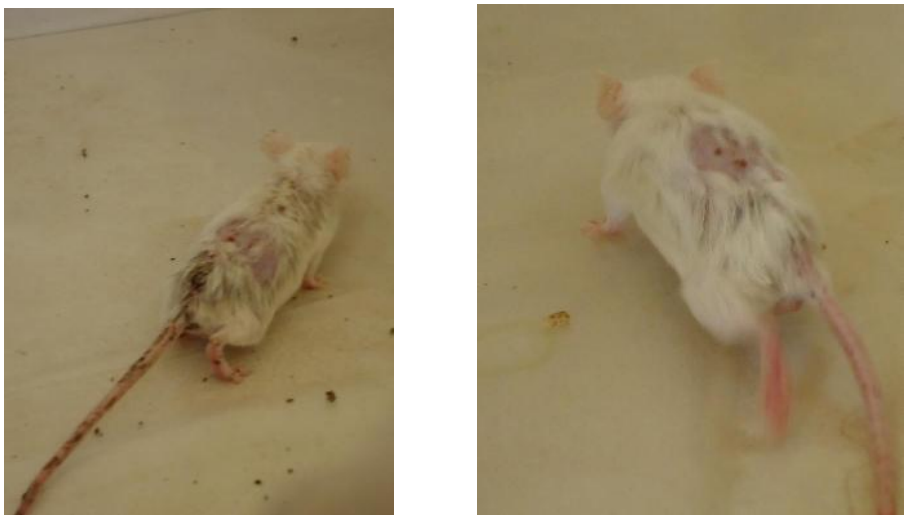
Figure(3-5)*Invitro* antifungal activity of methanolic extract of *Paliurus spina-christi* against *T.mentagrophytes* for seven days

3.6 Pathological study

3.6.1 Clinical features

After 12 days of infection with *Trichophyton mentagrophytes* figure(3- 6) certain degenerative changes were observed due to the adherence of the fungal spores into the keratinophilic layer of the skin producing skin infection .

Regular margin were seen , redness, flat, erythmatous plaques with a raised border .Scaly plaques may be studded with papules or crusts. Hairs are broken close to the skin, they may plug the hair follicle. This represent patches can remain stable for years or may enlarge if not treated (Lin *et al*,2004) .Some times ,especially when follicular pustules have developed and hair loss is observed ,the clinical morphology resembles bacterials folliculitis (Baran *et al*,2004).The specific clinical features in general depend on the inflammatory response of the host .Lesions usually appears with erythematous [illegible] with fine scales ,often in [illegible] and Summerbell



Figure(3-6)Mice infected with *T. mentagrophytes* for 12 days

3.6.2. Histological study

Histological analysis of the skin samples from all experimental groups included non-infected (-ve control),treated with Vaseline (vehicle), treated with recommended drug Clotrinazol-P (+ve control), and finally treated with *paliurus* methanolic extract (*P*.ME)on the 7^{th},14^{th},21^{1st} and 28^{th} days of lesion .

As mentioned previously +ve control and vehicle they were represent treatment of lesion by recommended drug and Vaseline respectively they shows moderate healing as compare with –ve control lesion sections which demonstrate a normal cytoarchitecture of skin (epidermis layer, dermis layer , sweet gland and hair follicle).

Sections made from skin lesions were stained by heamatoxilene and eocin (H and E) stain to detect cellularity ,fibroblast while periodic acid-schiff (PAS) stain was applied to show fungal hyphae. The last stain doesn't show the hyphae in skin but the hyphae appeared in H&E stain section and this is due to several factors such as the way of the biopsy taking, fixative Ph, storage period and temperature, affectivity of stain it self (Washington, 2006).

As we know normal skin in microscopic examination consist of two distinct but slightly attached layers ,figure (3-7)has shown that the skin consists of two distinct layers. The epidermis layer, an epithelial layer of ectodermal origin and The dermis layer, of mesodermal connective tissue. The junction of dermis and epidermis is irregular and projections of the dermis called dermal papillae interdigitate with evagination of the epidermis known as epidermal ridges. Epidermal derivatives include hairs, nails, sebaceous and sweat glands. Beneath the dermis lies the subcutaneous tissue or hypodermis, a loose connective tissue that may contain pads of adipocytes. The subc[illegible] anatomy(Mescher,2010).

After infection with the *T.mentagrophytes* the skin appeared with certain histological changes such as adherence of the fungal conidia to keratinocytes caused keratinolysis ,distribution of inflammatory cells in the dermal layer, odema and more inflammatory reactions figure(3-10),odema in dermal layer extend to hypodermal layer in addition to the appearance of polymorpho neutrophils and necrotic depris figure(3-11)damage of epidermal layer figure(3-13)and(3-15)the same results appeared with (Al-Ani,2006).Treatment of infected skin with plant extract for seven days, a clear healing demonstrated via moderate inflammatory cells in dermal layer figure(3-8)and (3-9).After 14 days the skin section appeared with normal shaped of epidermis with distribution of

collagene and fibroplast figure(3-12)melanocytes regeneration obvious under electron microscope figure (3-14).The regeneration of sweat glands and keratinocytes figure(3-16)after 21 days of treatment with plant extract .

Finally after 28 days of treatment by plant extract the skin section illustrate the normal shape of epidermal and dermal layers with its common structures (sweat glands ,hair follicle, keratin, collagen and fibroblast) figures(3-19)and(3-20 demonstrate the hair regeneration in treated mice by plant extract with disappear of fungal lesion.

Different active compounds were known to be responsible for healing like flavonoids ,volatile oils ,alkaloids and steroids (Abu-Al-basal,2001) ,(Al-Ani,2006),(Farageen,2007).

Hexoseamines are known to increase during early stage of wound healing and decrease hereafter (Peacock,1984). All plant extracts exhibited to certain degrees the promoting of collagen synthesis ,collagen is the predominant extracellular protein in the granulation tissue of a healing wound (Irvin,1984).

More over the collagen plays an important role in haemostasis and epithilization (Heggers *et al*,1993a;1995b) . Also similar effect has been observed with the ethanolic extract of *Centella asiatica* on rat dermal wound healing .This extract increased cellular proliferation and collagen synthesis at the wound site as well as quicker and better maturation of cross linking of collagen and enhancing the wound healing process(Suguna *et al*,1996). The

obtained results in this study were similar to the influence of *Aloe vera* on collagen characteristic in healing dermal wounds in rats .It was observed that *A.vera* increased the collagen content of the granulation tissue as well as its degree of cross linking which ultimately contributes to wound strength (Chithra *et al*,1998).

Healing in the current results may be attributed to phenolic compounds detected in our results such as (Gallic acid ,caffeic acid ,syringic acid and epichotechin)these compounds have an action on the growth of many dermatophytes. As compared with the treatment by recommended drug (Clotrinazole p-) figure (3-10)there are still inflammatory cell in dermal layer after 7 days of treatment .The same results also continue in skin section treated by recommended drug for 14 days, edema and necrotic cell reaction had been demonstrated figure(3-11),the inflammatory cell reactions extent to the 21 and 28 days of treatment by recommended drug figure (3-12)and figures (3-21) respectively. Histological studies of antifu

1. Drug that disrupt the cell membrane.
2. Drug that inhibit mitosis.
3. Drug that inhibit deoxy ribonucleic acid (DNA) synthesis (Laurence *et al*,1997).

The influence of some active compounds on the content of glycosaminoglycan and its types in the granulation tissue of healing dermal lesion have been studied. Results revealed higher levels of hyaluronic acid and dermatan sulfate in the treated wounds indicate increased turnover of the extracellular matrix and enhanced wound healing process(Chithra *et al*.,1998b). Flavonoids and phenolic compounds found in *P.spina-christi* may play the main

role in cross linking .This is un accordance with the results of Havesteen (1983)and Farageen(2007) who clarified that flavonoids are essential in activation the proline hydroxylase. Three general modes of action of different plant extract were recognized as

1. Inhibition of microbial cell wall formation or biosynthesis of some protein .
2. Disruption of deoxyribonucleic acid (DNA)metabolism.
3. Alteration of normal function of the cellular membrane(Tyler *et al*,1988).

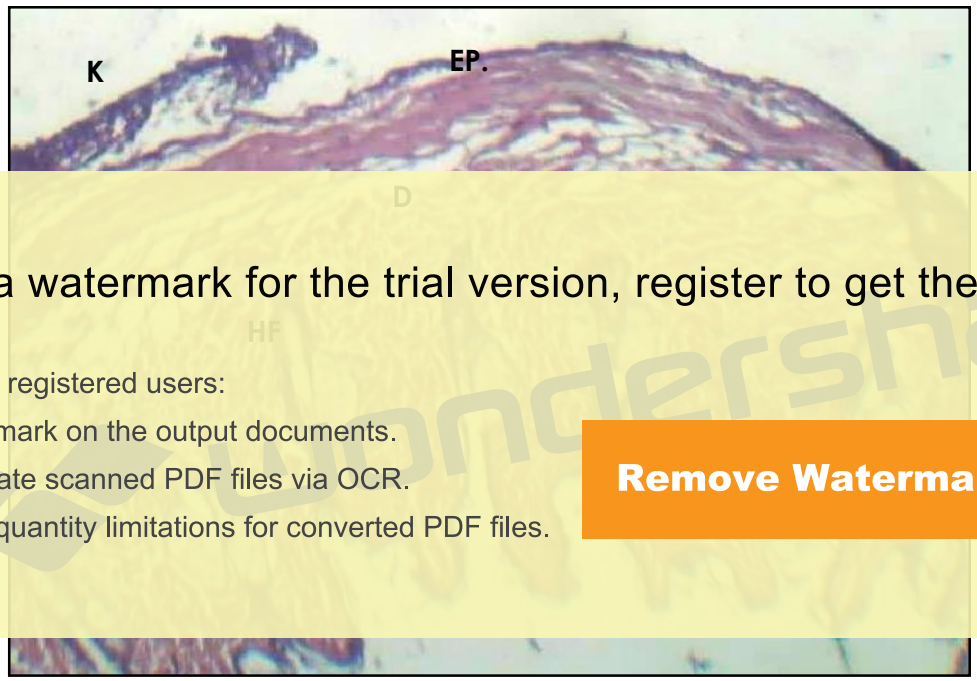
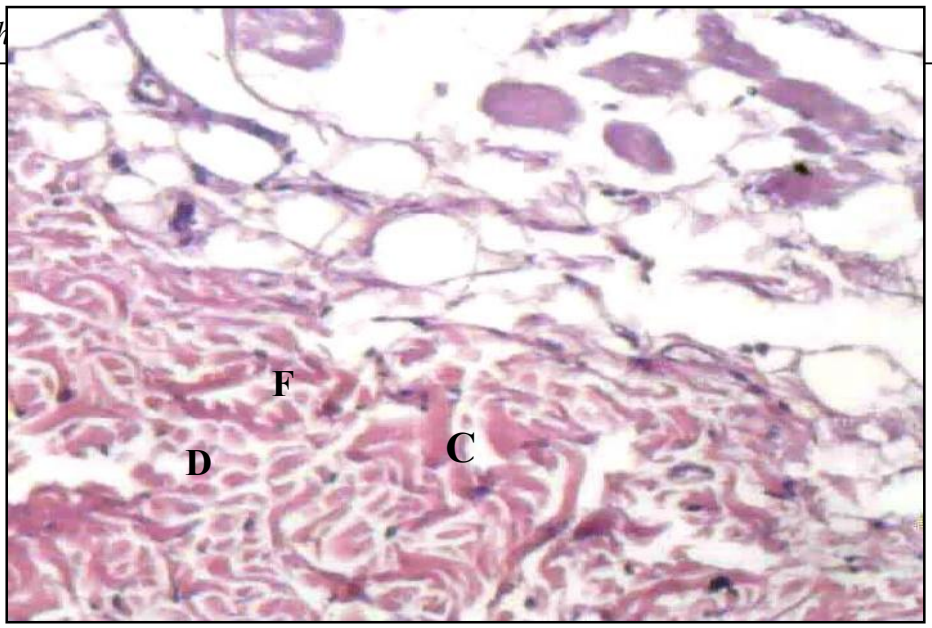
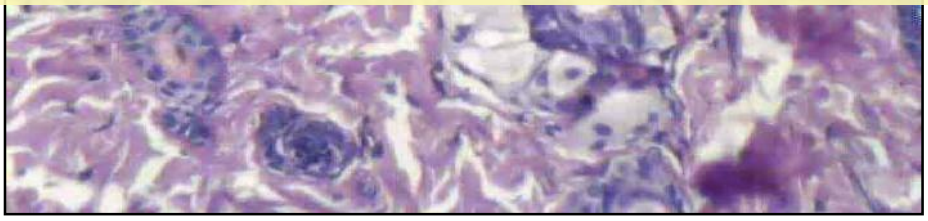


Figure (3-7)photomicrograph of normal section stained with HandE stain –ve control,EP:epidermis,D:dermis,HF:hair follicle .

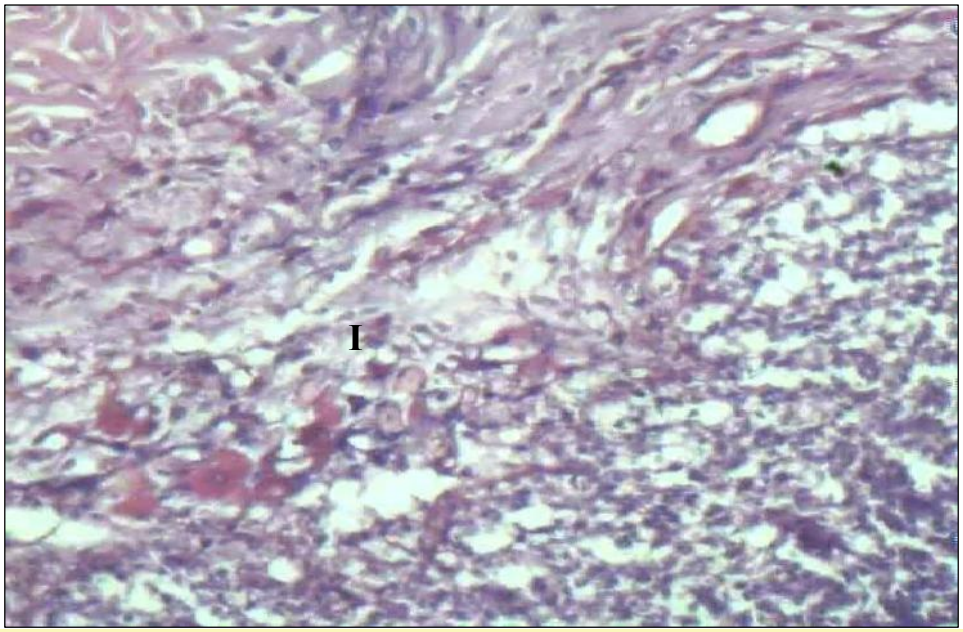


1:Hypodermis layer

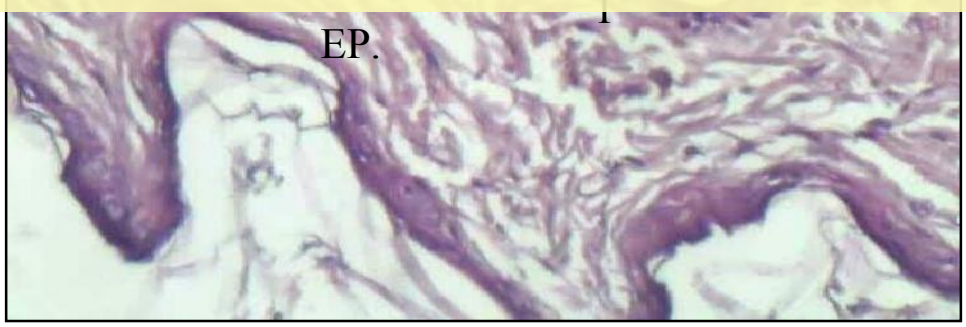


2 : upper layer

Figure(3-8)Photomicrographs of 7 days-old-lesion section stained with H and E stain 1and ,2:PME . C:collagen, F:fibroblast (Magnification: 200X).

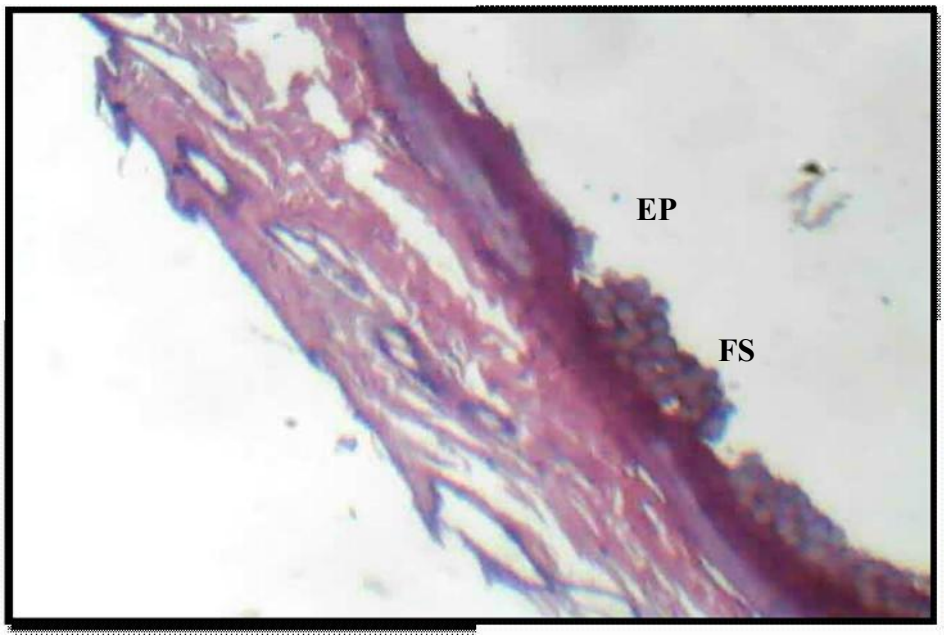


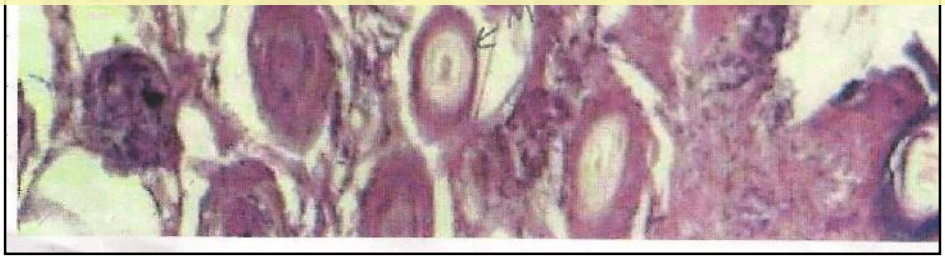
1 :Hypo dermis layer



2

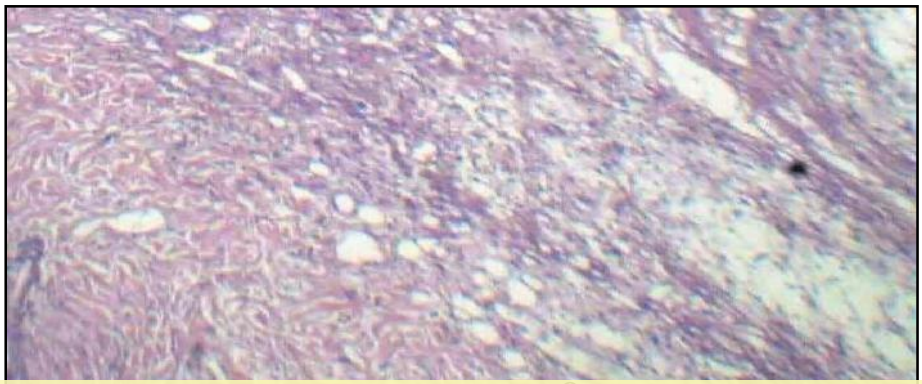
Figure(3-9)Photomicrographs of 7 days-old-lesion section stained with HandE stain 1and ,2:PME. Ep: Epidermis ,D:dermis I:inflammatory cells, C:collagen,F:fibroblast (Magnification: 200X).



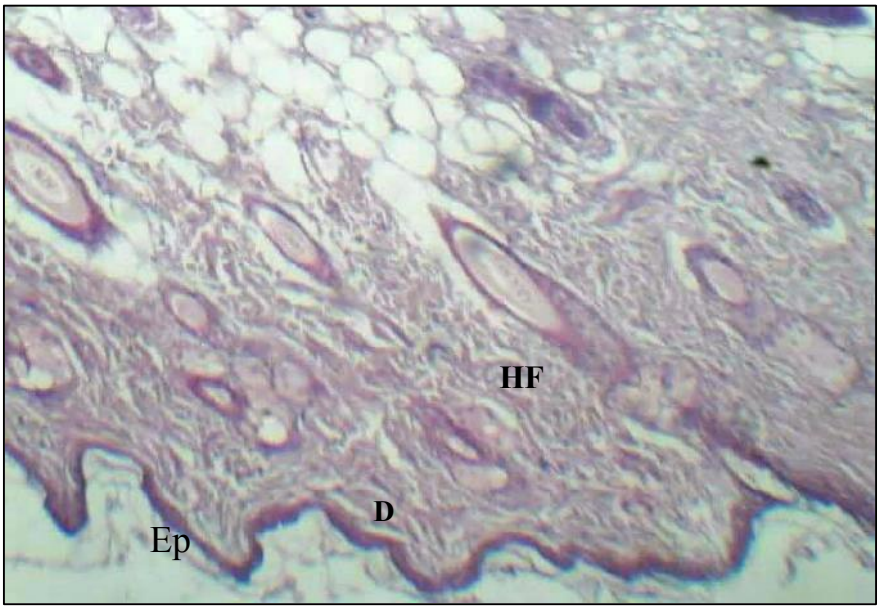


2: upper layer

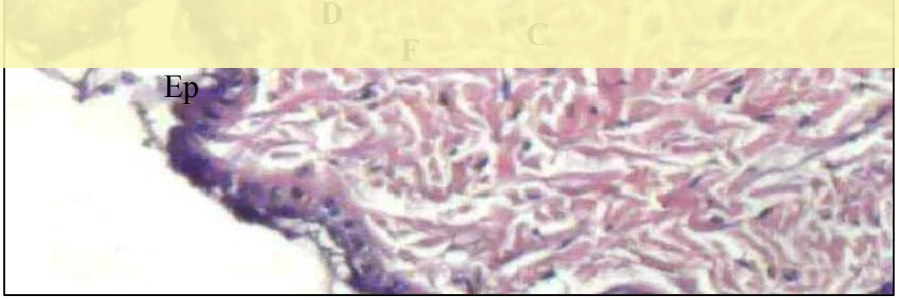
Figure(3-10)Photomicrographs of 7 days-old-lesion section stained with H and E stain 1:+control,2:vihcle, . Ep: Epidermis ,D:dermis I:inflammatory cells, FS:fungal spores **(**Magnification: 200X**).**



Figure(3-11)photomicrograph of 7 days-old-lesion section stained with H and E stain –ve control ,.Ep: Epidermis ,D:dermis ,I:inflammatory cell, O:odema **(**Magnification: 200X)

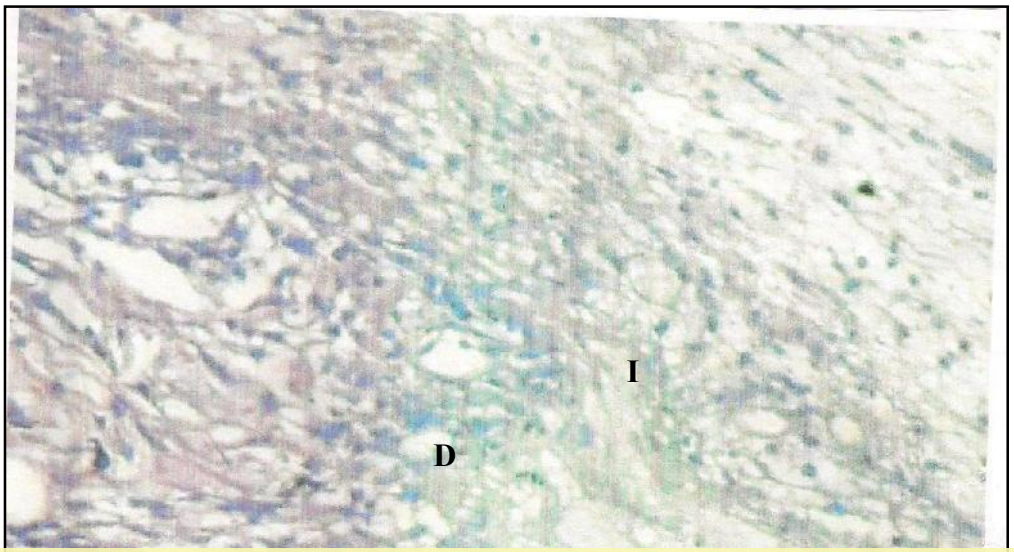


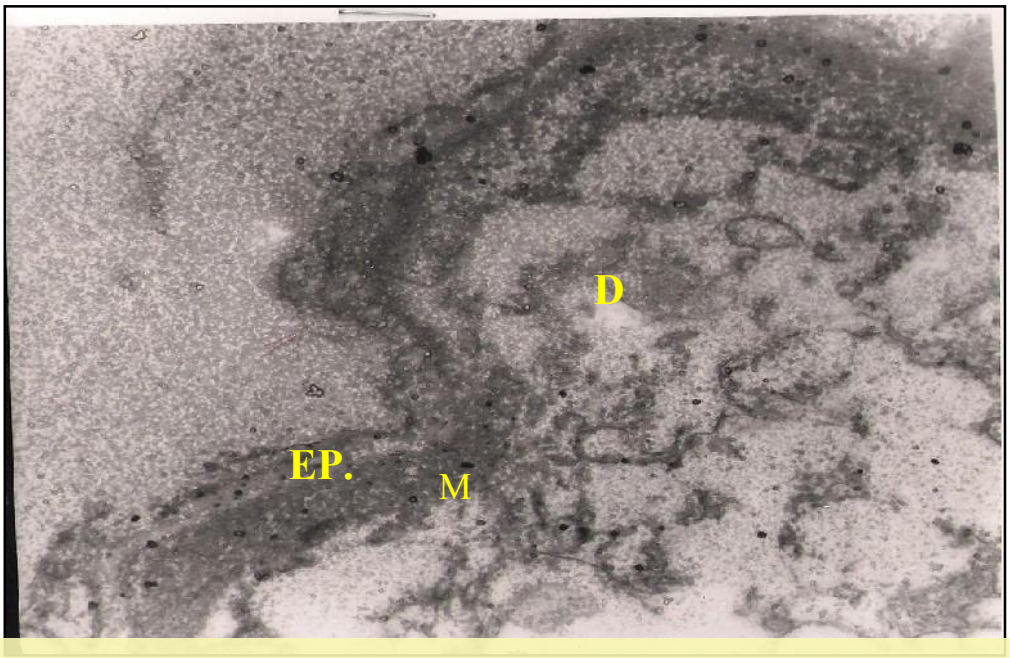
1



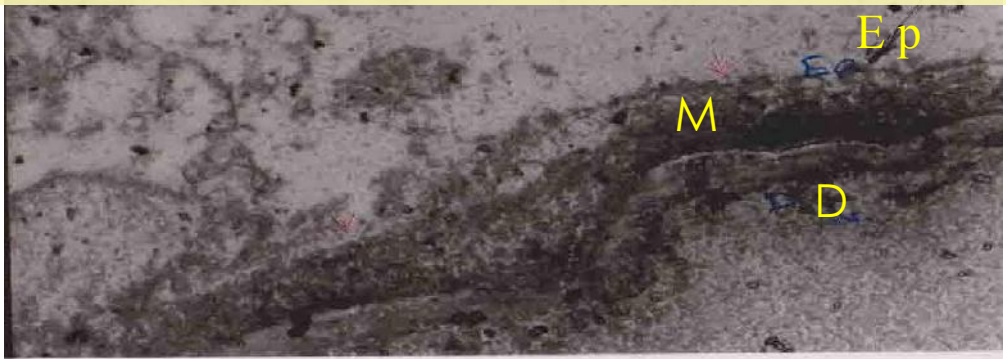
2

Figure(3-12)Photomicrographs of 14 days-old-lesion section stained with H and E stain 1and ,2:*P*.ME .Ep: epidermis ,D:dermis,HF:hair follicle C:collagen,F:fibroblast **(**Magnification: 200X**).**



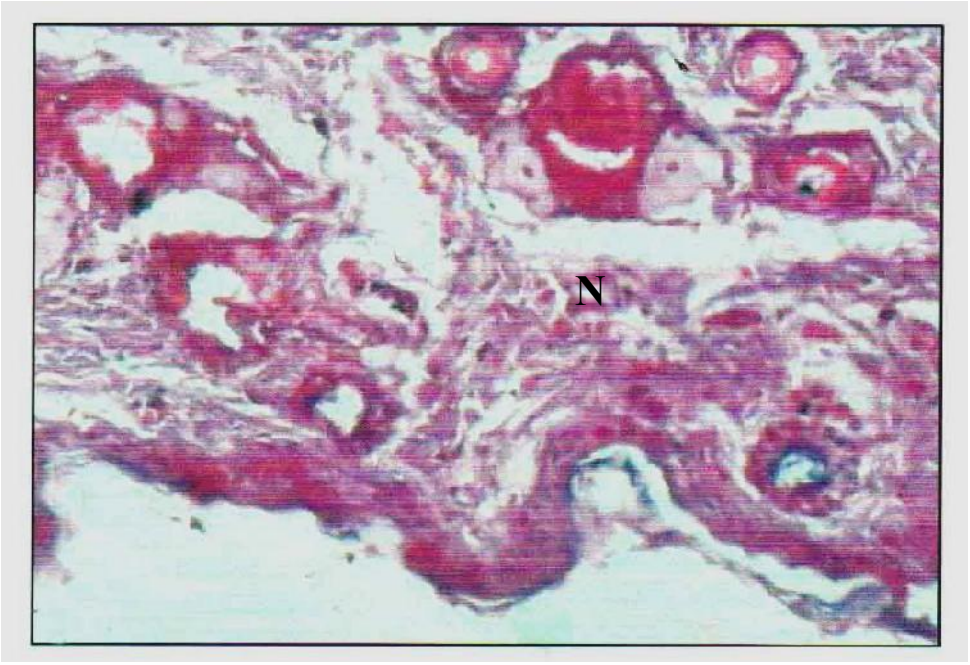


1



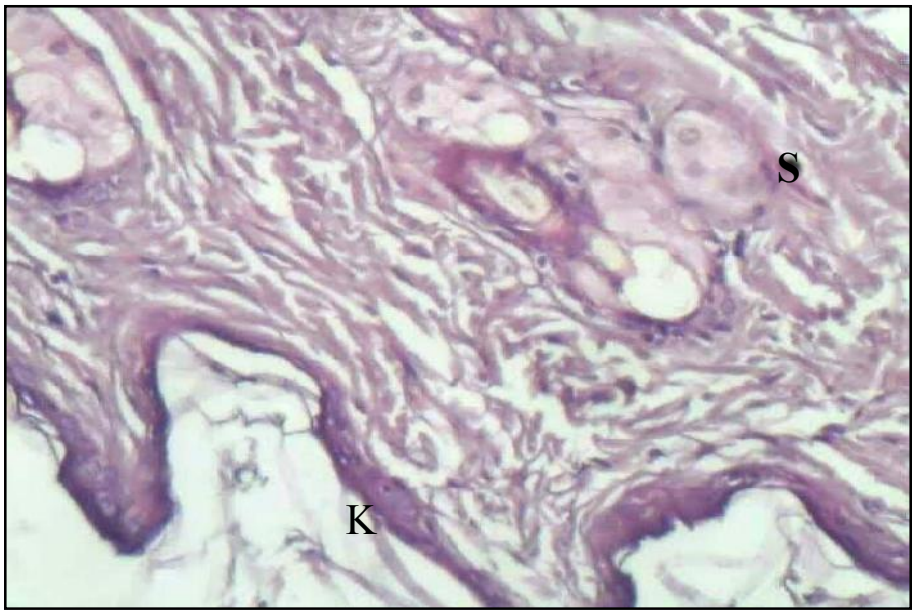
2

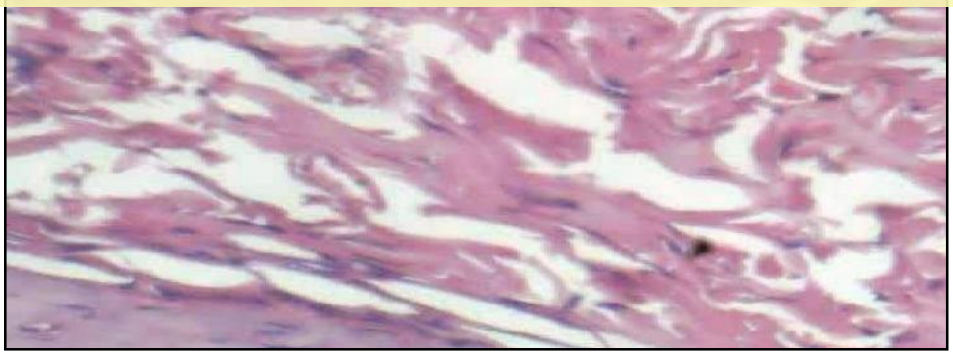
Figure(3-14)Photomicrograph of 14 days-old-lesion TEM section,*1and2: P.*ME lesion treated for 14 successive days . EP: epidermis, D:dermis,M:melanocytes. **(**Magnification: 15909X**).**



2

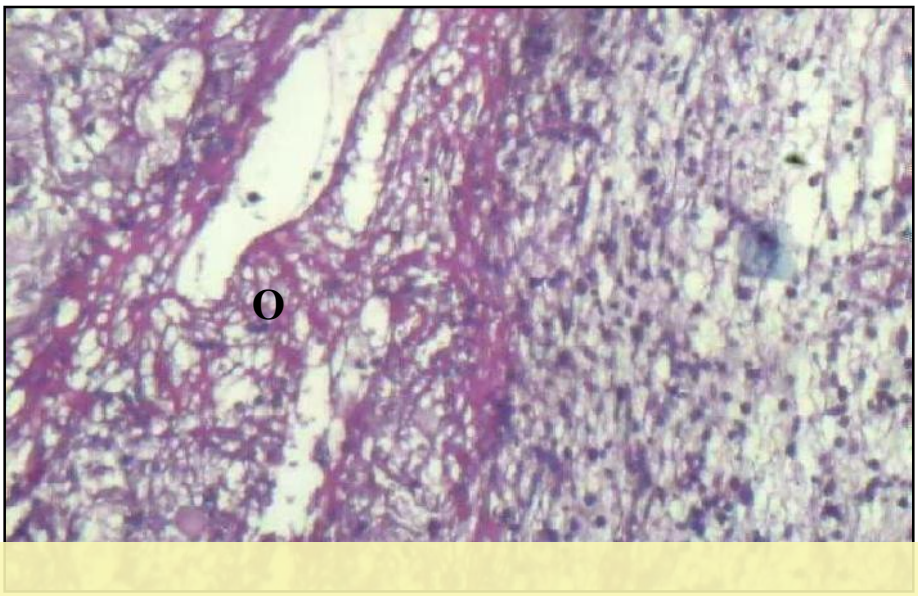
Figure(3-15)Photomicrographs of 21 days-old-lesion section stained with HandE stain 1:+ve control ,2:vehicle.N:necrosis.(Magnification: 200X)

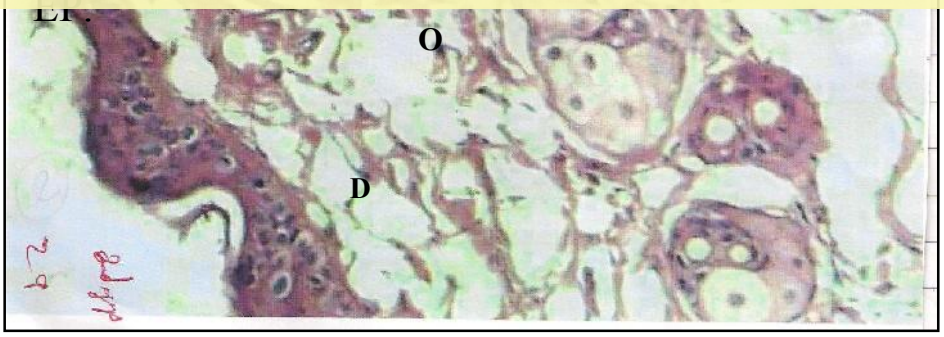




2

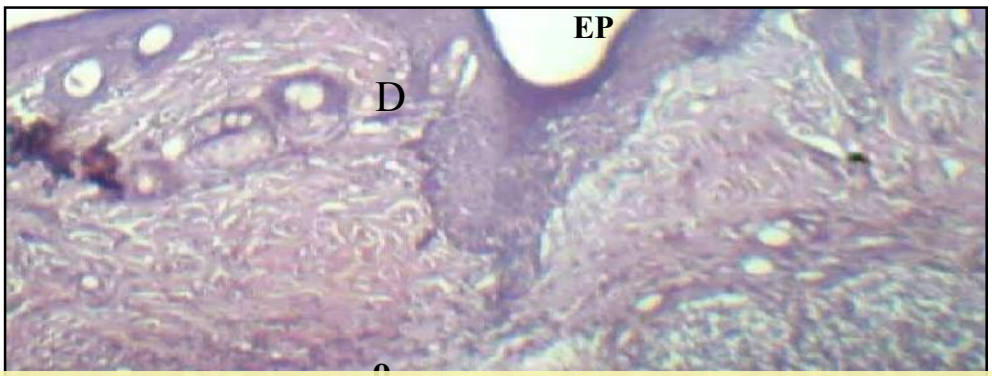
Figure(3-16)Photomicrographs of 21 days-old-lesion section stained with HandE stain 1and 2:*P*.ME EP:epidermis,D:dermis,K:keratin tissue, S:sweet gland.**(**Magnification: 200X**).**

