Eumycetoma and Plant-based Pharmacotherapy Candidates

By:

Hassabelrasoul Elfadil Hassan Abdelgadir

B.Pharm, University of Khartoum (1991)

M.Pharm, University of Gezira (2005)

A Thesis
Submitted in Fulfillment of the Requirements of the Degree of Doctor of Philosophy in

Pharmaceutical Microbiology

Department of Pharmaceutics

Faculty of Pharmacy

University of Gezira

Main supervisor: Prof. Elhadi Mohamed Mohamed Ahmed

Co-supervisor: Prof. Ahmed Hassan Fahal

Dr. Wendy Wilhelmina Johanna van de Sande

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Eumycetoma and Plant-based Pharmacotherapy Candidates
Hassabelrasoul Elfadil Hassan Abdelgadir

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- Date of Examination: Thursday 18/ April/ 2013
To the memory of my life supervisor and mentor

Professor: Asim Farouk Mustafa

To the memory of my father

Ostaz: Elfadil Hassan Abdelgadir Elsaeed

To the memory of my mother

Haja: Mahasin Ahmed Eljizouli

Everyone in life is destined to leave life with an incomplete project in mind,
today I am fulfilling one of my life-time dreams

To my wife Shaza and children: Elfadil, Hiba and Eltayeb

For making everything possible and worthwhile
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Eumycetoma and Plant-based Pharmacotherapy Candidates
Hassabelrasoul Elfadil Hassan Abdelgadir
Doctor of Philosophy in Pharmaceutical microbiology
Date of examination: Thursday 18/April /2013
Department of Pharmaceutics, Faculty of Pharmacy, University of Gezira

Abstract

Mycetoma is a chronic, inflammatory, granulomatous infection that is present worldwide and endemic in tropical and subtropical regions, caused by the traumatic inoculation of a fungus (eumycetoma) or a bacterium (actinomycetoma). Sudan is famous for being an endemic area of mycetoma and may be the homeland of the disease with Gezira state of central Sudan, being the focal point. Medical treatment of eumycetoma by conventional antifungal therapies is quite disappointing. Surgical debridement may be necessary, and amputation is sometimes the only solution. This study was an attempt to find a safe cure of natural origin. The methanolic extracts of seven selected local plants were screened for their antimycetomal activity using the Microbroth Dilution method. All seven plant extracts were able to inhibit Madurella mycetomatis growth at a concentration of 50 µg/ml or less, point to an important property that for the first time to be disclosed. Three plant species out of the seven managed to inhibit the growth of Madurella mycetomatis at a concentration as low as 0.781 µg/ml; these were: Boswellia papyrifera, Acacia nubica and Nigella sativa. MICs of these extracts and /or fractions on 13 mycetoma strains were determined using the standard cellular viability XTT-assay. Results revealed that almost all extracts and fractions exhibited antifungal activity (MIC-50 = 1-4 µg/ml). The methanolic extract from the gum resin of Boswellia papyrifera exhibited the best potential anti-mycetoma activity. Boswellia extract was fractioned using different organic solvents of variant polarities, and was then biological tested; results showed presence of good anti-mycetomal activity in the crude methanol and the ethyl acetate fraction. Gas-Liquid-Chromatography hybrid Mass-Spectrophotometer (GC-MS) analysis of these fractions; showed the occurrence of four phytoconstituents, triterpenoid in nature: Beta-amyrin, Beta-amyrone, Beta-Sitosterol and Stigmatriene. Biological evaluation of these triterpenes against 12 Madurella mycetomatis strains revealed that each individual triterpene phytoconstituent had some anti-mycetoma activity, but Stigmatriene was the best to show anti-mycetoma activity at an MIC equal to 32 µg/ml. In conclusion these results are quite encouraging for further evaluation of crude extracts and the identified isolates which represent an important class of drugs that have potential for clinical use for prevention and treatment of eumycetoma and other invasive fungal infections.
النبت الفطرى الأسود والمعالجة الدوائية المستنبطة من النباتات الطبية

حسب الرسول الفاضل حسن عبد القادر

دكتوراة الفلسفة في الأحياء الدقيقة الصيدلانية

الخميس 18 / أبريل 2013

شعبة الصيدلانيات، كلية الصيدلة، جامعة الجزيرة

الخلاصة

مرض النبت الفطرى الأسود هو عبارة عن عدوى حبيبية تسبب إلتهاباً مناعياً، يوجد في كل بقاع العالم بتركيز أعلى في المناطق المدارية وشبه المدارية. يدخل الفطر للجسم عبر فتحات في الجلد مسببًا المرض. أثبتت التجارب السريرية عدم جدوى المعالجة بمضادات الفطريات التقليدية للنبت الفطرى، وبدأت في التدخل الجراحي، مثل الحف بجل الكويت والأخوات في كل من الأحيان إلى بتر العضو المصابة. هذه الدراسة تهدف إلى إيجاد طريقة مناعية للمعالجة مستندة إلى المنتجات الطبيعية. تم اختبار سهولة نباتات طبية محلية وعمل استقصاء للمستخلص الميثانولي لمكوناتها ضد فطر (مادوريلا مايسيتوميتس). ثبت أن الدراسة الأولية قدرة المستخلصات السبع في تثبيط نمو الفطر عند تركيز أدنى 0.5 ميكروغرام/مل. أما، مما يشير إلى حاجة مهنية تدقيق الدراسات الأولية.

เอكتشافها لأول مرة في تاريخ الطب الحديث، تحت دعم المصادر الشهيرة، تمكن الكويت من تثبيط الفطر عند مستوى تركيز مثبط أدنى يصل إلى 0.781 ميكروغرام/مل. هذه النباتات هي: اللبان، اللعوت، والكمون الأسود. وvette لا تحديد التركيز المثبط أدنى لهذه المستخلصات الثلاث (والتي تم استنفها إلى سهولة مستخلصات باستخدام مذيبات ذات قطبية مختلفة). استخدمت في ذلك طريقة التعداد الحيوي الحاجة، بعد تخفيف المركض. أظهرت النتائج أن كل هذه المستخلصات ومشتقاتها تملك قدرة تثبيط الفطر عند تركيز مثبط أدنى يصل إلى 1 و 4 ميكروغرام/مل.

وأن المستخلص الميثانولي لنبات اللبان، يملك القدرة الأعلى في التثبيط، على ذلك تأثير مستخلص اللبان إلى أربعة أقسام باستخدام مذيبات مختلفة. أظهرت النتائج أن الفعال للنبات يكمن في المستخلص الميثانولي الخام ومشتقات الإيثايل أسيتيت. تبين ذلك تحليلاً كيميائياً للمكونات باستعمال مذيبات ذات قطبية مختلفة. استخدمت في ذلك طريقة التعداد الحيوي، بعد تخفيف المركض. نتجت النتائج أن كل هذه المستخلصات ومشتقاتها تملك قدرة تثبيط الفطر عند تركيز المثبط أدنى يصل إلى 0.5 ميكروغرام/مل.

من النتائج، نستطيع أن نتبني أن النباتات والنباتات النباتية التي تم الفحص عليها، يمكن أن تكون مفيدة في علاج مرض انتلاج ومريض الأمراض الفطرية المتعددة الأخرى.

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Chapter one

Introduction and Literature Review
1. Introduction and Literature Review

1.0 General Background:
Mycetoma or maduromycosis is a chronic, specific, granulomatous, progressive inflammatory, disfiguring and mutilating disease; it usually involves the subcutaneous tissue, most probably after traumatic inoculation of the causative organism (Fahal, 2006). Mycetoma may be caused by true fungi (eumycetes) or by certain bacteria (actinomycetes) and, hence, it is usually classified into eumycetoma and actinomycetoma, respectively (Fahal and Hassan, 1992). Mycetoma is not contagious and typically remains localized, involving cutaneous and subcutaneous tissue, fascia, and bone (Vanessa and Amor, 2006; Hay, 1999; McGinnis et al, 1997). Clinically, mycetoma is characterized by painless subcutaneous swelling with nodules that develop and drain through sinus tracts (Ahmed, et al, 2004; Fahal, 2004). The organisms causing mycetoma aggregate into grains or sclerotia and are found in the discharge (Hay, 1999; McGinnis, 1996). The mycetoma infection results in a granulomatous inflammatory response in the deep dermis and subcutaneous tissue, which can extend to the underlying bone. Mycetoma is characterized by the formation of grains containing aggregates of the causative organisms that may be discharged onto the skin surface through multiple sinuses. Mycetoma was described first in the mid 1800s and initially named Madura foot, after the region of Madurae in India; where it was first identified. Actinomycetomas are mycetomas caused by microaerophilic actinomycetes, and mycetomas caused by true fungi are called eumycetomas. These conditions are to be differentiated from actinomycosis; where actinomycosis is an endogenous suppurative infection caused by
*Actinomyces israelii* or other species of *Actinomyces* or related bacteria, affecting the cervical-facial, thoracic, and pelvic sites, which is usually associated with the use of intrauterine devices. The branching bacteria causing actinomycosis are non-acid-fast anaerobic or microaerophilic bacteria. These bacteria are less than one micrometer in diameter and are small compared to the larger diameter of eumycotic agents. On the other hand, the agents of actinomycetoma always are aerobic and sometimes are weakly acid-fast. More than 20 species of fungi and bacteria can cause mycetoma. The ratio of mycetomas that are caused by bacteria (actinomycetoma) to those that are caused by true fungi (eumycetoma) is 197:67 (Ahmed *et al* 2007; Ahmed *et al*, 2004).