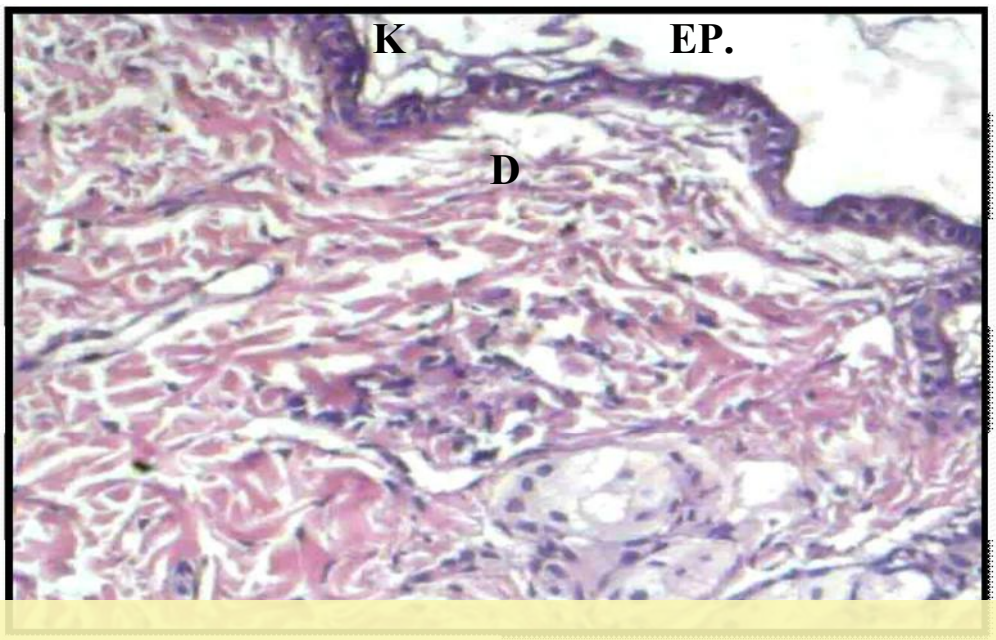


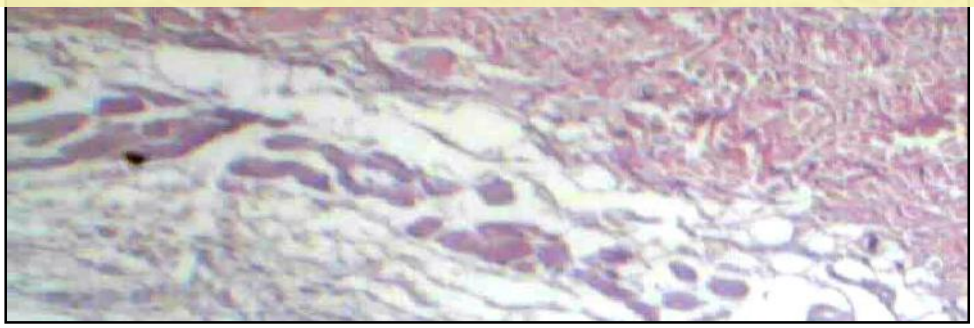


2:upper layer

Figure(3-17)Photomicrographs of 21 days-old-lesion section stained with HandE stain 1:+ve control ,2:vehicle.I:inflammatory cells , O:odema.(Magnification: 200X)

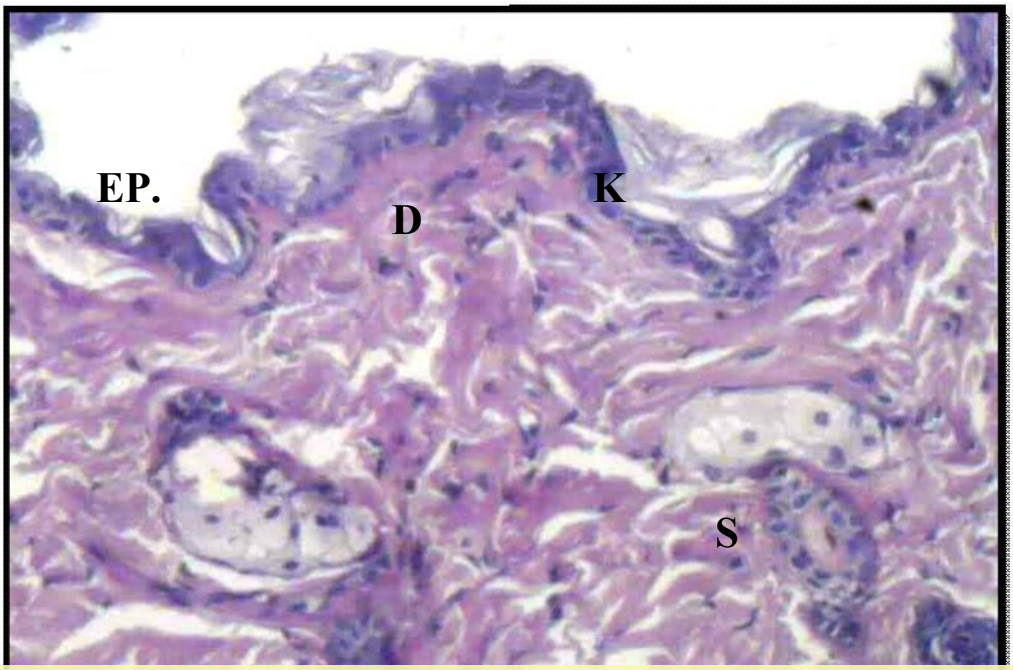


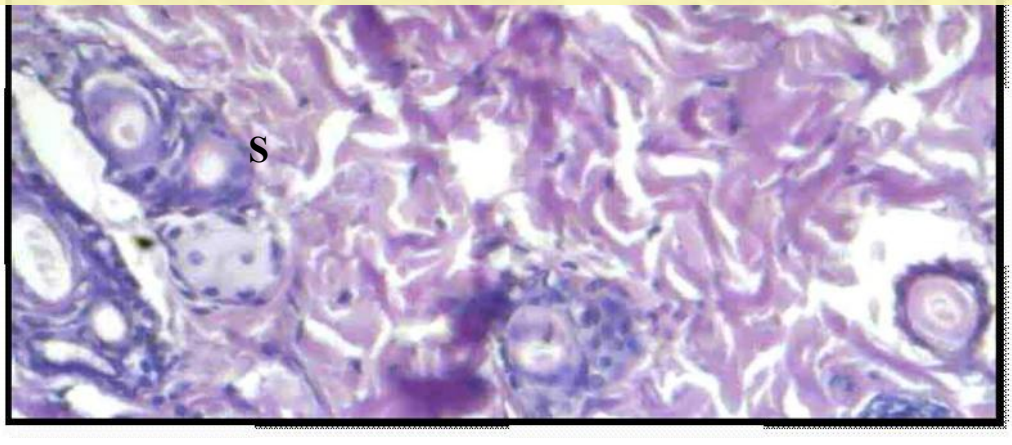




2

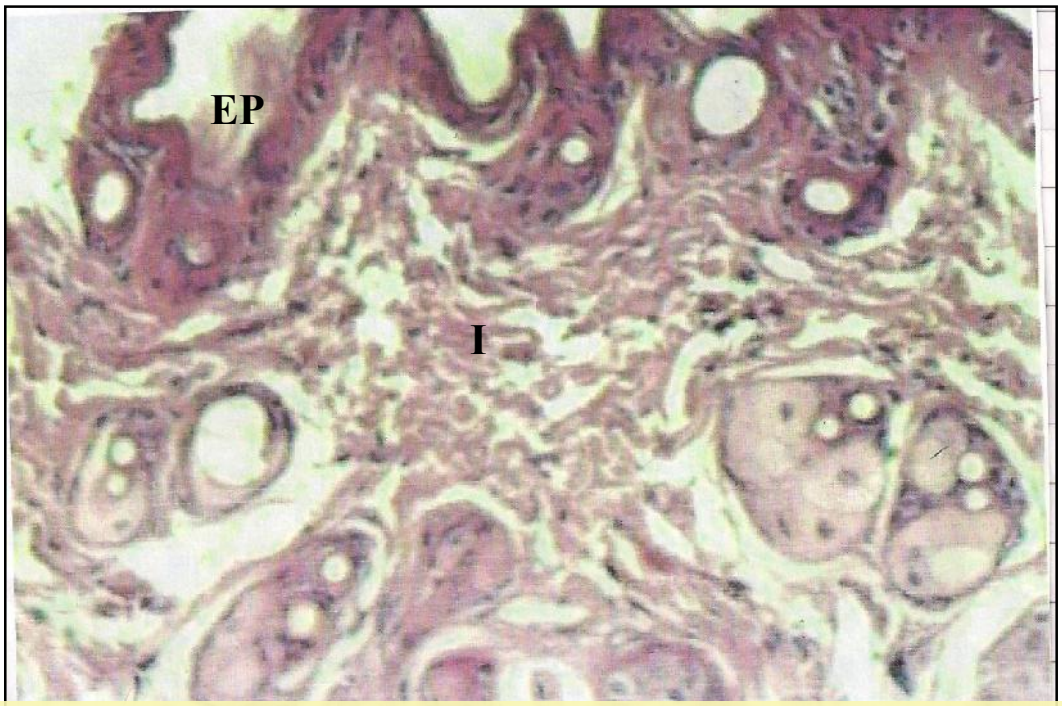
Figure (٣-١٩)Photomicrographs of 28days-old-lesion section stained with H and E stain 1and 2:*P.*ME .Ep: epidermis ,D:dermis, K:keratin tissue**.** HF: hair follicle **(**Magnification:200Xrespectively**).**

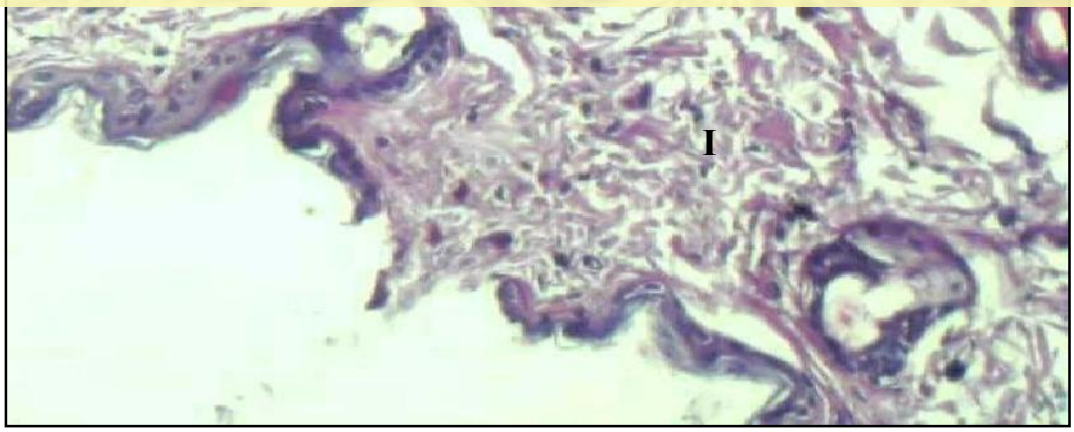




2

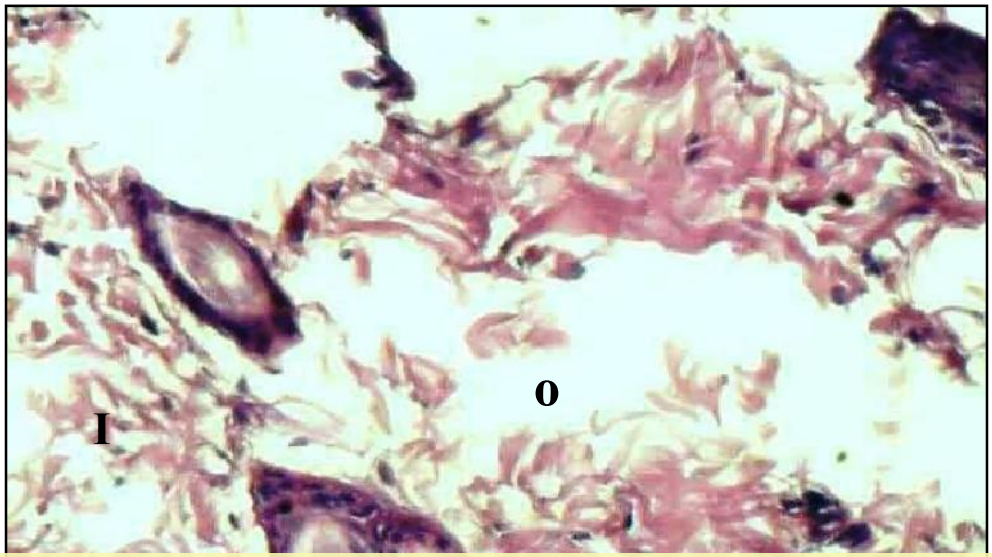
Figure(3-20)Photomicrograph of 28 days-old-lesion section stain with H and E stain 1and 2:*P.*ME . k:keratin tissue .Ep: epidermis ,D:dermis, S:sweet gland **(**Magnification: 200X**).**

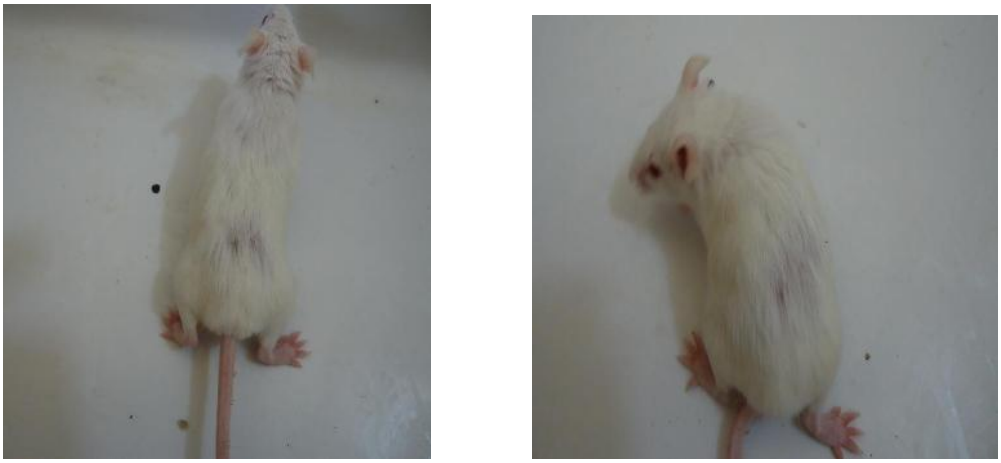




2

Figure(٣-2١)Photomicrographs of 28 days-old-lesion section stained with HandE stain 1:+control and 2:vehicle .EP: epidermis I:inflammatory cells .(Magnification: 200X)





Figure(3-23)Mice treated for 28 days with *P. spina-christi* methanolic extract

gene ,the gene was expressed in both non treated and treated *T.mentagrophytes* as shown in figure (3-25), this may be refer to several factors :-

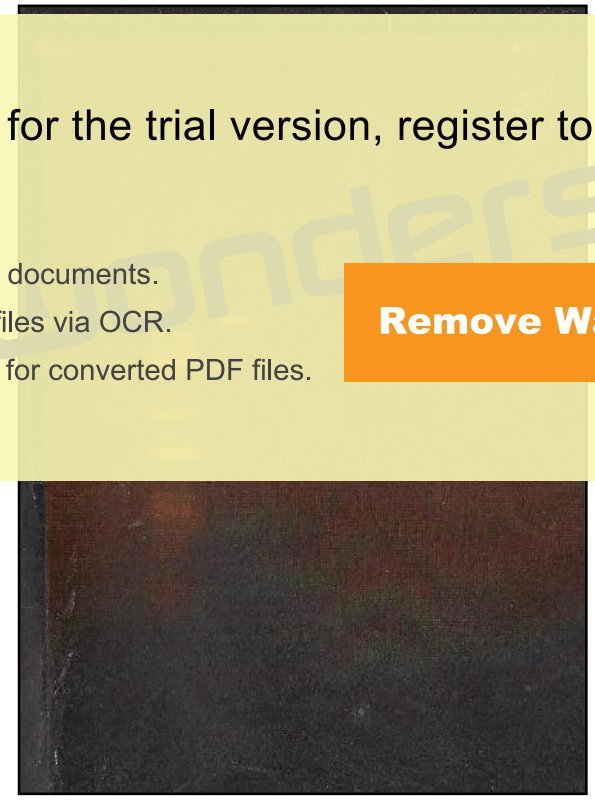
1-This gene expressed in media supplemented with salts, keratin and elastin as a sole source of carbon and nitrogen (Jousson *et al*,2004).

2-The primers were designed to detect the transcripts of several other candidates genes ,including the protease Tri m2 and Alp genes, metalloprotease Mep2 and Mep3 genes.

3-Tri m4 gave a strong signal which detected by RT-PCR from RNA isolated from cultures grown on blood plasma (Kaufman *et al*,2005).

The expression of the Tri m4 gene in presence of blood plasma with plant extract was confused they produce the same bands after 40 cycle of PCR. The combination of additives (glucose ,keratin and elastin) with plant extract doesn't recommended due to the effect of inhibition will be of little chance (Duke *et al*,2004).There must be an alternative way to regulate the expression of this gene without additives only the natural components of the fungal media e.g.(malt extract ,glucose ,peptone) then identified the effect of plant extract on the expression of this gene wither does it inhibit its expression or not?.

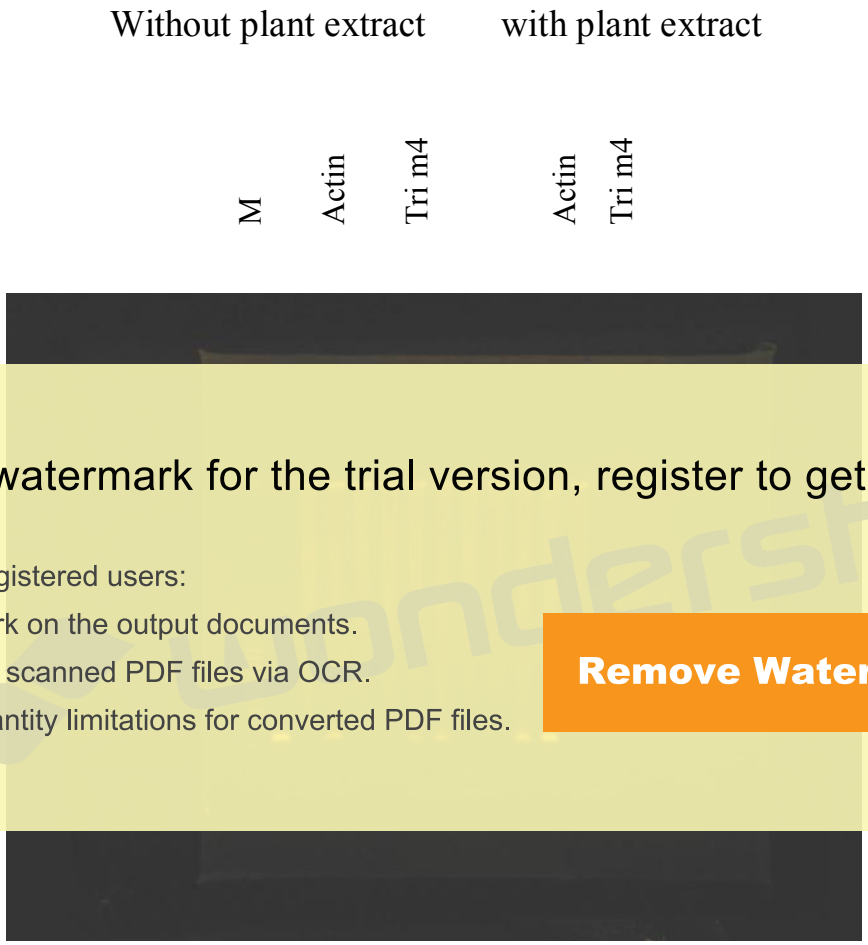
M 1



Figure(3-24) gel electrophoresis of *T. mentagrophytes* DNA Extract.

Lanes: M, molecular weight marker kappa (universal ladder), size range, (100-10000) Bp.

1: DNA Extract Of *T. mentagrophyte*(less than 10,000bp)



Figure(3-25)PCR Product of actin and Tri m4 gene of *T.mentagrophyte* species ,M:marker ,Actin gene,Tri m4 gene.

References

- **Abass,N. K**.(1995)Mycological and Bacteriological study of head and scalp lesions(Tinea capitis andImpetigo) .college of Medicine ,university of Baghdad.

- **Abu-Al-Basal, M**.(2001)The influence of some local medicinal plant extracts on skin wound healing activity ;evaluated by histological and ultra structural studies .faculty of graduate studies .university of Jordan .Ph.D in biological sciences

- **Adeleye I.A., Ogunniyi A.A., Omonigbehin E.A.,Antimicrobial** (200)activity of some local herbs on common skin pathogens ,

- **Agrawal A., S. Srivastava , J. N. Srivastava and M. M. Srivsava**(2004) Evaluation of Inhibitory effect of the Plant Phyllanthus Amarus on Hepatophytic

- **Agwa A.,M. Aly and R.Bonaly**(2000) Isolation and characterization of two Streptomyces species produced non polyenic antifungal agents. J.Union Arab Biol.,7:62-84.

- **Al-Abid, M. R**. (1985). Zur zusammem setrang der Absschlu Bmemtrame in phoenix dactily frawuzbury univ. Wurzburg F. R. of Germany.

- **Al-Ani, R.S.** (2006).Therapeutic activity of crud alkaloidal extract of *Fumaria officinalis* on dermatophyte(*Trichophyton rubrum)*,study by light microscope. MSc.Thesis, dept. of biotechnology, college of science ,University of Al-Nahrain.

- **Al-Farageen A.A**.(2007).Evaluation of ethanolic extract of *Mercurialis annua* L.(on skin wound healing of albino rats. MSc. thesis in biological science .Faculty of graduate studies .university of Jordan.
- **Ali,T. M**.(1990)Tinea capitis :clinical and mycological study ,MSc. In medical microbiology ,college of medicin ,University of Baghdad.
- **AL-Janabi A.A**.(1993)Tinea pedis clinic Epidemiological study of interdigital dermatophyte foot infection in Baghdad. Msc.in community Medicine .college of medicine.AL-Mustansirya univ.
- **Al-Jubori J.S**.(2005).Studying the efficiency of crude volatile oils extracted from some medicinal plants in inhibit some of pathogenic bacteria and dermatophytes. MSc.thesis ,Faculty of women education .Tikrit University.

- **Al-Kaisy ,K.S**.(1988)Election microscopic tudy of epethinal cells and their secretion activity in the thymus gland of mice after activation of growth hormone .MSc. Thesis .Department of Biology .Science college .Baghdad university.
- **AL-kayali, k.k.;w.m.Hassan and B.M.Razoogi**(2011)the rate of-Superficial fungal infections among patients with different skin diseases in Diyala Iraq.iraqi j.comm.Med.july.24(3):264-266.
- **Allen ,J.W.,Sheler,C.F.,Mendes,R.W. and Latt,S.A**.(1977).Asimplified technique for in vivo analysis of sister chromatid exchanges using 5-bromodepxy uridine tablets.Cell Genet.18:231-237.

- **Allen A.M and Taplin D.**(1973) Epidemic *Trichophyton mentagorphytes* infections in servicemen: source of infection , role of environment, host factors, and susceptibility. JAMA.ch.17; 226:864-867.
- **AL-Rawi,A.**(1988)Geographical disturibution of wiled type plant in Iraq.3rd E..Ministry of agricultural and water. (in Arabic)
- **AL-Rawi,A.M.,R.R.,Riyadh.**(1989).Modern chemistry of life.3rd Ed..ministry of higher education and scientific research printer.Baghdad.(in Arabic)
- **AL-sady A.A**(1995)some antibacterial and antifungal actions and - Myrtus cumis, M Sc.in clinical pharmacology, College of Medicine, university of Baghdad.
- Al-Sammaraie, B.A. (2008).Effect of Nerium oleander extract's on the growth of some fungi isolated from skin of patients in Samarraa city. MSc. Thesis. College of science. University of Tikrit.
- Al-Sammaraie K.W/Al-Rekabi,S. [illegible] extract of Withania somn[illegible] dermatophyte.seconed conference in medical and biological science .Faculty of allied medical science .Zarka private University.:40
- **Al-Suhaili,S**. (2002). Antifungal activity of some plant extracts of Al-Mafraq area .Faculty of science and Arts. University of Al-albait.
- **Al-Taie, S**.(2001).The effect of plant extracts on dermatophytes growth isolated from patients diagnosed with dermatitis and on the growth of some bacteria.MSc. thesis .College of Education .University of Tikrit.
- **Aly MM,** (1997). Potancy of selected actinomycete for certain antifungal production. Ph. D. Thesis, Tanta University, Cooperation system between Egypt and France, p. 375.

- **Aly, A.A. , Omer S.A., Zayed S.M., and Mansour ,M.T**.(2000).Use of saponin containing a triplex nummularia to suppress damping of cotton seedling.J.Agric.Sci.Mansoura University.25:7621-7631.
- **Aly, M.M. and Bafiel. S**.(2008).Screening for antimicrobial activity of some medicinal plants in Saudi Arabia. World conference on medical and aromatic .
- **Ambrogi, L.P.** (1960). Manual of histologic and special staining teqniques. 2nd Ed..McGraw-Hill Book company.Inc. New York.
- **Amer S., M. Aly and S. Sabbagh**.(2006) Biocontrol of dermatophytes using some plant extracts and actinomycetes filtrates. Egyptian J. Biotechnol, p: 330-315. 2006.
- **Araujo C.R.;K.C.Miranda ,O.F.Fernandes; A.J.Soares and M.R.Silva**(2009)*In vitro* susceptibility testing of dermatophytes isolated in Goiania, Brazil against five antifungal agents by broth microdilution
- **AuVera K. , R.Mozuraityte and I.S Rustad** (2009)Antioxidant activity of phenolic acids in lipid oxidation catalyzed by different prooxidants .J.of Agricultural and food chemistry .21:10377-103.

Backs, Chapman and Hall, New York.

- **Bancroft, J. D. and Stevens, A**.(1982).Theory and practice of histological techniques. 2nd Ed. .Churchill Living Stone:483-516.
- **Baran W., J. Szepietowski and R. Schwartz**.(2004). Tinea barbae. Acta dermatoven. APA. 13.3: 91-94.
- **Bogdanova V.P**.(1963)Chemical composition of the cortex of plants .the host of lac insects. Izwestija akademii Nauk SSSR 28;605-612.

- **Bokhari F. M**.(2009) Antifungal activity of some medicinal plants used in Jeddah, Saudi Arabia. Mycopathology. 7(1). P:51-57.
- **Branter A., Z.Males, S.Pepeljnjak and A.Antolic**.(1996)Antimicrobial activity of *Paliurus spina-christi* Mill.j.of ethnopharnacology 52.p:119-122.
- **BranterA., Z. Males, S.Pepeljnjak and A.Antolic**(1996)Antimicrobial activity of *Paliurus spina-christi* Mill.j.of ethnopharnacology 52.p:119-122.
- **Brantner A. and Z. Males**(1990) Investigations on the flavonoid glycosides of Paliurus spina-christi planta 56p: 582.
- **Brantner A.H.and Z.Males** (1999)Quality assessment of Paliurus spina-christi extracts) Journal of Ethnopharmacologylogy ,66p:175-179.
- **Brook G. J.S.Butel and S.Morse**(1998) Jawetz's medical microbiology
- **Browenlee, H. E., A. R. McEuen , J. Hedger and I. M. Scott.**(1990) Antifungal effects of cocoa tannin on the witches' broom pathogen *Crinipellis perniciosa*. Physiol. Mol. Plant Pathol. 36:39-48.
- **Burdick, E. M**.(1971) Carpaine, an alkaloid of *Carica papaya*. Its chemistry and pharmacology. Econ. Bot. 25:363-365.
- **Butler, L. G**.(1988) Effects of condensed tannin on animal nutrition, p. 553. In R. W. Hemingway, and J. J. Karchesy (ed.), Chemistry and significance of condensed tannins. Plenum Press, New York, N. Y.
- **Capek,P., Machova, E. and Turjan, J**.(2009).Scavenging and antioxidant activities of immune modulating polysaccharides isolated from salvia officinalis.Int.J.of Biological micromolecules .44:75-80.

- **Cardellina ; J. H. I.;M. H. G. Munro; R. W. Fuller; K. P. Manfredi; T. C. McKee; M. Tischler; H, R. Bokesch; K. R. Gustafson; J. A. Beutler; and M. R. Boyd**(1993) A chemical screening strategy for the dereplication and prioritization of HIV-inhibitory aqueous natural products extracts. J. Nat. Prod. 56: 1123-1129.
- **Casley-Smith ; J. R. and J. R. Casley-Smith.** (1997) Coumarin in the treatment of lymphoedema and other high-protein oedemas. In R. O'Kennedy, and R.D. Thornes, (ed.), Coumarins: biology, applications and mode of action. John Wiley and Sons, Inc. , New York, N. Y. : 348
- **Chabot, S. R.; R. Bel-Rhlid ; R. Chenevert, and Y. Piche**(1992) Hyphal growth promotion in vitro of the VA mycorrhizal fungus, Gigaspora margarita Becker and Hall, by the activity of structurally specific flavonoid compounds und[illegible] Phytol. 122: [illegible]-467.
- **Chaurasia, S. C. and K. K. Vyas**(1977) *In vitro* effect of some volatile oil against *Phytophthora parasiyica* var. piperina. J. Res.
- **Chen, J., Yi, J., Liu ,L., Yin, S., Chen, R., Li, M., Congxin, Y., Zhaug, Y.and Lai, W**.(2010).Substrate adaptation of *T. rubrum* secreted endoproteases microbial Pathogenesis.48:57-61.
- **Cheng J.*et al.***(2000) Naunyn schmiedebergs Arch pharmacology. 362(2). P:122-127, by net.
- **Chermette R.; L. Ferreiro and J. Guillot**(2008) Dermatophytoses in animal . Mycopathologia, 166 :385-405.
- **Chithra P.; Sajithalal G.B. and d. G. Chandrakasan** (1998b)Influence of Aloe vera on collagen characteristics in healing dermal wounds in rats .Mol.cell.Bioch.181:71-76.

- **Chung, E., T.Cardena-frey and JD. ,Clements**(1999)The role of cAMP in mucosal adjuvenicity of *Escherichia coli* heat –labile enterotoxin(lt).Vaccine.18(1):38-49.
- **Clark,A.M**.(1996)Natural products as a resource for new drugs pharm.Res.13.
- **Collee,J.G.;A.G.Fraser ;B.P.Mariman and S.A.Simmons**(1996)Practical medical microbiology .MackiandMacCartney(Eds)14th (Ed).churchill Linvington.
- **Cowan M.M.** (1999)Plant products as antimicrobial agents.clinical microbiology reviews.12.4:564-582.
- **Dalakishvili, T. M. , S. D. Gusakova , N. J. Chachanidze, K. G. Kuparadze and E. P. Kemertelidze**(1985) (Lipids of *Paliurus spina- christi* seed). Khimija prirodnii soedinenii5,:322-326.
- **Dalakishvili,TS., Zurabishvili, M. , T.S. and, Kemertelidze,** .Khimija priroduii Soedinenii.5:639.
- **De Bievre, C., C. Dauguet, V.H.Nguyen and** *M.abrum* isolates from clinical specimens.Ann.Inst.Pasteur Microbiol.138:719-727.
- **Dermatophytes**.(2008).Wikipedia free encyclopedia . Wikimedia Foundation .Inc.
- **Duek,L.,Kaufman,Y.Ulman and I.Berdicevsky**(2004)The pathogenesis of dermatophyte infections in human skin sections .J.Infect.48:175-180.
- **Elewski B. E.** (1999) Tina barbae .Clinical dermatology ,Demis DJ(ed) .Pheladelphia , Lippincott Williams and Wilkins, unit 17-8. p:1-4.

- **El-Fari ,M.,H.J.Tietz,W.Presber, w.Sterry and Y.Graser**(1999)Development of an oligonucleotide probe specific for T.rubrum Br.J.Dermatol.14:240-245.
- **Ellis D.,S.Davis, H.Alexion,R.Hanke,R.Bartly**(2007)Description of medical fungi .2nd Ed.published by the auyhors,Mycology unit .women's and children's hospital north Adelaide, Australia.
- **Ercan, A., Saracli, M. A. , Ahmed, A., Taner, Y., Zafer, K., and Ali Riza, G**.(2004). Polymerase chain reaction in the diagnosis of on-ychomycosis. Eur. J.Dermatol.14:52-55.
- **Ewing, G.W.**(1987)Instraumental methods of chemical analysis.5th Ed. McGraw-Hill Book Company.New York
- **Faggi, E., Pini, G., Campisi E., Bertellini C., Difonzo E. and Mancianti, F.**(2001).Application of PCR to distinguish between species of dermatophytes.J.Clin.Microbiol.39:3382-3385.
- [illegible] of bioscience a[illegible] Strathelyde. Glascow.U.K.
- Fieser, L.F., and Williamson, K.L.(1983).Gas chromatography. Ch.11.Organic experiments .5th Ed.D.C.Health Eds.
- Freeman, B. and Burrows, A. (Eds). 21st edition, W. B. Saunders N., (1995). Reduction effect of theanine on blood pressure and brain .
- **Glauert,A.M**.(1975)Fixation,dehydration and embedding of biological specimens.Practical methods in electronmicroscopy,North-Holland publishing company ,Amsterdam :2207.
- **Graser,Y.,A.F.Kuijpers,W.Presber and G.S.DeHoog**(2000)Molecular and conventional taxanomy of the T.rubrum complex J.Clin.Miceobiol.38:3329-3336.

- **Grlic,L.**(1986).Enciklopedija Samoniklog Jestivog Bilja.August Cesaree,Zagreb,P.165.
- **Gupta A.K., J.Q. Del Rosso, C.W Lynde, G.H .Brown and N.H .Shear**(1998) Hepatitis associated with terbinafine therapy : three case reports and a review of the literature . Clin Exp Dermatol 23 p:64-67 .
- **Han,B.H. and Park,J.H.**(1989)Chemical and pharmacological studies on sedative cyclopeptide alkaloids in some Rhamnaceae plants .Pure Appl.Chem.61:443-448.
- **Harborne, J. B.**(1973), Phytochemical methods. Science paper Backs, Chapman and Hall, New York.
- **Harborne, J.B.** (Editor) (1984). Phytochemical Methods, 2nd Edition.Chapman and Hall, U.K., pp. 37-99.
- **Hasegawa A.**(2000)Dermatophytes in animals Nippon Ishinkin Gakkia Zahi.41:1-4.
- **[illegible],L.Huang, J.P.Bouchara,R.Barton,H.C.Li and T.C.Chang** (2005)Identification of [illegible] oligonucleotide array. J.Clin Microb[illegible]
- **Havesteen, B.**(1983).Flavonoids, a class of natural products of high pharmacological potency. Biochemical. Pharmacology. 32.7:1142-1148.
- **Heggers,J.**P.,A.,Kucukelebi,C.J.Stabenan,F.Broemelling AND Robenson,M.C.(1995).Wound healing effects of Aloe vera gel and other topical antibacterial agents on rat skin .J.Phytoth.Res.9:455-457.
- **Heggers,J.**P.,Pelley ,R.P. and Robenson,M.C.(1993).Beneficial effect of Aloe vera in wound healing .J.Phytother.Res.7:548-562.
- **Heywood,V.**H.,T.G.TutinN.A.Burges,D.M.More,D.H.Valentine,S.M. Walters,D.A.Webb.(1968)Flora Europeae.Cambridge university press.

- **Holder, I. A. and Boyce, S. T**.(1994). Agar well diffusion assay testing of bacterial susceptibility to various antimicrobials in concentrations non-toxic for humans in culture. Burns 20.5.p:426-429.
- **Hu C.Q. and Chen, Z**.(1997).Sesquiterpenoid alcohols from Chrysanthummorifolium.phytochemistry,44:1287-1290.
- **Hubbard, T.W**.(1999).The predictive value of symptoms in diagnosing childhood Tinea capitis. Arch. Pediatr. Adolesc. Med. 153:1150-1153.
- **Hube,B., Mondo, M., Schofield, D.A., Brown, A.J. and Goow, N.A**.(1994).Expression of 7 members of the gene family encoding secretory aspartyl proteinases in *Candida albicans* .Mol.Mol.Microbiol.,14:87-99.
- **Hudson, L. and Hay, F**.(1989) Practical Immunology .Blackwell well scientific publication p:116.
- **Irvin T.T**.(1984)Wound healing principles and practice chapman and Halls .New york.
- **Issa, A**. (1981)Nomes des plantes.2nd Ed. Bierut –Lebanon.
- **Jessup C.T. ,J.Warner,N.Isham *etal*.**,(2000)Antifungal susceptibility testing of dermatophytesnn:establishing a medium for inducing conidial growth and evaluation of susceptibility of clinical isolates .J.Clin.Microbiol.38:341-344.
- **Jousson, O.*etal*.**(2004) Multiplication of an ancestral gene encoding secreted fungalysin preceded species differentiation in the dermatophytes *Trichophyton and Micrpsporum*. Microbiology .150p: 3001-3010.

- **Kakuda, T., Nozawa, A., Unno, T., Okamura, N.and Okai, O**.;(2000) Inhibiting effects of theanine on caffeine stimulation evaluated by EEG in the rat) .Bioscience Biotechnology and Biochemistry 64: 287–293.
- **Kanbe T.,Y.suznki, A.kamiya,T.Mochizuki,M .fujihior and A.Kikuchi** (2003)PCR-based identification of common dermatophyte sp.(Primer sets specific for the DNA topoisomerase π genes.j.Dermatol.sci.32:151-161
- **Kano R., K.Okabayashi, y.Nakamura, S.ooka .kashima ,M. Mizoguchi ,S. Watanabe and A. Hasegawa**(2000)Differences among chitin synthase1 gene sequences in *T. rubrum* and T.violaceum.med.Mycol.38:47-50.
- **Kaufman ,G.;I.Berdicevsky ,JA.Woodfolk [illegible] and B.A.Horwitz**(2005)Markers for host-induced gene expression in [illegible] dermatophytosis 73:1[illegible]
- **[illegible] Kawasaki M. , Aoki, M[illegible]** K.(1996).Phylogeny of epidermophyton floccosum and other dermatophytes. Mycopathologia. 134:121-128.

- **Ke-yue L**.(2011)Technology optimization of polysaccharide extraction from *Cyclocarya paliurus* leaves by ultrasonic assistance .International conference on new technology of agricultural :1125-1128.
- **Khalifa K. A., E.Al-Kasser, J.K.AL-Diwan and F.H.AL-Lami**(2011)Prevalence of Tinea capitis among primary schoolchildren in Baghdad governarate ,Iraq.Iraqi J.Comm.Med.jan.(1):9-12.
- **Kubo I. , Ping X. and Kenichi F**.(2000) Antifungal activity of octyl gallate :structural criteria and mode of action bioorganic and medicinal chemistry letters .v.11:347-350.

- **Kurihara, H., F. Harukazu, K., Aki,T., Yoshiko, S.Hiroshi, M.Yokichi ,A.,Sumio, and T.Takaharu.**(2003).Hypoglycemic action of *Cyclocarya paliurus* (Batal)Ijinskaja in normal and diabetic mice.Biosci.Biotechnol.Biochem.67.4:877-880.
- **Kustark, D., Z. Males, A. Brantner, I.Pitarevic.(**1990).Flavonoids of the leaves of christi's thorn (*Paliurus spina-christi*). Acta pharmaceutica jugoslovica .40:551-554.
- **Laurence D.R., D.N., Bennett, and Brown. M.J.(**1997).Clinical pharmacology. 8th Ed. Churchill living Stone.
- **Leaw ,S .N., Chang, H. C. , Sun, H .F. , Barton , R., Bouchara , J .P. and Chang, C.** (2006).Identification of medically important yeast species by sequence analysis of the internal transcribed spacer regions .J.Clin.Microbiol.,44:693-699.
- **Lee, S.S., Su., W.C., Karin, C.S. and Liu, C.**(20[illegible]).Cyclopeptide alkaloids [illegible] stems [illegible] Phytochemistry 58:1271-1276.

- **Lilian C. B., Alexandre, M.B., Clayton, L.B., Maristela, P. and Célia., M.S**. (2007).cDNA representational difference analysis used in the identification of genes expressed by *Trichophyton rubrum* during contact with keratin, Microbes and Infection.9:1415-1421.
- **Lin H. y., C. H. Chen, B. J. You, K. C. Lin and S. S. Lee**(2008) Cyclopeptide alkaloids from *Paliurus ramossisimus*, J. Natural product, 63 p:1338-1343.
- **Lin H. y., Chen, C. H. , You, B. J. , Lin, K. C., Lee, S. S**.(2008). Cyclopeptide alkaloids from *Paliurus ramossisimus*, J. Natural product. 63 p:1338-1343.

- **Lin, H.Y., Chen, C.H. , You, B.J. , Liu, K.C., , Lee, S.S**.(2000).Cyclopeptide alkaloids from *Paliurus ramosissimus*. J. N.at. Prod.63:1338-1343.
- **Lin, R.L., Szepietowski, J.G., Schwartz, R. A**.(2004). Tinea faciei an often deceptive facial eruption.Int. J. Dermatol. 43.
- **Liu D, Coloe S. ;Baird R. and Pedersen J.**(2000)Application of PCR to the identification of dermatophyte fungi .J Med Microbiol.49:493-497.
- **Mackie,R.M**.(1983)Clinical dermatology an illustrated text book.Oxford university press.London
- **Makimura K, Mochizuki T, Hasagawa A, Uchida K and Yamaguchi H**.(1998)Phylogenetic classification of Trichophyton mentagrophytes complex strains based on DNA sequences of nuclear ribosomal internal transcribed spacer 1 regions. J Clin Microbiol.36:2629-2633.

gawa,K.Uchida,and H.Yamaguchi.(2001)Cluster analysis of human and animal pathogenic *Microsporum* species and their teleomorphic states ,Arthroderma species ,based on the DNA sequences of nuclear ribosomal internal transcribed spacer 1 regions.J.Clin.Microbiol. Immunol.45:209-216.

- **Males, Z**.(1995).Structural characterization of the isolated flavonoids of christi's thorn (*Paliurus spina-christi*). Thesis. Faculty of pharmacy and biochemistry.University of Zegreb.
- **Marchand ,J., Pais, M.,Monseur,X. and Jarreau,F.X**.(1969)Peptide alkaloids .VII. Lasiodine A and B,alkaloids from Lasiodiscus marmoratus. Tetrahedron 25,937-954.

- **Masoko, P**.(2007).Characterization of antifungal compounds isolated from combretumand Terminalia species. Ph.D. Thesis in paraclinical science. Faculty of Vet. Sci. University of Pretoria.
- **Mescher, A.L.(**2010).Skin.ch8.Junquira'sbasic histology.text and atlas .12th Ed.. McGraw Hill.Eds..U.S.A..
- **Moaz M. and Neeman I**.(1998)Antimicrobial effects of aqueous plant extracts on the fungi *M.canis* and *Trichophyton rubrum* on three bacterial species .Lett.Appl.Microbial.,26:61-63.
- **Mochizuki, T., Sugie, N. and Vehara, M**.(1997).Random amplification of polymorphic DNA is useful for the differentiation of several anthropophilic dermatophytes. Mycoses ,40:405-409.
- **Moerman,D.E**.(1996)Analysis of the food plants and drug plants of
- **Mohammed K. Y**.(2004)Topical
- **Mondello F. ,De Bernardis F. , Girolamo A *et al.*,**(2003) *In vitro* and *in vivo* activity of tea tree oil against azole-susceptible and -resistant human pathogenis yeasts, J Antimicrob Chemother ,51:1223-9.
- **Mosaddegh M. , M. J. Khoshnood, M.Kamalinejad and E. Alizadeh** (2004) Study on the effect of *Paliurus spina-christi* on Cholesterol, Triglyceride and HDL Levels in Diabetic Male Rats Fed a High Cholesterol Diet, Iranian Journal of Pharmaceutical Research 3:51-54.

- **Muchuweti M.,Zenda G.,Ashwell R.and Abisha K**.(2005)Sugars ,organic acid and phenolic components of *ziziphus mauritiana* fruit .Eur food res technol.221:570-574.
- **Mustafa, P.L**.(2009)Epedimeological and diagnostic study of dermatophytes in Kirkuke city and the effect of onion extract on isolated fungi.College of science univ. of Tikrit, MSc. In Biological science.

Mycology , by McGinnis, M. R. (ed). Vol. 1 , Springer verlage .

- **Natarajan V.,P.Venugopal and T.Menon**(2003)Effect of azadirachta indica(neem)on the growth pattern of dermatophytes .Indian J.Med.Micro.21:98-100.
- **National committee for clinical laboratory standards** (NCCLS)(2000)Methods for dilution antimicrobial usceptibility tests for bacteria that grow aerobically .Approved standard 5th ed NCCL document M7-A5.
- **Nejad**[illegible] **and Deokule** [illegible] S.(200[illegible] drynaria quercifolia L..Jundishapur.J.[illegible]
- **Nwachukwu, E.O.,and Umechuruba,V**.(2006).Antifungal activities of some leaf extracts on seed-borne fungi of African yam bean seeds.seed germination and seedling emergenc.
- **Omer, S.A., and Abd-Elhalim,V**.(1992).Fungal growth response to alfalfa (Medicago sativa L.) saponin in extract .Egyptian J. Appl.Sci.,7:24-32.
- **Paundir R.K. and Jain P**.(2010)Antifungal activity of twenty ethanolic plant extracts against food associated fungi .J of pharmacy research.3(3).
- **Peacock, E**.(1984).Wound repair.3rd Ed..W.B.Saunders Co.. ,Philadeliphia. USA., :38-55.

- **Pina-Vaz C., A.Gon, Rodrigues and E.Pinto *et al*.** (2004)Antifungal activity of thymus oils and their major compounds, J Eur Acad Dermatol Venereol, 18: 73-8.
- **Polunin, O. and Huxley A.**(1981).Blumen am Mittelmer verlagsgesells chaft.Munchen.Wien and Zurich:157.
- **Ramar P. S. and P. Gopalakrishnakone** (2010)Therepeutic Potential of Plants as Anti-microbials for Drug Discovery. http://ecam.oxfordjournals.org by on October 19.
- **Rawat R. B. and R. C. Uniyal**(2003) National Medicinal plants Board committed for overall development of the sector. Agro Bios, Med. Plants, 1: 12-6.
- **Rhods J.C., B.G. Oliver, D.S. Askew and T.W.Amlny**(2001)Identification of genes of A.fumigatous up regulated during growth on endothelial cells .Med Mycol.39:253-260.
- **Ripp[illegible]** (1985) The changing ep[illegible] Curre[illegible] actinomycetes. In : Textbook of Microbiology , by Inc., New York, p: 208 – 229.

- **Roitt's I. M. and J.P. Delves**(2001)Essential Immunology .10th Ed.,by Blackwell science Ltd.USA.
- **Rossmando E.(1987)**Applications to the assay of enzymatic activity ch. In HPLC in enzymatic analysis.Eds Jhon Willey and sons .A wiley inter science publications.
- **Rushforth K. D.** (1999)Trees of Britain and Europe, ISBN.
- **Saadabi. A.**(2006) Antifungal activity of some Saudi plants used in traditional medicine. Asian J. Plant Sci., 5. p :907-909.

- **Samy, R.P., and Gopalakrishnakone, P.** (2010).Therepeutic Potential of Plants as Anti-microbials for Drug Discovery . http://ecam.oxfordjournals.org by on October 19.
- **SAS**. (2004). SAS. Statistical Analysis System, User's Guide. Statistical. Version 7th ed. SAS. Inst. Inc. Cary. N.C. USA.
- **Sharma K. K., R. Saikia, J. Kotoky, J. C. Kalita and R. Dive**(2011) Antifungal activity of *solanum melongena* L. *Lawsonia inermis* L. and *Justicia gendarussa* B. against Dermatophytes pharmatech.3.3. p :1635-1640.
- **Shelf ,L.A., Jyothi,V and Bulgarelli,V**.(1984).Effect of sage on growth of enteropathogenic and spoilage bacteria in sage-containing broths and foods. J. Food Sci.,49:737-740.
- **Shimizn K., Ozeki,M;Lino,A, Nakajyo,S.,Urakawa,N. and Atsuchi,M**.(2001)Structure-activity relation ships of triterpenoid derivatives extracted from gymnema inodorum leaves on glucose absorption.Jpn.J.pharmacolog,86,p:223-229.
- **Shmitt, M.E.,Brown,T.A.,Rumpower,B.L.(**1990).Arapid and simple method for preparation of RNA from Saccharomyces cervisiae.Nucl.Acid Res.,18.10:3091-3092.
- **Skoog A.D. and D.M.West**(1969)Fundementals of analytical chemistry .Holt ,Rinehart and Winston(Ed.),2nd Ed.U.S.A.
- Skoog,**A.D.,D.West, D.,James Haller, F.,Crouch, S.P**.(2004). Fundementals of Analyical chemistry

- **Smolenst, J. J., Silinis, H. and Faranssnoth, N. R.** (1972). Alkaloid screening coyadia, 35:11-34.
- **Soylu,E.M.,Toki,F.M.,Soylu,S. and Kaya,A.D.(**2005).Antifungal activities of essential oils on post harvest disease agent *Pencillium digitatum*.Pak.J.Biol.Sci.8:25-29.
- **Stahle, E**(1969). Thin layer chromatography a laboratory hand book, 2nd ed. , translated by Ashworth, M. Spring-verag,
- **Suguna L., Sivakuman, P. and Chandrakasan,V**.(1996).Effects of centella asiatica extract on dermal wound healing in Rats.Ind.J.Exp.Biol.,34:1208-1211.
- **Terashima, T.,Takido, J. and Yokogoshi, H.** (1999) Time-dependent changes of amino acids in the serum, liver, brain and urine of rats administered with theanine. Bioscience, Biotechnology and Biochemistry 63: 615–618.
- **Tsang P., T., Hopkins,V.,Jimenez-Lucho**(1996) Deep [illegible] dermatophytosis caused by T.rubrum in a patient with AIDS.V.34.NO.6:1090-1091.
- **Ts[illegible] W., Z. Y. C. Blomquist a[illegible]** [illegible] variations among common air[illegible] specific oligonucleotide probes for the detection of fungal isolates .Appl .Environ. Microbiol.69:5389-5397.
- **Udupa S.L.,A.L. Udupa and D.R. Kalkarni**(1991)Influence of Tridax procumbens on lysyl oxidase activity and wound healing .Planta Med.57:325-327.
- **Ulubelen,A.,G.Topcu,N.Tan,S.Olcal,C.Johansson, M.Ucer,H.Birman and S.Tamer**(1995).Biological activities of a Turkish medicinal plant prangos platycchlaena.J. of Ethnopharmacology.45:193-197.
- **Velcheva M.P**.(1993).Constituents of *Paliurus spina christi*.Fitoterapia.,46:284-285.
- **Venugopal, P.V. and Venugopal, T.V**.(1995)Antidermatophytic activity of garlic (*Allium sativum*) invitro.Int.J.Dermatol.34:278-289.
- **Vlietinck,A.J., L.Van Hoof, J.Totte,A.Laure, D.Vanden,P.Berghe and C.Rwangabo**(1995)screening of hundred Rwandese medicinal

plants for antimicrobial and antiviral properties .J.Ethnopharmacol.46:1-72.

- **Washington W.Jr, Allen, S.,Janda W., Koneman, E., Procop, G., Schreckenberger, P. and Woods, G**. (2006).Color Atlas and text book of diagnostic microbiology ,Lippincott Williams and wilkins(Ed.),6th Ed.Baltimore.U.S.A.
- **Weitzman I. and M.Crist**(1980)Studies with clinical isolates of cunninghamella.11.Physiological and morphological studies .Mycologia 72:661-669.
- **Weitzman I., M.R.McGinnis, A.A.Padhye, and L.Ajello**(1986)The genus arthroderma and its later synonym Nannizzia.Mycotaxon.25:505-509.
- **Weitzman, I., and [illegible] R.** (1996).The dermatophytes.Clin.Microbiol.Rev.,8:240-259.
- **Xie,J.H.,Ming-Young,X.,Ming-Yue,S.,Shao-Pin,N.,Chang, L. and Yuan-Xing,W**.(2010).Optimization of microwave –assisted extraction of polysaccharides from Cyclocarya paliurus (Batal)lljinskaja using response surface methodology.J.Sci.Food.Agric.90:1353-1360.
- **Yoshida, M., Fuchigami, M., Nagao, T., Okabe,H.etal.,**(2005). Antiproliferative constituents from Umbellieferae plants VII.Active triterpenes and rosmarinic acid from centella aciatica.Biol.Pharmacol.Bull.28:173-175.

- **Yunis,H**.(2007).The effect of Garlic extract on dermatophytes growth and the keratinized activity of the fungus .MSc.thesis .college of medicine .univ. of Tikrit. (in Arabic)
- **Zaug,C., Monod, M., Weber, J., Harshman, K., Pradervand, S., Thoma, J.etal**.(2009).Gene expression profiling in the human pathogenic dermatophyte T. rubrum during growth on proteins.Eucaryot cell.8:241-250.
- **Zhang, W., Yu, L., Yang, J. , Wang, L. , Peng, J. & Jin, Q.** (2009). Transcriptional profiles of response to terbinafine in *Trichophyton rubrum*. Appl Microbiol Biotechnol 82: 1123-1130.

Conclusions:

Results obtained in this study are established

The ethanolic extract contain numbers of biologically active compounds detected by preliminary test these compounds are (flavonoids ,phenols ,alkaloids,tannines,saponines).

2. The HPLC analysis of alcoholic extract revealed the presence of the following phenolic compounds (Gallic acid ,syringic acid ,caffeic acid and epichotechin).
3. Gas –Mass chromatograph analysis of alcoholic extract revealed the presence of numbers of biologically active compounds such as(sugars, amino acids, fatty acids ,phenols).
4. Age groups (20-30)years old shown the highest frequency among the patients group.
5. Maximum growth inhibition was statistically significant at 125µg/ml for both methanolic and ethanolic extract as compared to control group.
6. Stimulates the regeneration of the lesion ,skin hair , contraction and epithelialization.
7. Fibroblasts were more prominent in the developing granulation tissues of incision wound treated with PME at early stages of wound days 7-14 and less prominent in the developing granulation tissues at late stage (day 14), in

comparison to -ve control, reference drug, and vehicle groups , indicating faster healing process.

3. The applications of nucleic acid amplification technology to make a rapid precise identification of dermatophytes such as (restriction fragment length polymorphism (RFLP),random amplification of polymorphic DNA(RAPD),PCR finger printing .

الإهداء

إلى والدي المرحوم والمغفور له الذي

رباني وعلمني

إلى المستقبل الذي طالما حلمت به

لمى

Acknowledgement

I am extremely thankful to **Prof. KhuloodW.AL-Sammarae**, my supervisor, who always found time to assist me, and encouraged me to do the best, making continuous and fruitful discussion and directing me to the proper way.

My deepest and special thanks to my co-supervisor **Prof. Nabeel Khalaf AL-Ani**, for his valuable advice and help during this study.

Thanks are extended to the members of the committee for their helpful criticism. Also, my gratitude is to **Prof. Salim R.**, from whom I learned so much, for spending time in reading the thesis and being member of the committee.

I owe my special thanks to Assistant prof. Dr. Basem Jaber ,faculty of science ,university of Jordan for supporting my work in the department and for always having an open door and time for discussion.

I would like to express my appreciation to prof. Dr. Ahmed Al-Kaisi ,faculty of Science ,University of Jordan for his support .

My sincere regards to all instructors in Dhafar , who allowing me to finish my work in the college, microbiology laboratory.

My great thanks are extended to Mr. Muneer Khadhoor , Electron Microscope unit ,Al-Yarmook university, Jordan. for allowing me to photograph the studied slides in his lab . My sincere gratitude and deepest appreciation is to my greatest loving family, particularly my father(Mercy on him) ,and mum who gave me full encouragement and support. Without their prayers and guidance this project would not have been completed. Thanks to my sisters whom always I find them when I need. Finally, my appreciations also extend to Mrs.Mayada and Mrs,Dhafar , for their love, helping and sharing me in beautiful moments as well as suffering ones. Also, I would like to thank my friends and colleagues Heba Jarar, Amani Harb .

1.1 Introduction

The use of systemic drugs is limited to treat man or animal due to their high toxicity and problems of residues in products intended for human consumption (Araujo *et al*,2009).Different treatments have been recommended to control dermatophytes in general pharmacological treatment option include antifungal agents(Aly,1997;Agwa *et al*,2000).Recently the use of some natural plant products has been emerged to inhibit the causative organisms .

The antimicrobial and antitoxin properties of some plants ,herbs and their components have been documented since the late 19th century (Saadabi, 200 6).These natural plants involve garlic ,lemon grass, datura, acacia. A triplex ginger, black seed, neem, basil, eucalyptus and alfalfa(Omer and Abd-El-Hal- im,1992; Aly *et al*,2000 and Aly and Dafedar, 2008).They are safe to human and the ecosystem than the chemical fungicides for ailments and [illegible] used them for thousands of years to [illegible] foods as well as its economic value (Shelf *et al* ,1980; Shelf *et al* , 1984). Early cultures also recognized the value of these plant materials in medicine .Plant extract has been used traditionally to treat a number of infectious dise- ases including those caused by bacteria ,fungi, protozoa and viruses (Soylu *et a*l,2005;Yoshida *et al*,2005; Nejad and Deokule,2009).

The activity of plant extract against dermatophytosis i.e. the superficial infections of skin or keratinized tissue of man and animals can be very well visualized from the reports of Venugopal and Venugopal (1995). They reported the activity of plant extracts against 88 clinical isolates of dermatophytes which includes *Microsporum canis, Microsporum audouinii ,Trichophyton rubrum , Trichophyton*

mentagrophytes, Trichophyton violaceum, Trichophyton simii, Trichophyton verrucosum, Trichophyton erinacci ,Epedermophyton flocosum by agar dilution technique. While (Vlietinck *et al*,1995)reported clinical findings of Rawandese medicinal plants (267)plant extracts used by traditional healers to treat microbial infections and found 60% of these extracts were active against dermatophytes species.

Recently more than 200 different biologically active substances have been isolated from plant extract ,among them organosulphur compounds such as all- icin ,azoenes and diallyl trisulfide .Eugenol, phenolic compounds the most imp- ortant biologically active compound found in many plant extract (Aly and Bafiel,2008).

efficient non-toxic antifungal s and increasing number of treatment failures have motivated current searches for therapeutic alternatives to include the testing of medicinal plant and essential oils as potential antimicrobial agents (Mondello *et al*,2003; Pina *et al*,2004).

People used many plants and plant preparations to cure various skin infections and other disease .Though the recovery is slow ,the therapeutic use of medicinal plant is becoming popular because of less side effects. Unlike the synthetic drugs medicinal plants have the ability to control antibiotic resistant micro organism (Rawat *et al*,2003). Dermatophytoses are one of the most frequent skin diseases of human

pets and livestock .It's widely distributed all over the world with various degrees and more common in man than in women(Brook *et al*,1998).

Prangos platychlaena is used as a folk medicine in eastern Turkey to treat under tail wounds of cattle and to stop bleeding in man by application on wounds. The antifungal activity of 30 aqueous medicinal plant extract which distributed in Jordanian environment ,especially *Mentha viridis* and *Rosmarinus officinalis* aquous extract were studied.In Malaysia the antifungal activity of Gignoniaceae family was studied and it revealed that the extracts which obtained by dichloromethane solvent of the following plant species *Tacaranda filiciefolia, Tabeduia chrysantha,Oroxylum indicum and Fernadoa adenophyla* are most effective against dermatophytes (AL-Suhaili,2001).

Aloe vera 5%gel applied topically 3 times daily to the patient ... area of glabrous skin ... cure rates were noted from the first week of treatment. Moham... *officinale* and *Pimpella anisum* were examined against bacteria as well as two molds *Trichophyton mentagrophytes* and *Microsporum gypseum* and one yeast species *Candida albicans* and they revealed a good results especially *Thymus vulgaris* oil secondly the oil of Eucalyptus and zengiber becoming in 3rd degree while *mentha piperita* and *Pimpella anisum* oil had no inactivation effect(Al-Jubori,2005) .

The alcoholic extract of *Allium cepa* high antifungal activity rate against dermatophytes species *Trichophyton mentagrophytes* (Mohammed ,2009). *Paliurus spina-christi* belonging to Rhamnaceae family commonly known as Jersalem thorn ,Garland thorn or crown of thorns, (Rushforth,1999),Its used as diuretics, against diarrhea and

rheumatism in traditional Croatian herbal med- icine (Grlic,1986)Antibacterial activity mainly on G+ve bacteria (Brantner *et a*l,1996).

Paliurus spina-christi rich source of flavonoids isoquercitin ,rutin ,hyperoside (Dalakishvili *et al*,1986; Kustark *et al* ,1990).A quercetin-3-O-triglycoside (Brantner and Males,1990).This plant found in the north of Iraq ,Al-Sulaimanya governorate ,Rania village ,kaladizah ,Beramagron mountains it doesn't studied in Iraqi universities or by any researchers. For above reasons its interesting to study the chemical and biological properties of this plant.

The aims of the study were proposed for:

1. Isolation of dermatophytes species from patient infected with
2. In vitro and in vivo (histopathological examination)study of the antidermatophyte activity of the alcoholic *Paliurus spina-christi* extract .using light and electron microscope.
3. Molecular assessment of Tri m4 gene.

1.2-Literature Review:

1.2.1 History of medicinal plants

Historically, therapeutic results have been mixed, quite often cures or symptom relief resulted. Poisonings occurred at a high rate also. Clinical microbiologists have two reasons to be interested in the topic of antimicrobial plant extracts.

First its very likely that these phytochemicals will find their way into the arsenal of antimicrobial drugs prescribed by physicians ,several are already being tested in humans ,its reported that on average ,two or three antibiotics derived from microorganisms are lunched each year (Clark,1996).Plants play an important role in the medicinal practices of many ,if not all peoples .Thus plants are used not only in the diagnostic process ,but also in the physical and ritual purification procedures that commonly precede ceremonies and in the act of healing itself (Moerman,1996). After a downturn in the use in recent decades, the pace is a gain quickening as scientists seek to the effective life span of any [illegible]

Second, the public is becoming increasingly aware of problems with the prescription and misuse of traditional antibiotics .In addition ,many people are interested in having more autonomy over their medicinal care . Although several synthetic antimycotic drugs are available in the market, at present the use of these drugs has minimized because of a number of factors which includes low potency, poor solubility, development of resistant strains, drug toxicity and side effects, like gastrointestinal disturbances, cutaneous reaction, hepatotoxicity, leucopenia …etc (Brownlee *et al,*1990). The poor availability of efficient non toxic antifungals and increasing number of treatment failures have motivated current searches for therapeutic alternatives to include the testing of

Medicinal Plant and essential oils as potential antimicrobial agents (Butler,1988; Cardellina *et al*,1993).

The uses of traditional medicinal plants for primary health care have steadily increased worldwide in recent years. The goals of using plants as sources of therapeutic agents are

a) To isolate bioactive compounds for direct use as drugs, e.g., digoxin, digitoxin, morphine, vinblastine, vincristine.

b) To produce bioactive compounds of novel or known structures as lead compounds for semisynthesis to produce patentable entities of higher activity and/or lower toxicity, e.g., metformin,nabilone, oxycodon (and other narcotic analgesics), taxotere, teniposide,verapamil, and amiodarone, which

taxol, podophyllotoxin,khellin, and khellin.

c) To use agents as pharmacologic tools, e.g., lysergic acid diethylamide, [illegible]

echinacea, feverfew, garlic, ginkgo biloba, St. John's wort, saw palmetto.

Scientists are in search to discover a new bioactive compounds Scientists are in search of new phytochemicals that could be developed as useful anti-microbials for treatment of infectious diseases. Currently, out of 80% of pharmaceuticals derived from plants, very few are now being used as anti-microbials. Plants are rich in a wide variety of secondary metabolites that have found anti-microbial properties (Samy and Gopalakrishnakone,2010).

1.2.2 Scientific Classification of plant used in this study:-

Kingdome :Plantae

Division :magnoliopsida

subclass:Rosidae

Order:Roales

Family:Rhamnaceae

Genus and species: Paliurus spina-christi

Trees or shrubs .Leaves simple ,usually stipulate .Inflorescence ... Flowers perigynous .Calyx 4- to 5-lobed, lobes valvate in bud ... Petals 4-5, often small and sometimes absent ... hooded over the stamens ... with the calyx-lobes; anthers versatile. ovary superior, 2 to 4-locular ;ovules solitary .Fruit often fleshy(Heywood *et al*,1968) .

Rhamnaceae, the Buckthorn family, is a large family of flowering plants, mostly trees, shrubs and some vines. The family contains 50-60 genera and approximately 870-900 species. The Rhamnaceae have a worldwide distribution, but are more common in the subtropical and tropical regions. The earliest fossil evidence of Rhamnaceae is from the Eocene.

The simple leaves can be either alternate and spiraling, or opposite. Stipules are present. These leaves are modified into spines in many genera, in some (e.g. *Paliurus spina-christi* and *Colletia* cruciata)

spectacularly so. Colletia stands out by having two *axillary* buds instead of one, one developing into a thorn, the other one into a shoot(Rushforth,1999).

1.2.3Description of *Paliurus spina-christi*

Paliurus spina-christi Miller. Nearly glabrous ,much –branched shrub up to 3cm.Twigs flexuous ,puberulent when young .Leaves 2-4 cm, alternate and distichous ,ovate crenate –serrate ,shortly petiolate. Flowers in small, axillary,hortly peduculate cymes. Fruit 18-30 mm in diameter; wing undulate. Dry slopes ;also often used for hedges. Miditerranean region. Balkan peninsula and black sea coast(Heywood *et al*.1968)

It is a deciduous shrub or small tree growing to 3-4 m tall. The shoots are zig-zagged, with a leaf and two stipule spines (one straight, one curved) on the outside of each kink. The leaves are oval, 2-5 cm long and 1-4 cm broad, glossy green, with an entire margin. The fruit is a dry woody nutlet centered in a circular wing 2-3.5 cm diameter, The name reflects an old legend that the spiny branches were used to make the crown of thorns placed on Christ's head before his crucifixion (Rushforth,1999).

1.2.4 Distribution of *Paliurus spina-christi*

Paliurus spina-christi. is a traditional Mediterranean and Asiatic medicinal plant (Polunin and Huxley,1981). widely distributed in dry and rocky places in the Mediterranean region and Asia (Mosaddegh *et al*, 2004)

Cyclocarya paliurus (Batal.) Iljinskaja (*C. paliurus*) is a Juglandaceae plant, an endemic tree growing on cloudy and foggy highlands in the south of China.(Kurihara *et al*,2003) .*Cyclocarya paliurus* (Batal.) Iljinskaja, grown on cloudy and foggy highlands in southern China, is a well-known edible and medicinal plant and is commonly called the 'sweet tea tree' because of the flavour of its leaves (Liu *et al*, 2009).

1.2.5 Chemical constituents

Chemical investigation of *Paliurus spina-christi* fruits and leaves indicated the presence of the flavonoides isoquercitin,rutin, hyperoside (Dalakishvili *et al*, 1986, Kustark *et al*,1990) .Sterols (Dalakishvili *et al*,1985) .In addition, the fruits contain alkaloids (Velcheva,1993). Generally plant parts contain flavonoids and tannins (Males,1995).

1.2.6 Biological potentials

Paliurus spina-christi commonly used as a diuretic and against diarrhea and rheumatism (Grlic, 1986). A remarkable antibacterial activity, mainly on Gram-positive bacteria, of the ethanolic extracts from different plant parts was found ,*Paliurus spina-christi* demonstrate a clear inhibition zone against gram positive and negative bacteria (*Staph*

aureus, Streptococcus faecalis, Micrococcus luteus, Shigella sonnei, Proteus mirabilis, E.coli) (Brantner *et al*., 1996) .Also used against rheumatism in ethnomedicine (Wichtl,1997). *Paliurus spina-christi* fruit could be a good candidate for clinical investigation towards the treatment of hyperlipidemia. This can explain the reason for its use in traditional medicine as a lipid-lowering plant. (Mosaddegh *et al*,2004) The leaves of *Cyclocarya paliurus* have been a food source for maritime people for a long time, and are known to have beneficial effects on health and used as a traditional remedy for ailments, the enhancement of mental efficiency, and recovery from mental fatigue (Kurihara *et al*,2003).

1.2.7 High performance liquid Chromatography

The newest addition to the chromatographic family of analytical procedures, most commonly known as HPLC. This form of chromatography resembles column chromatography except that the process involves pumping liquid eluant under pressure instead of depending on gravity for the eluant to pass through a number of fractions to detect separated products ,The experimenter runs the eluant through a detector ,most commonly an ultraviolet detector or a refractive index detector. The detector is connected to a chart recorder that gives a record of U.V. absorbance or refractive index versus ml of eluant –a chromatogram .The U.V. detector can detect as little as 10^{-10} g of solute ,while the refractive index detector can detect 10^{-6} g of solute .

1.2.7.1 Types of High performance liquid chromatography

- Exclusion chromatography
- Ion exchange chromatography
- adsorption chromatography

- ❖ Partition chromatography
- ❖ Reversed phase chromatography

In general each type of separation uses a different kind of packing material, and since each type of separation exploits a different property of the molecules, the choice of packing really comes down to which property of the molecules would be most useful in achieving the separation (Rossomando,1987)

1.2.8Gas chromatography

Gas chromatography (GC) ,also called vapor phase chromatography is a means of separating volatile mixture ,the components of which may

Williamson,1983). The Gas chromatograph was introduced by James and Martin in 1952(Skoog *et al*,2004). The GC process is similar to fractional distillation and used a glass column or a coiled stainless steel sponge the GC column used which is a3-10m long coiled metal tube (diameter 6 mm)packed with ground firebrick .

The firebrick serves as an inert support for a very high boiling liquid is essentially non volatile such as silicon oil and low molecular weight polymers like carbo wax these are the liquids of gas liquid chromatography and are referred to us the stationary phase the sample (1-52 µl) which is injected through silicon rubber septum into the column ,which is being swept with a current of helium(ca,200ml/min). Gas chromatography determines the number of components and their relative amounts in a very small sample. The small sample size is an advantage in many cases, but it precludes isolating the separated components .Some specialized chromatographs can separate samples as large as 0.5 ml per injection and automatically collect each fraction in a separate container .

At the other extreme gas chromatographs equipped with flame ionization detectors can detect micrograms of sample and are used to analyze for traces of drugs in blood and urine .Clearly a gas chromatograph gives little information about the chemical nature of the sample being detected. However ,its sometimes possible to collect enough sample at the exit port of the chromatograph to obtain an infrared spectrum .The sample ,if its not too volatile ,will condense in the gold glass tube. Subsequently the sample is washed out with a drop or two of solvent and an infrared spectrum obtained (Fieser and Williamson, 1983**).**

Gas chromatography (G.C.) is one of the most widely used techniques for qualitative and quantitative analysis. In(G.C.), the components of a vaporized sample are separated as a consequence of being partitioned between a mobile gaseous phase and a liquid or a solid stationary phase held in a column. An important fact of the G.C is the use of a carrier gas such as hydrogen or helium, to transfer the sample from the injector through the column and [illegible] to most other types of chromatography, the mobile phase does not interact with molecules of the analyte; its only function is to transport the analyte through the column.

1.2.8.1Two types of gas chromatography are encountered

1.2.8.1.1 Gas-liquid chromatography (GLC) and

1.2.8.1.2 Gas-solid chromatography (GSC)

Gas-liquid chromatography finds widespread use in all fields of science; in 1955, the first commercial apparatus for gas-liquid. Chromatography appeared on the market. Since that time, the growth in applications of this technique has been phenomenal. Currently, several

hundred thousand gas chromatographs are in use throughout the world (Skoog *et al*,2004).

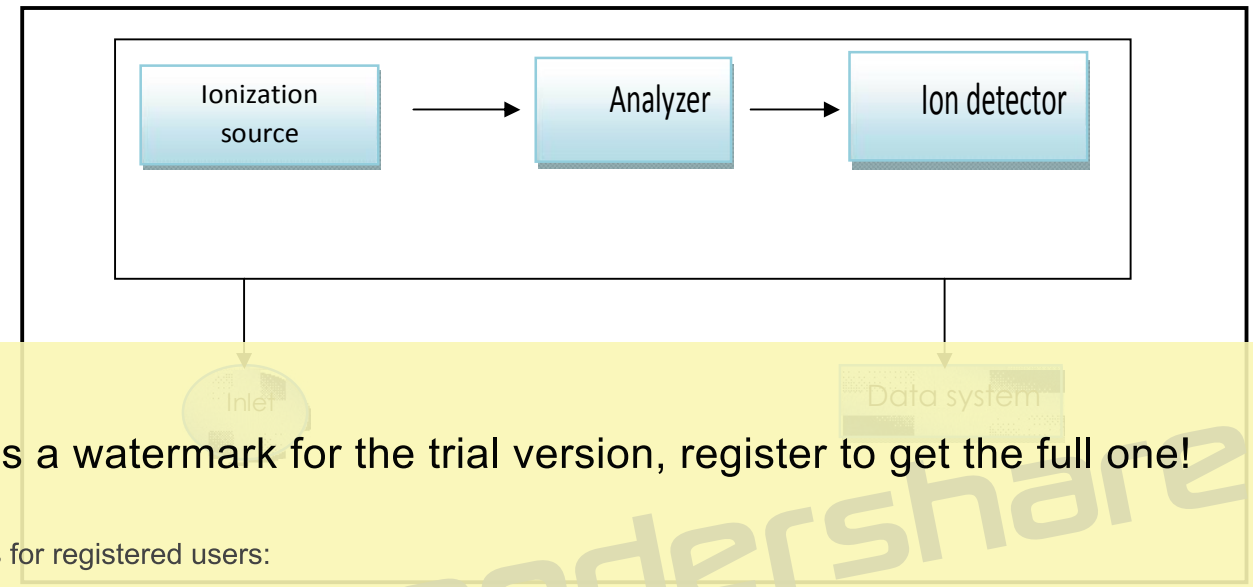
1.2.9 Mass Spectrometry

One of the most powerful detectors for gas chromatography is the mass spectrometer. The combination of gas chromatography and mass spectrometry is known as GC/MS. A mass spectrometer measures the mass-to charge ratio (m/z) of ions that have been produced from the sample. Most of the ions produced are singly charged (z = 1), so that mass spectrometer is often speak of measuring the mass of ions when mass-to-charge ratio is actually measured. A block diagram of a typical molecular-mass spectrometer is shown in Figure(1-1).

Sample molecules enter the mass spectrometer through an inlet system. In the case of GC, the sample is in the form of a vapor, and the inlet must interface between the atmospheric pressure GC system and the low-pressure (10-4 to 10-6 torr) mass spectrometer. In the ion source of the mass spectrometer, sample molecules enter an ionization source, which ionizes the sample. The ionization sources for molecular mass spectrometry are energetic enough to break chemical bonds in the sample molecules but not so energetic as to decompose the sample molecules into their constituent atoms, as is done in atomic mass spectrometry. The ionization sources in GC/MS produce fragments, which can also be ionized. Hence, leaving the ion source are ions of the sample molecules, called molecular ions, fragment ions, and un-ionized molecules. The uncharged molecules and fragments are normally

pumped out of the ion source by the vacuum pumps used to produce the low pressure environment. The next part of the mass spectrometer is an analyzer stage. The analyzer serves to sort the ions according to their

m/Z; values, just as in atomic mass spectrometry. The separated ions are then detected, and a plot of the ion intensity versus mi; value is produced by the data system (Skoog, 2004).



ions strike an ion detector, where they produce an electrical signal that is recorded and plotted by the data system.

1.2.10 Dermatophyte infections

Defenition. Cutaneous lesions due to dermatophytes ,presenting most commonly as athlete's foot(tinea mannum) ,nail infections(tinea unguium) ,body infection (tinea corporis) ,and scalp ringworm(tinea capitis). These pathogenic fungi inhabit the keratinized tissue of skin ,hair and nails, and generally do not invade living tissue ,in active

infections they are seen as branched hyphae .They grow down the pilosebaceous follicles towards the hair bulb ,but are arrested before reaching the bulb itself and fan out to form multiple hyphal fronds known as Adamson's fringe(Mackie,1983).

Dermatomycoses due to *Trichophyton mentagrophytes Trichophyton.rubrum ,Microsporum gypseum* and *Epidermophyton floccosum* occur commonly world wide and these are one of the most common skin infections in north east region of India. Unlike other superficial fungal infections ,the incidence of dermatophytoses ,commonly called ring worm or tinea , (Weitzman and Summerbell,1995; Jessup *et al*,2000)

The prevalence of different types of superficial fungal infections of the skin is variable among different age group ,gender ,socio-economic levels and countries . In Iraq ,tinea pedis infection age group 15-45 years showed the highest frequency ,male :female ratio was 1.37:1(Habbu and industrial workers formed the largest group (42.4%) of the patients and the rate of ability was seems USA is 8.1%and in general it is more prevalent in male's children (Al-Kayali *et al*,2011).

Three genera of dermatophyte fungi cause tinea infections (tinea worm):

- Trichophyton (skin, hair and nail infections)
- Microsporum (skin and hair)
- Epidermophyton (skin and nails)

Dermatophytes invade keratin only, and the inflammation they cause is due to metabolic products of the fungus or to delayed hypersensitivity. In general ,zoophilic fungi (those transmitted to humen by animals)are responsible for the majority of the cases (Hasegawa,2000).

1.2.10.1 Tinea types

1.10.1.1 Tinea pedis (athlete's foot). This is the commonest type of fungal infection in man (Hunter *et al*,1995). Ring worm of the feet it is the most prevalent of all dermatophytoses .It occurs as a chronic infection of the toe webs. other varieties are the vesicular ,ulcerative and moccasin types ,with hyper keratosis of the sole ,initially there is itching between the toe webs becomes chronic ,peeling and cracking of the skin are the principle manifestations ,accompanied by pain and pruritus (Brook *et al*,1998).

Infections are most common during the warm ,humid months, *T.mentagrophytes,T.rubrum and E.floccosum* are the dermatophyte species most commonly recovered .The increased amount of keratin on the soles of the feet and the palms of the hands makes these two sites selectively vulnerable to infection with *T.mentagrophytes* and other dermatophytes (Washington *et al*,2006).

1.2.10.1.2Tinea cruris : When the infection occurs in the groin area, Its called tinea cruris, or jock itch. frequ[illegible] [illegible] infection [illegible] m[illegible] *rubrum,T.mentagrophytes,E.floccosum* (Al-Ani,2006).The theory is that the feet get infected first from contact with the ground. The fungus spores are carried to the groin from scratching (Dermatophytes , 2008).

1.2.10.1.3Tinea Corporis: Typically appears as single or multiple annular ,scaly lesions with clearing ,a slightly elevated ,reddened edge and sharp margin on the trunk, extremities or face(Hubbard,1999). *Tinea corporis* was a major problem for U.S. troops during the Vietnam conflict, particularly those assigned to combat units exposed to wet terrain. In the United States, the most common organism recovered is *T. rubrum*; however, during the Vietnam conflict, the most common isolated species recovered from

lesions of tinea corporis and tinea cruris was a zoophilic strain of *T. mentagrophytes* that accounted for 73% of fungal infections in combat servicemen (Mondello *et al*,2003) .

1.2.10.1.4 Tinea capitis: Hair infectied with one of the following fungi *Microsporum ferrugineum, Microsporum audouinii and cause tinea capitis* . *Microsporum canis* var. *distortum* is a zoophilic fungus known to cause infections in cats, dogs and other animals. It is a rare cause of tinea capitis in New Zealand, Australia and North America. Clinical disease is similar to *M. canis* . Invaded hairs show an ectothrix infection and fluoresce a bright greenish-yellow under Wood's ultraviolet light (Ellis *et al*,2007).

which becomes yellow and crumbly .Subungual hyperkeratosis ,separation of the nail from its bed ,and thickening may then follow .Usually only a few nails are infected but rarely all are .finger nail lesions are similar ,but less common ,and are seldom seen without a chronic *T.rubrum* infection of the skin of the hands(Hunter,1995) . Onychomycosis , its caused by members of the fungal triad, namely dermatophytes, yeasts and other molds. It is estimated that fungi cause 50% of all nail disorders, and onychomycosis accounts for 30% of all superficial fungal infections (Ercan *et al*, 2004).