One of the characteristic features of mycetoma is the presence of a painless subcutaneous mass, with multiple sinuses and purulent or seropurulent discharge that may contain grains is. It usually spreads to involve the skin and the deep structures resulting in destruction, deformity and loss of function; occasionally it could be fatal (Ahmed *et al* 2007; Ahmed *et al*, 2004).

1.1 The history of mycetoma:

The oldest known case of mycetoma dates back to the Byzantine period (300-600 AD), with possible evidence from an adult skeleton found suggesting mycetoma; this was being based on morphologic changes in the bone (Hershkovitz *et al*, 1992). The first written reference to mycetoma was found in the ancient Indian religious book *Atharva Veda*, where it was mentioned as “Pada valmita” or “Anthill foot” (Kwon-Chung, Bennett, 1992). On the other hand the earliest scientific medical description of mycetoma seems to be the doctoral thesis of the German physician and notorious traveler Engelbert Kaempfer (1651-1716) presented to Leyden University of Netherlands in 1694 entitled
**Disputatio physica inauguralis exhbbens decadem observationum**, this scientist gave the mycetoma cases a different name as: Perical or Ulcerous hypersarcosis of the feet (Fahal, 2006). Kaenpfer first reports showed the disease to be indigenous to Malabar of South India, but later he reported other cases very similar to mycetoma from Ceylon and Japan (Fahal, 2006). In 1812 new cases of mycetoma were reported by the French missionaries in Ponchicherry. Gill, who worked at Madurai dispensary in the Southern Indian province of Madras, described the condition as (a foot covered with large fungoid excrescence discharging an offensive ichorous fluid). In fact Gill was not the person who introduced the term madura, but his successor in Mudrai the scientist Colebrook was first to introduce the term Madura foot in 1848 (Fahal, 2006). In the year 1840, military physicians of the Royal Army stationed in Madura region in India reported an invasive disease which severely affected the foot with about 80% of cases affecting the dorsal part of the foot. The foot degenerated into one mass of disease of fibrocartilaginous nature, with entire destruction of the joints, cartilages and ligaments (Fahal, 2004 c.f Rippon, 1988). Godfrey, a surgeon from Bellary in India 1846 reported four patients with a subcutaneous tumefaction with bone involvement in two of them while the other two showed cysts filled with melanotic matter, he gave the condition the name” morbus tuberculosis Pedis” (Fahal, 2006). In the year 1855 Billingall described the microscopic details of the disease in relation to bone changes and tissue damage. In 1859 Eyre from Madras mentioned the significance of small pigmented grains seen within the lesion and recommended amputation to be the best remedy. In 1860 Minas from Hisaar district reported on hand involvement (Fahal, 2006). On the other hand the scientist Van Dyke Carter (1860), managed to play a major role in understanding the course of the
mysterious disease, he was the first person who emphasized the importance of the parasitic origin of the disease and was the first to coin the name mycetoma, also he was the first person to describe the two varieties of mycetoma, the melanoid type with black granules and the ochroid variety with white granules for which he suggested that the latter variety is produced by different species of fungi. Efforts of Carter were culminated in producing a detailed monograph on: (Mycetoma and the Fungus Diseases of India) (Fahal, 2006).

The history of the mycetoma in Sudan goes back to years before the advent of modern medicine where the present common name for mycetoma of “Nebit” meaning growth has been known in Sudan long years back. The first scientific documented report of a case of mycetoma in north Sudan was published by Balfour in 1904. Then in 1908 Wenyon reported on the first case of black madura from Bor Upper Nile, Republic of South Sudan. Some other reports showed evidence for madura infection management since the time of Mahdeya (1885-1899), where the native doctors of Sudan practiced treatment of mycetoma using cautery for drastic excision and amputation (Fahal, 2004).

In 1910 a pioneer, well-organized and systematic myco-pathological studies on the causal mycetoma organism was carried out by Chalmers, Director of Welcome Tropical Research Laboratories at Khartoum in collaboration with Mr. Archibald the director of Khartoum Civil Hospital and Mr. Christopherson the director of Omdurman Civil Hospitals. This study culminated in excellent findings enabled this brilliant group to give an elaborative, specific definition to mycetoma and for the first time in history to introduce the terms Maduromycoses and Actinomycoses and then afterwards to classify the mycetomas into Actinomycetoma with grains composed of the fine non-segmented
mycelia and Maduromycetoma the grains of which are large segmented mycelia (Fahal, 2004). In 1931, Grantham-Hill the senior surgeon, Khartoum Hospital made a comprehensive clinical study on 184 patients, out of which 64% were diagnosed as being black madura, the rest is the yellow type; in this study Grantham-Hill managed, skillfully to discuss the relative virulence of the two types. His inquisitive findings was that the actinomycotic infection was more virulent, and gradually infiltrates into tissues, but once it penetrates the periosteum it disseminates rapidly in bone, on the other hand the black madura infection forms a chronic, localized subcutaneous nodule. Grantham-Hill was thinking at that time, the only way for management for both types of mycetoma is surgery and the medical treatment is quite doubtful if not useless and the way to success lies in early detection and complete removal of the lesion. This brilliant surgeon specified the question of complete removal by stating that “in case of black maduromycosis, the pseudo-capsule can readily be identified by its bluish colour and dissection follows its outer surface. In the absence of sinuses an incision is made into the tumour to identify its nature. If it is found to be mycetoma, fresh instruments are taken and a circumscribing incision is made at a distance of about one centimeter from the apparent margins of growth (Grantham, 1931). Grantham-Hill was so keen to encourage early detection and management in order to avoid drastic amputation, because he was thinking of amputation to be one of the factors that deter patients from attending hospitals. He therefore defended local removal of the mycetoma lesion as much as possible, and out of his 184 patients, there are 141 patients treated by local removal, constituting 77% while the rest (23%) treated with amputation. He also highlighted the involvement of a thorn prick in causation of the disease, he showed based on actual clinical data that in 30% of cases for
patients infected for less than six months the thorn was found to be embedded in the excised lesion (Granatham, 1931). In the year 1972, an outstanding clinical study was conducted by Mahgoub, Gumaa and Elhassan on the immunological status of the mycetoma patients, who were found to be partially deficient in cell-mediated immunity. This was in turn a giant step towards the proper serological diagnosis of mycetoma and a better understanding to the mycetoma immunology. Also on that year (1972) Elhassan and Mahgoub confirmed beyond doubt the spread of mycetoma through the lymphatics and in 1978 Mahgoub managed to produce mycetoma grains in the thymectomized, (Nu-Nu) nude mice which are deficient in cell-mediated immunity (Fahal, 2004).

In 1976 Mahgoub reported on the medical management of 144 actinomycotic mycetoma patients (Mahgoub, 1976). The first mycetoma clinic in Sudan was established in 1972 at the Khartoum North Civil Hospital. The clinic was an initiative of Professor Elsheikh Mahgoub Gaafar and Prof. Julian Taylor, professor of surgery, Faculty of Medicine, University of Khartoum. The initiative was supported by Federal Ministry of Health and World Health Organization and the Ministry of Overseas Development of the United Kingdom. The clinic was focusing on provision of clinical service. In 1992 the Mycetoma Research Center was established at Soba University Hospital, Khartoum, Sudan. With the objectives of providing high quality clinical service as well as training of medical and paramedical staff and to conduct research in all aspects of mycetoma. Since that date the Mycetoma Research Center at Soba is enthusiastically leading research and developing multidisciplinary systems to find the appropriate medical cure to the cumbersome issue of mycetoma.
Gezira state, Central Sudan became one of the most notorious endemic areas for eumycetoma worldwide. The actual history of mycetoma in Gezira may date back to the year 1954 when Abbot, the senior surgeon of Wad Medani Civil Hospital, was awarded the degree of M.D. from Cambridge University on the *Clinical and Epidemiological Studies of Mycetoma*. During this study he carried out *in vitro* trial with some antibiotics against *Madurella mycetomatis* and *Streptomyces somaliensis*. His work was the seminal work for identifying the causative organism of black madura of Sudan: *Madurella mycetomatis* and the yellow mycetoma: *Streptomyces somaliensis*. The name of *Madurella mycetomatis* was introduced into medical literature to replace the old name coined by Chalmers and Archibald in 1916 of *Glenospora khartoumensis*. In (1967) Maghraby the famous Sudanese surgeon from Wad Medani Civil Hospital reported on the Clinical Aspects of Mycetoma in the area of the Gezira, where he described for the first time Gezira as being an area of highly endemicity (Fahal, 2004).