1.2.10.1.6 Tinea mannum: it's the infection of the hands this is usually asymmetrical and associated with tinea pedis .*T.rubrum* may cause a barely perceptible erythema of one palm with characteristic powdery scales in the creases (Hunter,1995).

1.2.11 Diagnoses using molecular and Immunological techniques

1.2.11.1 Immunological tests

Trichophyton is a crude Anti gene preparation that can be used to detect immediate or delayed type hypersensitivity to dermatophytic anti gene. Many patients who develop chronic ,non inflammatory dermatophyte infections have poor-cell mediated immune response to dermatophyte Anti gene in the normal host immunity to dermatophyte infection a[illegible] Serologic tests for Anti body detection are generally considered to be in adequate for use in diagnoses .In only 25%of known cases are immunodiffusion and complement fixation assays +ve .Enzyme immunoassays (EIAs)are more sensitive than immunodiffusion (ID),but less specific (Washington *et al*,2006).

1.2.11.2 Molecular techniques

The application of nucleic acid amplification technology has made rapid and precise identification of dermatophytes possible. Morphological and physiological characteristic can frequently vary in fact, the phenotypic feature can be easily influenced by outside factor such as temperature variations medium and chemotherapy

(Mondello,2003). Molecular approaches have been developed to provide more rapid and accurate alternatives for dermatophyte identification. These methods include restriction fragment length polymorphism (RFLP)analysis (Kawasaki *et al* ,1996; Kanbe *et al* ,2003) sequencing of protein encoding genes (Kano *et al* ,2000) random amplification of poly morphic DNA (RAPD) (Mochizuki *et al*,1997). PCR finger printing and amplified fragment length polymorphism analysis (Graser *et al*,2000),and dot blot hybridization (El Fari *et al*,1999). Internal transcribed spacer (ITS) region of the RNA gene(Graser *et al*,1999). Faggi *et al* (2001)reported that the application of PCR finger printing for the identification of species and verities of common dermatophytes and related fungi utilizing arbitrary single primer the simple repetitive oligonucleotide (GACA)4,the primer was able to amplify all the strains ,producing species –specific profiles for *M.canis,M.gypseum T.mentagrophytes,T.rubrum,E. floccosum* (dpsD1)for these species and species –specific primer sets(PsT-PsME)for each species were designed based on the genomic sequences of the DNA topoisomerase II genes of the dermatophytes. Using dPsD1,a DNA fragment of 3390pb was amplified from the genomic DNA of all the dermatophyte species .second subsequent nested PCR using species-specific primer sets (psT-PsME),both sets amplified unique sizes of PCR products ,all of which corresponded to a species of the dermatophytes even in the presence of other fungal DNA.PCR-based identification targeting the DNA topoiomerase II gene is rapid and simple and is a available as a tool for the identification of the major dermatophyte species .

Developing of light cycler PCR method for the rapid detection and differentiation of fungal DNA in dermatological specimens such as skin scales and skin swabs light cycler melting points were defined by amplification of DNA from 21 fungi and sensitivity was determined by amplification of serial dilutions of fungal DNA .Two subsequent light cycler PCR reactions and one RFLP reaction allowed the differentiation of dermatophytes and non-dermatophytes molds and subclassification of yeasts .analysis of clinical samples from 38 patients with fungal skin diseases provide conclusive new diagnostic information in 9/38 cases (23.7%)by this PCR protocol that was not equally provided by direct microscopy and mycological culture(Gutzmer, 2004).

and invasive infections. Of 397 fungal strains (290 target and107non-target strains)tested ,the sensitivity and specificity of the array was 98.3% and 98.1%respectively.

When one of the 4 random primers (OPAA11, OPD18, OPAA17 and OPU15) was used to arbitrarily primed polymerase chain reaction (AP-PCR)up to 20 of the 25 of dermatophyte species or subspecies under investigation could be distinguished on the basis of characteristic band patterns detected in agarose gel electrophoresis (Liu *et al,* 2000).

1.2.12 Treatment of Tinea by herbs

Cure ringworm with Garlic capsules:Garlic has been used since Biblical times for treating infections. Garlic has both antiseptic and immune stimulant properties. It is most effective if crushed and eaten raw also Cure for ringworm with Grapefruit Seed Extract.

Grapefruit (*Citrus paradisi*) has strong antimicrobial properties as anti-bacterial, anti-viral and anti-fungal activity. It has been often used in the topical and internal treatment of different fungal infections. Therefore grapefruit seed extract is a powerful ringworm remedy both orally and topically(Capek *et al*,2009).

1.2.13 Treatment of Ringworm: Anti-fungal Ginger Paste

1.2.14 In vitro Antifungal activity evaluations

Plant extract has been used traditionally to treat a number of infectious diseases including those caused by bacteria ,fungi, protozoa and viruses (Soylu *et al*,2005)A number Of reports are available *invitro* and *invivo* efficacy of plant extract against plant and human pathogens causing fungal infections (Natarajan *et al*,2003). The antifungal activity of methanolic extract of different types of zygophyllum plant species (venugopal and venugopal, 1995) reported the activity of plant extracts against 88 clinical isolates of dermatophytes which includes *Microsporum canis*, *M. audouinii*,*Trichophyton rubrum* , *T.*

mentagrophytes , *T. violaccum* , *T. simii*, *T. verrucosum*, *Trichophyton erinacci* and *Epidermophyton floccosum*.While Vlietinck *et al*(1995)revealed clinical findings of Rwandese medicinal plants (267)plant extracts used by traditional healers to treat microbial infections and found 60% of these extracts were active against dermatophytes.Inhibition effect of 30 aqueous extract of medicinal plants were studied against *Alternaria solani* and *Fusarium* oxysporum especially *Menta viridis* and *Rosmarinus* officinalis are the highest inhibition rate. (AL-Suhaili,2002)Found that the extracts of 9 plant species in Jordan extracted with different solvents have antifungal activity against 3 types of fungi, especially *Bryonia syriaca* and *Onion spinosa*. Al-Taie (2001)studied the antifungal activity of water and alcoholic extracts of (*Mentha longifolia*, *Matricarica chemomilla*, *Albezia lebbeck* and *Citrus sinensis*)the alcoholic extract showed the highest antifungal activity against (*Microsporum gypseum* and *Trichophyton mentagrophytes* (Mohammed ,2004,repo[illegible] [illegible] [illegible] d s[illegible] after 4 weeks treatment of tinea corporis patients with *Aloe vera* 5%gel.

Ethanolic extract of *Fumaria officinalis* showed a good therapeutic when used to treat mouse skin infected with *Trichophyton rubrum* ,after 11 days of treatment the incision was completely covered with newly formed epithelial and hair seen in comparison with fugidin ointment (Al-Ani,2006).

The antidermatophytic efficacy of other plant extracts also studied such as *Nerium oleander* extract against Tinea corporis and tinea capitis (Heba,2007) Acetone extract of leaves *combretum nelsonii*, *combretum albopunctatum*, *combretum imberbe*and *Terminalia sericea* possessed remarkable growth inhibitory activities against fungal pathogens(Masoko,2007) for example

Garlic sativum extract (Mustafa,2009).Methanolic extract of lemon grass,lanta,nerium,basil,olive oil(Bokhari,2009) .

Chloroform ,methanol and aqueous extracts of *Solanum melongena*, *Lawsonia inermis*, and *Justicia gendarussa* were evaluated against four common dermatophytic species (T. *metagrophytes*, *T. rubrum*, *M. gypseum and M. fulvum*(Sharma *et al* , 2011).

1.2.15 Gene expression of dermatophytes virulence factors

In host pathogen interactions the gene expression of the pathogen is modulated by signals from the host, and understanding the expression patterns may provide insight into the mechanisms of disease .Little or no information is available on the transcription patterns of dermatophyte [illegible] *Cryptococcus neoformans*_and pathogens of plant (Hube *et al*,1994 and (Rhodes *et al* 2001) Kaufman characterized the expression of an [illegible] *mentagrophyte* is closely related to TRI 14. Trim4 resembles other protease other protease encoding genes though to be virulence factors ,Expression of Trim4 gene was induced several fold when *T.mentagrophyte* was grown on keratin and elastin. Exvivo ,strong induction was observed after culture on blood plasma ,differential cDNA screening was performed to identify additional gene encoding putative virulence factors .By this method a fungal thioredoxin and a cellulase homolog were identified and both genes were found to be strongly induced by skin extracellular matrix proteins .Induction by superficial (keratin)and deep (elastin)skin elements suggest that the products of these genes may be important in both superficial and deep dermatophytosi and models for their function are proposed .(Kaufman *et al*, 2005)Secreted proteolytic activity is considered a virulence trait of these fungi .In a

medium containing protein as a sole N.A. and C source *T.rubrum* secretes a m*etal*locarboxy peptidase (TruMcpA)of the M14 family according to the MEROPS proteolytic enzyme database. TruMcpA is homologus to human pancreatic carboxy peptidase A, and is synthesized as a precursor in a preproprotein form In addition *T.rubrum* was shown to possess 2 genes (TruSCP A and TruSCP B)encoding serine carboxypeptidases of the S10 family while are homologues of the previously characterized *Aspergillus* and *Penecillium* secreted acid carboxypeptidases (Zaugg *et al*, 2008).

Chen *et al*,(2010)reported the secreted proteases were considered to be the most important virulence factors .first time they used the keratins from human nail and skin stratum corneum as the growth medium to investigate the different expression patterns of *T.rubrum* secreted endoprotease genes .so they found that SUB7 and MEP2 were the highest expressed gene in each family during growth in both keratin containing media. Also they found that [illegible] structures of SUB1,SUB4 and MEP4 may be more suitable for degradation of nail original keratins.

Chapter Two

2 Materials and Methods

2.1 Materials

2.1.1 Equipments and Apparatus

The following equipments and apparatus were used through out this study:

Apparatus	Company
Autoclave	**SES Little Sister(England)**
Centrifuge	**Beckman(England)**
Incubator	**Memmert (Germany)**
Microscope	**Olympus(Japan)**
Oven	**Jan rkamp**
Rotary Evaporator	**Buchi (Switzerland)**
Spectrophotometer	**Cecil(France)**
Thermal regulator	**Eppendorf**
Water bath	**Gallen kamp(England)**

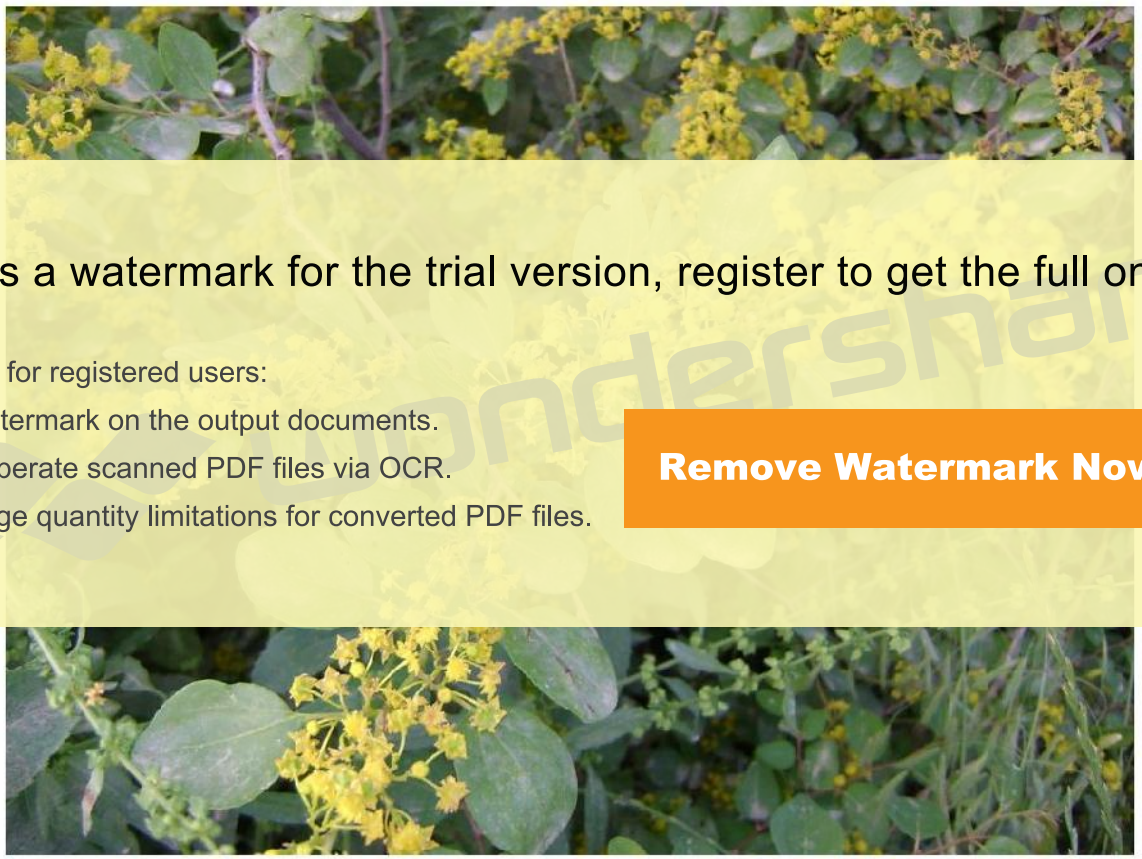
2.1.2.Chemical reagents

MATERIALS	COMPANY
Acetone	**BDH(England)**
Benzen	**BDH**
Cephalexin	**BDH**
Chloroform	**BDH**
Dermatophyte test media	**Conda, Spain**
Diethyl ether	**BDH**
Ethanol	**BDH**
Ethyl acetate	**BDH**
Formaline	**BDH**
Glacial acetyle	**BDH**
HCl	**Fluka**
Methanol	**BDH**
Saboraoud dextrose agar media	**Oxoid(England)**
Standard of phenolic compounds	**Sigma(England)**
Vaseline	**Kawa-Erbil**

2.2 .Methods

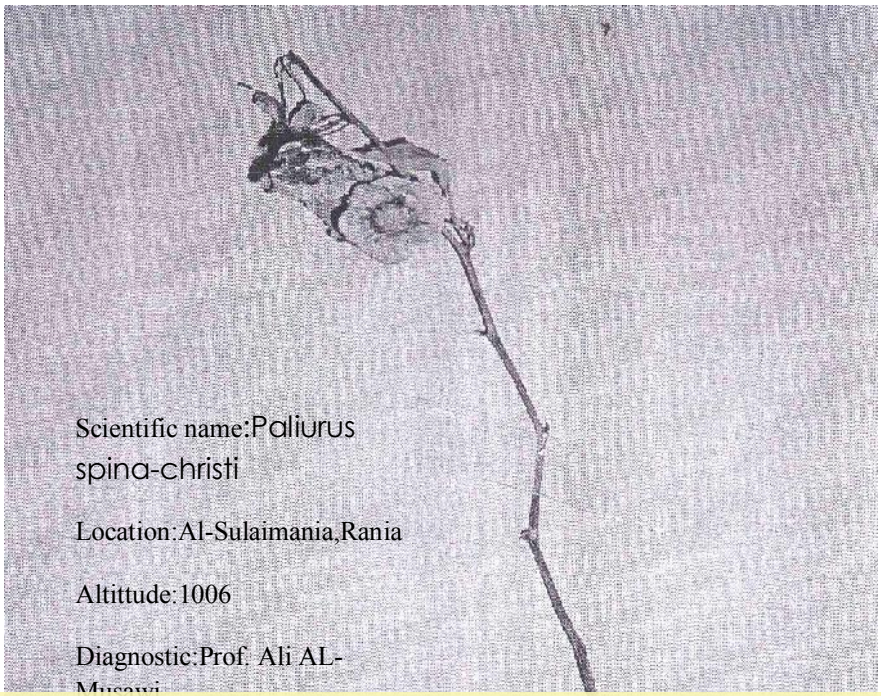
2.2.1.Collection of *Paliurus spina-christi*

The plant *Paliurus spina- cheristi*, was collected from Al-Sulaimanyia the mountain regions(Rania Village) in may 2010,and was identified by Professor Dr.Ali AL-Mosawy, Biology Department, College of science ,Baghdad University .Aerial parts of the plant were air dried at room temperature and grinded into powder form as shown in figures (2-1)and (2-2).



(Figure 2-1) *Paliurus spina-christi* during may in flowering period **.**

The photo captured by author



Figure(2- 2):Dried flowers of *Paliurus spina-christi* captured by

2.2.2. Plant Extraction

Solutions

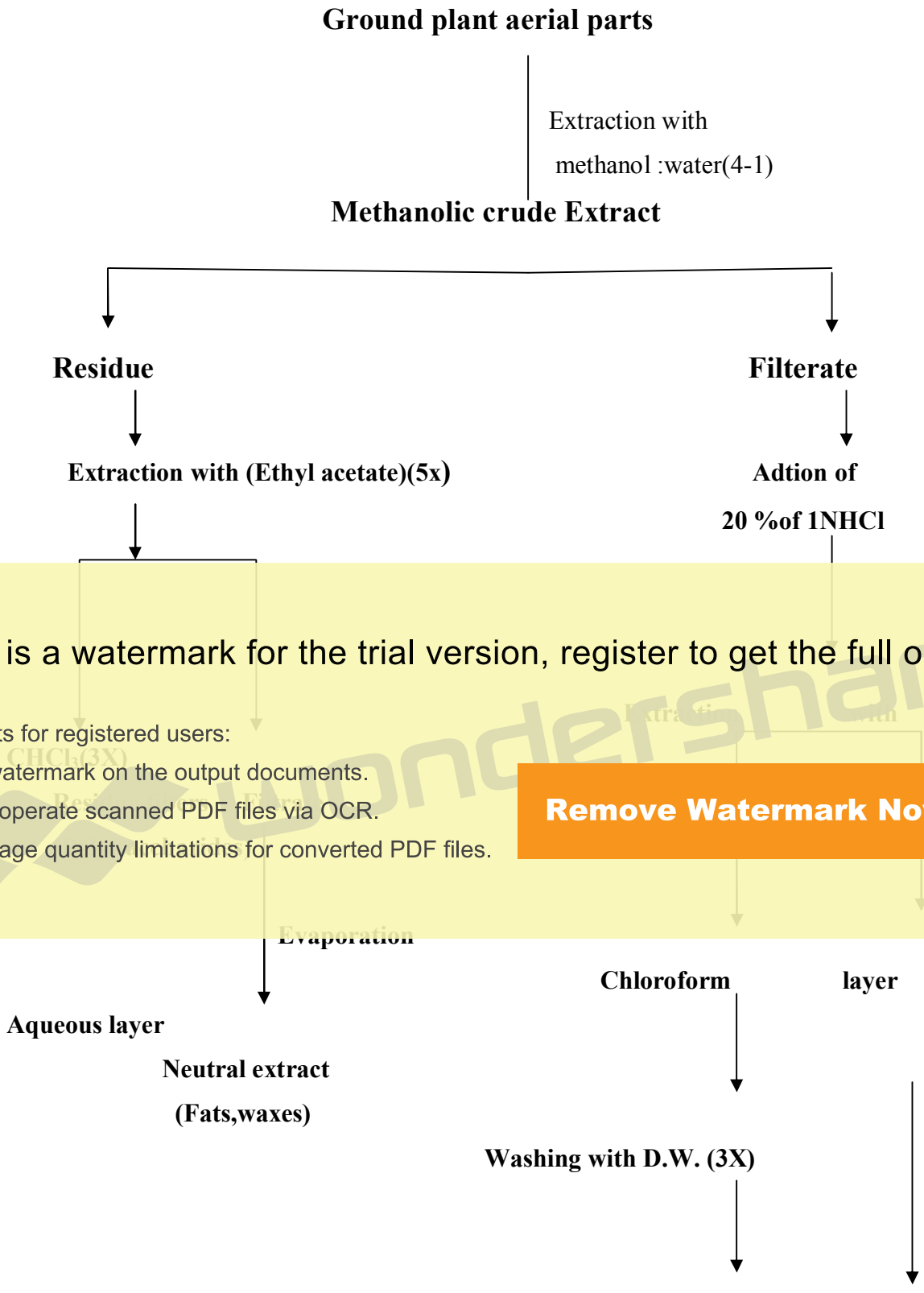
i. Ethanol 80%
ii. Methanol 80%

Method

The dried aerial parts of the plant (stem) were powdered using a seeds grinder for 5 minutes, and two extract were obtained using two types of solvents (Ethanol and methanol). In both cases, 50 grams of the processed plant were extracted in 250 ml of the solvent apparatus and the source of heating was a hot plate (70°C). The obtained extract was then evaporated at 40°C using a rotary evaporator, and the resultant crude extract was

lyophilized then used to prepare the required doses and concentrations (Arokiyaraj *et al*, 2007).

The ethanol or methanol crude extract was dissolved in sterile distilled water to prepare the dose by which the infected mice were treated.



Scheme(2-1):Extraction of phenols from *Paliurus spina-christi* (Harborne,1973)

2.3. Extracts preparation

Stock solution was prepared by (1mg) of plants extract powder in 0.1ml ethanol and then the volume was completed to 10ml sterile distilled water .The plant extract was prepared at different concentration started with (25, 50, 75, 100, 125) µg/ml. These concentrations were prepared according to the equation (Al-Ani,2006).

$$C_1V_1 = C_2V_2$$

Plant Extracts was sterilized by filtration using Millipore filter 0.22µm size .Then each concentration was poured into plates in addition to the control plates.

2.4. *In vitro* antifungal evaluation

Plant extracts were added to modified sabouraud dextrose agar [illegible] petridishes were inoculated with spore and incubated at 30 C for 3-7 days (Al-Sammarei *et al* ,2001). The diameter of fungal colonies was [illegible] incubation.

2.5.Detection of Some active compounds:

2.5.1 Detection of tannins:

Solutions:

lead acetate

Method:

(10)g of the plant powder was mixed with 50 ml distilled water in a magnetic stirrer .The mixture was boiled in a boiling water bath for few minutes .Then filtered ,and the filtrate was treated with few drops of 1% lead acetate solution .The development of greenish –blue precipitate is an indicator for the presence of tannins(Shihata,1951).

2.5.2 Detection of Saponins :

This method was done according to method described by Stahl,(1969).

Saponines were detected by two methods :

i. Aqueous extract of plants powder was shaken vigorously with D.W. in a test tube .The formation of foam standing for a time indicate a positive result.

ii. Five milli liters of aqueous extract of the plant was added to 1-3ml of 3%ferric chloride solution ,a white precipitate was developed which indicates a positive result.

2.5.3 Detection of terpenes and steroids :

Solutions:

a) chloroform

b) acetic anhydride

c) sulphuric acid

Method

(1ml)of ethanolic extract was mixed with few drops of chloroform, then a drop of acetic anhydride and drop of concentrated sulphuric acid were added , brown precipitate appeared which presenting the presence of terpene., whith the appearance of dark blue color after few minutes would represent the presence of steroids(Al-Abid,1985).

2.5.4 Detection of flavonoids:

Solutions

a) petroleum ether
b) ammonia solution

Method

Ethanolic extract of the plant was partitioned with petroleum ether ,the aqueous layer was mixed with ammonia solution. The appearance of dark color is an evidence for the presence of flavonoids (Harborn,1973).

2.5.5 Detection of Alkaloids:

Solutions

a) Mayer's reagent: Two solutions were firstly prepared; the first one was prepared by dissolving 1.58 g of mercuric chloride (HgCl2) in 60 ml of distilled water, while the second solution was prepared by dissolving 5 grams of potassium iodide (KI) in 10 ml of distilled water. Both solutions were mixed and the volume was made-up to 100ml with distilled water (Smolenst *et al*, 1972).

2.5.6 Detection of Flavonoid Compounds by TLC

The procedure of Harborne (1984) was followed, in which a silica gel TLC plate (200×200×0.25mm) was activated in the oven at 105°C for one hour, and after cooling the plate at room temperature (around 30 minutes), a marginal line was drawn on the upper and lower side of the plate with a distance of 2 centimeters from the margins. On the lower line of the plate, 0.25 ml of concentrated sample (methanol extract) and a flavonoid standard (rutin) was applied. After around 5-7 minutes, the plate was placed in a jar-closed-system containing a solvent (n-Butanol:

Acetic Acid :H2O at a ratio 4:1:5), and the system was left for two hours at room temperature.

Then plate was examined under UV light to detect the separated flavonoid compounds in the methanol extract samples as compared to the flavonoid standard and the RF value .The RF value is the distance compound moves in the chromatography relative to the solvent front. It is obtained by measuring the distance from the origin to the center of the spot produced by the substance, and this is divided by the distance between the origin and the solvent front (i.e. the distance the solvent travels). This always appears as a fraction and lies between 0.01 and 0.99. It is convenient to multiply this by 100 and RF is quoted as RF (×100) (Harborne, 1984). Such value was used to assess the presence of flavonoid compounds in the plant extracts.

2.6. High Performance Liquid Chromatography

The chemical analysis of methanolic extract of *Paliurus spina-christi* was carried out in the laboratories of science [illegible] technology center,the equipment type is Agilent 1100/1200 Binary pump column ,flow 0.750ml/min and stop time 60 min. solvents A:95%(0.2%TFA),solvent B:5%(MEOH), For pressure limits; minimum pressure :0 bar, maximum pressure:400 bar. Column diameter(0.2-0.17)mm And the flow of solvent B for different concentration(5,80,5)at different time (0,50,60) was 0.750. The sample injection volume was 50 µL.

Peak purity detection at wave length 280nm , signal polarity +ve and the scan from190nm to 400nm in 2 step scan was accomplished using an Agilent 1100 .

2.7. Gas chromatography mass spectrometry

Sample preparation

Weight about three of the dry sample was dissolved in 1 ml dichloromethane, then sonicate for 5 min, centrifuge at 4000 rpm for 3 min, the upper layer was transfer to test tube and evaporated at 35C° under N_2 gas then 100µl of BSTFA was added and put on oven for 20 min at 70C°finally 1 µl was injected on GC/MS.

Method

Chemical separation was in the same above place .Gas chromatography Ajilent type of Varian 450, composed of two parts A-Seperator and B-Detector (MS model Varian320), injected volume: 1µl,splitless injection type ,250C° injection temperature ,helium gas was

2.8 Isolation of the Dermatophytes species

2.8.1 Sampling of the dermatophytes species

i. Sabouraud dextrose agar media.

ii. Dermatophytes test media.

iii. Potassium hydroxide solution: It was prepared by dissolving 10 grams of potassium hydroxide in 100 ml of distilled water (Collee *et al*, 1996).

Method

Skin scales ,nail clippings , and hairs were examined after KOH preparation is made by emulsifying the specimen in a drop of 10 %KOH on a microscope slide .Lactophenol was added to stain the specimen after that the specimen was examined under light microscope at (10,40)X powers respectively (Washington *et al*,2006).

Isolation of dermatophytes were done by culturing above specimen(skin scales ,nail scrapings , and hairs directly on the media and incubated at 30C° for 7 days (Washington,2006) after that sub culturing of resulted growth into selective media (dermatophytes test media).

2.9 Laboratory Animals

Balb/C male mice were the laboratory animals that were employed in carrying out the experiments of the study. They were supplied by the Drug surveillance centre /Ministry of Health .Their age at the start of experiments was 8-10 weeks, and their weight was 25-27grams. They were divided into groups, and each group was kept in a separate plastic cage (details of these groups are given in the section of experimental design). The animals were maintained at a temperature of 20-25°C, and

experimental work.

The experiments were designed to evaluate antidermatophytic effects of *Paliurus spina-christi* (methanol)

2.9.1 Experiment

The experiment was designed to assess the histopathological effects of *Paliurus spina -christi* extracts (methanol)on skin. The animals were divided into four groups each group consist of six animals in comparison with negative and positive composed.

- Group I (negative controls): The animals without any treatment.
- Group II (positive control): The animals were treated with clotrinazol-p powder + thin layer of vaselin. (0.23 mg/ml) .
- Group III: The animals treated with dose (0.23 mg/ml) of methanolic extract .
- Group IV: The animals were treated with vaselin.

The animals were treated with the plant extract topically by applying the extract topically to the infected area twice daily then the insicion biopsy was taken from the mouse each week for histopathological manifestation .

2.9.2 Spore suspension preparation

Spore suspensions were prepared according to Faraj method (Faraj,1990),spores were harvested by adding 5ml/slant of sterilized water containing 0.1 % tween 80 to aid wetting and separation of spores ,The suspension was filtered through sterile cotton wool ,the filtrate was centrifuged at 3000 r.p.m for 5 minutes. The supernatant was removed and the spores were washed twice by resuspending in sterile distilled water and further centrifuged .Then 5 ml of sterile distilled water was added to precipitate and mixed vigorously by the vortex for [illegible] drop of the suspension was added to the haemocytometer by Pasteur pipette, spores were calcul[illegible] under high [illegible] 40 of light microscope using the following equation:

Concentration of spores =(Z×4×10^6)/n spores/ml

Where n: total No. of small squares.

Z: total No. of spores. (Faraj,1990).

2.10. Histopathological Study

2.10.1 light microscopic examination

Solutions

i. Phosphate Buffer Saline (PBS): The solution was prepared by dissolving the following chemicals in 950 ml of distilled water and then the volume was made-up to 1000 ml:

- Sodium chloride (NaCl): 8.0 g
- Potassium chloride (KCl): 0.2 g

- Di-sodium hydrogen phosphate (Na2HPO4): 1.15 g
- Potassium dihydrogen phosphate (KH2 PO4): 0.2 g

The pH was adjusted to 7.4, and the solution was completed to 1000ml with D.W, autoclaved and stored at 4 C (Hudson and Hay,1989).

ii. Potassium chloride (KCl) hypotonic solution (0.075M): was prepared by dissolving (5.75g) in 1000 ml of distilled water, and then the solution was autoclaved and stored at 4°C (Allen *et al*, 1977).

iii. Haematoxylin stain: The stain solution was ready supplied by the Histo- pathology Department at Educational Laboratories of Medical Baghdad City.

Method

The tissue was fixed in 10% formalin for 48 hours and then prepared as of Bancroft and Stevens (1982) was followed to prepare sections for histopathological examinations. The procedure is outlined as the following:

i. Fixation: The sample was fixed in 10% formalin for 48 hours.

ii. Dehydration: The sample was dehydrated with ascending concentrations (50, 70, 90 and 99)% of ethanol (two hours for each concentration).

iii. Clearing: The sample was placed in xylene for two hours.

iv. Infiltration: The sample was first placed in paraffin-xylene (1:1) for 30 minutes at (57-58)ºC, and then in paraffin alone for 2 hours at (60-70)ºC.

v. Embedding: The sample was embedded in pure paraffin wax (melting temperature: (60-70)ºC and left to solidified at room temperature.

vi. Sectioning: The paraffin block was sectioned (rotary microtome) at a thickness of 5 microns, and then the sections were transferred to a

slide with Mayer's albumin. The section of tissue was placed in a water bath (35-40)ºC for few seconds.

vii. Staining: The slide was first placed in xylene for 15-20 minutes, descending concentrations (90, 80 and 70)% of ethanol (two minutes for each concentration) and finally distilled water. After that, the slide was stained with haematoxylin for 10-20 minutes and then washed with distilled water for 5 minutes. Then, the slide was placed in acidic alcohol for one minutes and washed with distilled water. After washing, the slide was placed in eosin stain for 10-15 seconds, and then in ascending concentrations (70, 80, 90 and 99)% of ethanol (two minutes for each concentration). Finally, the slide was cleared with xylene for 10 minute.

viii. Mounting: The slide was mounted with Canada balsam and was covered with a cover slip. Then, the slide was examined microscopically to inspect the histopathological changes.

i. Cut paraffin sections at 5 µm.

ii.Deparffinzed by xylene.

iii.Absolute alcohol.

iv.Add alcohol 95%.

v. Rinsed in distilled water.

vi. Periodic acid solution for 5minutes (oxidizer).

vii. Rinsed in distilled water.

viii. placed in schiff's leuco. Fuchsin for 15 minutes for pink color to develop.

ix. Stained in Harries's Hematoxyline for 6 minutes ,or light green counter stain for few seconds .Light green recommended for counter staining sections in which fungi are to be demonstrated .

x. Rinsed in tap water .

xi. Differentiated in acid alcohol- quick dips.

xii. Washed in tap water .

xiii. Dip in ammonia water to blue sections.

xiv. Wash in running tap water 10 minutes.

xv.Alcohol,95%.

xvi. Absolute alcohol,2 changes .

xvii.Xylene,2 changes.

Mountin in permount(Ambrogi,1960).

2.10.2 .Preperation for transmission electron microscope (T.E.M)

A-Fixation ,dehydration and embedding

The fixation, dehydration and embedding were performed by using the method of Glauret(1975) and AL-Kaisy,(1988).

1-Samples were obtained from skin and cut into small pieces (0.1-0.2) cm fixed in 2.5% glute[illegible] (pH 7.4),the buffer was rinsed for several times and left in the PBS for 12 hours.

2-The specimens were post fixed in 1% osmium tetraoxide for 1 hour.

3- The specimens were dehydrated through a series of ethanol concentrations (30,50,70,80,90 and 100)%.

4-The specimens were cleared in propylene oxide for 15 minutes (two times).

5-The specimens were placed in mixture of propylene oxide and embedding materials (araldite) for 1 hour, then left in araldite for 12 hours at room temperature.

6-Each specimen was cleaned from adherent araldite by filter papers then placed in plastic capsule and filled with araldite and left in oven for 48 hours at 60 °C, left at room temperature for sectioning and staining .

B-Sectioning and staining

The glass knives prepared by cutting the sheet of glass plate by the knife maker .The specimen blocks were cut electrically by ultra microtome. Semi-thin sections of (0.5-1)Mm were collected on a dry glass slide to which a drop of water was added and placed on a hot plate at 60°C to dry out for few minutes ,then stained with 1 % methylene blue.

The ultra –thin sections that have silver color (60-90mmthickness) with uranyl acetate that saturated in 70% ethanol then stained with lead citrate for 20 minutes and washed again in distilled water then dried by special filter paper.

The sections examined by electron microscope(Boch,1981) using special films which photographed and printed in electron microscope unit/Al-Yarmouk university ,Jordan.

2.11.Molecular identification

2.11.1.DNA Extraction

The genomic DNA was extracted according to the method of intron biotechnology company kit**.** Mycelial samples of *T. mentagrophytes* were grounded in liquid N_2 and transferred into eppendorf tubes.

I. Pre- lyses step

1. 100 µl of MP buffer and 3µl lysozyme solution were added into sample tube ,and mix well by vortex for 30 sec or pipetting vigorously .

2.Incubation of the lysate for 15 min. at 37 °C.

during this time the tubes inverted 5-6 times .

II. Lyses step

3. Aquantity of 200µl buffer MG ,10µL proteinase K, and 5µl Rnase A solution were added into sample tube, and mixed by vortexing vigorously.

4. Lysis sample was incubated for (30-45)min. at 65C° ,during this period the tube inverted 5-6 times .

III. Binding step

5. Add 250 µl ethanol (96-100%) to the lysate ,and mix by pipetting or gently inverting 5~6 times. After mixing the tube spin down to remove drops from inside the lid.

6. 250 µl of 80% EtOH were added gently and mixed or inverted **.**

7. 750 µl of the mixture was transferred to the spin column .then centrifuged at 13.000 rpm for 1 minute.

8. The collection tube was Discarded then replaced by new one ,after that 700 µl of buffer MW was added to the spin column.

9. The spin column was centrifuged at 13,000 rpm for 1 minute.

10.The collection tube was replaced with new eppendorf tube, then 50µl of buffer ME was added followed by incubation for one minute at room temperature.

11.The spin column was centrifuged at 13,000 rpm for 1 min. then the resulted precipitant is DNA template can be use in the new eppendorff tube.

2.11.2.Spectrophotometric analysis and electrophoresis

The extracted DNA was quantified using spectrophotometer .To do that, extracted DNA was diluted 100X using nuclease free water then DNA was analyzed spectrophoto metrically at λ=260 nm. The formula used to calculate DNA concentration is as follows:OD_{260}=50 µg/ml(Faggi

purity of DNA preparation .DNA quality was tested by running

flame ,the flask was shaken repeatedly to ensure complete dissolution of agarose .The molten agarose was cooled to about 60°C and 2.5µl of red safe solution was added ,mixed thoroughly. The warmed agarose was carefully poured into a previously prepared horizontal casting tray .After the gel was completely set (40 minutes at room temperature),the comb was carefully removed .The agarose tray was submerged in 1X TBE running buffer such that a level of around 0.5 cm above the gel. The voltage was set for 120 volts and timed for 1 hour. The gel was visualized under UV light and photographed using gel documentation system.

2.11.3 PCR of Template DNA

PCR was performed according to (chung *et al*,1999).Ready mix Taq PCR was carried out in reaction volume of 25 µl of ready mix Kapa(2 G fast ready PCR) and 1.25 of 10µM of each specific *(T.menragrophytes)* primers (Table2-1) and 2µl(0.1 µg/µl)template DNA .Blank which contained only water instead of DNA ,was used in each PCR run to check for DNA contamination.

DNA amplification was carried out by thermal cycler .the sample was denatured at 95 °C for 3 minutes ,followed by 25 cycles of 95°C for 10 seconds (denaturation step),55°C for 30 seconds (annealing step),72°C

Following amplification, PCR product was kept at -20 °C until tested by 1% agarose gel electrophoresis (as described in section 3.12.2).

Table 2-1) Primers used for *T.mentagrophytes*

Primer name	Sequences (5' to 3')	Target gene	PCR product/bp	Aim and References
T.mentagrophytes	F)5'-CGA GCG TGG CTA CAG CTT CT-3' R)5'-CTCCTTGAT ACG GAC GAT-3'		<120	Kaufman *etal*.,2005
M4#804	f)5'-CAG	Tri	<120	Kaufman

	GACTTCAAC GGA ACC TTC T-3'	m4		*etal.*,2005
M4#1859	R)5'-CAA TCC CAG CGG TCA TAG TTC T-3'	Tri m4	120	=
#219	F)5'-CGA GCG TGG CTA CAG CTT CT-3'	Actin	120	=
#279	R)5'-CTC CTT GAT GTC ACG-3'	Actin	120	=

transferred to eppendorf tubes and was centrifuged at 20,000 g for one minute. Then the supernatant was discarded and pelleted cells were frozen in liquid nitrogen and stored at -80°C until used.Frozen cells were resuspended in 0.5 ml of lysis buffer TES (10 mM Tris-HCl (pH8.0),1 mM EDTA (pH8),SDS2%)and incubated at 65 °C for 2 minutes. Then 5µl of 1 M sodium acetate(Ph5.0) and 0.5 ml of acid phenol (Ph5.2)was added and vortexed vigorously for 30 seconds. The mixture was incubated at 65°C for 6 minutes followed by centrifugation at 20,000 g for 10 minutes. The aqueous layer was aspirated which was approximately 0.5 ml, then transferred to a new eppendorf tube. Equal volume of chloroform was added and vortexed for 30 seconeds and

centrifuged at 20,000 g for 10 minutes. The upper layer was aspirated and transferred to a new eppendorf tube,RNA was precipitated by addition of 50 µl of 3 M sodium acetate (pH5) and 1 mM EDTA (pH8.0). Isopropanol (1ml)was added and mixed gently by inverting the tube several times. To precipitate nucleic acid, the mixture was incubated at -80 °C for 20 minutes or 1 hr at -20°C.After that ,the tubes were centrifuged at 12,000 g for 20 minutes and the supernatant was discarded .Nucleic acid pellet was washed with 1 ml of cold 70% ethanol and centrifuged again at 12,000g for 5 minutes.RNA pellet was air-dried for 10 minutes then dissolved by addition of 30-50µl nuclease –free water and stored at -20°C until use.

2.11.5 Power cDNA Synthesis

cDNA synthesis was according to the kit protocol of Intron Biotechnology ,Inc.and as follwing:

briefly in order to collect the solution at the bottom of the tube. and then

the plates were placed on ice for at least 1 min..A group of reagents were added in the order and mixed gently they are (RNase inhibitor(1µl),5Xrt buffer(4µl),dntP 2µl,DTT 2µl and AMV RT 0.5µl. followed by incubation at 42°C for 60 min. and heating to 70°C.Finally the above reactant diluted by adding 50-80 µl sterile water .

The resulted product electrophoresis according to the method (2.11.2) and examined under U.V.

2.11.6 Statistical analysis:

The Statistical Analysis System- SAS (2004) was used to find the effect of the different concentrations in study percentage. Least significant difference –LSD test was estimated to compare between means.

3.1 Detection of Some Active compounds in *Paliurus spina-christi* extract:

In this study the active constituents of alcoholic extract of *Palliurus spina-christi* were determined .Depending on results there were classes of active compounds using different specific reagents they were(Alkaloids, Tannins, Flavonoids, Saponin, Terpenes and Steroids) were detected (Table 3-1). Previous work on the same plant indicated the presence of the same classes of compounds (Lee *et al*,2001) found that 13-memberd cyclopeptide alkaloids ,paliurines G,H and I ,together with six known alkaloids, were isolated from the stem of *Paliurus* species .

Another study indicated that *Paliurus spina- christi* alcoholic extract was rich of flavonids contents of different plant parts and the different composition of the flavonoid pattern , rutin are the main flavonoids compounds present in [illegible] parts (leaves, flowers, fruits) investigated (Bra[illegible]tner and Ma[illegible]es, 1999).

[illegible] phytochemical screening of compoun[illegible] flavonoids, tannins in all plant parts of *P.* s[illegible] 1999).

Table (3-1): Some active compounds in *Paliurus spina- christi* methanolic extract.

Chemical compounds of alcoholic extract	Reagents used for detection	Result
Alkaloids	Mayer's reagent	White PPt.
Tannins	FeCl2	Greenish blue
Flavonoids	Ammonia	Yellow color
Saponin	Ferric chloride	White PPt.
Terpenes and steroids	H2SO4	Brown PPt.

3.2. HPLC and TLC Analysis of *Paliurus spina-christi* Extract

HPLC analysis was done to detect the most important active compounds phenols found in the plant ,using phenols as a standard reference .

Table (3-2) revealed a major peak with different retention times of phenolic compounds which detected in our results including :(Gallic acid, caffiec acid, syringic acid and epecotechin) .

Gallic acid is the basic unit of tannines (Al-Rawi and sulaiman,1984).Caffeic acid is a simplest bioactive phytochemical consist of a single substituted phenolic ring(Cowan,1999). Gallic acid and epicotechin are the major phenolic compounds (Terashima *et al*,1999; Kakuda *et al*,2000).

spina-christi. This may be due to different plant parts used. While other study revealed the presence of amino acids (Bogdanova,1963);alkaloids (Velcheva ,1993)and (Lin *et al*,2000). Methanolic extract of leaves of another species of *Paliurus* resulted in caffeic acid with 10 known phenolic compounds(Zhang *et al*,2009).

Table (3-2) phenolic compounds assessed by HPLC in *Paliurus spina-christi* extract.

Compounds	Retaintion Time	Area	Area%
Gallic acid	4.632	7411.067	82.3599
Caffeic acid	19.418	278.787	3.0982
Syringic acid	20.408	217.197	2.4137
Epichotichen	21.871	1090.168	12.115

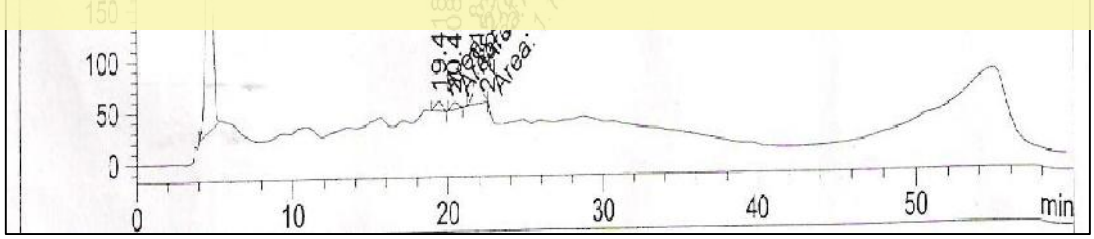


Figure (3-1)HPLC Analysis Of *Paliurus spina -christi* extract

3.3. Gas chromatograph mass spectrometry analysis of the *Paliurus spina-christi* extract

Using gas chromatograph revealed a numbers of chemicals such as fatty acids (Myristic acid), sugars (arabinofuranose,xylose and glucose), amino acids (1-aminocyclopentancarboxylic acid), phenols (benzoic acid) were identified as shown in table(3-3).

In general there are similarities in some classes of active compounds between genera belonging to the same family , Soluble sugars ,non-volatile acids and phenolic compounds were identified in *Ziziphus mauritiana* fruits by using TLC and HPLC techniques, sugars were galactose ,fructose and glucose whereas phenolic compounds were ,caffeic acids, p-hydroxy benzoic acid and coumaric acid in addition organic acids were identified malonic acid, malic acid and citric acid(Muchuweti *et al*,2005).Polysaccharides were extracted from Cyclocarya paliurus by ultrasonic wave technique (Ke-yue,2011).

Table (3-3): Biologically active constituents of *Paliurus spina-christi* extract assessed by GC/MS chromatogram.

No	Retention time/min.	Name
1	6.181	Propanoic acid, 2-[(trimethylsi[illegible]
2	[illegible]	Trisiloxane, octamathyl
3	[illegible]	Acetic acid, [(-[(trimethylsilyl)oxy], trimethylsilyl ester
4	7.832	Silane, trimethyl (4-methylphenoxy)
5	8.953	3,7-Dioxa-2,8-disilanonan-5-one, 2,2,8,8-tetramethyl
6	9.408	Arabinofurannose,1,2,3,5-tetrakis-o-(trimethyl)
7	9.467	Benzoic acid trimethyl ester
8	9.729	Trimethylsilyl ether of glycerol
9	10.048	Acetoacetic acid, bis(trimethylsilyl)
10	10.209	1-Aminocyclopentancarboxylic acid, N-methoxycarbonyl-, heptyl
11	10.488	Butanedioic acid, bis(trimethylsilyl)ester
12	10.682	Propanoic acid, 2,3 – bis[(trimethylsilyl)oxy], trimethyl ester
13	11.286	Nonanoic acid. trimethyl ester
14	11.988	Hexadecane
15	12.640	2-Isopropyl-3-ketobulyrate, bis(O-trimethylsilyl)
16	12.858	Decanoic acid, trimethyl ester
17	13.244	Malic acid, (O-trimethylsilyl), bis(trimethylsilyl)ester
18	13.640	Benzoic acid,2-[(trimethylsillyl)oxy], trimethylester
19	13.767	n-octanoic acid, 2-[(trimethylsillyl) amino], trimethylsilyl ester

20	**13.844**	**N,O-Bis(trimethylsilyl)-2-pyrrolidone carboxylic acid**
21	**14.288**	**L-threonic acid, trimethylsilyl)ether, tnmethylsilyl ester**
22	**15.174**	**D-Xylofuranose, 1,2,3,5-tetrakis-O-(trimethylsilyl)**
23	**15.784**	**Lauric acid**
24	**15.893**	**Mannoic acid, 2,3,5,6-tetrakis-O-(trimethylsilyl), lactone**
25	**17.132**	**D-Glycero-L-manno-Heptonic acid 2,3,5,6,7-pentakis-O-(trimethylsilyl), gamma-lactone**
26	**17.680**	**Arabinofurannose,1,2,3,5-tetrakis-(trimethylsilyl)**
27	**17.782**	**D-Glycero-L-manno-Heptonic acid 2,3,5,6,7-pentakis-O-(trimethylsilyl), gamma-lactone**
28	**17.857**	**Mannoic acid, 2,3,5,6-tetrakis-O-(trimethylsilyl), lactone**
29	**17.932**	**1,2,3,-Propanetricarboxylic acid, 2-[(tetrakis-(trimethylsilyl)oxy], tris(tetrakis-(trimethylsilyl) ester**
30	**18.268**	**Glucofurancoside, methyl 2,3,5,6-tetrakis-O-(trimethylislyl)**
31	**18.319**	**2-Doxy-galactopyranose,tetrakis-(trimethylsilyl)**
32	**18.445**	**Myristic acid**
33	18.778	Xylulose tetraki tetrakis-(trimethylsilyl)
34	18.846	Glucose
35	18.959	Beta-D-Galactopyranoside, methyl 2,3,4- tris-O-(trimethylsilyl)
36	18.972	2,3,4,6-tetra-Otrimethylsilyl-1-t1,7-dicarbacloso dodecaborane-yl]-D-glucopyranose

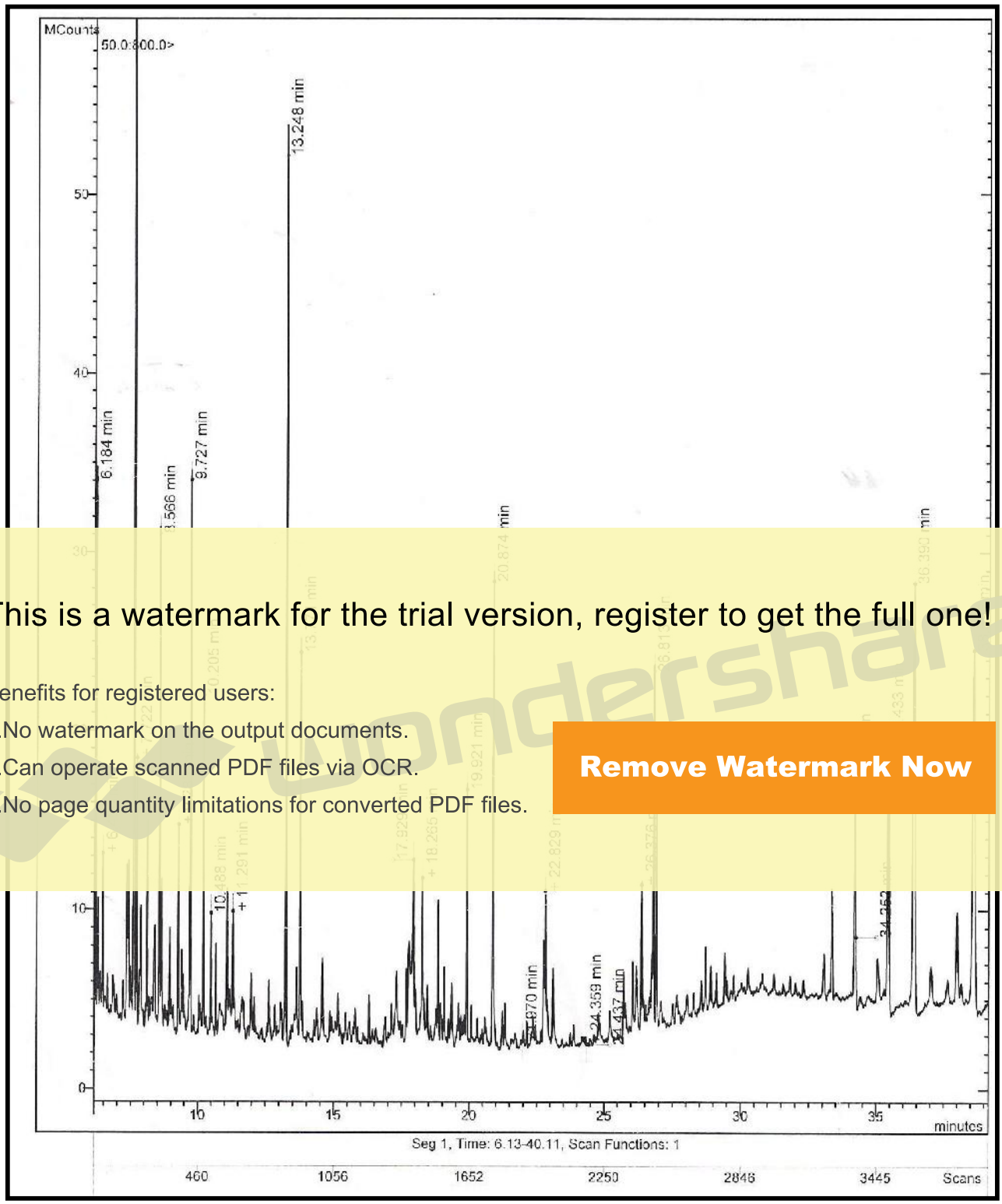


Figure 3-3 :Gas-mass spectrometer chromatograph analysis of *Paliurus spina -christi* methanolic extract

3.4. Diagnosis of Dermatophytes species

A total of fifty five samples (male and female) were collected from the tinea diagnosed patients who submitted to the consultant of Baquba Teaching Hospital of Diyala City from the period June 2010 to September 2010 .

All cases were successfully assessed regarding its clinical types of tinea. The direct KOH mount smear showed the presence of fungal elements in all of the cases while the growth of dermatophytes was positive in 18(32.27%) of these 55 patients .

Depending on species identification of the positive dermatophytes cultures, *Trichophyton mentagrophytes* percentage was (13.63%) for male and (22.72%)for female ,*Trichophyton rubrum* the percentage was (12.5%)for male only, followed by *Microsporum gypsum* (35.29%)detected in male and for female(23.52%). The detection was based on macroscopic colonial morphology and microscopic features as well . The age range was between (4 to 66)years. The age group (20-40)years old showed the highest frequency . Male : female ratio was 1:1, labor and industrial workers formed the largest group of the patients as well as house wives and the rate among military personnel seems to be high. Persons living in villages near by the animals , big families, public bathes attendants those with positive family history and persons with history of atopic dermatitis were at high risk of developing the disease.

These results agree with, (Abass,1995) who reported that the predominant fungal species were *T. mentagrophytes* (35.6%),of the positive dermatophytes culture followed by *Microsporum* canis species in (22.3%) . while disagree with(Ali,1990) and (Al-Janabi,1993) and (Al-Samarrai ,2008) who reported that *T. rubrum* were the predominant species(63.26%) followed by *T.*

mentagrophytes (19%)and *E. floccosum* (14.28%)this due to several factors such as climate differences, period of sampling, type of samples(hand, groins, foot skin scrub) (Ellis *et al*,2007).

The fungal species *Trichophyton mentagrophytes* is zoophilic dermatophytes which transmitted from the animal to human and cause a number of tinea types especially tinea capitis ,tinea unguium .The prevalence of different types of superficial fungal infections of the skin is variable among different age group ,gender, socio-economic levels and countries (Khalifa, 2011).

Table(3-4) Percentage of Dermatophytes species isolated from patient diagnosed with Tinea.

Fungal species	No. of samples	Male No.	Male %	Female No.	Female %
Trichophyton rubrum	16	2	12.5	-	-	٢
Microsporum gypseum	17	4	35,29	4	23.53	٨
Total	55	9	٦١.٤٢	9	٤٦.٢٥	١٨

Figure(3- 4)*Trichophyton mentagrophytes* growing on dermatophytes test media

Using the five different concentrations of methanolic extract maximum growth inhibition was at 125µg/ml while minimum inhibitor concentration observed at 25µg/ml. Table(3-5)shows the means of *T. mentagrophytes* growth diameter with percentage of inhibition ,the differences between means statistically non significant (P≥0.05) with the concentrations (25 and 50) µg/ml while the concentrations(75,100 and 125)µg/ml the effect of extract on growth diameter is significantly at level p≤0.05 .The differences between percentage of inhibition is statistically significant (P<0.05) for all concentrations of *P.spina-christi* extract. At the concentration 25µg/ml of methanolic plant extract *T. mentagrophytes* growth diameter after period of incubation was(6.90) cm and

the percentage of inhibition in contrast was (16%) .Using concentration 50 µg/ml of *P. spina- christi* methanolic extract the *T. mentagrophytes* growth diameter after incubation for seven days was (6.50)cm as demonstrated in Table 3-5.The inhibition percentage at this concentration was (21%) .

By increasing the concentration to 75 µg/ml of plant extract ,the mean diameter of *T. mentagrophytes* after seven days of incubation was (4.56)cm this indicated the ability of this concentration to inhibit the growth of *T. mentagrophytes* .The inhibition percentage was (45%) .

The *T. mentagrophytes* maximum growth diameter was (2.73)cm after incubation for seven days in media treated with 100 µg/ml of plant extract concentration. The inhibition percentage was statistically significant (67%).

While at the 125 µg/ml of plant methanolic extract concentration *T. mentagrophytes* growth diameter aft [illegible] cm. The effect was statistically [illegible] inhibition concentration was statistically significant(82%).

Table 3-5. The *In Vitro* antifungal activity of Methanolic extract (diameter of the growth, cm) of *Paliurus spina-christi* against *Trichophyton mentagrophytes* after seven days.

Concentration (µgl/ml)	Average of colonial diameter (cm) ± SE	Percentage of inhibition (%)
0	8.30 ± 0.87	0 ± 0.0
25	6.90 ± 0.62a	16 ± 0.94a
50	6.50 ± 0.49a	21 ± 1.02a
75	4.56 ± 0.31b	45 ± 2.37b
100	2.73 ± 0.25c	67 ± 2.93c
125	1.50 ± 0.03d	82 ± 3.77d
LSD value	2.508 *	9.366 *

A numbers of plant extract were tested for their antifungal activity against dermatophytes species *T.mentagrophytes* or indirectly treated dermatophytes caused tinea (Tinea corporis ,Tinea cruris and Tinea facie)(Sharma2011)This inhibition may be attributed to different compounds detected in methanolic extract such as flavonoids, phenols (including tannins, gallic acid and other) .

These ingredients were known to have antimicrobial activity and were found in many plant species and genera(Cown,1999).

Aloe vera gel acts as anti-inflammatory drug reducing the pruritus and the scales of the lesion caused by Tinea corporis and tinea cruris (Mohammed ,2004)*Aloe vera* is known to contain flavonoids, glycosides(Chithra *et al* ,19

98).while the methanolic extract of *Lemon grass* ,*Lanta* and *Nerium* followed by their ethyl acetate extracts showed the highest activities against *T.mentagrophytes* ,extract of lemon were the most effective followed by *Lanta* , *Nerium* and Basil showed moderate activities this affectivity may be due to free and bound flavonoid fractions (Bokhari,2009). In addition the presence of phenolic compounds which can be hold a good promise as a natural fungicide against common pathogens of crops (Nwachukwu and Umechuruba,2006). A wide variety of flavonoids, sesquiterpenoid alcohols, triterpenoids and quinic acid caffeates product from plants may also be useful as antimicrobials. The activity is probably due to their ability to form a complex with extra cellular and soluble proteins, which then binds to bacterial cell wall . More lipophilic flavonoids may also disrupt microbial membranes (Hu and Chen, 1997).

3.5.2 *In Vitro* Antifungal activity of *Paliurus spina-christi* Ethanolic extract

Different concentrations of ethanolic extract (25, 50, 75, 100)µg/ml are statistically significant (P≤0.05) as compared with control, while statistically non significant (P≥0.05) at concentration 25 µg/ml. The differences between percentage of inhibition is statistically significant (P≤0.05)for all concentrations after incubation period as shown in table(3-6).the *T. mentagrophytes* growth diameter after incubation for seven days in media non treated with *P. spina- christi* ethanolic extract the maximum growth diameter was at seventh day (8.3)cm which is consider control group for the growth of the fungal species.

By using the 25 µg/ml of plant ethanolic extract concentration *T. mentagrophytes* growth diameter after incubation for seven days was (7.30)cm table(3-6).The inhibition concentration was (12%).The *T. mentagrophytes*

maximum growth diameter was (5.2)cm after incubation for seven days in media treated with 50 µg/ml of ethanolic plant extract concentration. The inhibition percentage was (37%) which .

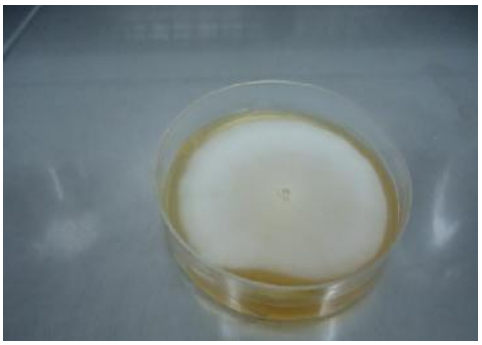
Using concentration *75* µg/ml of Plant ethanolic extract the *T.mentagrophytes* growth diameter after incubation for seven days was (4.6)cm as demonstrated in Table 3-6. The inhibition percentage was (44%).The *T. mentagrophytes* maximum growth diameter was (3.5)cm after incubation for seven days in media treated with 100 µg/ml of plant extract concentration. The inhibition percentage was (58%). The mean diameter of *T. mentagrophytes* growth after incubation for seven days in media treated with 125 µg/ml of *P. spina- christi* ethanolic extract concentration was(2.4) cm as seen in table(3-6). The inhibition percentage was (71%).

Table 3-6. The antifungal activity of *Paliurus spina-christi* Ethanolic extract (Mean of the growth, cm) against *Trichophyton mentagrophytes*

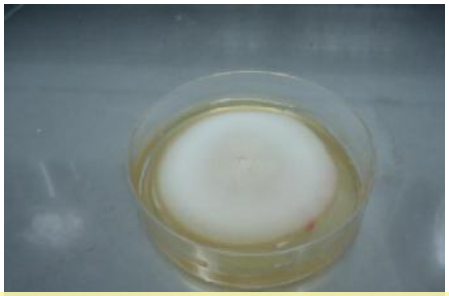
[illegible]	Average of colon diameter (cm) ± SE	inhibition (%)
0	8.30 ± 0.87	0 ± 0.0
25	**7.30 ± 0.65a**	**12 ± 0.67a**
50	**5.2 ± 0.49ab**	**37 ± 1.28b**
75	**4.6 ± 0.32c**	**44 ± 2.66c**
100	**3.5 ± 0.14d**	**58 ± 2.81d**
125	**2.4 ± 0.09e**	**71 ± 2.93e**
LSD value	**2.574 ***	**12.633 ***
***** **(P<0.05)**		

The inhibition may be attributed to different compounds found in the ethanolic extract in addition to the anti microbial activity of these compounds were found in many plant species and genera.

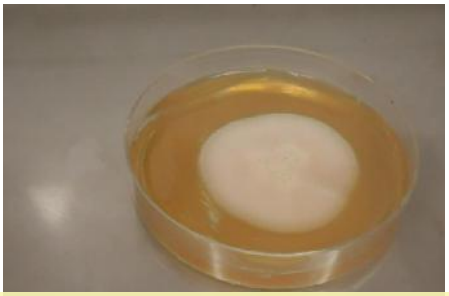
The ethanolic extract of Pomegranate peel at 5mg /ml concentration was the potent extract in complete inhibition the *T. mentagrophytes*(Al-Jumaily,2008)another study revealed that red and white onion extracts have highest inhibition rate on dermatophytes growth ,the alcoholic extracts were better than the aqueous extract in their effect against *T.mentagrophytes* at concentration 40mg/ml of alcoholic extract of red onion which give complete inhibition (Mustafa,2009).Of 22 plant extracts clove and ginger ethanolic extracts showed inhibitory activity against ten food associated fungal isolates by poisoned food technique. While garlic ethanolic extract also showed inhibitory activity against all the tested fungi except *pencilium expticum* (Ruqaia and Jalil,2010).



Control



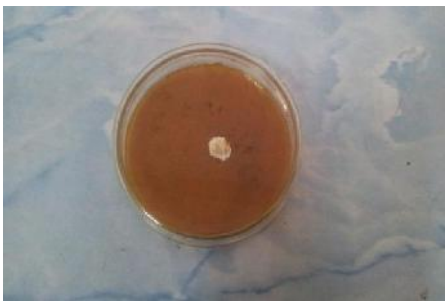
25µg/ml



50µg/ml

75µg/ml

100µg/ml



125 µg/ml

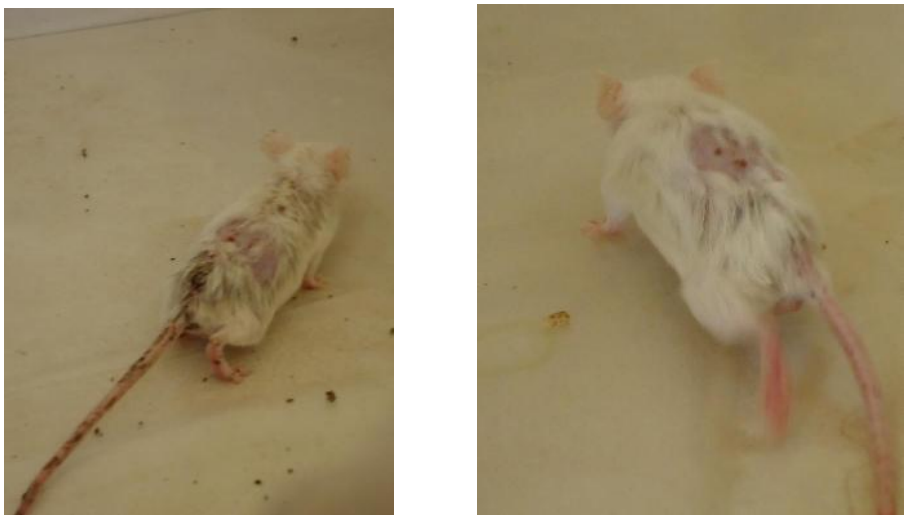
Figure(3-5)*Invitro* antifungal activity of methanolic extract of *Paliurus spina-christi* against *T.mentagrophytes* for seven days

3.6 Pathological study

3.6.1 Clinical features

After 12 days of infection with *Trichophyton mentagrophytes* figure(3- 6) certain degenerative changes were observed due to the adherence of the fungal spores into the keratinophilic layer of the skin producing skin infection .

Regular margin were seen , redness, flat, erythmatous plaques with a raised border .Scaly plaques may be studded with papules or crusts. Hairs are broken close to the skin, they may plug the hair follicle. This represent patches can remain stable for years or may enlarge if not treated (Lin *et al*,2004) .Some times ,especially when follicular pustules have developed and hair loss is observed ,the clinical morphology resembles bacterials folliculitis (Baran *et al*,2004).The specific clinical features in general depend on the inflammatory response of the host .Lesions usually appears with erythematous [illegible] with fine scales ,often in [illegible] and Summerbell



Figure(3-6)Mice infected with *T. mentagrophytes* for 12 days

3.6.2. Histological study

Histological analysis of the skin samples from all experimental groups included non-infected (-ve control),treated with Vaseline (vehicle), treated with recommended drug Clotrinazol-P (+ve control), and finally treated with *paliurus* methanolic extract (*P*.ME)on the 7^{th},14^{th},21^{1st} and 28^{th} days of lesion .

As mentioned previously +ve control and vehicle they were represent treatment of lesion by recommended drug and Vaseline respectively they shows moderate healing as compare with –ve control lesion sections which demonstrate a normal cytoarchitecture of skin (epidermis layer, dermis layer , sweet gland and hair follicle).

Sections made from skin lesions were stained by heamatoxilene and eocin (H and E) stain to detect cellularity ,fibroblast while periodic acid-schiff (PAS) stain was applied to show fungal hyphae. The last stain doesn't show the hyphae in skin but the hyphae appeared in H&E stain section and this is due to several factors such as the way of the biopsy taking, fixative Ph, storage period and temperature, affectivity of stain it self (Washington, 2006).

As we know normal skin in microscopic examination consist of two distinct but slightly attached layers ,figure (3-7)has shown that the skin consists of two distinct layers. The epidermis layer, an epithelial layer of ectodermal origin and The dermis layer, of mesodermal connective tissue. The junction of dermis and epidermis is irregular and projections of the dermis called dermal papillae interdigitate with evagination of the epidermis known as epidermal ridges. Epidermal derivatives include hairs, nails, sebaceous and sweat glands. Beneath the dermis lies the subcutaneous tissue or hypodermis, a loose connective tissue that may contain pads of adipocytes. The subc[illegible] to the underlying tissues and corresponds [illegible] anatomy(Mescher,2010).

After infection with the *T.mentagrophytes* the skin appeared with certain histological changes such as adherence of the fungal conidia to keratinocytes caused keratinolysis ,distribution of inflammatory cells in the dermal layer, odema and more inflammatory reactions figure(3-10),odema in dermal layer extend to hypodermal layer in addition to the appearance of polymorpho neutrophils and necrotic depris figure(3-11)damage of epidermal layer figure(3-13)and(3-15)the same results appeared with (Al-Ani,2006).Treatment of infected skin with plant extract for seven days, a clear healing demonstrated via moderate inflammatory cells in dermal layer figure(3-8)and (3-9).After 14 days the skin section appeared with normal shaped of epidermis with distribution of

collagene and fibroplast figure(3-12)melanocytes regeneration obvious under electron microscope figure (3-14).The regeneration of sweat glands and keratinocytes figure(3-16)after 21 days of treatment with plant extract .

Finally after 28 days of treatment by plant extract the skin section illustrate the normal shape of epidermal and dermal layers with its common structures (sweat glands ,hair follicle, keratin, collagen and fibroblast) figures(3-19)and(3-20 demonstrate the hair regeneration in treated mice by plant extract with disappear of fungal lesion.

Different active compounds were known to be responsible for healing like flavonoids ,volatile oils ,alkaloids and steroids (Abu-Al-basal,2001) ,(Al-Ani,2006),(Farageen,2007).

Hexoseamines are known to increase during early stage of wound healing and decrease hereafter (Peacock,1984). All plant extracts exhibited to certain degrees the promoting of collagen synthesis ,collagen is the predominant extracellular protein in the granulation tissue of a healing wound (Irvin,1984).

More over the collagen plays an important role in haemostasis and epithilization (Heggers *et al*,1993a;1995b) . Also similar effect has been observed with the ethanolic extract of *Centella asiatica* on rat dermal wound healing .This extract increased cellular proliferation and collagen synthesis at the wound site as well as quicker and better maturation of cross linking of collagen and enhancing the wound healing process(Suguna *et al*,1996). The

obtained results in this study were similar to the influence of *Aloe vera* on collagen characteristic in healing dermal wounds in rats .It was observed that *A.vera* increased the collagen content of the granulation tissue as well as its degree of cross linking which ultimately contributes to wound strength (Chithra *et al*,1998).

Healing in the current results may be attributed to phenolic compounds detected in our results such as (Gallic acid ,caffeic acid ,syringic acid and epichotechin)these compounds have an action on the growth of many dermatophytes. As compared with the treatment by recommended drug (Clotrinazole p-) figure (3-10)there are still inflammatory cell in dermal layer after 7 days of treatment .The same results also continue in skin section treated by recommended drug for 14 days, edema and necrotic cell reaction had been demonstrated figure(3-11),the inflammatory cell reactions extent to the 21 and 28 days of treatment by recommended drug figure (3-12)and figures (3-21) respectively. Histological studies of antifu

1. Drug that disrupt the cell membrane.
2. Drug that inhibit mitosis.
3. Drug that inhibit deoxy ribonucleic acid (DNA) synthesis (Laurence *et al*,1997).

The influence of some active compounds on the content of glycosaminoglycan and its types in the granulation tissue of healing dermal lesion have been studied. Results revealed higher levels of hyaluronic acid and dermatan sulfate in the treated wounds indicate increased turnover of the extracellular matrix and enhanced wound healing process(Chithra *et al*.,1998b). Flavonoids and phenolic compounds found in *P.spina-christi* may play the main

role in cross linking .This is un accordance with the results of Havesteen (1983)and Farageen(2007) who clarified that flavonoids are essential in activation the proline hydroxylase. Three general modes of action of different plant extract were recognized as

1. Inhibition of microbial cell wall formation or biosynthesis of some protein .
2. Disruption of deoxyribonucleic acid (DNA)metabolism.
3. Alteration of normal function of the cellular membrane(Tyler *et al*,1988).

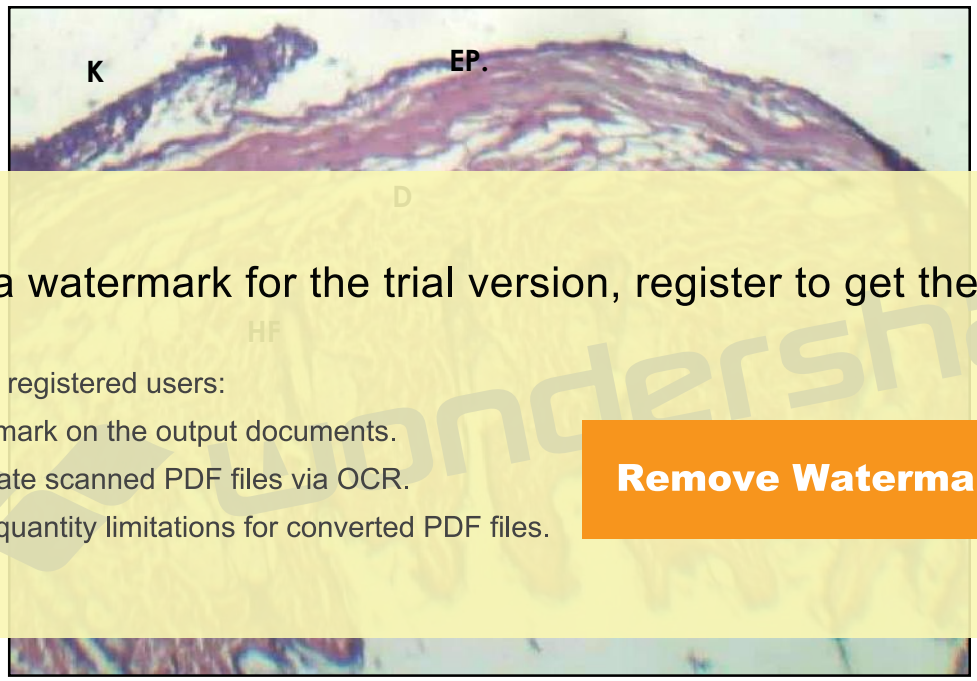
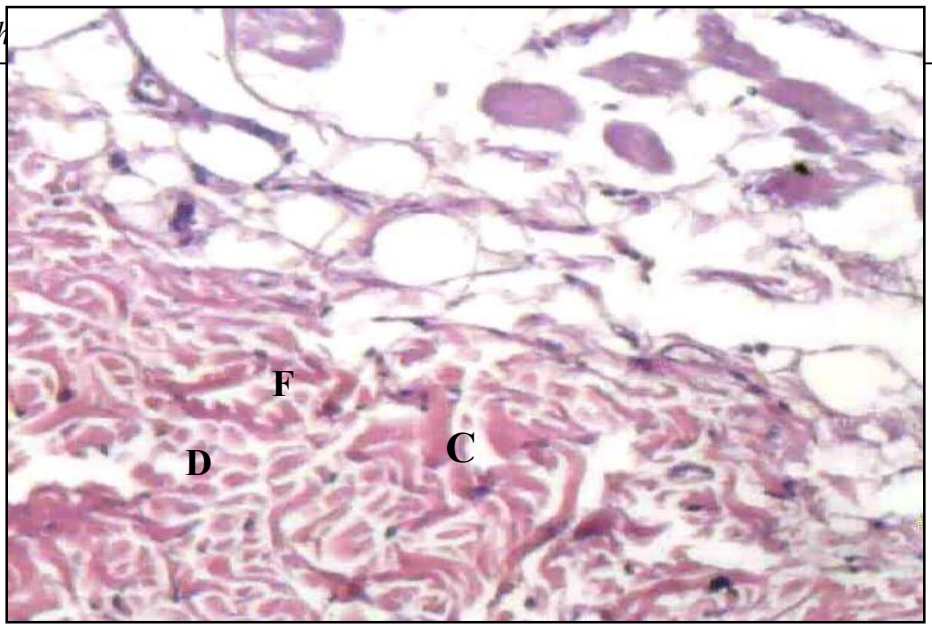
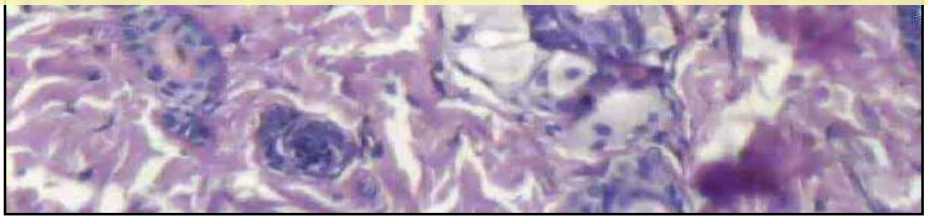


Figure (3-7)photomicrograph of normal section stained with HandE stain –ve control,EP:epidermis,D:dermis,HF:hair follicle .

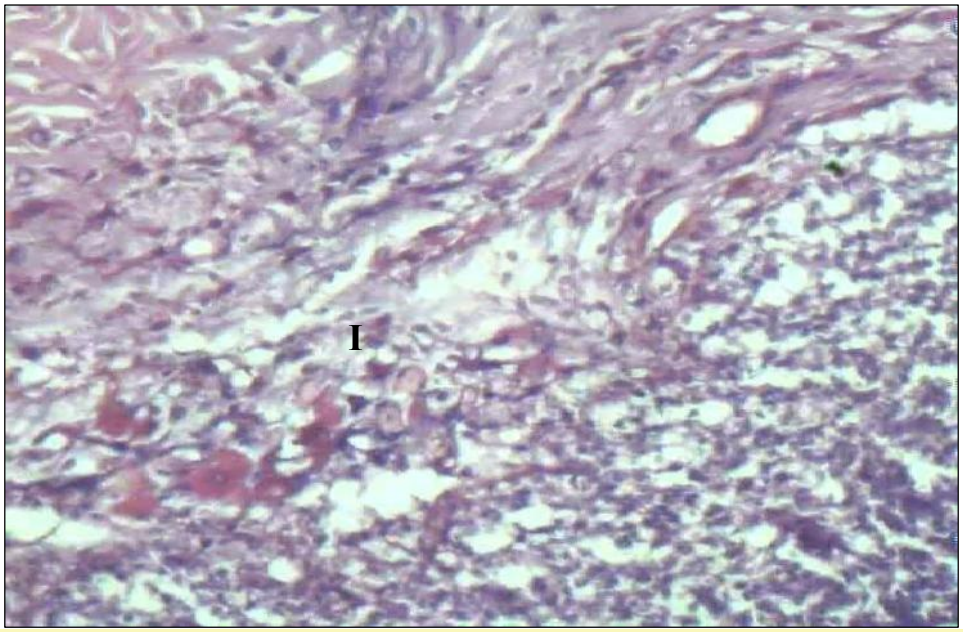


1:Hypodermis layer

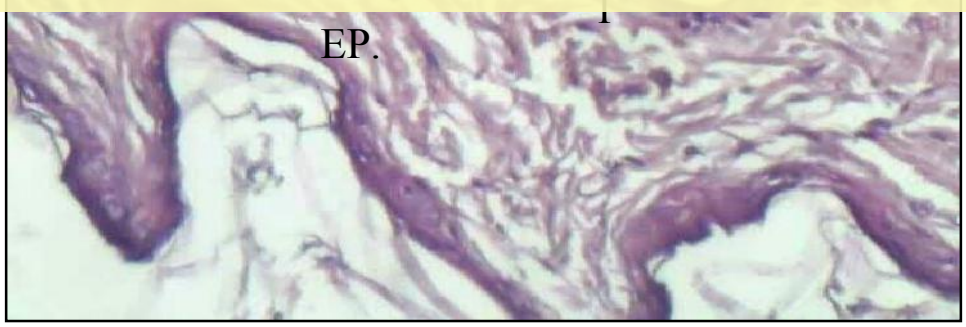


2 : upper layer

Figure(3-8)Photomicrographs of 7 days-old-lesion section stained with H and E stain 1and ,2:PME . C:collagen, F:fibroblast (Magnification: 200X).