### 1.2 Clinical course of mycetoma:

The clinical course is slow and progressive, cases having been reported with duration of up to twenty years. Early symptoms may consist of painless subcutaneous mass, then a hard, deep-seated, fixed nodule develops. Swelling gradually extends peripherally about the nodule and other nodules appear; these nodules normally soften and after a few days rupture spontaneously, discharging fluid containing the characteristic granules. Drainage continues for a few days and then gradually diminishes, the fistula crusting and healing over. As the disease progresses, all stages may be present, draining sinuses, encrusted lesions, and small scars from old lesions. The individual sinuses lead to deep-seated abscesses. The swelling gradually involves the entire foot, leading to a massive globular
deformity. The subjective symptoms are usually slight. There may be some pain just before rupture of a nodule, and patients sometimes complain of a deep aching or sensation of fullness (Caroll, 1949).

Fig. 1: Typical presentation of Mycetoma of the foot, with the tumefaction, deformity and discharging sinuses

Fig. 2: Actinomycetoma attacking the forehead
Fig. 3: Eumycetoma with marked damage of the gluteal muscle with rectal region involvement

Fig. 4: Eumycetoma attacking on the left foot with massively discharging sinuses

(Fahal, 2004)
Male predominance is a constant finding in mycetoma with a sex ratio of 3.7:1. This is commonly attributed to the greater risk of exposure to organisms in the soil during outdoor activities (Abbott, 1965). However in some areas where mycetoma is endemic females are more committed to outdoor activities than males. It is interesting to note that during pregnancy mycetoma become more active and aggressive; changes in hormonal environment and decreased immune response during pregnancy may be the explanation for this observation. No age is exempted but mycetoma commonly affects adults between 20-40 years of age and these are the most active members of the society especially in underdeveloped countries (Fahal and Suliman, 1994). Mycetoma is seen more conventionally in farmers, field workers and in herdsmen and in endemic areas people of other occupations are also affected. The characteristic clinical triad for mycetoma is swollen tissue, draining sinuses, and identified grains from the discharge (Lupi et al, 2005). Initially, some individuals may report a feeling of pain or discomfort at the site (McGinnis, 1997). Others may not recall any direct trauma, (Foltz, and Fallat, 2004). Inoculation is followed by development of painless subcutaneous nodule that spreads slowly (Ahmed, 2004; Hay1999; McGinnis et al, 1997). Usually, the formed nodule is round and firm, but it may also be soft, lobulated or, rarely, cystic (Fahal, 2004; Fahal et al, 1998). As the nodule increases in size, secondary nodules and papules may develop with accompanying sinuses that drain serous, serosanguineous, or purulent discharge (Ahmed et al, 2004; Fahal, 2004; Hay, 1999). Over time, some of the sinuses close and heal (Fahal, 2004). The overlying skin may be shiny with local hyperhidrosis and is usually hyperpigmented but may also be hypopigmented (Welsh et al, 2005; Fahal, 2004; Richardson and Warnock, 1997). Abscesses occur under the surface of the skin and as the
disease progresses, the lesions extend into bony tissue, causing small cavities of about 2 to 10 mm, to develop (Fahal, 2004). If the condition is not treated, bony involvement can be extensive and devastating, leading to complete bone destruction (McGinnis, 1997). Later presentation and more rarely in the disease, lesions may destroy nerves and tendons (Fahal, 2004; Boiron, et al, 1998). Local lymphadenopathy is common with small and shotty lymph nodes, and may result from secondary bacterial infection, spread of mycetoma, or immune complex deposition as part of the local immune response to infection; continual lymphatic spread is rare about 1 to 3% of cases (Fahal, 2004; Hay, 1999; Boiron et al, 1998). Chronic infection may lead to disability, distortion, and deformity, and may be fatal if untreated (Fahal, 2004). Throughout active disease, the sinus discharge is composed of organisms that produce distinctively colored grains, or sclerotia when observed under a microscope (Fahal, 2004; Sobera and Elewski, 2003). These grains, which help to identify the causative organism and guide treatment of the disease, vary in size and consistency and may be black, white, yellow, red, or a mix of colors. Mycetoma is typically unilateral, and most often (70 - 80%) the foot is the primary site of infection, followed by the hands about (12%), legs, and knee joints (Ahmed et al 2004; Fahal, 2004; Sobera and Elewski, 2003). Individuals infected with actinomycetes exhibit a more rapid, progressive clinical disease with increased inflammation, local destruction, and quicker bone invasion compared with those infected with eumycetes, who have well-defined, slower growing lesions that stay encapsulated longer (Fahal, 2004; Pang, 2004). The clinical presentation of mycetoma is identical in both types fungal and bacterial lesions. However, actinomycetoma has a rapid progressive course compared to eumycetoma. In the latter, the lesion grows slowly with
clear defined margins and remains encapsulated for a long period, whereas, in actinomycetoma the lesion is more inflammatory, more destructive and invades the bone at an earlier period. Mycetoma presents as a slowly progressive painless subcutaneous swelling, which is usually firm and rounded but it may be soft, lobulated, rarely cystic and it is often mobile (Fahal et al., 1998). Multiple secondary nodules then develop; the nodules may suppurate and drain through multiple sinus tracts. The sinuses may close transiently after discharge during the active phase of the disease. Fresh adjacent sinuses may open while some of the older ones may heal completely. They are connected with each other, with deep sterile abscesses and with the skin surface. The discharge is usually serous, serosanguinous or purulent. During the active phase of the disease the sinuses discharge grains, the colour of which depends on the causative organism (Fahal et al., 1998). The grains can be black, yellow, white or red and they are of variable size and consistency. Pus, exudates, the dressing gauze and biopsy material should be examined for the presence of the grains (Fahal et al., 1998). Mycetoma is usually painless in nature. It has been suggested that the mycetoma produces substances that have an anesthetic action (Gumaa, 1983) or that the lack of pain may be due to nerve damage at a later stage of the disease (Gumaa, 1983). Pain may be produced by the expansion of the bone by the mycetoma granuloma and grains or it may be due to secondary bacterial infection (Gumaa, 1983). Skin changes are common in mycetoma, in some patients, there may be areas of local hyperhidrosis, confined only to the mycetoma lesion and the skin around it; the reason for this is unclear. For unknown reasons, the tendons and the nerves are curiously spared until very late in the disease process, this may explain the rarity of neurological and trophic changes even in patients with long standing mycetoma. The
absence of trophic changes may also be explained by the adequate blood supply in the mycetoma lesion (Fahal et al., 1997). In the majority of patients, the regional lymph nodes are small and shotty; regional lymph adenopathy is not uncommon, this may be due to secondary bacterial infection, genuine lymphatic spread of mycetoma or it may be due to immune complex deposition as part of a local immune response to mycetoma infection (El Hassan and Mahgoub, 1972). The infection remains localized and constitutional disturbances are rare but when they do occur, they are generally due to septicemia or to immuno-suppression. Cachexia and anemia may be seen in late mycetoma (Gumaa et al., 1986). This is often due to malnutrition, sepsis and mental depression. Mycetoma can produce many disabilities, distortion and deformity. It can be fatal especially if it affects the skull (Gumaa et al., 1986).

1.2.1 Susceptible population and risk factors of mycetoma:

Anyone living in an endemic area could become infected, but infection is more common in herdsmen, farmers, and other field labourers who are in frequent and direct contact with the field environment. Males are about five times more often affected by mycetoma than females, even in areas where both sexes spend a lot of time outdoors (McGinnis, 1996; Fahal and Suliman, 1994). Mycetoma is seen in all age groups, but it usually affects adults between 20 and 40 years old. The infection is not considered to be transmissible from person to person or from animals to people. There are some conflicting reports about the role of the immune status of the susceptible population. Some investigators reported partial impairment of the cell mediated immune-response (CMI) in patients severely infected or not responding to medical treatment (Mahgoub et al., 1977). As in patients with HIV infection, pulmonary mycetoma is associated with
*Pneumocystis carinii* pneumonia and HIV infected individuals show a tendency towards accelerated mycetoma progression. The small number of case studies available and HIV patients presenting with various forms of mycetoma does not yet allow for definite conclusions to be drawn on the relation between immune status and mycetoma susceptibility and progression (Castro *et al.*, 1999; Neumeister *et al.*, 1995). In animals, some evidence supports the partial CMI hypothesis e.g. the more successful induction of mycetoma in athymic mice compared to immunocompetent mice. The more rapid progression of induced mycetoma in immunosuppressed goats compared to healthy goats provided another important piece of evidence (Gumma and Abu-Samra, 1981; Bendl *et al.*, 1987). The Humoral immune response has been evaluated by Wethered and his colleagues using an ELISA system. High IgM levels were seen in most patients with mycetoma due to *Madurella mycetomatis*, whereas low levels of IgA were detected in some of the patients. Sera from patients did better in the ELISA compared with western blots, indicating possible involvement of polysaccharide antigens in *Madurella mycetomatis* infection (Elhassan *et al.*, 2001). IgM and IgG and complement factors have been identified on the surface of the grains, and on the filaments inside the grains in case of actinomycetoma (Elhassan *et al.*, 2001).