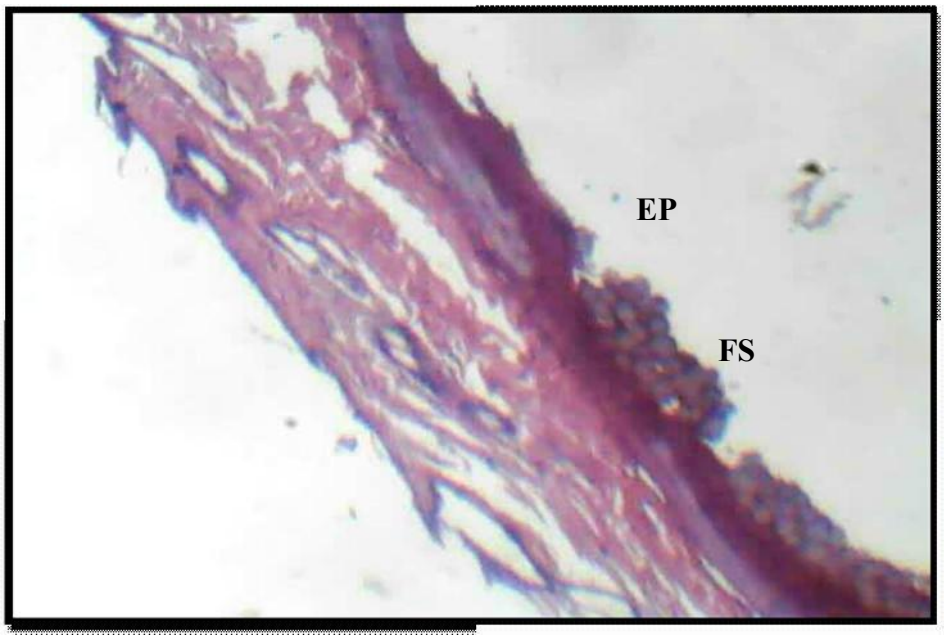


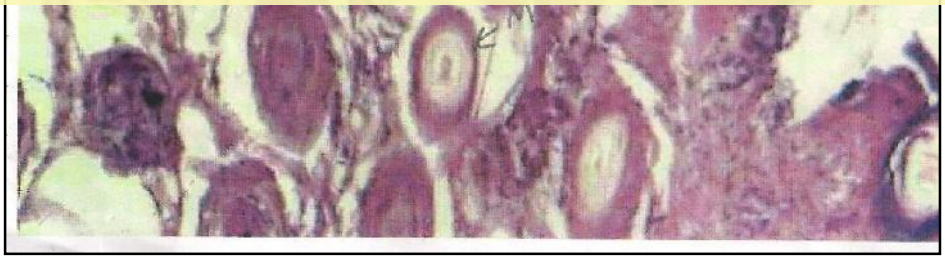
1 :Hypo dermis layer



2

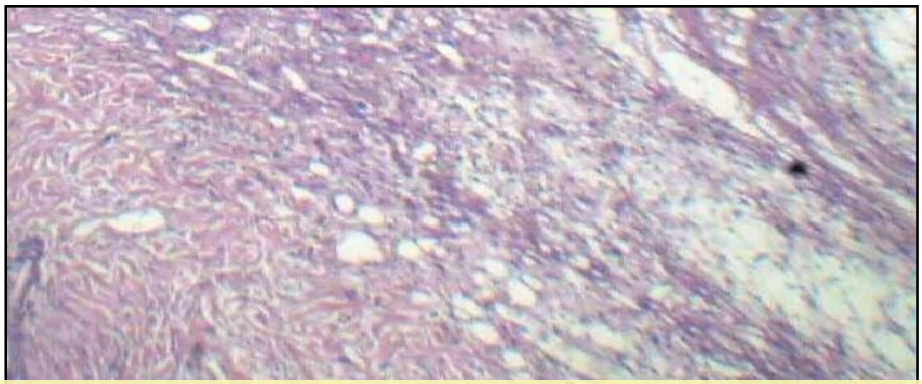
Figure(3-9)Photomicrographs of 7 days-old-lesion section stained with HandE stain 1and ,2:PME. Ep: Epidermis ,D:dermis I:inflammatory cells, C:collagen,F:fibroblast (Magnification: 200X).



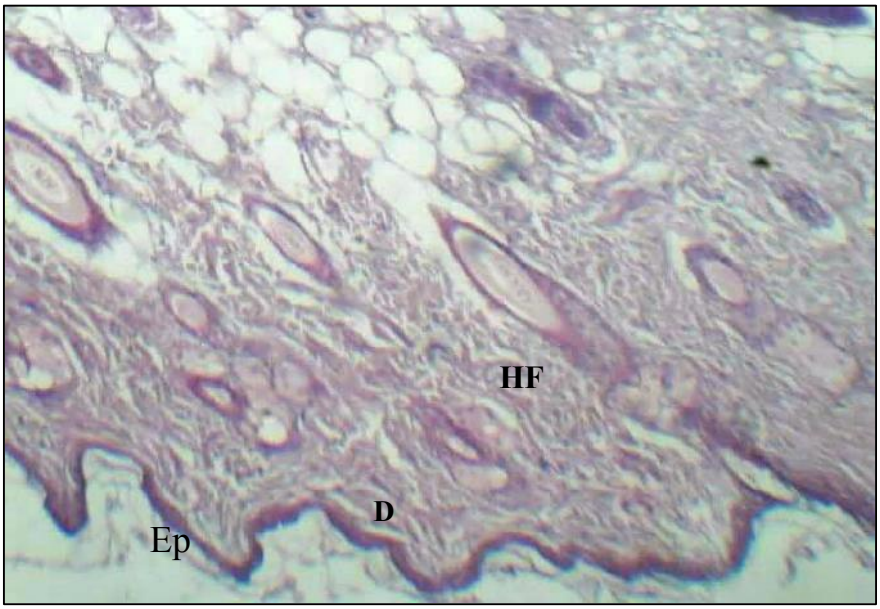


2: upper layer

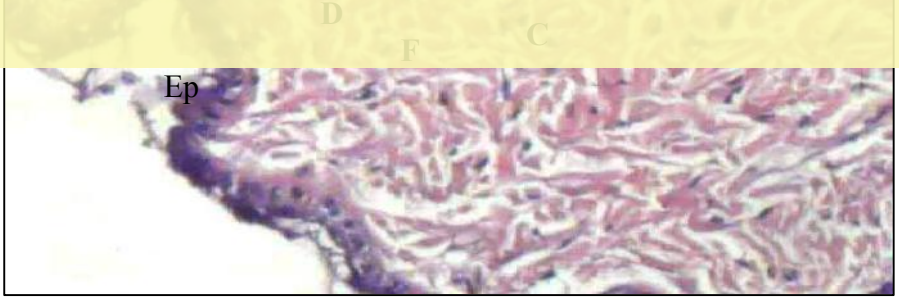
Figure(3-10)Photomicrographs of 7 days-old-lesion section stained with H and E stain 1:+control,2:vihcle, . Ep: Epidermis ,D:dermis I:inflammatory cells, FS:fungal spores **(**Magnification: 200X**).**



Figure(3-11)photomicrograph of 7 days-old-lesion section stained with H and E stain –ve control ,.Ep: Epidermis ,D:dermis ,I:inflammatory cell, O:odema **(**Magnification: 200X)

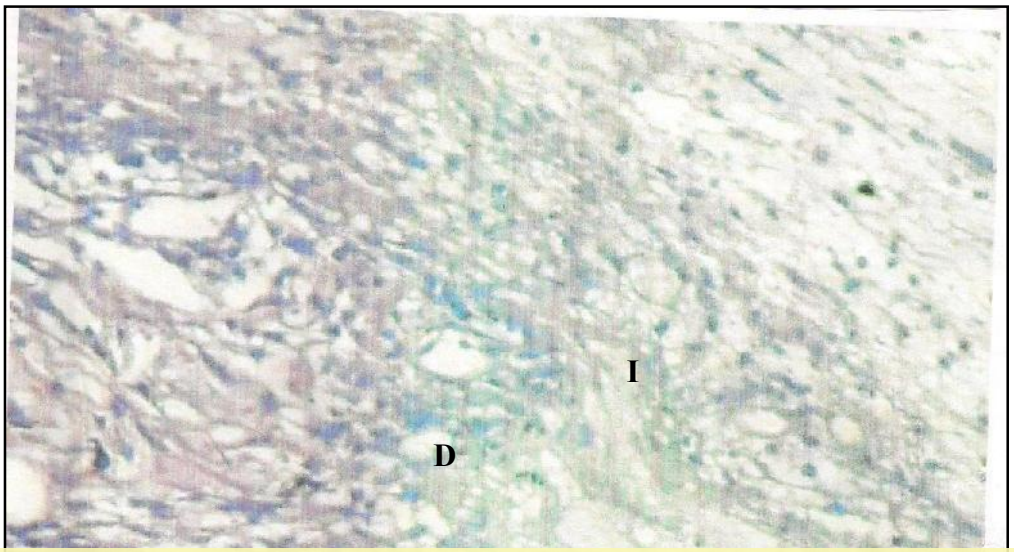


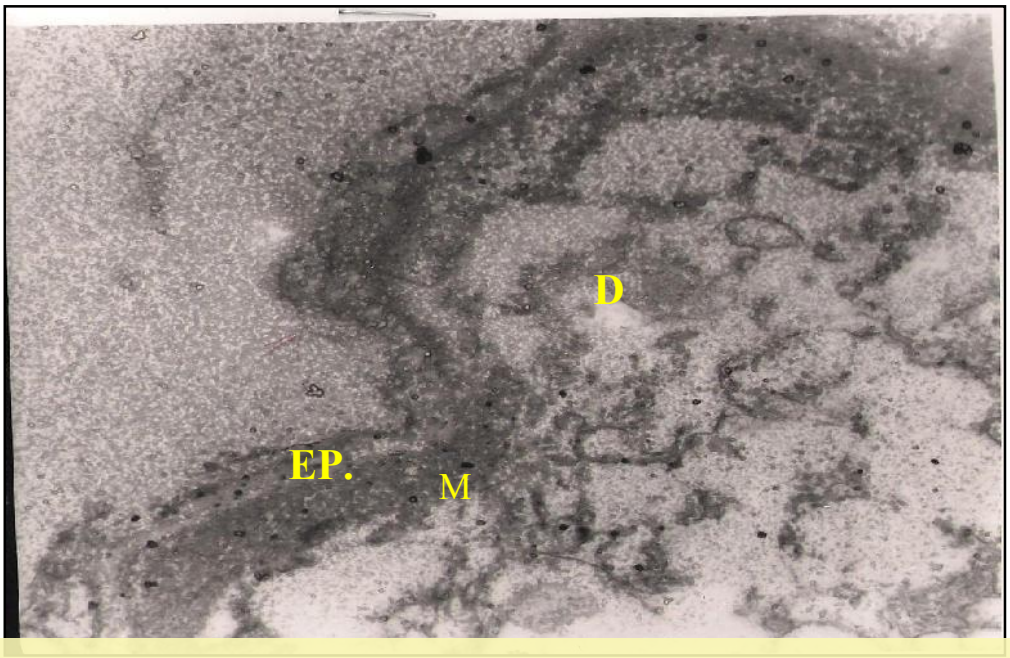
1



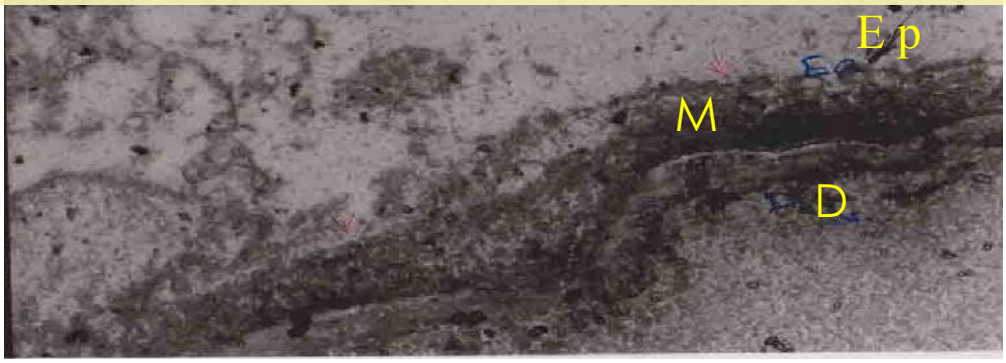
2

Figure(3-12)Photomicrographs of 14 days-old-lesion section stained with H and E stain 1and ,2:*P*.ME .Ep: epidermis ,D:dermis,HF:hair follicle C:collagen,F:fibroblast **(**Magnification: 200X**).**



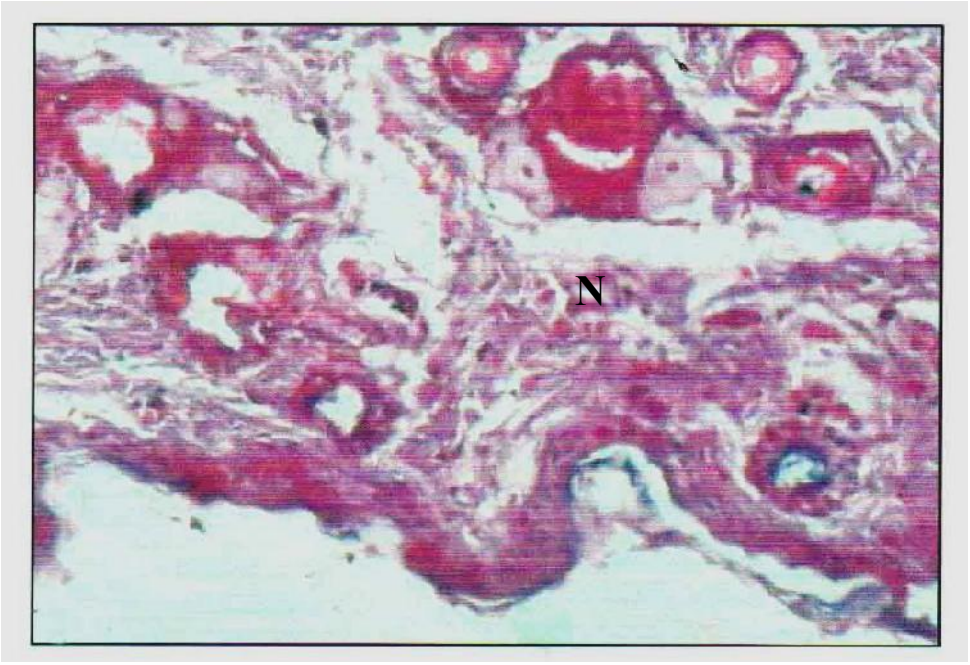


1



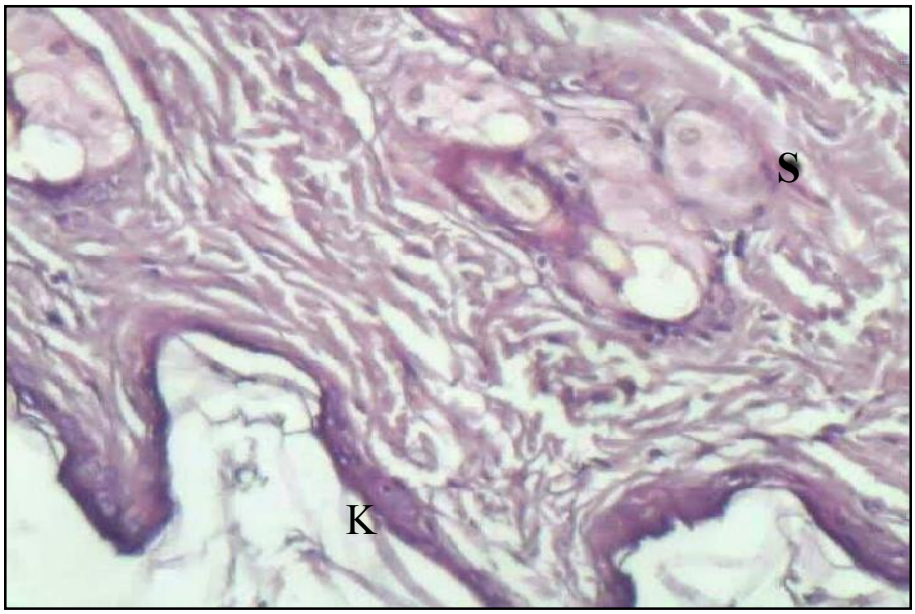
2

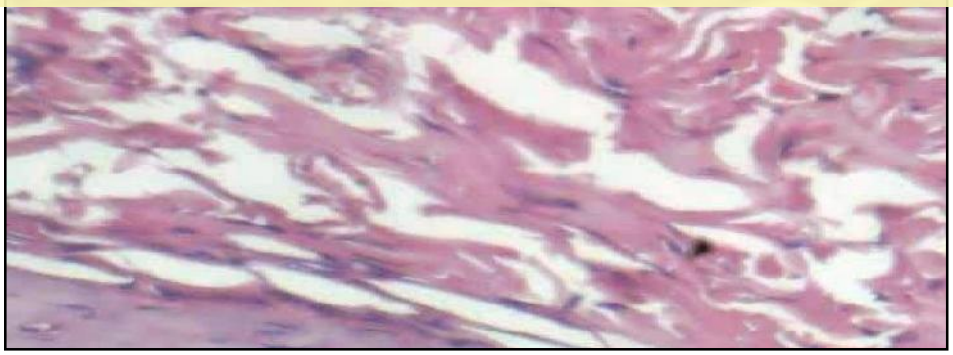
Figure(3-14)Photomicrograph of 14 days-old-lesion TEM section,*1and2: P.*ME lesion treated for 14 successive days . EP: epidermis, D:dermis,M:melanocytes. **(**Magnification: 15909X**).**



2

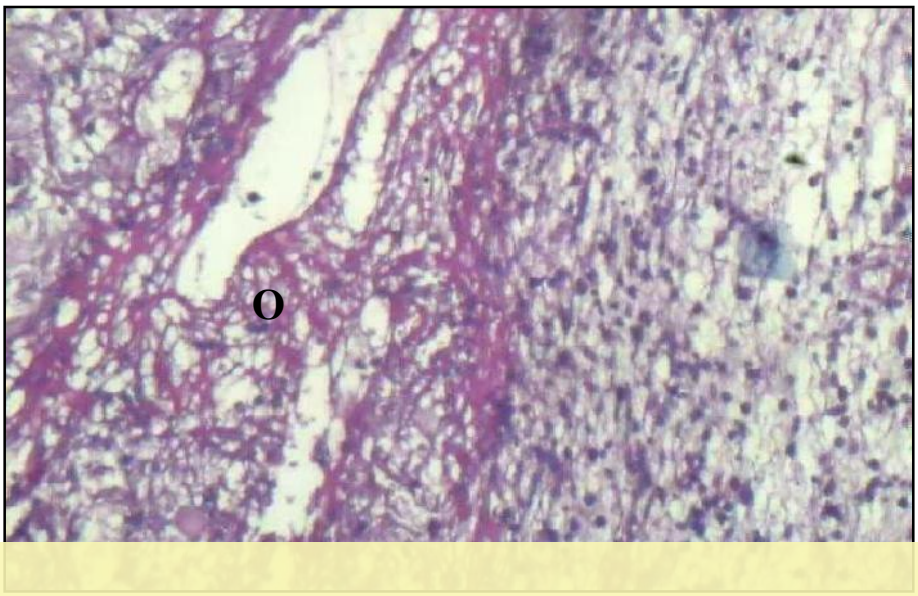
Figure(3-15)Photomicrographs of 21 days-old-lesion section stained with HandE stain 1:+ve control ,2:vehicle.N:necrosis.(Magnification: 200X)

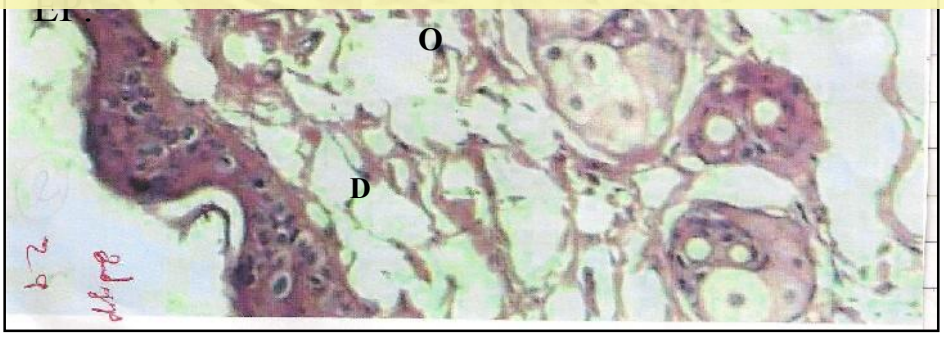




2

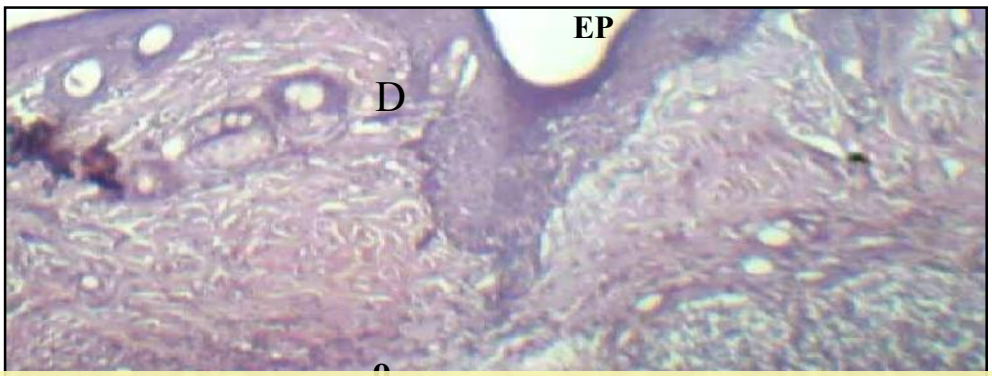
Figure(3-16)Photomicrographs of 21 days-old-lesion section stained with HandE stain 1and 2:*P*.ME EP:epidermis,D:dermis,K:keratin tissue, S:sweet gland.**(**Magnification: 200X**).**

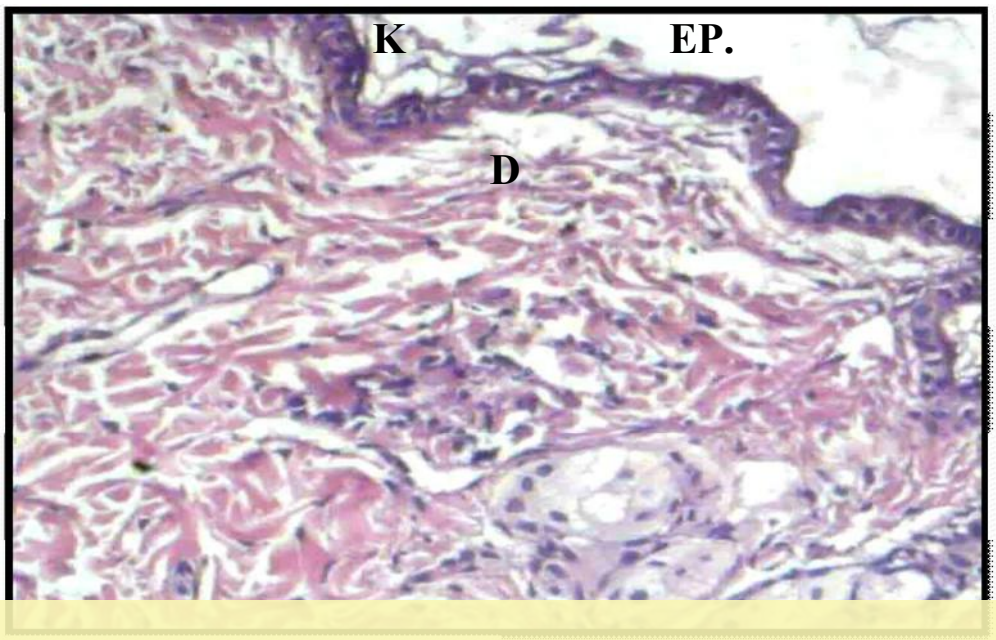


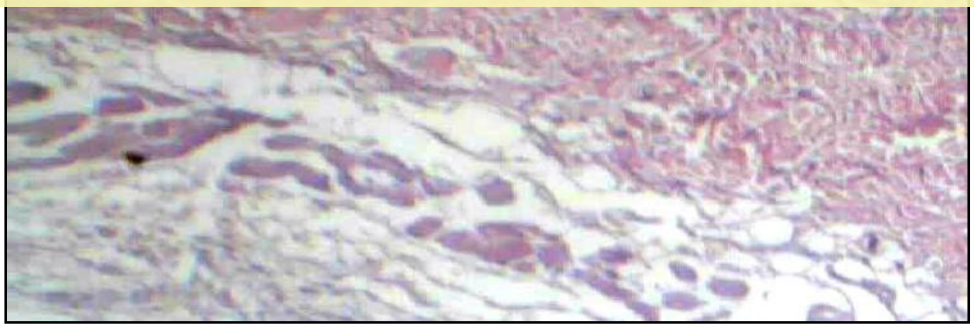


2:upper layer

Figure(3-17)Photomicrographs of 21 days-old-lesion section stained with HandE stain 1:+ve control ,2:vehicle.I:inflammatory cells , O:odema.(Magnification: 200X)

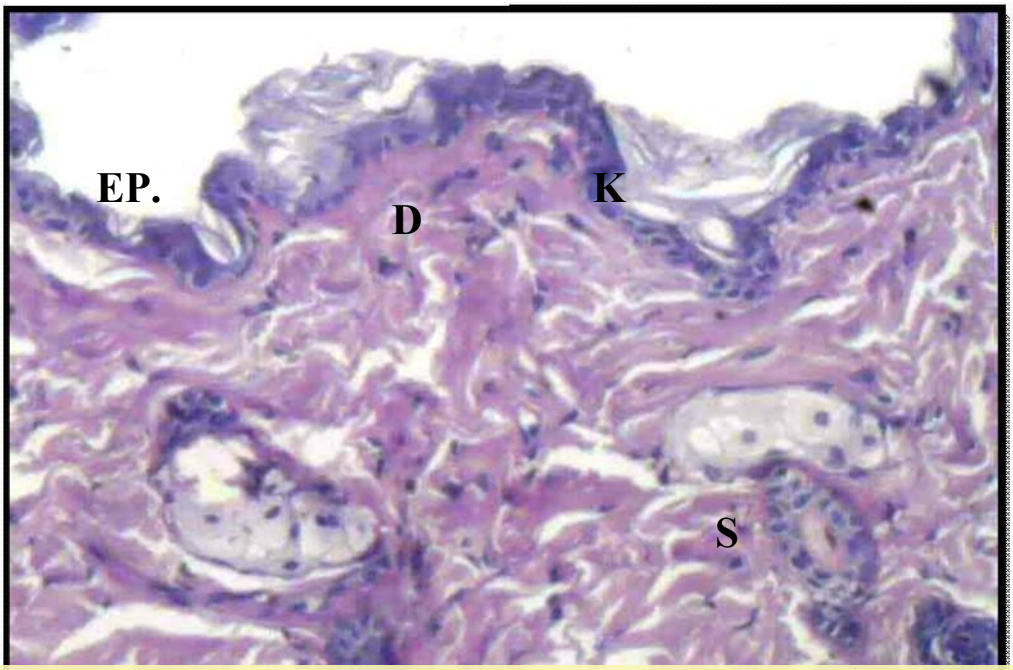


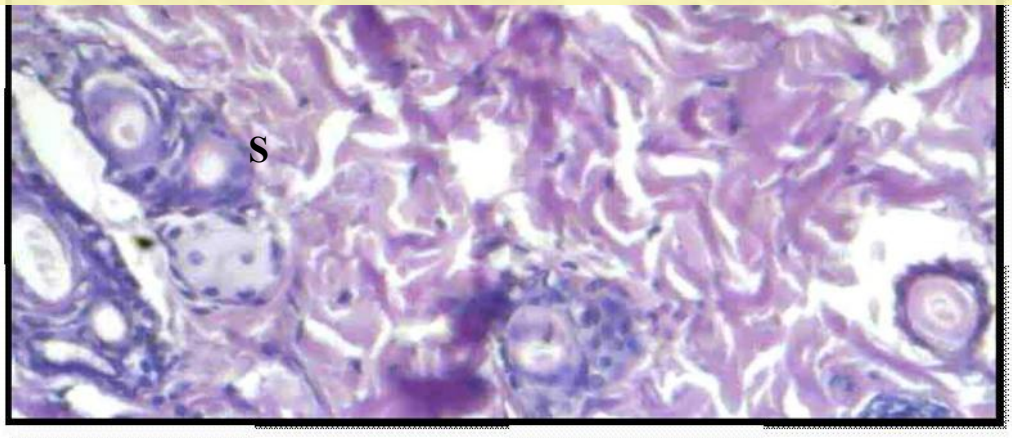




2

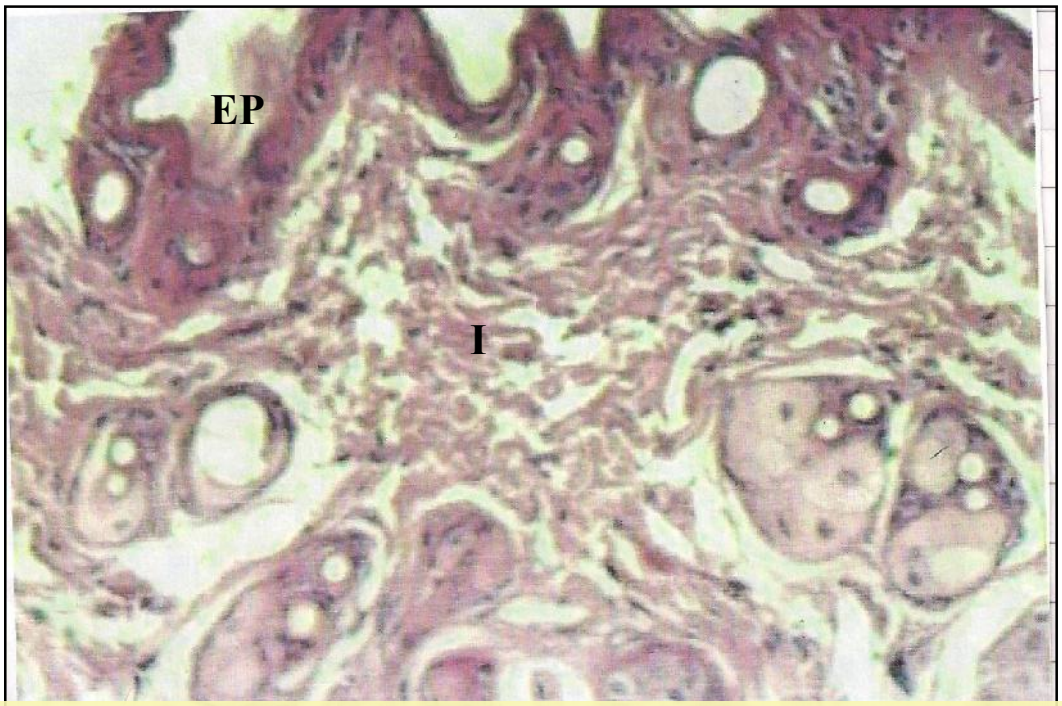
Figure (٣-١٩)Photomicrographs of 28days-old-lesion section stained with H and E stain 1and 2:*P.*ME .Ep: epidermis ,D:dermis, K:keratin tissue**.** HF: hair follicle **(**Magnification:200Xrespectively**).**

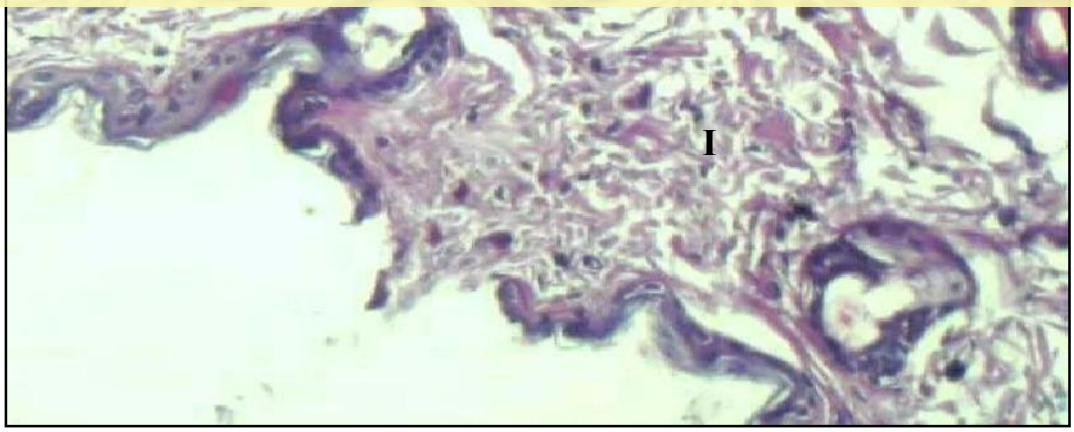




2

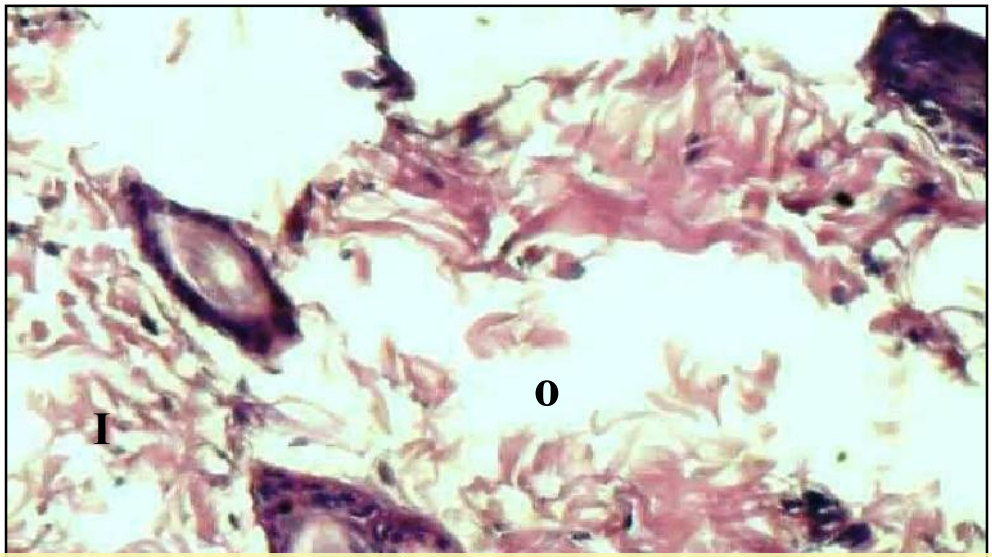
Figure(3-20)Photomicrograph of 28 days-old-lesion section stain with H and E stain 1and 2:*P.*ME . k:keratin tissue .Ep: epidermis ,D:dermis, S:sweet gland **(**Magnification: 200X**).**

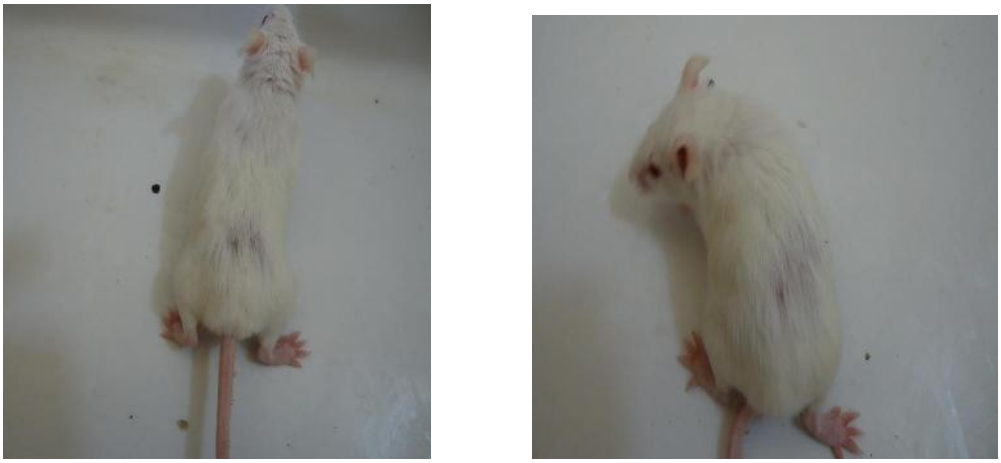




2

Figure(٣-2١)Photomicrographs of 28 days-old-lesion section stained with HandE stain 1:+control and 2:vehicle .EP: epidermis I:inflammatory cells .(Magnification: 200X)





Figure(3-23)Mice treated for 28 days with *P. spina-christi* methanolic extract

gene ,the gene was expressed in both non treated and treated *T.mentagrophytes* as shown in figure (3-25), this may be refer to several factors :-

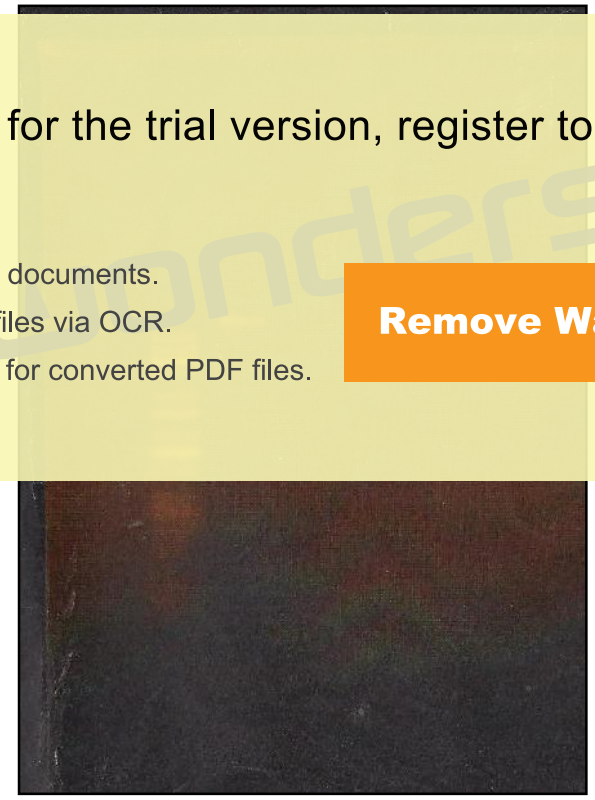
1-This gene expressed in media supplemented with salts, keratin and elastin as a sole source of carbon and nitrogen (Jousson *et al*,2004).

2-The primers were designed to detect the transcripts of several other candidates genes ,including the protease Tri m2 and Alp genes, metalloprotease Mep2 and Mep3 genes.

3-Tri m4 gave a strong signal which detected by RT-PCR from RNA isolated from cultures grown on blood plasma (Kaufman *et al*,2005).

The expression of the Tri m4 gene in presence of blood plasma with plant extract was confused they produce the same bands after 40 cycle of PCR. The combination of additives (glucose ,keratin and elastin) with plant extract doesn't recommended due to the effect of inhibition will be of little chance (Duke *et al*,2004).There must be an alternative way to regulate the expression of this gene without additives only the natural components of the fungal media e.g.(malt extract ,glucose ,peptone) then identified the effect of plant extract on the expression of this gene wither does it inhibit its expression or not?.