### 1.2.2 Human body sites mostly affected by mycetoma infection:

The foot is affected most often (80% of cases) in mycetoma. The lesions are seen commonly on the dorsal aspect of the forefoot and for unexplained reasons the left foot is affected more than the right (Fahal *et al.*, 1994). The hand ranks as the second commonest site of about, (6.6%), the right hand is more often affected (Fahal *et al.*, 1994; Fahal and Suliman, 1994; Mahgoub, 1985). In endemic areas other parts of the body may be
involved but less frequently and these include the knee, arm, leg, head and neck, thigh and the perineum. Rare sites such as the chest and abdominal walls, fascial bones, mandible, paranasal sinuses, eyelid, vulva, orbit, scrotum and surgical incisions may be affected (Fahal et al, 1996)

1.3 Epidemiology of mycetoma:

Mycetoma has a worldwide but unequitable distribution. Mycetoma is endemic in tropical and subtropical areas, and the majority of disease occurs in the ‘mycetoma belt,’ which stretches between the latitudes of 15° south and 30° north (Ahmed et al, 2004; Hay, 1999; Fahal, 1998). Within this belt are countries such as Sudan, Somalia, Senegal, India, Yemen, Mexico, Venezuela, Colombia, and Argentina (Ahmed et al, 2004; Fahal, 1998). Regions in the mycetoma belt are characterized by short rainy seasons that last for 4 to 6 months with fairly consistent daily temperatures ranging (30 to 37°C) and relative humidity of 60-80%, followed by dry seasons of 6 to 8 months with variable daytime temperatures (45 to 60°C) and relative humidity of 12 to 18%, (Ahmed et al, 2004). These alternating weather conditions may contribute to the survival of the causative organisms (Ahmed et al, 2004). While most Mycetomas are limited to these regions, there have been occasional sporadic case reports outside the mycetoma belt, including the United States of America (Foltz and Fallat, 2004; Green and Adams, 1964) and Europe, (Rigopoulous et al, 2000). The disease is endemic in tropical and subtropical regions and the African continent has the highest prevalence (Fig.5). The true incidence and the geographical distribution of mycetoma throughout the world is not exactly known due to the nature of the disease which is usually painless, slowly progressive which may lead to the late presentation of the majority of patients (Gonzalez-Ochoa, 1975). The organisms
are usually present in the soil in the form of grains. The infecting agent is implanted into the host tissue through a breach in the skin produced by trauma caused by sharp objects such as thorn pricks, stone or splinters (Boiron et al, 1998; Magana, 1984; Mariat and Sur La, 1963). In Africa, mycetoma is most frequently seen in Sudan, Senegal, Mauritania, Kenya, Niger, Nigeria, Ethiopia, Chad, Cameroon, Djibouti, and Somalia, (Singh, 1978; Abbot, 1965). Mycetoma has also been extensively reported in India (Bocarro, 1909).

Due to the chronicity of mycetoma and the fact that most of the imported cases are usually involving immigrant patients who probably contracted the infection in their home countries years before diagnosis, this usually complicates the clinical diagnosis, since it is frequently unexpected (De Hoog et al, 1993). Reports on mycetoma have come from the USA, Ceylon which is now Sri Lanka, Germany, Egypt, Turkey, Philippines, Japan, Lebanon, Thailand, Iran, Netherlands, and Saudi Arabia (DeHoog et al, 1993; Tight and Bartlett, 1981). The geographical distribution of the mycetoma causative agents shows considerable variation that could be explained by environmental factors, especially lack of rainfall (Boiron et al, 1998; Mahgoub and Murry, 1973; Mariat, 1963). Optimal areas where mycetoma prevails are reasonably arid with a short rainy season of 4-6 months. Rainfall is 50 -1000 mm per year, with a relative humidity of 60 - 80%, and fairly constant temperatures of 30 - 37°C, throughout the day and night (Mahgoub and Murry, 1973). This extreme alteration in weather conditions might be a prerequisite to the survival of the causative organism in its natural habitat or niche. Many microorganisms are capable of causing mycetoma and several of these organisms seem to have their natural habitat in soil or plant materials including thorns (Welsh et al 2005; Lupi et al, 2005; Ahmed, 2004; Maiti et al, 2002; Fahal, 1998). In Sudan, Abbot reported on the
admission of 1231 mycetoma cases to outpatient clinics throughout the country within a period of 2-5 years, (Abbot, 1965). In 1964, Lynch gave an estimation of 300 to 400 new cases per year in Sudan.

**Fig.5: Global distribution of mycetoma** (Heiman et al, 2010)
1.4 Etiology of mycetoma and causative organisms:

Mycetoma is caused by two principal groups of microorganisms:

1.4.1 Actinomycotic mycetoma:

Is caused by actinomycetes, including: *Streptomyces, Actinomadurae*, and *Nocardia*.

1.4.2 Eumycotic mycetoma:

Is caused by true fungi, including species of *Madurella, Exophiala, Pseudallescheria, Curvularia, Neotestudina, Pyrenochaeta, Aspergillus, Leptosphaeria, Plemodomus, Polycytella, Fusarium, Phialophora, Corynespora, Cylindrocarpon, Pseudochaetosphaeronema, Bipolaris, and Acremonium*.

The most prevalent causative agent of eumycetoma worldwide and in Africa in particular, is *Madurella mycetomatis* (McGinnis, 1997). In some parts of central Africa, including Sudan, *Madurella mycetomatis* causes more than 70% of all mycetoma infections (Boiron
Although mycetoma is found worldwide, its exact mode of incidence is still unknown because of the slow and chronic nature of the disease in addition to the late presentation by the majority of individuals with the condition (Fahal, 2000). The organisms are usually present in the soil in the form of grains. Traumatic inoculation of the subcutaneous tissue caused by sharp objects such as thorn pricks, or splinters is thought to be the route of entry. However, this theory has been recently disputed, as many patients have no history of trauma at the infection site. In areas where mycetoma is frequent the habit of going barefoot is common and thorns are plentiful and as the result of that, natural infection is expected to be more frequent than it actually is. Mycetoma in deep tissues without skin involvement is frequently reported. Furthermore, the isolation of the causative organisms from the soil is difficult; all these suggest that, the presence of an intermediate host is important to produce infection in man (Ahmed et al, 2002). Mycetoma is especially endemic and severely debilitating in Sudan, where Abbott reported 1231 cases occurring over a 2.5 year period (Abbott, 1956). Other causative organisms (Table I) include *Madurella grisea, Leptosphaeria senegalensis,* and *Scedosporium apiospermum* (Ahmed et al, 2004, Pang, 2004). Interestingly, the predominant source also varies by region. For example, in Central America and Mexico, mycetoma is more commonly caused by the actinomycetes *Nocardia brasiliensis,* *Streptomyces somaliensis, Actinomadura madurae,* and *Actinomadura pelletierii* (Pang, 2004; Hay, 1999). In the US, mycetoma is most commonly caused by the fungus *Pseudallescheria boydii* (Pang, 2004; Sobera and Elewski, 2003).
Table 1: Causative agents of eumycetoma and actinomycetoma grouped by appearance of typical intralesional grains (Padhye and McGinnis, 1999; McGinnis, 1996; Guma’a, 1994; Ribbon, 1988)

<table>
<thead>
<tr>
<th>Species</th>
<th>Colour of grains</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Eumycotic mycetoma</strong></td>
<td></td>
</tr>
<tr>
<td>Acremonium falciforme</td>
<td>White</td>
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<tr>
<td>Acremonium kiliense</td>
<td>White</td>
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<tr>
<td>Acremonium recifei</td>
<td>White</td>
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<tr>
<td>Cylindrocarpon Cyanescens</td>
<td>White</td>
</tr>
<tr>
<td>Cylindrocarpon destructans</td>
<td>White</td>
</tr>
<tr>
<td>Pseudallescheria boydii</td>
<td>White</td>
</tr>
<tr>
<td>Fusarium oxysporum</td>
<td>White</td>
</tr>
<tr>
<td>Fusarium solani</td>
<td>White</td>
</tr>
<tr>
<td>Fusarium moniliforme</td>
<td>White</td>
</tr>
<tr>
<td>Hormonema spp ..</td>
<td>White</td>
</tr>
<tr>
<td>Aspergillus flavus</td>
<td>Green</td>
</tr>
<tr>
<td>Aspergillus nidulans</td>
<td>White</td>
</tr>
<tr>
<td>Polycytella hominis</td>
<td>White</td>
</tr>
<tr>
<td>Plenodomus avramii</td>
<td>Black</td>
</tr>
<tr>
<td>Corynespora cassiicola</td>
<td>Black</td>
</tr>
<tr>
<td>Curvularia geniculate</td>
<td>Black</td>
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<tr>
<td>Curvularia lunata</td>
<td>Black</td>
</tr>
<tr>
<td>Leptosphaeria senegalensis</td>
<td>Black</td>
</tr>
<tr>
<td>Leptosphaeria thompkinsii</td>
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</tr>
<tr>
<td>Madurella grisea</td>
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</tr>
<tr>
<td>Pseudochaetosphaeronema Larense</td>
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</tr>
<tr>
<td>Pyrenochaeta mackinnonii</td>
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</tr>
<tr>
<td>Pyrenochaeta romeroi</td>
<td>Black</td>
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</tbody>
</table>
### 1.4.2.1 Madurella mycetomatis:

*Madurella mycetomatis* is the most prevalent etiological agent of black grain eumycetoma worldwide (McGinnis, 1996). Contarction of this organism by the human leads to a chronic, granulomatous infection of subcutaneous and deep tissues known as eumycetoma (Boiron *et al*, 1998). In some areas where *Madurella mycetomatis* is endemic, it has been held responsible for more than 70% of all mycetoma infections, although such studies have been done without molecular verification of the identity (Boiron *et al*, 1998).
Madurella mycetomatis cultured on Sabraud’s agar plus Gentamycin growth medium

Madurella mycetomatis the main causative agent of human eumycetoma, the severe debilitating disease endemic in Sudan. It has been suggested that eumycetoma has a soil-borne or thorn prick-mediated origin. For this reason, efforts were undertaken to culture Madurella mycetomatis from soil samples (n = 43) and thorn collections (n = 35) derived from areas in which it is endemic. However, ribosomal sequencing data revealed that the black fungi obtained all belonged to other fungal species. In addition polymerase chain reaction, PCR-mediated detection was performed, followed by restriction fragment length polymorphism (RFLP) analysis for the identification of Madurella mycetomatis DNA from the environmental samples, as well as biopsies from patients with mycetoma (DeHoog et al, 2000). In the case of the Sudanese soil samples, 17 out of 74 (23%) samples were positive for Madurella mycetomatis DNA. Among the thorn collections, 1 out of 22 (5%) was positive in the PCR. All PCR and RFLP patterns clearly indicated the presence of Madurella mycetomatis; in contrast, 15 Dutch and English control soil samples were all negative. Clinically and environmentally obtained fungal PCR products share the same PCR-RFLP patterns, suggesting identity, at least at the species level. These observations support the hypothesis that eumycetoma is primarily environmentally
acquired and suggest that *Madurella mycetomatis* needs special conditions for growth, as direct isolation from the environment seems to be impossible (DeHoog et al, 1993). *Madurella* species in general are slow-growing fungi that produce dark colonies composed of a dense, melanised, and mostly sterile mycelium. *Madurella* species are well known agents of black-grain mycetoma. Two species are recognized, *Madurella mycetomatis* and *Madurella grisea* (DeHoog et al, 1993). In 1905, Brumpt placed an organism described by Laveran in 1902 as *Streptothrix mycetomi* in the genus *Madurella*. The fungus was known as *Madurella mycetomi* for more than seven decades. In 1977, the British Medical Research Council published memorandum number 23 named, *(Nomenclature of fungi pathogenic to humans and animals)* in which the name was corrected to *Madurella mycetomatis*. The name was corrected because the specific species must be in the genitive case (the *Madurella* of mycetoma). Since mycetoma is a Greek neuter noun, the correct citation is *mycetomatis* (Kwon-Chung, 1992). In different culture media, *Madurella mycetomatis* strains show moderate, and the colonies are white and woolly at first, becoming olivaceous, yellow, or brown, generally producing a brownish, diffusing pigment. In poor media, sclerotia of 750 μm diameter can develop; colonies are mostly sterile, composed of dense melanised mycelium. No efficient sporulation has ever been seen. However, phialides with minute conidia in short chains and collarettes may be seen (Padhye and McGinnis, 1999; McGinnis, 1996). Some investigators reported that better sporulation was noted when *Madurella mycetomatis* was cultured in straw extract agar, wheat extract agar, soil extract agar, or water agar (Rajendran et al, 1999; Kwon-Chung, 1992). In most cases, the microscopic appearance of *Madurella mycetomatis* and *Madurella grisea* is quite similar. Some isolates of
Madurella grisea were reported to form conidia. Such isolates are indistinguishable from Pyrenochaeta mackinnonii (Padhye and McGinnis, 1999). Species differentiation of Madurella mycetomatis and Madurella grisea can be made by differences in sugar assimilation and optimal growth temperature. Madurella mycetomatis assimilates lactose but not sucrose, whereas Madurella grisea assimilates sucrose but not lactose. Madurella mycetomatis grows well at 37°C; while Madurella grisea does not grow at 37°C (growth is seen at 30°C) (DeHoog et al, 2000; Padhye and McGinnis, 1999). This finding might also explain the observed difference in virulence.

![Figure 8: Cultures of Madurella mycetomatis clinical isolates on Sabouraud’s dextrose agar after three weeks incubation at 37°C (Ahmed et al, 2004)](image)

In tissues, Madurella mycetomatis forms numerous black sclerotia (grains). Grains are vegetative aggregates of the fungal mycelia embedded in a hard brown matrix, (Fig.8) (Elhassan, 1994). This matrix consists of extra-cellular cement that seems to be 1, 8-dihydroxynaphthalene melanin in combination with host tissue debris. This rigid matrix might act as a barrier protecting the fungus from the natural immunity of the host and antifungal agents (McGinnis, 1996; Elhassan, 1994). Melanin-like pigments in Cryptococcus neoformans or the dimorphic fungal pathogen Paracoccidioides
*brasiliensis* are detectable *in vitro* and *in vivo* and have an important role in the pathogenesis of various mycoses (Gomez and Nosanchuk, 2003; Gomez et al, 2001). Melanins are thought to be protective in circumstances of host-induced oxidative stress (Hamilton and Gomez, 2002; Jacobson, 2000). The grains have different shapes and sizes. In stained sections, the grain is mostly rounded, oval, or trilobed. Two types of grains have been identified: filamentous and, less commonly, vesicular. The filamentous type of grain consists of brown, septate and branched hyphae that might be slightly more swollen and thick towards the periphery of the colony (McGinnis, 1996; Fahal et al, 1995; Elhassan, 1994). The vesicular type of grain, which is less common, has a cement-like matrix in the cortex and a central area filled with numerous vesicles of about 6 to 14 μm in diameter; and light-coloured hyphae (Padhye and McGinnis, 1999). Triple layered tissue reaction zones have been described around the grains (Fahal et al, 1995). An inner neutrophil zone immediately around the grain, an intermediate zone containing mainly macrophages, and an outer zone consisting of lymphocytes and plasma cells mainly can be seen under the microscope (Elhassan et al, 2001; Fahal et al, 1995; Elhassan et al, 1994). Studies on the ultrastructure of *Madurella mycetomatis* showed an elaborate development of mesosomes as the most striking ultrastructural component. In tissue sections, the hyphae show a thick cell wall, which becomes thicker in the periphery of the grain (Fahal, 2004; Elhassant et al, 1994; Hay and Collins, 1983). It seems that excessive production of polysaccharides in the fungal cell wall renders the fungus less susceptible to the action of antifungal agents and more resistant to the host immune system, owing to possible interference with leukocyte interaction with specifically recognized cell wall antigens (Wethered et al, 1987; Hay and Collins, 1983).
1.4.2.1.1 Fungal viability and antifungal susceptibility of *Madurella mycetomatis*:

The vegetative cells of *Madurella mycetomatis* die rapidly in conditions of moist heat (60°C for 30 min) and are easily killed by 70% ethanol. Homogenized mycelia from fresh agar cultures survive well at 4°, -20°, and -80°C for several weeks (Ahmed, 2004). Little is known about the *in vitro* and *in vivo* susceptibility of the fungus to different anti-fungal agents, especially to the new generation azoles and the Echinocandins. In the past, some reports described the effectiveness of *Ketoconazole* in treating mycetoma patients. Ketoconazole treatment usually leads to some degree of improvement (Mahgoub and Guma’a, 1984; Cuce *et al.*, 1980). Few investigators have described *in vitro* susceptibility testing results. Preliminary data have shown that azoles were capable of inhibiting the growth of at least 50% of all *Madurella mycetomatis* strains included in the studies. Unfortunately, the *in vivo* activity of azoles is often poor (Venugopal and Venugopal, 1993; Bayles, 1992). Still, long-term treatment with itraconazole seems to be the best therapeutic regimen at present. However, variable responses to itraconazole have also been described (McGinnis, 1996; Bayles, 1992). On the other hand a report that eumycetoma due to *Madurella mycetomatis* responds poorly to medical treatment with ketoconazole, especially in late, advanced cases, but lesions of patients under ketoconazole treatment remain localised and well encapsulated (Fahal, 2004). Until recently, no rigorous study using a standard protocol had been done to assess the susceptibility of large numbers of *Madurella mycetomatis* clinical isolates. In general, most investigators agree that early diagnosis followed by both surgery and chemotherapy with appropriate antifungal agents results in the most successful outcome (Boiron *et al.*, 1998; McGinnis, 1996; Fahal and Elhassan, 1994; Andreu, 1986). Mendez, Tovar and
colleagues proved that progesteron can inhibit the growth of *Madurella mycetomatis* and *Pyrenoachaeta romeroi*, which might contribute to the sex bias in the incidence of mycetoma (Fahal, 2004; McGinnis, 1996). Addition of hormone therapy to the standard chemotherapeutic regimen has not yet been assessed in clinical trials. Two antifungal susceptibility testing protocols were developed (Ahmed et al, 2004). One was based on guidelines approved by NCCLS, which was previously the National Committee for Clinical Laboratory Standards, but now a global organisation, for assessing culture base viability, whereas the other was based on XTT (2,3-Bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) chemistry. Susceptibility data for a large number of clinical isolates were obtained for the antifungals, amphotericin-B and itraconazole. Most isolates were more susceptible to itroconazole, which might be the most obvious current choice for therapy of mycetoma. This approach is confirmed by recent clinical results (Ahmed et al, 2004).