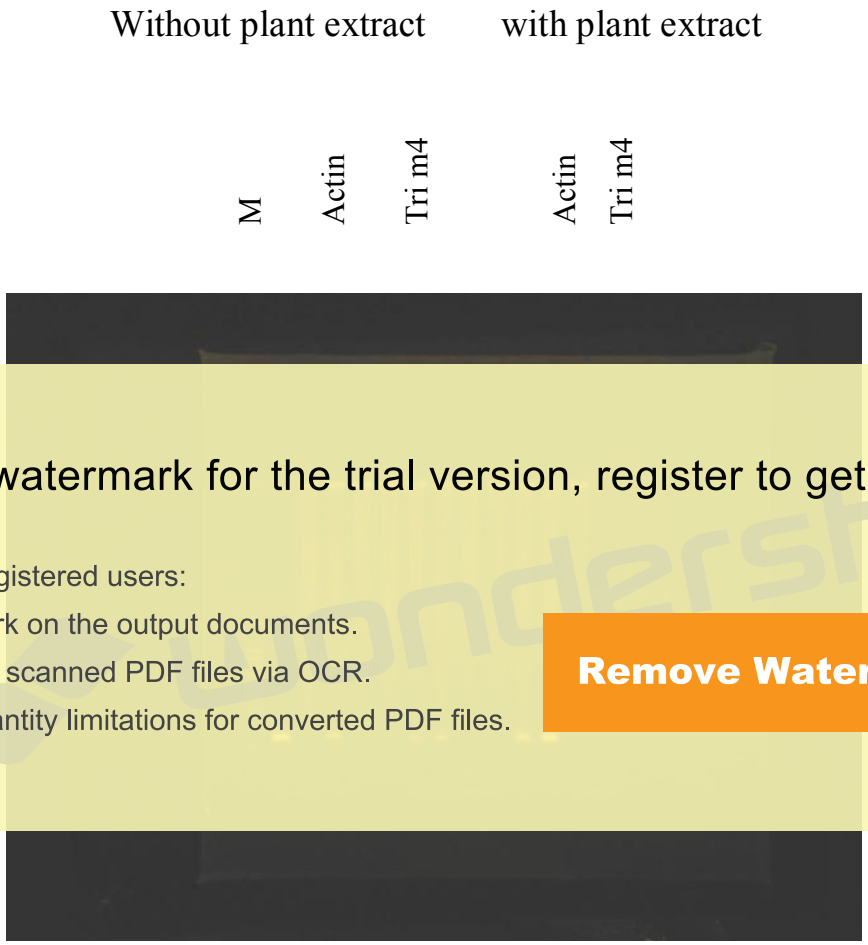
M 1



Figure(3-24) gel electrophoresis of *T. mentagrophytes* DNA Extract.

Lanes: M, molecular weight marker kappa (universal ladder), size range, (100-10000) Bp.

1: DNA Extract Of *T. mentagrophyte*(less than 10,000bp)



Figure(3-25)PCR Product of actin and Tri m4 gene of *T.mentagrophyte* species ,M:marker ,Actin gene,Tri m4 gene.

References

- **Abass,N. K**.(1995)Mycological and Bacteriological study of head and scalp lesions(Tinea capitis andImpetigo) .college of Medicine ,university of Baghdad.

- **Abu-Al-Basal, M**.(2001)The influence of some local medicinal plant extracts on skin wound healing activity ;evaluated by histological and ultra structural studies .faculty of graduate studies .university of Jordan .Ph.D in biological sciences

- **Adeleye I.A., Ogunniyi A.A., Omonigbehin E.A.,Antimicrobial** (200)activity of some local herbs on common skin pathogens ,

- **Agrawal A., S. Srivastava , J. N. Srivastava and M. M. Srivsava**(2004) Evaluation of Inhibitory effect of the Plant Phyllanthus Amarus on Hepatophytic

- **Agwa A.,M. Aly and R.Bonaly**(2000) Isolation and characterization of two Streptomyces species produced non polyenic antifungal agents. J.Union Arab Biol.,7:62-84.

- **Al-Abid, M. R**. (1985). Zur zusammem setrang der Absschlu Bmemtrame in phoenix dactily frawuzbury univ. Wurzburg F. R. of Germany.

- **Al-Ani, R.S.** (2006).Therapeutic activity of crud alkaloidal extract of *Fumaria officinalis* on dermatophyte(*Trichophyton rubrum)*,study by light microscope. MSc.Thesis, dept. of biotechnology, college of science ,University of Al-Nahrain.

- **Al-Farageen A.A**.(2007).Evaluation of ethanolic extract of *Mercurialis annua* L.(on skin wound healing of albino rats. MSc. thesis in biological science .Faculty of graduate studies .university of Jordan.
- **Ali,T. M**.(1990)Tinea capitis :clinical and mycological study ,MSc. In medical microbiology ,college of medicin ,University of Baghdad.
- **AL-Janabi A.A**.(1993)Tinea pedis clinic Epidemiological study of interdigital dermatophyte foot infection in Baghdad. Msc.in community Medicine .college of medicine.AL-Mustansirya univ.
- **Al-Jubori J.S**.(2005).Studying the efficiency of crude volatile oils extracted from some medicinal plants in inhibit some of pathogenic bacteria and dermatophytes. MSc.thesis ,Faculty of women education .Tikrit University.

- **Al-Kaisy ,K.S**.(1988)Election microscopic tudy of epethinal cells and their secretion activity in the thymus gland of mice after activation of growth hormone .MSc. Thesis .Department of Biology .Science college .Baghdad university.
- **AL-kayali, k.k.;w.m.Hassan and B.M.Razoogi**(2011)the rate of-Superficial fungal infections among patients with different skin diseases in Diyala Iraq.iraqi j.comm.Med.july.24(3):264-266.
- **Allen ,J.W.,Sheler,C.F.,Mendes,R.W. and Latt,S.A**.(1977).Asimplified technique for in vivo analysis of sister chromatid exchanges using 5-bromodepxy uridine tablets.Cell Genet.18:231-237.

- **Allen A.M and Taplin D.**(1973) Epidemic *Trichophyton mentagorphytes* infections in servicemen: source of infection , role of environment, host factors, and susceptibility. JAMA.ch.17; 226:864-867.
- **AL-Rawi,A.**(1988)Geographical disturibution of wiled type plant in Iraq.3rd E..Ministry of agricultural and water. (in Arabic)
- **AL-Rawi,A.M.,R.R.,Riyadh**.(1989).Modern chemistry of life.3rd Ed..ministry of higher education and scientific research printer.Baghdad.(in Arabic)
- **AL-sady A.A**(1995)some antibacterial and antifungal actions and -Myrtus cumis, M Sc.in clinical pharmacology, College of Medicine, university of Baghdad.
- Al-Sammaraie, B.A. (2008).Effect of Nerium oleander extract's on the growth of some fungi isolated from skin of patients in Samarraa city. MSc. Thesis: College of science. University of Tikrit.
- Al-Sammaraie K. W. Al-Rekabi,S. [illegible] leaves extract of Withania [illegible] dermatophyte.seconed conference in medical and biological science .Faculty of allied medical science .Zarka private University.:40
- **Al-Suhaili,S**. (2002). Antifungal activity of some plant extracts of Al-Mafraq area .Faculty of science and Arts. University of Al-albait.
- **Al-Taie, S**.(2001).The effect of plant extracts on dermatophytes growth isolated from patients diagnosed with dermatitis and on the growth of some bacteria.MSc. thesis .College of Education .University of Tikrit.
- **Aly MM,** (1997). Potancy of selected actinomycete for certain antifungal production. Ph. D. Thesis, Tanta University, Cooperation system between Egypt and France, p. 375.

- **Aly, A.A. , Omer S.A., Zayed S.M., and Mansour ,M.T**.(2000).Use of saponin containing a triplex nummularia to suppress damping of cotton seedling.J.Agric.Sci.Mansoura University.25:7621-7631.
- **Aly, M.M. and Bafiel. S**.(2008).Screening for antimicrobial activity of some medicinal plants in Saudi Arabia. World conference on medical and aromatic .
- **Ambrogi, L.P.** (1960). Manual of histologic and special staining teqniques. 2nd Ed..McGraw-Hill Book company.Inc. New York.
- **Amer S., M. Aly and S. Sabbagh**.(2006) Biocontrol of dermatophytes using some plant extracts and actinomycetes filtrates. Egyptian J. Biotechnol, p: 330-315. 2006.
- **Araujo C.R.;K.C.Miranda ,O.F.Fernandes; A.J.Soares and M.R.Silva**(2009)*In vitro* susceptibility testing of dermatophytes isolated in Goiania, Brazil against five antifungal agents by broth microdilution
- **AuVera K. , R.Mozuraityte and I.S Rustad** (2009)Antioxidant activity of phenolic acids in lipid oxidation catalyzed by different prooxidants .J.of Agricultural and food chemistry .21:10377-103.

Backs, Chapman and Hall, New York.

- **Bancroft, J. D. and Stevens, A**.(1982).Theory and practice of histological techniques. 2nd Ed. .Churchill Living Stone:483-516.
- **Baran W., J. Szepietowski and R. Schwartz**.(2004). Tinea barbae. Acta dermatoven. APA. 13.3: 91-94.
- **Bogdanova V.P**.(1963)Chemical composition of the cortex of plants .the host of lac insects. Izwestija akademii Nauk SSSR 28;605-612.

- **Bokhari F. M**.(2009) Antifungal activity of some medicinal plants used in Jeddah, Saudi Arabia. Mycopathology. 7(1). P:51-57.
- **Branter A., Z.Males, S.Pepeljnjak and A.Antolic**.(1996)Antimicrobial activity of *Paliurus spina-christi* Mill.j.of ethnopharnacology 52.p:119-122.
- **BranterA., Z. Males, S.Pepeljnjak and A.Antolic**(1996)Antimicrobial activity of *Paliurus spina-christi* Mill.j.of ethnopharnacology 52.p:119-122.
- **Brantner A. and Z. Males**(1990) Investigations on the flavonoid glycosides of Paliurus spina-christi planta 56p: 582.
- **Brantner A.H.and Z.Males** (1999)Quality assessment of Paliurus spina-christi extracts) Journal of Ethnopharmacologylogy ,66p:175-179.
- **Brook G. J.S.Butel and S.Morse**(1998) Jawetz's medical microbiology
- **Browenlee, H. E., A. R. McEuen , J. Hedger and I. M. Scott.**(1990) Antifungal effects of cocoa tannin on the witches' broom pathogen *Crinipellis perniciosa*. Physiol. Mol. Plant Pathol. 36:39-48.
- **Burdick, E. M**.(1971) Carpaine, an alkaloid of *Carica papaya*. Its chemistry and pharmacology. Econ. Bot. 25:363-365.
- **Butler, L. G**.(1988) Effects of condensed tannin on animal nutrition, p. 553. In R. W. Hemingway, and J. J. Karchesy (ed.), Chemistry and significance of condensed tannins. Plenum Press, New York, N. Y.
- **Capek,P., Machova, E. and Turjan, J**.(2009).Scavenging and antioxidant activities of immune modulating polysaccharides isolated from salvia officinalis.Int.J.of Biological micromolecules .44:75-80.

- **Cardellina ; J. H. I.;M. H. G. Munro; R. W. Fuller; K. P. Manfredi; T. C. McKee; M. Tischler; H, R. Bokesch; K. R. Gustafson; J. A. Beutler; and M. R. Boyd**(1993) A chemical screening strategy for the dereplication and prioritization of HIV-inhibitory aqueous natural products extracts. J. Nat. Prod. 56: 1123-1129.
- **Casley-Smith ; J. R. and J. R. Casley-Smith.** (1997) Coumarin in the treatment of lymphoedema and other high-protein oedemas. In R. O'Kennedy, and R.D. Thornes, (ed.), Coumarins: biology, applications and mode of action. John Wiley and Sons, Inc. , New York, N. Y. : 348
- **Chabot, S. R.; R. Bel-Rhlid ; R. Chenevert, and Y. Piche**(1992) Hyphal growth promotion in vitro of the VA mycorrhizal fungus, Gigaspora margarita Becker and Hall, by the activity of structurally specific flavonoid compounds und[illegible] Phytol. 122:[illegible]-467.
- **Chaurasia, S. C. and K. K. Vyas**(1977) *In vitro* effect of some volatile oil against *Phytophthora parasiyica* var. piperina. J. Res.
- **Chen, J., Yi, J., Liu ,L., Yin, S., Chen, R., Li, M., Congxin, Y., Zhaug, Y.and Lai, W**.(2010).Substrate adaptation of *T. rubrum* secreted endoproteases microbial Pathogenesis.48:57-61.
- **Cheng J.*et al.***(2000) Naunyn schmiedebergs Arch pharmacology. 362(2). P:122-127, by net.
- **Chermette R.; L. Ferreiro and J. Guillot**(2008) Dermatophytoses in animal . Mycopathologia, 166 :385-405.
- **Chithra P.; Sajithalal G.B. and d. G. Chandrakasan** (1998b)Influence of Aloe vera on collagen characteristics in healing dermal wounds in rats .Mol.cell.Bioch.181:71-76.

- **Chung, E., T.Cardena-frey and JD. ,Clements**(1999)The role of cAMP in mucosal adjuvenicity of *Escherichia coli* heat –labile enterotoxin(lt).Vaccine.18(1):38-49.
- **Clark,A.M**.(1996)Natural products as a resource for new drugs pharm.Res.13.
- **Collee,J.G.;A.G.Fraser ;B.P.Mariman and S.A.Simmons**(1996)Practical medical microbiology .MackiandMacCartney(Eds)14th (Ed).churchill Linvington.
- **Cowan M.M.** (1999)Plant products as antimicrobial agents.clinical microbiology reviews.12.4:564-582.
- **Dalakishvili, T. M. , S. D. Gusakova , N. J. Chachanidze, K. G. Kuparadze and E. P. Kemertelidze**(1985) (Lipids of *Paliurus spina- christi* seed). Khimija prirodnii soedinenii5,:322-326.
- **Dalakishvili,TS., Zurabishvili, M. , T.S. and, Kemertelidze,** .Khimija priroduii Soedinenii.5:639.
- **De Bievre, C., C. Dauguet, V.H.Nguyen and** *M.abrum* isolates from clinical specimens.Ann.Inst.Pasteur Microbiol.138:719-727.
- **Dermatophytes**.(2008).Wikipedia free encyclopedia . Wikimedia Foundation .Inc.
- **Duek,L.,Kaufman,Y.Ulman and I.Berdicevsky**(2004)The pathogenesis of dermatophyte infections in human skin sections .J.Infect.48:175-180.
- **Elewski B. E.** (1999) Tina barbae .Clinical dermatology ,Demis DJ(ed) .Pheladelphia , Lippincott Williams and Wilkins, unit 17-8. p:1-4.

- **El-Fari ,M.,H.J.Tietz,W.Presber, w.Sterry and Y.Graser**(1999)Development of an oligonucleotide probe specific for T.rubrum Br.J.Dermatol.14:240-245.
- **Ellis D.,S.Davis, H.Alexion,R.Hanke,R.Bartly**(2007)Description of medical fungi .2nd Ed.published by the auyhors,Mycology unit .women's and children's hospital north Adelaide, Australia.
- **Ercan, A., Saracli, M. A. , Ahmed, A., Taner, Y., Zafer, K., and Ali Riza, G**.(2004). Polymerase chain reaction in the diagnosis of on-ychomycosis. Eur. J.Dermatol.14:52-55.
- **Ewing, G.W.**(1987)Instraumental methods of chemical analysis.5th Ed. McGraw-Hill Book Company.New York
- **Faggi, E., Pini, G., Campisi E., Bertellini C., Difonzo E. and Mancianti, F.**(2001).Application of PCR to distinguish between species of dermatophytes.J.Clin.Microbiol.39:3382-3385.
- [illegible] of bioscience a[illegible] Strathelyde. Glascow.U.K.
- Fieser, L.F., and Williamson, K.L.(1983).Gas chromatography. Ch.11.Organic experiments .5th Ed.D.C.Health Eds.
- Freeman, B. and Burrows, A. (Eds). 21st edition, W. B. Saunders N., (1995). Reduction effect of theanine on blood pressure and brain .
- **Glauert,A.M**.(1975)Fixation,dehydration and embedding of biological specimens.Practical methods in electronmicroscopy,North-Holland publishing company ,Amsterdam :2207.
- **Graser,Y.,A.F.Kuijpers,W.Presber and G.S.DeHoog**(2000)Molecular and conventional taxanomy of the T.rubrum complex J.Clin.Miceobiol.38:3329-3336.

- **Grlic,L**.(1986).Enciklopedija Samoniklog Jestivog Bilja.August Cesaree,Zagreb,P.165.
- **Gupta A.K., J.Q. Del Rosso, C.W Lynde, G.H .Brown and N.H .Shear**(1998) Hepatitis associated with terbinafine therapy : three case reports and a review of the literature . Clin Exp Dermatol 23 p:64-67 .
- **Han,B.H. and Park,J.H**.(1989)Chemical and pharmacological studies on sedative cyclopeptide alkaloids in some Rhamnaceae plants .Pure Appl.Chem.61:443-448.
- **Harborne, J. B**.(1973), Phytochemical methods. Science paper Backs, Chapman and Hall, New York.
- **Harborne, J.B.** (Editor) (1984). Phytochemical Methods, 2nd Edition.Chapman and Hall, U.K., pp. 37-99.
- **Hasegawa A**.(2000)Dermatophytes in animals Nippon Ishinkin Gakkia Zahi.41:1-4.
- **Hsiao,C.R.,L.Huang, J.P.Bouchara,R.Barton,H.C.Li and T.C.Chang**(2005)Identification of [illegible] oligonucleotide array. J.Clin.Microb[illegible]
- **Havesteen, B**.(1983).Flavonoids, a class of natural products of high pharmacological potency. Biochemical. Pharmacology. 32.7:1142-1148.
- **Heggers,J**.P.,A.,Kucukelebi,C.J.Stabenan,F.Broemelling AND Robenson,M.C.(1995).Wound healing effects of Aloe vera gel and other topical antibacterial agents on rat skin .J.Phytoth.Res.9:455-457.
- **Heggers,J**.P.,Pelley ,R.P. and Robenson,M.C.(1993).Beneficial effect of Aloe vera in wound healing .J.Phytother.Res.7:548-562.
- **Heywood,V**.H.,T.G.TutinN.A.Burges,D.M.More,D.H.Valentine,S.M. Walters,D.A.Webb.(1968)Flora Europeae.Cambridge university press.

- **Holder, I. A. and Boyce, S. T**.(1994). Agar well diffusion assay testing of bacterial susceptibility to various antimicrobials in concentrations non-toxic for humans in culture. Burns 20.5.p:426-429.
- **Hu C.Q. and Chen, Z**.(1997).Sesquiterpenoid alcohols from Chrysanthummorifolium.phytochemistry,44:1287-1290.
- **Hubbard, T.W**.(1999).The predictive value of symptoms in diagnosing childhood Tinea capitis. Arch. Pediatr. Adolesc. Med. 153:1150-1153.
- **Hube,B., Mondo, M., Schofield, D.A., Brown, A.J. and Goow, N.A**.(1994).Expression of 7 members of the gene family encoding secretory aspartyl proteinases in *Candida albicans* .Mol.Mol.Microbiol.,14:87-99.
- **Hudson, L. and Hay, F**.(1989) Practical Immunology .Blackwell well scientific publication p:116.
- **Irvin T.T**.(1984)Wound healing principles and practice chapman and Halls .New york.
- **Issa, A**. (1981)Nomes des plantes.2nd Ed. Bierut –Lebanon.
- **Jessup C.T. ,J.Warner,N.Isham *etal*.**,(2000)Antifungal susceptibility testing of dermatophytesnn:establishing a medium for inducing conidial growth and evaluation of susceptibility of clinical isolates .J.Clin.Microbiol.38:341-344.
- **Jousson, O.*etal*.**(2004) Multiplication of an ancestral gene encoding secreted fungalysin preceded species differentiation in the dermatophytes *Trichophyton and Micrpsporum*. Microbiology .150p: 3001-3010.

- **Kakuda, T., Nozawa, A., Unno, T., Okamura, N.and Okai, O**.;(2000) Inhibiting effects of theanine on caffeine stimulation evaluated by EEG in the rat) .Bioscience Biotechnology and Biochemistry 64: 287–293.
- **Kanbe T.,Y.suznki, A.kamiya,T.Mochizuki,M .fujihior and A.Kikuchi** (2003)PCR-based identification of common dermatophyte sp.(Primer sets specific for the DNA topoisomerase π genes.j.Dermatol.sci.32:151-161
- **Kano R., K.Okabayashi, y.Nakamura, S.ooka .kashima ,M. Mizoguchi ,S. Watanabe and A. Hasegawa**(2000)Differences among chitin synthase1 gene sequences in *T. rubrum* and T.violaceum.med.Mycol.38:47-50.
- **Kaufman ,G.;I.Berdicevsky ,JA.Woodfolk and B.A.Horwitz**(2005)Markers for host-induced gene expression in Trichophyton dermatophytosis 73.[illegible]
- **Kawasaki M. , Aoki, M**[illegible] K.(1996).Phylogeny of epidermophyton floccosum and other dermatophytes. Mycopathologia. 134:121-128.

- **Ke-yue L**.(2011)Technology optimization of polysaccharide extraction from *Cyclocarya paliurus* leaves by ultrasonic assistance .International conference on new technology of agricultural :1125-1128.
- **Khalifa K. A., E.Al-Kasser, J.K.AL-Diwan and F.H.AL-Lami**(2011)Prevalence of Tinea capitis among primary schoolchildren in Baghdad governarate ,Iraq.Iraqi J.Comm.Med.jan.(1):9-12.
- **Kubo I. , Ping X. and Kenichi F**.(2000) Antifungal activity of octyl gallate :structural criteria and mode of action bioorganic and medicinal chemistry letters .v.11:347-350.

- **Kurihara, H., F. Harukazu, K., Aki,T., Yoshiko, S.Hiroshi, M.Yokichi ,A.,Sumio, and T.Takaharu.**(2003).Hypoglycemic action of *Cyclocarya paliurus* (Batal)Ijinskaja in normal and diabetic mice.Biosci.Biotechnol.Biochem.67.4:877-880.
- **Kustark, D., Z. Males, A. Brantner, I.Pitarevic.(**1990).Flavonoids of the leaves of christi's thorn (*Paliurus spina-christi*). Acta pharmaceutica jugoslovica .40:551-554.
- **Laurence D.R., D.N., Bennett, and Brown. M.J.(**1997).Clinical pharmacology. 8th Ed. Churchill living Stone.
- **Leaw ,S .N., Chang, H. C. , Sun, H .F. , Barton , R., Bouchara , J .P. and Chang, C.** (2006).Identification of medically important yeast species by sequence analysis of the internal transcribed spacer regions .J.Clin.Microbiol.,44:693-699.
- **Lee, S.S., Su., W.C., Karin, C.S. and [illegible] C.**(200[illegible]).Cyclopeptide alkaloids [illegible] stems [illegible] Phytochemistry.58:1271-1276.
- **Lilian C. B., Alexandre, M.B., Clayton, L.B., Maristela, P. and Célia., M.S**. (2007).cDNA representational difference analysis used in the identification of genes expressed by *Trichophyton rubrum* during contact with keratin, Microbes and Infection.9:1415-1421.
- **Lin H. y., C. H. Chen, B. J. You, K. C. Lin and S. S. Lee**(2008) Cyclopeptide alkaloids from *Paliurus ramossisimus*, J. Natural product, 63 p:1338-1343.
- **Lin H. y., Chen, C. H. , You, B. J. , Lin, K. C., Lee, S. S**.(2008). Cyclopeptide alkaloids from *Paliurus ramossisimus*, J. Natural product. 63 p:1338-1343.

- **Lin, H.Y., Chen, C.H. , You, B.J. , Liu, K.C., , Lee, S.S**.(2000).Cyclopeptide alkaloids from *Paliurus ramosissimus*. J. N.at. Prod.63:1338-1343.
- **Lin, R.L., Szepietowski, J.G., Schwartz, R. A**.(2004). Tinea faciei an often deceptive facial eruption.Int. J. Dermatol. 43.
- **Liu D, Coloe S. ;Baird R. and Pedersen J.**(2000)Application of PCR to the identification of dermatophyte fungi .J Med Microbiol.49:493-497.
- **Mackie,R.M**.(1983)Clinical dermatology an illustrated text book.Oxford university press.London
- **Makimura K, Mochizuki T, Hasagawa A, Uchida K and Yamaguchi H**.(1998)Phylogenetic classification of Trichophyton mentagrophytes complex strains based on DNA sequences of nuclear ribosomal internal transcribed spacer 1 regions. J Clin Microbiol.36:2629-2633.

gawa,K.Uchida,and H.Yamaguchi.(2001)Cluster analysis of human and animal pathogenic *Microsporum* species and their teleomorphic states ,Arthroderma species ,based on the DNA sequences of nuclear ribosomal internal transcribed spacer 1 regions.J.Clin.Microbiol. Immunol.45:209-216.

- **Males, Z**.(1995).Structural characterization of the isolated flavonoids of christi's thorn (*Paliurus spina-christi*). Thesis. Faculty of pharmacy and biochemistry.University of Zegreb.
- **Marchand ,J., Pais, M.,Monseur,X. and Jarreau,F.X**.(1969)Peptide alkaloids .VII. Lasiodine A and B,alkaloids from Lasiodiscus marmoratus. Tetrahedron 25,937-954.

- **Masoko, P**.(2007).Characterization of antifungal compounds isolated from combretumand Terminalia species. Ph.D. Thesis in paraclinical science. Faculty of Vet. Sci. University of Pretoria.
- **Mescher, A.L.(**2010).Skin.ch8.Junquira'sbasic histology.text and atlas .12th Ed.. McGraw Hill.Eds..U.S.A..
- **Moaz M. and Neeman I**.(1998)Antimicrobial effects of aqueous plant extracts on the fungi *M.canis* and *Trichophyton rubrum* on three bacterial species .Lett.Appl.Microbial.,26:61-63.
- **Mochizuki, T., Sugie, N. and Vehara, M**.(1997).Random amplification of polymorphic DNA is useful for the differentiation of several anthropophilic dermatophytes. Mycoses ,40:405-409.
- **Moerman,D.E**.(1996)Analysis of the food plants and drug plants of
- **Mohammed K. Y**.(2004)Topical
- **Mondello F. ,De Bernardis F. , Girolamo A *et al.*,**(2003) *In vitro* and *in vivo* activity of tea tree oil against azole-susceptible and -resistant human pathogenis yeasts, J Antimicrob Chemother ,51:1223-9.
- **Mosaddegh M. , M. J. Khoshnood, M.Kamalinejad and E. Alizadeh** (2004) Study on the effect of *Paliurus spina-christi* on Cholesterol, Triglyceride and HDL Levels in Diabetic Male Rats Fed a High Cholesterol Diet, Iranian Journal of Pharmaceutical Research 3:51-54.

- **Muchuweti M.,Zenda G.,Ashwell R.and Abisha K**.(2005)Sugars ,organic acid and phenolic components of *ziziphus mauritiana* fruit .Eur food res technol.221:570-574.
- **Mustafa, P.L**.(2009)Epedimeological and diagnostic study of dermatophytes in Kirkuke city and the effect of onion extract on isolated fungi.College of science univ. of Tikrit, MSc. In Biological science.

Mycology , by McGinnis, M. R. (ed). Vol. 1 , Springer verlage .

- **Natarajan V.,P.Venugopal and T.Menon**(2003)Effect of azadirachta indica(neem)on the growth pattern of dermatophytes .Indian J.Med.Micro.21:98-100.
- **National committee for clinical laboratory standards (NCCLS)**(2000)Methods for dilution antimicrobial usceptibility tests for bacteria that grow aerobically .Approved standard [illegible] ed NCCL [illegible] M7-A5.
- **Nejad [illegible] and Dorfule [illegible] S.**(200[illegible] [illegible] dryopteria quercifolia L..Jundishapur.J. [illegible]
- **Nwachukwu, E.O.,and Umechuruba,V**.(2006).Antifungal activities of some leaf extracts on seed-borne fungi of African yam bean seeds.seed germination and seedling emergenc.
- **Omer, S.A., and Abd-Elhalim,V**.(1992).Fungal growth response to alfalfa (Medicago sativa L.) saponin in extract .Egyptian J. Appl.Sci.,7:24-32.
- **Paundir R.K. and Jain P**.(2010)Antifungal activity of twenty ethanolic plant extracts against food associated fungi .J of pharmacy research.3(3).
- **Peacock, E**.(1984).Wound repair.3rd Ed..W.B.Saunders Co.. ,Philadeliphia. USA., :38-55.

- **Pina-Vaz C., A.Gon, Rodrigues and E.Pinto** ***et al***. (2004)Antifungal activity of thymus oils and their major compounds, J Eur Acad Dermatol Venereol, 18: 73-8.
  - **Polunin, O. and Huxley A.**(1981).Blumen am Mittelmer verlagsgesells chaft.Munchen.Wien and Zurich:157.
- **Ramar P. S. and P. Gopalakrishnakone** (2010)Therepeutic Potential of Plants as Anti-microbials for Drug Discovery. http://ecam.oxfordjournals.org by on October 19.
- **Rawat R. B. and R. C. Uniyal**(2003) National Medicinal plants Board committed for overall development of the sector. Agro Bios, Med. Plants, 1: 12-6.
- **Rhods J.C., B.G. Oliver, D.S. Askew and T.W.Amlny**(2001)Identification of genes of A.fumigatus up regulated during growth on endothelial cells. Med Mycol.39:253-260.
- **Rippon J. W.**(1985) The changing ep[illegible] [illegible]ophyte species. In : Curre[illegible] actinomycetes. In : Textbook of Microbiology , by Inc., New York, p: 208 – 229.

- **Roitt's I. M. and J.P. Delves**(2001)Essential Immunology .10th Ed.,by Blackwell science Ltd.USA.
- **Rossmando E.(1987)**Applications to the assay of enzymatic activity ch. In HPLC in enzymatic analysis.Eds Jhon Willey and sons .A wiley inter science publications.
- **Rushforth K. D.** (1999)Trees of Britain and Europe, ISBN.
- **Saadabi. A.**(2006) Antifungal activity of some Saudi plants used in traditional medicine. Asian J. Plant Sci., 5. p :907-909.

- **Samy, R.P., and Gopalakrishnakone, P.** (2010).Therepeutic Potential of Plants as Anti-microbials for Drug Discovery . http://ecam.oxfordjournals.org by on October 19.
- **SAS**. (2004). SAS. Statistical Analysis System, User's Guide. Statistical. Version 7th ed. SAS. Inst. Inc. Cary. N.C. USA.
- **Sharma K. K., R. Saikia, J. Kotoky, J. C. Kalita and R. Dive**(2011) Antifungal activity of *solanum melongena* L. *Lawsonia inermis* L. and *Justicia gendarussa* B. against Dermatophytes pharmatech.3.3. p :1635-1640.
- **Shelf ,L.A., Jyothi,V and Bulgarelli,V**.(1984).Effect of sage on growth of enteropathogenic and spoilage bacteria in sage-containing broths and foods. J. Food Sci.,49:737-740.
- **Shimizn K., Ozeki,M;Lino,A, Nakajyo,S.,Urakawa,N. and Atsuchi,M**.(2001)Structure-activity relation ships of triterpenoid derivatives extracted from gymnema inodorum leaves on glucose absorption.Jpn.J.pharmacolog,86,p:223-229.
- **Shmitt, M.E.,Brown,T.A.,Rumpower,B.L.(**1990).Arapid and simple method for preparation of RNA from Saccharomyces cervisiae.Nucl.Acid Res.,18.10:3091-3092.
- **Skoog A.D. and D.M.West**(1969)Fundementals of analytical chemistry .Holt ,Rinehart and Winston(Ed.),2nd Ed.U.S.A.
- Skoog,**A.D.,D.West, D.,James Haller, F.,Crouch, S.P**.(2004). Fundementals of Analyical chemistry

- **Smolenst, J. J., Silinis, H. and Faranssnoth, N. R.** (1972). Alkaloid screening coyadia, 35:11-34.
- **Soylu,E.M.,Toki,F.M.,Soylu,S. and Kaya,A.D.(**2005).Antifungal activities of essential oils on post harvest disease agent *Pencillium digitatum*.Pak.J.Biol.Sci.8:25-29.
- **Stahle, E**(1969). Thin layer chromatography a laboratory hand book, 2nd ed. , translated by Ashworth, M. Spring-verag,
- **Suguna L., Sivakuman, P. and Chandrakasan,V**.(1996).Effects of centella asiatica extract on dermal wound healing in Rats.Ind.J.Exp.Biol.,34:1208-1211.
- **Terashima, T.,Takido, J. and Yokogoshi, H.** (1999) Time-dependent changes of amino acids in the serum, liver, brain and urine of rats administered with theanine. Bioscience, Biotechnology and Biochemistry 63: 615–618.
- **Tsang P., T., Hopkins,V.,Jimenez-Lucho**(1996)Deep [illegible] dermatophytosis caused by T.rubrum in a patient with AIDS.V.34 NO.6:1090-1091.
- **Tsu[illegible] W., Z. Y., C. Blomquist a[illegible]** [illegible] variations among common air[illegible] specific oligonucleotide probes for the detection of fungal isolates .Appl .Environ. Microbiol.69:5389-5397.
- **Udupa S.L.,A.L. Udupa and D.R. Kalkarni**(1991)Influence of Tridax procumbens on lysyl oxidase activity and wound healing .Planta Med.57:325-327.
- **Ulubelen,A.,G.Topcu,N.Tan,S.Olcal,C.Johansson, M.Ucer,H.Birman and S.Tamer**(1995).Biological activities of a Turkish medicinal plant prangos platycchlaena.J. of Ethnopharmacology.45:193-197.
- **Velcheva M.P**.(1993).Constituents of *Paliurus spina christi*.Fitoterapia.,46:284-285.
- **Venugopal, P.V. and Venugopal, T.V**.(1995)Antidermatophytic activity of garlic (*Allium sativum*) invitro.Int.J.Dermatol.34:278-289.
- **Vlietinck,A.J., L.Van Hoof, J.Totte,A.Laure, D.Vanden,P.Berghe and C.Rwangabo**(1995)screening of hundred Rwandese medicinal

plants for antimicrobial and antiviral properties .J.Ethnopharmacol.46:1-72.

- **Washington W.Jr, Allen, S.,Janda W., Koneman, E., Procop, G., Schreckenberger, P. and Woods, G**. (2006).Color Atlas and text book of diagnostic microbiology ,Lippincott Williams and wilkins(Ed.),6th Ed.Baltimore.U.S.A.
- **Weitzman I. and M.Crist**(1980)Studies with clinical isolates of cunninghamella.11.Physiological and morphological studies .Mycologia 72:661-669.
- **Weitzman I., M.R.McGinnis, A.A.Padhye, and L.Ajello**(1986)The genus arthroderma and its later synonym Nannizzia.Mycotaxon.25:505-509.
- **Weitzman, I., and [illegible] R.** (1996).The dermatophytes.Clin.Microbiol.Rev.,8:240-259.
- **Xie,J.H.,Ming-Young,X.,Ming-Yue,S.,Shao-Pin,N.,Chang, L. and Yuan-Xing,W**.(2010).Optimization of microwave –assisted extraction of polysaccharides from Cyclocarya paliurus (Batal)lljinskaja using response surface methodology.J.Sci.Food.Agric.90:1353-1360.
- **Yoshida, M., Fuchigami, M., Nagao, T., Okabe,H.etal.,**(2005). Antiproliferative constituents from Umbellieferae plants VII.Active triterpenes and rosmarinic acid from centella aciatica.Biol.Pharmacol.Bull.28:173-175.

- **Yunis,H**.(2007).The effect of Garlic extract on dermatophytes growth and the keratinized activity of the fungus .MSc.thesis .college of medicine .univ. of Tikrit. (in Arabic)
- **Zaug,C., Monod, M., Weber, J., Harshman, K., Pradervand, S., Thoma, J.etal**.(2009).Gene expression profiling in the human pathogenic dermatophyte T. rubrum during growth on proteins.Eucaryot cell.8:241-250.
- **Zhang, W., Yu, L., Yang, J. , Wang, L. , Peng, J. & Jin, Q.** (2009). Transcriptional profiles of response to terbinafine in *Trichophyton rubrum*. Appl Microbiol Biotechnol 82: 1123-1130.

Conclusions:

Results obtained in this study are established

The ethanolic extract contain numbers of biologically active compounds detected by preliminary test these compounds are (flavonoids ,phenols ,alkaloids,tannines,saponines).

2. The HPLC analysis of alcoholic extract revealed the presence of the following phenolic compounds (Gallic acid ,syringic acid ,caffeic acid and epichotechin).
3. Gas –Mass chromatograph analysis of alcoholic extract revealed the presence of numbers of biologically active compounds such as(sugars, amino acids, fatty acids ,phenols).
4. Age groups (20-30)years old shown the highest frequency among the patients group.
5. Maximum growth inhibition was statistically significant at 125µg/ml for both methanolic and ethanolic extract as compared to control group.
6. Stimulates the regeneration of the lesion ,skin hair , contraction and epithelialization.
7. Fibroblasts were more prominent in the developing granulation tissues of incision wound treated with PME at early stages of wound days 7-14 and less prominent in the developing granulation tissues at late stage (day 14), in

comparison to -ve control, reference drug, and vehicle groups , indicating faster healing process.

3. The applications of nucleic acid amplification technology to make a rapid precise identification of dermatophytes such as (restriction fragment length polymorphism (RFLP),random amplification of polymorphic DNA(RAPD),PCR finger printing .

الإهداء

إلى والدي المرحوم والمغفور له الذي

رباني وعلمني

إلى المستقبل الذي طالما حلمت به

لمى

Acknowledgement

I am extremely thankful to **Prof. KhuloodW.AL-Sammarae**, my supervisor, who always found time to assist me, and encouraged me to do the best, making continuous and fruitful discussion and directing me to the proper way.

My deepest and special thanks to my co-supervisor **Prof. Nabeel Khalaf AL-Ani**, for his valuable advice and help during this study.

Thanks are extended to the members of the committee for their helpful criticism. Also, my gratitude is to **Prof. Salim R.**, from whom I learned so much, for spending time in reading the thesis and being member of the committee.

I owe my special thanks to Assistant prof. Dr. Basem Jaber ,faculty of science ,university of Jordan for supporting my work in the department and for always having an open door and time for discussion.

I would like to express my appreciation to prof. Dr. Ahmed Al-Kaisi ,faculty of Science ,University of Jordan for his support .

My sincere regards to all instructors in Dhafar , who allowing me to finish my work in the college, microbiology laboratory.

My great thanks are extended to Mr. Muneer Khadhoor , Electron Microscope unit ,Al-Yarmook university, Jordan. for allowing me to photograph the studied slides in his lab . My sincere gratitude and deepest appreciation is to my greatest loving family, particularly my father(Mercy on him) ,and mum who gave me full encouragement and support. Without their prayers and guidance this project would not have been completed. Thanks to my sisters whom always I find them when I need. Finally, my appreciations also extend to Mrs.Mayada and Mrs,Dhafar , for their love, helping and sharing me in beautiful moments as well as suffering ones. Also, I would like to thank my friends and colleagues Heba Jarar, Amani Harb .