1.4.2.1.2 *Madurella mycetomatis* in animals:

In addition to human, natural mycetoma has also been reported in dogs, cats, horses, cows, goats, and even dolphins (VanAmstel et al, 1984; Guma’a and AbuSamra, 1981; Boomker et al, 1977). However, in contrast with actinomycetoma, the induction of eumycetoma in animals in laboratory conditions is very difficult. Many investigators have reported reproducible animal models for bacterial mycetoma, but few have been successful in developing the fungal *Madurella mycetomatis* infection in small animals in the laboratory. Occasionally, the characteristic black grains due to *Madurella mycetomatis* have been reproduced in an animal. This was done either by inoculation of *Madurella mycetomatis* mixed with killed tubercle bacilli or by the use of congenitally
athymic nude mice. In immunocompetent mice such lesions were sometimes seen after repeated intraperitoneal inoculation (Cremer et al, 1995; Mahgoub, 1978; Murray et al, 1960). A reproducible Madurella mycetomatis infection model in BALB/c mice was developed (Ahmed, 2003). Different routes of inoculation, adjuvant usage, host immune status, and sex of the mice were all screened for effect on the infection rate. The infection seemed to be inoculum-dependent with increasing infection rates for larger inocula. Adjuvants were needed for induction of infection and autoclaved soil from an endemic region seemed to be a very effective adjuvant. Small black grains were produced in the peritoneal cavity of infected mice, once more showing the validity of the mouse model. These findings suggest that in people too, an intact immune system might not be able to eliminate the infectious agent.

1.4.3 The other Madurella species:

Recently it appeared that the genus Madurella contains more species with ability to cause mycetoma. Based on single case, Madurella pseudomyctomitis, Madurella fahalli and Madurella Tropicana, were identified as new causative agents of human mycetoma. This is a newly described Mycetoma Causative Agent, where a male Sudanese patient was presented to Soba Mycetoma Research Center (MRC) outpatient clinic with a massive foot black-grain mycetoma. Grain culturing revealed a fungus which resembled Madurella mycetomatis (Sybren de Hoog et al, 2011). Based on the sequences of the genes encoding for the internally transcribed spacer (ITS), β-tubulin (BT2) and the (RPB2) gene, it appeared that although this fungus was closely related to Madurella mycetomatis, it was not identical. Based on the sequence data this newly described mycetomacausative agent was placed in the genus Madurella and named it Madurella
This newly described fungus, *Madurella fahalli* had a growth optimum at 30° C, which is at a slightly lower temperature than *Madurella mycetomatis*. It is a nonsporulating fungus, and characterized by phenotypic and molecular criteria. Multigene phylogenies based on the ribosomal DNA (rDNA) internal transcribed spacer (ITS), the partial β-tubulin gene (*BT2*), and the RNA polymerase II subunit 2 gene (*RPB2*) indicate that *Madurella fahalli* is closely related to *Madurella mycetomatis* and *Madurella pseudomyctomatis*; the latter name is validated according to the rules of botanical nomenclature.

*Madurella ikedae* was found to be synonymous with *Madurella mycetomatis*. An isolate from Indonesia was found to be different from all known species based on multilocus analysis and is described as *Madurella tropicana*. Madurella is nested within the order Sordariales, with Chaetomium as its nearest neighbor. *Madurella fahalli* is less susceptible to the azoles than other Madurella species, with voriconazole and posaconazole MICs of 1 μg/ml, a ketoconazole MIC of 2 μg/ml, and an itraconazole MIC of >16 μg/ml. Since eumycetoma is still treated only with azoles, correct species identification is important for the optimal choice of antifungal therapy, (Sybren de Hoog *et al.*, 2011). *Madurella fahalli* was more resistant to antifungal agents than *Madurella mycetomatis* (Wendy, 2011). It was completely resistant to fluconanazole, itraconazole, 5-flucytosine, caspofungin and had high MICs for ketoconazole (MIC of 2 μg/ml), posaconazole (MIC of 1 μg/ml), voriconazole (1 μg/ml) and amphotericin B (0.5 μg/ml) (Wendy, 2011)
1.5 Diagnosis of mycetoma:

A variety of modalities exist to diagnose mycetoma, the most definite of which is to obtain a sample of mycetoma sinus discharge and visualize its grains (Pang, 2004). This sample can be obtained from any open sinus or by deep surgical biopsy (Ahmed et al 2004; Pang, 2004). Use of a variety of stains, including 20% potassium hydroxide, acid-Schiff, or Grocott’s methenamine silver, allows visualization of grains and fungal and actinomycetes filaments (Pang, 2004). Actinomycetes are usually observed with granules 100μm in diameter and delicate, branched filaments about 1μm in diameter; eumycetes are usually seen as a mass of hyphae embedded in intercellular cement with filaments wider than 1μm (Welsh, 2005). Mycetoma is categorized as a subcutaneous infection because it is primarily limited to subcutaneous tissue and dermis with minimal, rare systemic disease (Pang et al, 2004; Hay, 1999). The incubation period for the disease is variable and not well-defined; some individuals present with symptoms lasting several weeks and others with symptoms lasting years (Foltz and Fallat, 2004; Verdolini, et.al, 2000; Turner 1989). Often, the patient’s exact recall of the trauma is not reliable (Maiti and Ray, 2002).

1.5.1 Differential Diagnosis of mycetoma:

Many soft tissue tumors such as lipoma, fibroma, fibrolipoma, sarcomas, malignant melanoma as well as thorn and foreign body granuloma resemble mycetoma clinically. Estrogenic sarcoma and bone tuberculosis have radiological features similar to advanced mycetoma (Fahal and Hassan, 1992). Primary osseous mycetoma has a radiological appearance like chronic osteomyelitis, osteoclastoma, bone cysts and syphilitic osteitis. The differential diagnosis of mycetoma includes soft tissue tumors such as lipoma,
fibroma, fibrolipoma, sarcomas, and malignant melanoma, as well as chronic osteomyelitis (Lupi, 2005; Fahal, 2004). Further considerations include tuberculosis, Kaposi sarcoma, and other subcutaneous mycoses such as sporotrichosis and chromoblastomycosis (Lupi et al., 2005; Fahal, 2004).

**1.5.2 Radiology in mycetoma:**

In early mycetoma lesions, there is a soft tissue granuloma, which is shown as a dense shadow or as multiple scattered soft tissue shadows. Calcification and obliteration of the fascial planes may sometimes be seen. As the disease progresses, the cortex may be compressed from outside by the granuloma leading to bone scalloping, this is followed by a variable amount of periosteal reaction. Periosteal new bone spicules are laid down at right angle to the cortex to create a sun ray appearance and Codman triangle, an appearance that may be indistinguishable from that due to osteogenic sarcoma. Late in the disease, there may be multiple cavities. They are large in size, few in number with well-defined margins in eumycetoma, whereas, in actinomycetoma, they are usually smaller in size, numerous and have no definite margins (Abbott, 1965; Davies, 1958). The cavities are produced by the replacement of the osseous tissue by the grains. Their size is due to the size of the grains of the causative organism. The cavities are usually filled with solid masses of grains and fibrous tissue, which provides bone support. This may explain the rarity of pathological fractures in mycetoma (Abd Bagi et al., 2003; Fahal et al., 1996). The bony changes in the skull are unique: they are purely sclerotic with dense bone formation and loss of trabeculation. With chemotherapy bone changes in the form of remoulding, absorption of the sclerotic bone and reappearance of the normal
trabecular pattern are observed, hence radiological follow-up of patients is essential to ascertain cure.

1.5.3 Ultrasonic imaging of mycetoma:

The mycetoma grains, the capsule and the accompanying inflammatory granuloma have characteristic ultrasonic appearances. Ultrasound imaging can differentiate between eumycetoma and actinomycetoma and between mycetoma and other non-mycetomous lesions. In eumycetoma lesions, the grains produce numerous sharp bright hyper-reflective echoes, which are consistent with the black grains. The grain cement substance is most probably the origin of these sharp echoes. Also there are multiple thick-walled cavities with absent acoustic enhancement. In actinomycetoma lesion, the findings are similar but the grains are less distinct. This may be due to their smaller size and consistency, individual embedding of the grains or the absence of the cement substances.

The ultrasonic diagnosis of mycetoma is more precise and accurate in lesions with no sinuses. The size and extent of the lesion can be accurately determined ultrasonically and this is useful in planning surgical incisions and procedures (Fahal et al., 1997).

1.5.4 Fine needle aspiration cytology of mycetoma:

Mycetoma can be accurately diagnosed by fine needle aspiration (FNA) cytology, (El hag et al, 1996). Mycetoma lesion has a distinct appearance in a cytology smear and is characterized by the presence of polymorphous inflammatory cells consisting of an admixture of neutrophils, lymphocytes, plasma cells, histiocytes, macrophages and foreign body giant cells and grains. In sections, the grain is closely surrounded by and occasionally infiltrated by neutrophils causing its fragmentation. Outside the neutrophil zone, monocytic cells and giant cells are seen. This is surrounded by granulation tissue
rich in fibroblasts. Different grains have distant appearance, which allows morphological identification and classification of mycetoma. The technique is simple, cheap, rapid and sensitive and it is tolerated by patients. It can be used in routine diagnosis and as an effective means of collection of material for culture and immunological studies. Due to the simplicity of the technique it can be used in epidemiological survey of mycetoma and for detection of early cases in which radiological and serological techniques may not be helpful (El hag et al, 1996).

1.5.5 Culture of mycetoma:

A large variety of microorganisms are capable of producing mycetoma. They can be identified by their textural description, morphological and biological activities in pure culture. The biological activity may include acid fastness, optimal temperature, proteolytic activity, utilization of sugars and nitrogenous compounds (Rippon, 1988). The grains are the source of the culture and they should be alive and free of contaminants. Many culture media are in use e.g. Sabouraud, blood agar and malt extract agar. The culture technique is often cumbersome, time consuming and chance contamination may give a false positive result. It also requires experience to identify the causative organisms (Rippon, 1988).

1.5.6 Histology of eumycetoma lesion:

Stained sections usually show the grain morphology and the tissue reaction to the organisms. Three types of tissue reactions have been described (Fahal et al, 1995); this includes:
1.5.6.1 Type I tissue reaction:

In Type I reaction, the grains are usually surrounded by a layer of polymorphonuclear leucocytes. The innermost neutrophils are closely attached to the surface of the grain. They sometimes invade the substance of the grain causing its fragmentation. The hyphae and cement substance usually disappear and only remnants of brown-pigmented cement are left behind. Outside the zone of neutrophils there is granulation tissue containing macrophages, lymphocytes, plasma cells and few neutrophils. The mononuclear cells increase in number towards the periphery of the lesion. Many of the macrophages have plentiful vacuolated cytoplasm. Some macrophages are phagocytising nuclear debris and neutrophils. The vacuolated macrophages give a positive reaction for lipid and Russell’s bodies are observed. Capillaries and venules are surrounded by concentrically arranged layers of fibrin giving them an onion skin appearance. The outermost zone of the lesion consists of fibrous tissue. The arterioles show hypertrophied muscles. The intima is thickened and edematous and the lumen is narrowed, the nerves showed edema and sometimes a mononuclear cell infiltrate. Some of the sweat glands may show hypertrophy and hyperplasia. Type I reaction involves grains surrounded by a layer of polymorphonuclear leukocytes, with the innermost neutrophils closely attached to the surface of the grain or sometimes invading the substance of the grain (Fahal, 2004).

1.5.6.2. Type II tissue reaction:

The neutrophils have largely disappeared and are replaced by macrophages and multinucleated giant cells. The latter have engulfed grain material. This consists largely of pigmented cement substance although hyphae are sometimes identified. Other
inflammatory cells and histological changes are the same as in type I reaction (Fahal, 2004).

1.5.6.3. Type III tissue reaction:
At this stage there is formation of a well-organized epithelioid granuloma with Langhans’s giant cells. The centre of the granuloma sometimes contains remnants of fungal material but in some no fungal elements can be identified. Inflammatory and histological changes are the same as described for both types I and II reactions (Fahal, 2004).

1.5.7 Immunodiagnosis of mycetoma:
Recently the immune responses in mycetoma lesions caused by *Streptomyces somaliensis* were characterized by immunohistochemistry (El Hassan et al, 2001). In the haematoxylin and eosin sections, the inflammatory reaction around the grain is of two types. In type I there are three zones: a neutrophil zone immediately around the grain, an intermediate zone containing mainly macrophages and a peripheral zone consisting of lymphocytes and plasma cells. By immunohistochemistry, zone1 stained positive for CD15 (neutrophils), zone 2 was positive for CD68 (macrophages) and CD3, (T lymphocytes) while zone 3 contained CD20+ cells, (B lymphocytes). In type II reaction there was no neutrophil zone, the grains being surrounded by macrophages and giant cells. This was confirmed by immunohistochemistry, which also showed the presence of CD3+ cells. IgG, IgM and complements were demonstrated on the surface of the grain and on the filaments inside the grain. Neutrophils and macrophages are recruited in the lesion by complement and are involved in the damage of the grain. The cytokine profile in the lesion and regional lymph nodes is of a dominant (Th2) pattern (IL-10 and IL-4).
1.5.8 Serodiagnosis in mycetoma:

The demonstration of significant antibodies titres against the causative organism may be of diagnostic value. Serodiagnosis is of a great help in identification and classification of the various organisms, which is an essential prerequisite for medical treatment, and is mandatory for the follow-up of patients. It has many advantages over culture and histopathological techniques, as both require surgical biopsy, which may enhance the spread of the organism. The common serodiagnostic tests for mycetoma are the immunodiffusion and counter-immuno-electrophoresis (Gumaa and Mahgoub, 1975; Gumaa and Mahgoub, 1973). Unless antigens used in these tests are quite pure, the tests can be negative in early cases. Cross-reactivity between actinomycetes is quite common and this limits the value of these tests in the diagnosis of the different types of actinomycetes. However these tests and their antigens preparation take a considerable time. Sero-epidemiological survey could give valuable information on the distribution and prevalence of exposure to mycetoma (Taha, 1983).

1.5.9 Novel diagnostic approaches:

A new method of diagnosis of mycetoma is to culture the grains on media such as Sabourauds agar, blood agar, and malt extract agar to isolate fungi or bacteria (Lupi et al, 2005; Fahal, 2004). This is one of the most cumbersome ways to diagnose mycetoma as it is time-consuming and easily contaminateable (Fahal, 2004). Fine needle aspiration cytology smears enable diagnosis of mycetoma through visualization of the characteristic lesion of polymorphous inflammatory cells mixed with grains, neutrophils, lymphocytes, plasma cells, histiocytes, macrophages, and foreign body giant cells (Fahal, 2004). Depending on the stage of infection, radiology can be used to diagnose mycetoma. The
early x-ray appearance of the lesion resembles a soft tissue granuloma. With progression, x-rays show bone scalloping with variable periosteal reaction and, finally, multiple cavities (Fahal, 2004). Bone scans or magnetic resonance imaging may detect bone lesions earlier than x-rays and have been shown to be more sensitive and specific diagnostic modalities than plain x-rays (Abd Bagi, 2003; Czechowski, 2001; Hay, 1999). Ultrasonic imaging is another method for diagnosing mycetoma because the grains, capsule, and granuloma display characteristic echoes on ultrasound. Immunodiagnosis uses stains for CD15 (neutrophils), CD68 (macrophages), and CD3 (T lymphocytes) to diagnose mycetoma while serodiagnosis evaluates levels of antibodies against the causative agent (Fahal, 2004). Recently, molecular tests have been developed to improve the quality of diagnosis of mycetoma. These include polymerase chain reaction, which identifies species based on amplification of a region of ribosomal gene complex (Ahmed et al, 2004; Pang, 2004; Yera, 2003; Ahmed, 2002). These tests give hope for better identification and detection of the disease, which will lead to improvements in patient care and facilitate research into the pathogenesis and epidemiology of the disease (Ahmed et al, 2004)

1.6 The management of mycetoma:

The management of mycetoma depends mainly on its etiological agent and the severity of the disease. Until recently the only available treatment for mycetoma was amputation or mutilating surgical excision of the affected part. No case of self-cure has ever been reported in the medical literature. Meticulous patient clinical examination, imaging, cytological, bacteriological and pathological investigations are mandatory to confirm the diagnosis and to determine the disease extent (Abd el-bagi, 2009; Bonifaz et al, 2008).
However, spontaneous lesion regression was observed in some patients. Combined medical and surgical treatment is the gold standard in eumycetoma (Fahal, 2007; Ahmed, 2006). It is interesting to note that although mycetoma is a common problem in many regions of the world, reports on the treatment of mycetoma are few and limited. All of them are based on personal clinical experience rather than controlled clinical trials, and this is probably due to the fact that mycetoma is one of the badly neglected diseases.