Conclusions:

Results obtained in this study can establish the claimed wound healing activity for *Paliurus spina-christi* that is used in folk medicine in the North of Iraq . This is supported by the following

1. The alcoholic extract content numbers of biologically active compounds detected by preliminary test these compounds are (flavonoids ,phenols ,alkaloids,tannines,saponines).
2. The HPLC analysis of alcoholic extract revealed the presence of the following phenolic compounds (Gallic acid ,syringic acid ,caffeic acid and epichotechin).
3. Gas –Mass chromatograph analysis of alcoholic extract revealed the presence of numbers of biologically active compounds such as(sugars, amino acids, fatty acids ,phenols).
4. Direct KOH mount smear showed the presence of fungal elements while dermatophytes were positive in(32.27%)of 55 cases (42.1%). was *T. mentagrophytes* species and
5. Age groups (20-30)years old shown the highest frequency among the patients group
6. Maximum growth inhibition was statistically significant (12 mm) for both methanolic and ethanolic extract as compared to control group.
7. Stimulates the keratinocyte lesion ,
8. fibers,regeneration of sweet glands and hair follicles were observed in PME in comparison to -ve control, reference drug, and vehicle groups.
9. Fibroblasts were more prominent in the developing granulation tissues of incision wound treated with PME at early stages of wound days 7-14 and less prominent in the developing granulation tissues at late stage (day 14), in comparison to -ve control, reference drug, and vehicle groups , indicating faster healing process.

Recommendations:

1. Determination of the chemical structure of phenolic extract of *Paliurus spina-christi* using NMR technique.
2. Study the immunological effects of *Paliurus spina-christi invitro* and *invivo*.
3. study the Genotoxic and cytotoxic effect of active components of *Paliurus spina-christi* .
4. Study the molecular effects of *Paliurus spina-christi* extract on cancer cells.
5. The applications of nucleic acid amplification technology to make a rapid precise identification of dermatophytes such as (restriction fragment length polymorphism (RFLP) random amplification of polymorphic DNA (RAPD) PCR fingerprinting

Chapter one

Chapter Two

Literature Review

Chapter three

Materials

Chapter Four

Results

and

Chapter Five

Conclusion

List of Contents

Itm No.	Title	Page
Chapter two:Materials and methods		
2.1	**Materials**	25
2.1.1.	**Equipments and Apparatus**	25
2.2	**Methods**	27
2.2.1	**Collection of *Paliurusspina-christi***	27
2.2.2	**Plant extraction**	28
2.3	**Extracts preparation**	28
2.4	***In vitro* antifungal evaluations**	30
2.5	**Detection of some active compounds**	31
2.5.1	**Detection of tannins**	31
2.5.2	**Detection of Saponins**	32
2.5.3	**Detection of terpenes and steroids**	32
2.5.4	**Detection of flavonoids**	32
2.5.5	**Detection of Alkaloids**	33
2.5.6	**Detection of flavonoids by TLC**	33
2.6	**HPLC**	34
2.7	**Gas chromatograph mass spectrometer**	35
2.8	**Isolation of the dermatophytes species**	35
2.8.1	**Sampling of the dermatophytes**	35
2.9	**Lab animals**	36
2.9.1	**Experiment**	36
2.9.2	**Sporesuspension preparation**	37
2.10	**Histopathological study**	37
2.10.1	**Light Microscopic examination**	37
2.10.2	**Preparation for transmission electron microscope**	40

List of tables

List of figures

List of Abbreviations

µl	Micro liter
BSTFA	Drevatizing reagent
cDNA	Complementary DNA
EDTA	Ethylene diamine tetra acetic acid
ETOH	Ethanol
µg	Micro gram
H & E	Hematoxilin and Eosin
HPLC	High performance liquid chromatography
Ig	Immunoglobulin
MeOH	Methanol
*P.*ME	Paliurusmethanolic extract
Pb	Base pair
PCR	Polymerase chain reaction
SDS	Sodium dodecyl sulfate
SUB	Subtilicin gene
TEB	Tris buffer
TFA	
Tr m4	*Trichophytonmentagrophytes* 4 gene
Tr r4	*Trichophytonrubrum 4* gene
U.V.	Ultraviolet

Supervisors Certification

We, certify that this thesis entitled "**Histopathological Study on the Antifungal Activity of *Paliurus spina-christi* Active Compounds and Assessing the Molecular Mechanism of their Antifungal Activity**"

was prepared by "**LumaT.Ahmed**" under our supervision at the College of Science/ Al-Nahrain ,University as a partial fulfillment of the requirements for the Degree of Doctorate of Science in Biotechnology.

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

Signature:

Name: Dr. Majid Hussain AL-Jelawi

Scientific Degree: Professor

Title: Head of Biotechnology Department

Date: /7/2012

Summary

Fifty five samples of patients with skin infections by dermatophytes have been studied during the period from June 2010 to September 2010.Eighteen isolates were isolated. The most common species were *Trichophyton mentagrophytes*, *Trichophyton rubrum*, and *Microsporum gypsum* .The relation between dermatophytes infections and the age and gender of patients were studied .The first group age (20-40 years old)showed the highest rate of infections .Tinea corporis was the most common one. The percentage of infection with *Trichophyton mentagrophytes* was(13.63%) for male and(22.72%) for female *Trichophyton rubrum* percentage was (12.5%) for male only. This was followed by *Microsporum gypsum*(23.53%) detected in male and female .The clinical examination revealed that tinea corporis was most common among other tineas such as tinea cruris,tin

High performance liquid chromatography(HPLC) analysis of methanolic extracts of *Paliurus spina-christi* revealed the presence of Gallic acid,caffeic acid,syringic acid & epicotichen acid , while Gas chromatography(GC) revealed a wide range of chemical compounds (36).They were varied in types (sugar,amino acids ,phenols, fatty acids and esters).The *Paliurus spina-christi* methanolic extract was significantly the most effective in comparison with the ethanolic extract ,125µg/ml of *Paliurus spina-christi* methanolic extract was the most effective in inhibiting the mycelial growth of *T.mentagrophytes* .The mean diameter of *T. mentagrophytes* growth after incubation for 7 days in media treated with 125µg/ml of *Paliurus spina-christi* methanolic extract concentration was (1.5±0.03)cm. This effect was statistically

significant(P<0.05),as compared with the control group whose mean diameter was (8.3 ±0.87)cm .On the other hand ,the mean diameter of *T.mentagrophytes* growth after incubation for 7 days in medium treated with125µg/ml of ethanolic *Paliurus spina-christi* extract was (2.4±0.09). This effect was statistically significant(P<0.05) as compared with the control group which recorded (8.3±0.87).The ethanolic extract of *spina-christi* had a moderate effect in comparison with the methanolic extract at concentration 125 µg/ml on growth inhibiting .Clinical features of mice skin infected with *T. mentagrophytes* were characterized by redness ,swelling , regular margin with ulceration and loss of hair. The light microscopic study had shown certain degenerative changes in the infected area. These changes included edema with heavy and acute inflammatory cells in the dermis area which may extend to hypodermis causing abscess .Infected mice treated with *Paliurus spina-christi* methanolic extract showed the disappearance of redness and hair regeneration after 7 days leaving [illegible] treatment of Clotrnazole-P ,where healing was characterized by slower regenerative changes and the incision area was less in comparison with the area treated with *Paliurus spina-christi* methanolic extract.

Light microscopic study of the infected area after 14 days revealed that the moderate inflammatory cells were present in the dermis along with a normal epidermis with its common structures in comparison to the other groups (+ve and vehicle)which demonstrated the moderate healing after21 days of treatment :keratin tissue ,sweat glands, hair follicle regeneration in the dermal layer. Healing progress was more observed in the treated skin section after 28 days of treatment ;complete healing was observed with the absence of inflammatory

cells,edema in contrast with the abundance of collagen and fibroblast cells in the dermis layer with sweat glands and hair follicles .

The electron microscope study revealed that the normal shape of the epidermal layer with the dermal layer showed the melanocytes regeneration to its normal shape and size .The molecular assessment was characterized by evaluating the effect of methanolic extract on Tri m4 gene expression .The study found that the methanolic extract at concentration (125µg/ml) did nothave any effect on the expression of this gene; its expression was the same in the absence and presence of *P.spina-christi* extract by using PCR.

الملخص

تم جمع (٥٥) عينة من مرضى مصابين بفطريات جلدية للفترة من حزيران ٢٠١٠ الى ايلول ٢٠١٠، وجرى عزل وتشخيص ١٨ عزلة فطرية وكانت أكثرا لأنواع هي *Trichophyton mentagrophytes* و *Trichophytonrubrum* و *Microsporum gypsum*.

تم دراسة العلاقة بين الإصابات الفطرية بالجلد وتبين أن ،الأعمار بين (٢٠-٤٠) سنة هي الأكثرعرضة للإصابة.الإصابات الموجبة كانت،سجلت الإصابةبالفطر , (*Trichophytonmentagrophytes*) ١٣.٦٣)% للذكور وبالنسبة للاناث كانت النسبة (٢٢.٧٢)% واما إصابات الفطر *Trichophyton rubrum* كانت النسبة للذكور (١٢.٥)% ولم تسجل أصابات للإناث تبعها نسبة *Microsporum gypseum* (٢٣.٥٣)%للذكور وللإناث . وأظهر الفحص السريري أن سعفة الجسم كانت أكثر الأنواع شيوعا بين الأنواع الأخرى مثل سعفة الفخذ، سعفة الشعر ،سعفة الأظافر وسعفة اليدين) .وبين الفصل الكيميائي باستخدام جهاز الـ HPLC ان المركبات الفعالة للمستخلص الكحولي لنبات الـ *P.spina-christi* (Gallic acid, caffeicacid,syringic acid &epicotichen acid) ونسبة مساحة %(area)

ماده كيميائية تتوعت بين (سكريات واحماض دهنية واحماض امينية واسترات) وان المستخلص الميثانولي أكفأ معنويا بالمقارنة مع المستخلص الكحولي وبلغ التركيز ١٢٥ مايكروغرام /مل من نبات *P. spina-christi* بنسبة تثبيط(٣.٧٧± ٨٢ %) هذا التثبيط أكثر فعالية من باقي التراكيز بمستوى (P≤0.05) ولكلا المستخلصين .متوسط قطر نمو الفطر (*T.mentagrophytes*) بعد سبعة أيام من الحضن في وسط معامل ب ١٢٥ مايكروغرام /مل من المستخلص الميثانولي لنبات *P.spina-christi* كان (٠.٠٣ ±١.٥) سم . وهذا التأثير كان معنويا بمستوى (P≤0.05) بالمقارنة مع مجموعة السيطرة والتي كان متوسط نمو الفطر عندها هو (٠.٨٧±٨.٣)سم من جهة ثانية متوسط نمو الفطر*T.mentagrophytes* بعد سبعة أيام من ألحظن في وسط معامل ب١٢٥ مايكروغرام /مل من المستخلص الكحولي لنبات *Paliurus spina-christi*كان(٠.٠٩ ±٢.٤) وهذا التأثير معنوي بمستوى (P≤٠.٠٥)بالمقارنة مع مجموعة السيطرة التي سجلت (٠.٨٧±٨.٣).ومستوى التثبيط سجل (٧١±٢.٩٣) حيث كان معنويا بمستوى (P≤0.05).

المستخلص الكحولي لنبات *Paliurus spina-christi* كان ذا تأثير متوسط بالمقارنة مع المستخلص الميثانولي على تركيز ١٢٥مايكروغرام /مل في تثبيط النمو .وتميزت الصفات السريرية لجلد الحيوانات المصابة *T.mentagrophytes* بالاحمرار وانتفاخ موضع الإصابة و حافة غير منتظمة وتساقط للشعر.فيما يخص فحص المجهر الضوئي بين أن هناك تغيرات تنكسية واضحة في المناطق المصابة وهذه التغيرات تتمثل بالاستسقاء مع تركيز عالٍ من الخلايا الالتهابية في منطقة البشرة وتمتد إلى منطقة تحت البشرة وتكون الخراج.

كذلك بينت الدراسة إن الحيوانات المصابة والمعالجة ب المستخلص الميثانولي تميزت باختفاء الاحمرار وظهور الشعر بعد سبعة أيام من ترك ندبة بيضاء وتكون مضغوطة على طول حافتها.المنطقة المخدوشة من قبل والمصابة بالفطر كانت مغطاة بشكل كامل بطبقة فوق البشرة الجديدة والشعر كان واضحا بالمقارنة مع المنطقة المصابة في مجموعة الحيوانات المعاملة بالعقار Clotrinazole-P والذي

ولوحظ التقدم بالشفاء لوحظ في مقاطع الجلد المعاملة بعد ٢٨ يوماً حيث كان شفاء تاما مع اختفاء للخلايا الالتهابية والاستسقاء على العكس لوحظ غزارة في الكولاجين والفايبروبلاست في طبقة فوق البشرة مع الغدد العرقية والبصيلات الشعرية.

وفيما يخص المجهر الالكتروني أظهر الفحص الشكل الطبيعي لطبقة البشرة وفوق البشرة مع تولد خلايا الميلانين بشكلها وحجمها الطبيعي.أما دراسة تأثير المستخلص الميثانولي على تعبير جين Tri m4فلم يكن له تأثير على التعبير الجيني لهذا الجين وباستعمال أعلى تركيز (١٢٥ مايكروغرام/مل) من المستخلص الميثانولي حيث تتابع الجين ظهر بنفس ما هو عليه في خلايا الفطر النامي بوسط خالٍ من المستخلص الميثانولي بما يؤكد عدم تأثره بالمستخلص.

Chapter one

Introduction

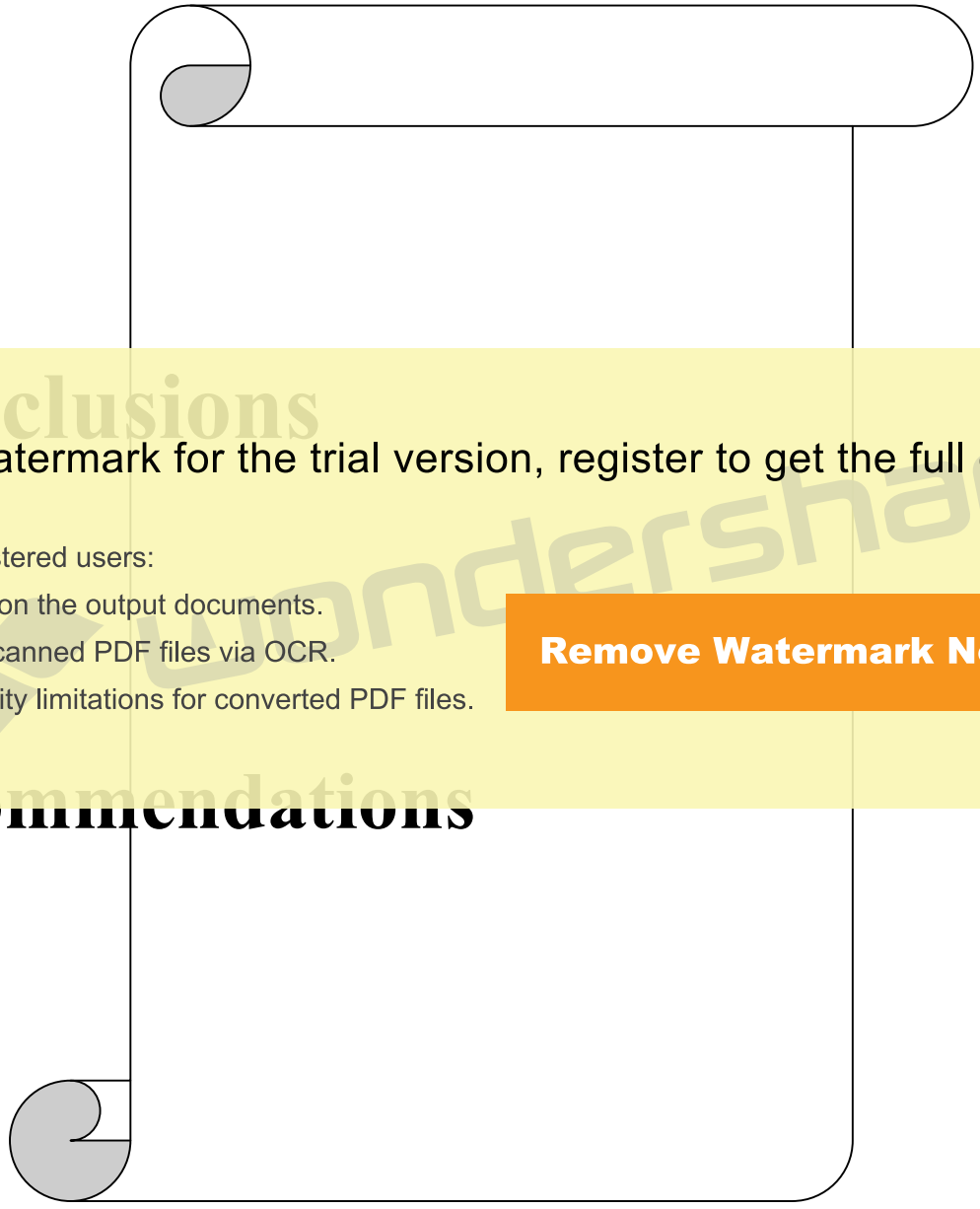
Chapter Two

Methods

Chapter three

Results and Discussion

Chapter Four



Conclusions and Recommendations

Republic of Iraq
Ministry of Higher Education
and Scientific Research
Al-Nahrain University
College of Science

Detection of some active compounds in *Paliurus spina-christi* plant :their histopathological effect and molecular mechanism on dermatophytes

LumaTaha Ahmed

B.Sc.Biotechnology, Al-Nahrain University,1999
M.Sc.Biotechnology, Al-Nahrain University,2001

Supervised By

Dr.Khulood W. Al-Sammarrae — Professor

Dr. Nabil K.Al-Ani — Assistant Professor

July2012

Shaaban 1432

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة النهرين

كلية العلوم

قسم التقانة الإحيائية

الكشف عن بعض المواد الفعالة للنبات *Paliurus spina-christi* وتأثيراته النسيجية والجزيئية على الفطريات الجلدية

بكالوريوس تقانة احيائية،جامعة النهرين،١٩٩٩
ماجستير تقانة احيائية,الجامعة النهرين،٢٠٠٢

بإشراف

د.نبيل خلف العاني
أستاذ مساعد

د.خلود وهيب السامرائي
أستاذ

شعبان ١٤٣٢
تموز٢٠١٢

بسم الله الرحمن الرحيم

(وقل رب زدني علماً)

Supervisors Certification

We, certify that this thesis entitled "**Detection of some active compounds in *Paliurus spina-christi* plant:their histopathological effect and molecular mechanism on dermatophytes**"

Was prepared by "LumaTaha Ahmed" under our supervision at the College of Science, Al-NahrainUniversity as a partial fulfillment of the requirements for the Degree of Doctorate of Philosophy in Biotechnology.

Signature:

Supervisor:**Dr.Khulood W. Al-Sammarrae**

Scientific Degree: Professor

Date:/ /2012

Signature:

Supervisor : **Dr. Nabil K.Al-Ani**

Scientific Degree: Assistance professor

Date:/ /2012

In view of the available recommendations, I forward this dissertation for debate by the examining committee.

Signature:

Name: Dr. Majid Hussein Al-Jailawi

Title: Head of Biotechnology Department

Date: / /2012

Committee Certification

We the examining committee certify that we have read this dissertation entitled " **Detection of some active compounds in *Paliurus spina-christi* plant:their histopathological effect and molecular mechanism on dermatophytes** " an examined the student **Luma Taha Ahmed** in this content and that in our opinion, it is accepted for the Degree of Doctorate of philosophy in Biotechnology.

Chairman:
Signature:
Name:
Scientific:
Date:

(Member)
Signature:
Name:
Scientific Degree:
Date:

(Member)
Signature:
Name:
Scientific Degree:
Date:

I ,Hereby Certify upon the decision of the examining committee.

Signature:
Name: Khulood W. AL-Sammarrae
Scientific degree: Professor
Title: Dean of college of science.

Conclusions:

Results obtained in this study can establish the claimed wound healing activity for *Paliurus spina-christi* that is used in folk medicine in the North of Iraq . This is supported by the following

1. The alcoholic extract content numbers of biologically active compounds detected by preliminary test these compounds are (flavonoids ,phenols ,alkaloids,tannines,saponines).
2. The HPLC analysis of alcoholic extract revealed the presence of the following phenolic compounds (Gallic acid ,syringic acid ,caffeic acid and epichotechin).
3. Gas –Mass chromatograph analysis of alcoholic extract revealed the presence of numbers of biologically active compounds such as(sugars, amino acids, fatty acids ,phenols).
4. Direct KOH mount smear showed the presence of fungal elements while dermatophytes were positive in(32.27%)of 55 cases (42.1%). was *T. mentagrophytes* species and
5. Age groups (20-30)years old shown the highest frequency among the patients group
6. Maximum growth inhibition was statistically significant (12 mm) for both methanolic and ethanolic extract as compared to control group.
7. Stimulates the keratinocyte lesion ,
8. fibers,regeneration of sweet glands and hair follicles were observed in PME in comparison to -ve control, reference drug, and vehicle groups.
9. Fibroblasts were more prominent in the developing granulation tissues of incision wound treated with PME at early stages of wound days 7-14 and less prominent in the developing granulation tissues at late stage (day 14), in comparison to -ve control, reference drug, and vehicle groups , indicating faster healing process.

Recommendations:

1. Determination of the chemical structure of phenolic extract of *Paliurus spina-christi* using NMR technique.
2. Study the immunological effects of *Paliurus spina-christi invitro* and *invivo*.
3. study the Genotoxic and cytotoxic effect of active components of *Paliurus spina-christi* .
4. Study the molecular effects of *Paliurus spina-christi* extract on cancer cells.
5. The applications of nucleic acid amplification technology to make a rapid precise identification of dermatophytes such as (restriction fragment length polymorphism (RFLP) random amplification of polymorphic DNA (RAPD) PCR fingerprinting

Chapter one

Chapter Two

Literature Review

Chapter three

Materials

Chapter Four

Results

and

Chapter Five

Conclusion

List of Contents

Itm No.	Title	Page
Chapter two:Materials and methods		
2.1	**Materials**	25
2.1.1.	**Equipments and Apparatus**	25
2.2	**Methods**	27
2.2.1	**Collection of *Paliurusspina-christi***	27
2.2.2	**Plant extraction**	28
2.3	**Extracts preparation**	28
2.4	***In vitro* antifungal evaluations**	30
2.5	**Detection of some active compounds**	31
2.5.1	**Detection of tannins**	31
2.5.2	**Detection of Saponins**	32
2.5.3	**Detection of terpenes and steroids**	32
2.5.4	**Detection of flavonoids**	32
2.5.5	**Detection of Alkaloids**	33
2.5.6	**Detection of flavonoids by TLC**	33
2.6	**HPLC**	34
2.7	**Gas chromatograph mass spectrometer**	35
2.8	**Isolation of the dermatophytes species**	35
2.8.1	**Sampling of the dermatophytes**	35
2.9	**Lab animals**	36
2.9.1	**Experiment**	36
2.9.2	**Sporesuspension preparation**	37
2.10	**Histopathological study**	37
2.10.1	**Light Microscopic examination**	37
2.10.2	**Preparation for transmission electron microscope**	40

List of tables

List of figures

List of Abbreviations

µl	Micro liter
BSTFA	Drevatizing reagent
cDNA	Complementary DNA
EDTA	Ethylene diamine tetra acetic acid
ETOH	Ethanol
µg	Micro gram
H & E	Hematoxilin and Eosin
HPLC	High performance liquid chromatography
Ig	Immunoglobulin
MeOH	Methanol
*P.*ME	Paliurusmethanolic extract
Pb	Base pair
PCR	Polymerase chain reaction
SDS	Sodium dodecyl sulfate
SUB	Subtilicin gene
TEB	Tris buffer
TFA	
Tr m4	*Trichophytonmentagrophytes* 4 gene
Tr r4	*Trichophytonrubrum 4* gene
U.V.	Ultraviolet

Supervisors Certification

We, certify that this thesis entitled "**Histopathological Study on the Antifungal Activity of *Paliurus spina-christi* Active Compounds and Assessing the Molecular Mechanism of their Antifungal Activity**"

was prepared by "**LumaT.Ahmed**" under our supervision at the College of Science/ Al-Nahrain ,University as a partial fulfillment of the requirements for the Degree of Doctorate of Science in Biotechnology.

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

Signature:

Name: Dr. Majid Hussain AL-Jelawi

Scientific Degree: Professor

Title: Head of Biotechnology Department

Date: /7/2012

Summary

Fifty five samples of patients with skin infections by dermatophytes have been studied during the period from June 2010 to September 2010.Eighteen isolates were isolated. The most common species were *Trichophyton mentagrophytes*, *Trichophyton rubrum*, and *Microsporum gypsum* .The relation between dermatophytes infections and the age and gender of patients were studied .The first group age (20-40 years old)showed the highest rate of infections .Tinea corporis was the most common one. The percentage of infection with *Trichophyton mentagrophytes* was(13.63%) for male and(22.72%) for female *Trichophyton rubrum* percentage was (12.5%) for male only. This was followed by *Microsporum gypsum*(23.53%) detected in male and female .The clinical examination revealed that tinea corporis was most common among other tineas such as tinea cruris,tin

High performance liquid chromatography(HPLC) analysis of methanolic extracts of *Paliurus spina-christi* revealed the presence of Gallic acid,caffeic acid,syringic acid & epicotichen acid , while Gas chromatography(GC) revealed a wide range of chemical compounds (36).They were varied in types (sugar,amino acids ,phenols, fatty acids and esters).The *Paliurus spina-christi* methanolic extract was significantly the most effective in comparison with the ethanolic extract ,125µg/ml of *Paliurus spina-christi* methanolic extract was the most effective in inhibiting the mycelial growth of *T.mentagrophytes* .The mean diameter of *T. mentagrophytes* growth after incubation for 7 days in media treated with 125µg/ml of *Paliurus spina-christi* methanolic extract concentration was (1.5±0.03)cm. This effect was statistically

significant(P<0.05),as compared with the control group whose mean diameter was (8.3 ±0.87)cm .On the other hand ,the mean diameter of *T.mentagrophytes* growth after incubation for 7 days in medium treated with125µg/ml of ethanolic *Paliurus spina-christi* extract was (2.4±0.09). This effect was statistically significant(P<0.05) as compared with the control group which recorded (8.3±0.87).The ethanolic extract of *spina-christi* had a moderate effect in comparison with the methanolic extract at concentration 125 µg/ml on growth inhibiting .Clinical features of mice skin infected with *T. mentagrophytes* were characterized by redness ,swelling , regular margin with ulceration and loss of hair. The light microscopic study had shown certain degenerative changes in the infected area. These changes included edema with heavy and acute inflammatory cells in the dermis area which may extend to hypodermis causing abscess .Infected mice treated with *Paliurus spina-christi* methanolic extract showed the disappearance of redness and hair regeneration after 7 days leaving [illegible] treatment of Clotrnazole-P ,where healing was characterized by slower regenerative changes and the incision area was less in comparison with the area treated with *Paliurus spina-christi* methanolic extract.

Light microscopic study of the infected area after 14 days revealed that the moderate inflammatory cells were present in the dermis along with a normal epidermis with its common structures in comparison to the other groups (+ve and vehicle)which demonstrated the moderate healing after21 days of treatment :keratin tissue ,sweat glands, hair follicle regeneration in the dermal layer. Healing progress was more observed in the treated skin section after 28 days of treatment ;complete healing was observed with the absence of inflammatory

cells,edema in contrast with the abundance of collagen and fibroblast cells in the dermis layer with sweat glands and hair follicles .

The electron microscope study revealed that the normal shape of the epidermal layer with the dermal layer showed the melanocytes regeneration to its normal shape and size .The molecular assessment was characterized by evaluating the effect of methanolic extract on Tri m4 gene expression .The study found that the methanolic extract at concentration (125µg/ml) did nothave any effect on the expression of this gene; its expression was the same in the absence and presence of *P.spina-christi* extract by using PCR.

الملخص

تم جمع (٥٥) عينة من مرضى مصابين بفطريات جلدية للفترة من حزيران ٢٠١٠ الى ايلول ٢٠١٠، وجرى عزل وتشخيص ١٨ عزلة فطرية وكانت أكثرا لأنواع هي *Trichophyton mentagrophytes* و *Trichophytonrubrum* و *Microsporum gypsum*.

تم دراسة العلاقة بين الإصابات الفطرية بالجلد وتبين أن ،الأعمار بين (٢٠-٤٠) سنة هي الأكثرعرضة للإصابة.الإصابات الموجبة كانت،سجلت الإصابةبالفطر , (*Trichophytonmentagrophytes*) ١٣.٦٣)% للذكور وبالنسبة للاناث كانت النسبة (٢٢.٧٢)% واما إصابات الفطر *Trichophyton rubrum* كانت النسبة للذكور (١٢.٥)% ولم تسجل أصابات للإناث تبعها نسبة *Microsporum gypseum* (٢٣.٥٣)%للذكور وللإناث . وأظهر الفحص السريري أن سعفة الجسم كانت أكثر الأنواع شيوعا بين الأنواع الأخرى مثل سعفة الفخذ، سعفة الشعر ،سعفة الأظافر وسعفة اليدين) .وبين الفصل الكيميائي باستخدام جهاز الـ HPLC ان المركبات الفعالة للمستخلص الكحولي لنبات الـ *P.spina-christi* (Gallic acid, caffeicacid,syringic acid &epicotichen acid) ونسبة مساحة %(area)

ماده كيميائية تتوعت بين (سكريات واحماض دهنية واحماض امينية واسترات) وان المستخلص الميثانولي أكفأ معنويا بالمقارنة مع المستخلص الكحولي وبلغ التركيز ١٢٥ مايكروغرام /مل من نبات *P. spina-christi* بنسبة تثبيط(٣.٧٧± ٨٢ %) هذا التثبيط أكثر فعالية من باقي التراكيز بمستوى (P≤0.05) ولكلا المستخلصين .متوسط قطر نمو الفطر (*T.mentagrophytes*) بعد سبعة أيام من الحضن في وسط معامل ب ١٢٥ مايكروغرام /مل من المستخلص الميثانولي لنبات *P.spina-christi* كان (٠.٠٣ ±١.٥) سم . وهذا التأثير كان معنويا بمستوى (P≤0.05) بالمقارنة مع مجموعة السيطرة والتي كان متوسط نمو الفطر عندها هو (٠.٨٧±٨.٣)سم من جهة ثانية متوسط نمو الفطر*T.mentagrophytes* بعد سبعة أيام من ألحظن في وسط معامل ب١٢٥ مايكروغرام /مل من المستخلص الكحولي لنبات *Paliurus spina-christi*كان(٠.٠٩ ±٢.٤) وهذا التأثير معنوي بمستوى (P≤٠.٠٥)بالمقارنة مع مجموعة السيطرة التي سجلت (٠.٨٧±٨.٣).ومستوى التثبيط سجل (٧١±٢.٩٣) حيث كان معنويا بمستوى (P≤0.05).

المستخلص الكحولي لنبات *Paliurus spina-christi* كان ذا تأثير متوسط بالمقارنة مع المستخلص الميثانولي على تركيز ١٢٥مايكروغرام /مل في تثبيط النمو .وتميزت الصفات السريرية لجلد الحيوانات المصابة *T.mentagrophytes* بالاحمرار وانتفاخ موضع الإصابة و حافة غير منتظمة وتساقط للشعر.فيما يخص فحص المجهر الضوئي بين أن هناك تغيرات تنكسية واضحة في المناطق المصابة وهذه التغيرات تتمثل بالاستسقاء مع تركيز عالٍ من الخلايا الالتهابية في منطقة البشرة وتمتد إلى منطقة تحت البشرة وتكون الخراج.

كذلك بينت الدراسة إن الحيوانات المصابة والمعالجة ب المستخلص الميثانولي تميزت باختفاء الاحمرار وظهور الشعر بعد سبعة أيام من ترك ندبة بيضاء وتكون مضغوطة على طول حافتها.المنطقة المخدوشة من قبل والمصابة بالفطر كانت مغطاة بشكل كامل بطبقة فوق البشرة الجديدة والشعر كان واضحا بالمقارنة مع المنطقة المصابة في مجموعة الحيوانات المعاملة بالعقار Clotrinazole-P والذي

ولوحظ التقدم بالشفاء لوحظ في مقاطع الجلد المعاملة بعد ٢٨ يوماً حيث كان شفاء تاما مع اختفاء للخلايا الالتهابية والاستسقاء على العكس لوحظ غزارة في الكولاجين والفايبروبلاست في طبقة فوق البشرة مع الغدد العرقية والبصيلات الشعرية.

وفيما يخص المجهر الالكتروني أظهر الفحص الشكل الطبيعي لطبقة البشرة وفوق البشرة مع تولد خلايا الميلانين بشكلها وحجمها الطبيعي.أما دراسة تأثير المستخلص الميثانولي على تعبير جين Tri m4فلم يكن له تأثير على التعبير الجيني لهذا الجين وباستعمال أعلى تركيز (١٢٥ مايكروغرام/مل) من المستخلص الميثانولي حيث تتابع الجين ظهر بنفس ما هو عليه في خلايا الفطر النامي بوسط خالٍ من المستخلص الميثانولي بما يؤكد عدم تأثره بالمستخلص.

Chapter one

Introduction

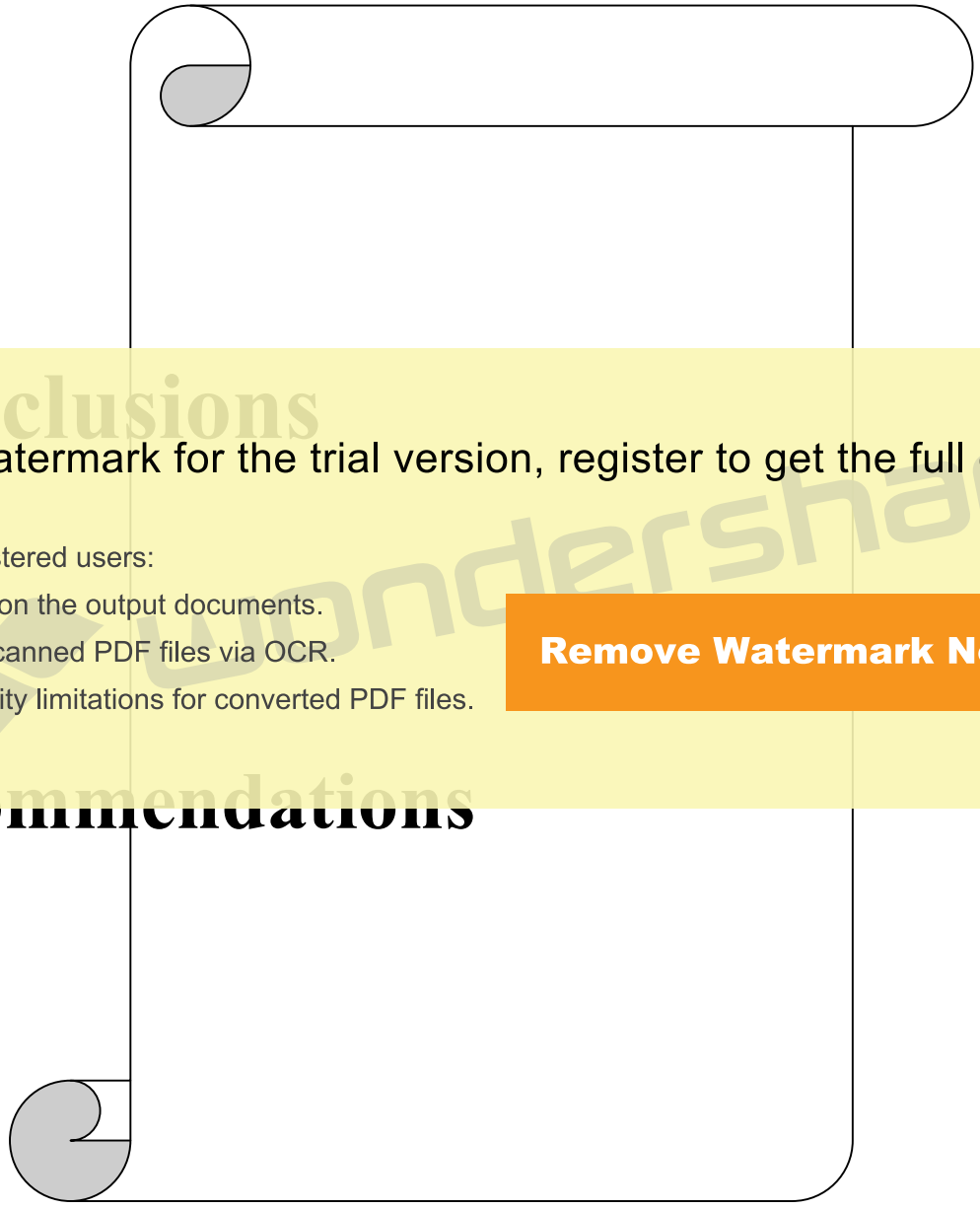
Chapter Two

Methods

Chapter three

Results and Discussion

Chapter Four



Conclusions and Recommendations

Republic of Iraq
Ministry of Higher Education
and Scientific Research
Al-Nahrain University
College of Science

Detection of some active compounds in *Paliurus spina-christi* plant :their histopathological effect and molecular mechanism on dermatophytes

LumaTaha Ahmed

B.Sc.Biotechnology, Al-Nahrain University,1999
M.Sc.Biotechnology, Al-Nahrain University,2001

Supervised By

Dr.Khulood W. Al-Sammarrae — Professor

Dr. Nabil K.Al-Ani — Assistant Professor

July2012

Shaaban 1432

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة النهرين

كلية العلوم

قسم التقانة الإحيائية

الكشف عن بعض المواد الفعالة للنبات *Paliurus spina-christi* وتأثيراته النسيجية والجزيئية على الفطريات الجلدية

بكالوريوس تقانة احيائية،جامعة النهرين،١٩٩٩
ماجستير تقانة احيائية,الجامعة النهرين،٢٠٠٢

بإشراف

د.نبيل خلف العاني
أستاذ مساعد

د.خلود وهيب السامرائي
أستاذ

شعبان ١٤٣٢
تموز٢٠١٢

بسم الله الرحمن الرحيم

(وقل رب زدني علماً)

Supervisors Certification

We, certify that this thesis entitled "**Detection of some active compounds in *Paliurus spina-christi* plant:their histopathological effect and molecular mechanism on dermatophytes**"

Was prepared by "LumaTaha Ahmed" under our supervision at the College of Science, Al-NahrainUniversity as a partial fulfillment of the requirements for the Degree of Doctorate of Philosophy in Biotechnology.

Signature:

Supervisor:**Dr.Khulood W. Al-Sammarrae**

Scientific Degree: Professor

Date:/ /2012

Signature:

Supervisor : **Dr. Nabil K.Al-Ani**

Scientific Degree: Assistance professor

Date:/ /2012

In view of the available recommendations, I forward this dissertation for debate by the examining committee.

Signature:

Name: Dr. Majid Hussein Al-Jailawi

Title: Head of Biotechnology Department

Date: / /2012

Committee Certification

We the examining committee certify that we have read this dissertation entitled " **Detection of some active compounds in *Paliurus spina-christi* plant:their histopathological effect and molecular mechanism on dermatophytes** " an examined the student **Luma Taha Ahmed** in this content and that in our opinion, it is accepted for the Degree of Doctorate of philosophy in Biotechnology.

Chairman:
Signature:
Name:
Scientific:
Date:

(Member)
Signature:
Name:
Scientific Degree:
Date:

(Member)
Signature:
Name:
Scientific Degree:
Date:

I ,Hereby Certify upon the decision of the examining committee.

Signature:
Name: Khulood W. AL-Sammarrae
Scientific degree: Professor
Title: Dean of college of science.