1.6.1. Actinomycetoma therapeutic management:

In general, actinomycetoma is amenable to medical treatment with antibiotics and other chemotherapeutic agents. Combined drug therapy is always preferred to a single drug to avoid drug resistance and for disease eradication (Ahmed et al, 2004; Hassan and Fahal, 2004). In the past, the treatment of actinomycetoma was by combination of streptomycin sulfate 14 mg/kg daily for 1 month, then on alternate days, and 4, 4´-diaminodiphenyl sulfone (dapsone) 1.5 mg/kg twice daily until cure. The cure rate varied between 60% and 90%. This treatment regime needs a long duration to achieve cure and has many side effects (Mahgoub, 1985; Mahgoub, 1976). For patients who do not respond to treatment or those who had persistent side effects to dapsone, the dapsone is replaced by cotrimoxazole 1.5 mg/kg twice daily (Mahgoub, 1972). Certain actinomycetes, such as Actinomadura pelletierii, had good response to this combination, and it was used as the first-line treatment. Many other drugs, such as Rifampin, sulfadoxine and pyrimethamine (Fansidar), and sulfonamides, had been tried as a second line of treatment for actinomycetoma for patients who did not respond to the first-line treatment or who developed serious drug side effects. However, these drugs take a long time to achieve cure, the mean duration was approximately one year and the recurrence rate was high.
They have many side effects, and some of these were serious, such as Stevens Johnson’s syndrome (Mahgoub1985; Mahgoub1976). Gentamicin and kanamycin, members of the aminoglycosides family, have been tested in combination with sulfonamides, but the response was not very remarkable. Netilmicin, another member of the aminoglycosides group, at a dose of 300 mg/day combined with cotrimoxazole, was used for patients who developed complications or resistance to amikacin sulfate, but all of them had demonstrated lower efficacy against actinomycetoma and had many side effects (Gomes Flores et al, 2004). Many reports showed excellent clinical response to the combination of amikacin sulfate and cotrimoxazole, (The Welsh regimen). When given in a form of cycles; each one consisted of amikacin sulfate 15 mg/kg twice daily for 3 weeks and cotrimoxazole in a dose of 1.5 mg/kg twice daily for 5 weeks; the cycles were repeated until cure (Welsh et al, 1987). The number of cycles ranged between five and ten cycles. Renal failure and ototoxicity are well-recognized complications, and resistant cases to this regimen have been recently reported (Welsh et al, 1987). Damle and his associates reported on 18 patients with actinomycetoma with poor response to the Welsh regimen. In that report, rifampin was added to the amikacin sulfate and cotrimoxazole combination. In total, 16 patients (88.9%) were followed up for 18 months and all of them showed remission (Damale et al, 2008). Recently, the experimental oxazolidinone DA-7867 proved to be active in vitro against Nocardia brasiliensis, which opens the possibility of using this drug once it is accepted for human application. The in vitro activity of linezolid, a novel oxazolidinone, was studied and compared with amikacin, trimethoprim/sulfamethoxazole and amoxicillin/clavulanic acid against total of (25) strains of Nocardia brasiliensis isolated from patients with actinomycetoma in Mexico.
All of the tested strains showed high sensitivity to linezolid. This antimicrobial may be a good option for treatment of actinomycetoma, but due to its high cost, this drug is used only for cases unresponsive to other treatments (Chacon and Moreno, 2009; Vera-Cabrera et al, 2008). The efficacy of ciprofloxacin and moxifloxacin against *Nocardia brasiliensis* was tested in *Nocardia Brasiliensis* infected BALB/c mice. A statistically significant difference was observed only with moxifloxacin. The combination of moxifloxacin/trimethoprim/sulfamethoxazole also proved to be effective for actinomycetoma treatment (Chacon-Moreno et al, 2009). The combination of amoxicillin/clavulanic acid has shown effectiveness against strains of *Nocardia brasiliensis*, *in vitro* and *in vivo*. It is a second line of treatment for patients with advanced actinomycetoma involving the bone or vice versa or who had developed resistance to the commonly used drugs, especially the Welsh regime. The recommended dose is 500 mg of amoxicillin and 125 mg of clavulanic acid three times per day. The mean treatment duration is 6 months. This drug combination is well tolerated and the side effects are minimal (Bonifaz et al, 2007; Gomez et al, 1993). Meropenam, a carbapenem with broad bactericidal activity, which resists hydrolysis by β-lactamases, is another treatment option. Some *in vitro* studies have demonstrated its activity against various strains of *Nocardia* including *Nocardia brasiliensis*. The drug has been successfully used to treat systemic nocardiosis in the immunocompromised patients (Fahal et al, 1997). In addition, imipenem, another carbapenem, has demonstrated clinical efficacy against Nocardia actinomycetoma when given as monotherapy or in combination with amikacin sulfate (Fuentes, 2006; Yazwa, 1992). The results of a recent *in vitro* study suggested that nemonoxacin, linezolid and tigecycline can be good treatment options for the
actinomycetoma causative bacteria rapidly become resistant to various antibiotics and antimicrobials; therefore, it is necessary to regularly test the sensitivity of the available drugs, both \textit{in vitro} and \textit{in vivo}, and clinicians should be on the search for novel treatments (Ameen, 2009; Ameen, 2008). Whichever regimen is used, regular and close observation and follow-up of patients, along with renal, hepatic and hematological assessments and evidence of ototoxicity, are mandatory (Welsh \textit{et al}, 1987; Damale \textit{et al}, 2008). In actinomycetoma, combined medical and surgical treatments are beneficial. This regime facilitates surgery, accelerates healing and reduces the chance of relapse; however, a good number of patients respond to medical treatment alone (Ahmed, 2007; Fahal, 2006). Medical treatment is effective in all stages of actinomycetoma, even in patients with advanced disease and with a great deal of bone damage. Cure is possible, although a prolonged period of treatment is needed. Patients with joint involvement usually develop joint fibrosis, and this leads to deformities and disabilities. Recurrence is more common after an incomplete or irregular course of medical treatment. With drug incompliance, there is a good chance for the organism to develop drug resistance (Ahmed, 2007; Fahal, 2006). Local administration of mycetoma chemotherapeutic agents was used, but the results were not impressive with a high rate of failure and complications. Recently, López-Cervantes and his associates reported on the development of transdermal patches with two different formulations: one with free kanamycin and the other one with Kanamycin adsorbed in silica along with an emulgel to be used as a Kanamycin-based auxiliary system. This system was intended to be used in the treatment of mycetoma caused by \textit{Actinomadura madurae}. However, further studies
are needed to evaluate this to be used as an auxiliary system in the treatment of actinomycetoma (Lopes Cervantes et al, 2009).

1.6.2 Therapeutic management of eumycetoma:

Reports on medical treatment in eumycetoma are scarce and disappointing. Over the years the treatment of eumycetoma was based on personal clinical experience and on the results of sporadic case reports, rather than controlled clinical trials. Still, in many centers, massive surgical excisions or amputation of the affected part is the treatment of choice (Hassan and Fahal, 2004; Fahal, 1997). Various antifungal agents have been tried with little success. This is perhaps surprising, as the eumycetoma causative agents are low grade infective organisms and their eradication should be readily achieved by the administration of safe systemically antifungal drugs. Amphotericin B, has been used with limited success, and it is no longer popular due to its serious toxic side effects (Lupi et al, 2005). The combination of griseofulvin 1.5 g/day given orally and 60,000 to 80,000 IU of procaine penicillin was a popular regime for a while, but it had low cure and high recurrence rates. The most popular treatment regimes nowadays for eumycetoma are ketoconazole 400 to 800 mg/day or itraconazole 400 mg/day for extended periods of time with a mean duration of 9 to 12 months (Fahal, 2006; Fahal, 2004). These drugs have many serious side effects. The side effects are more noticeable with ketoconazole, and these include: hepatotoxicity, gynecomastia, lip dryness and ulceration, skin darkness and decreased libido. Both drugs are probably excreted in the milk, and therefore mothers who are under treatment should not breast feed. There are no adequate and well-controlled studies on the effects of these drugs in pregnant women. Therefore, they should only be used during pregnancy if the potential benefit justifies the potential risk to
the fetus (Mahgoub and Gumaa, 1984). Both of these drugs alone are not curative in most eumycetoma patients, but they help in localizing the disease. In most patients, after completion of treatment with ketoconazole or itraconazole, the lesions were found to be well localized, encapsulated within thick capsules and were easily excised surgically. Grain colonies in these lesions were consistently encountered and they were viable on culture. In these patients, the postoperative surgical biopsies showed no significant changes in the morphology of the grains, and the presence five to seven grains in every cavity, which was well walled by fibrous tissue. The reaction surrounding the grains was commonly type I and II tissue reactions. Type III tissue reactions, indicating healing, were rarely encountered (Fahal, 2006). In vitro susceptibilities of Madurella mycetomatis, the most common eumycetoma causative organism, to amphotericin B, fluconazole, itraconazole, ketoconazole, 5-flucytosine and voriconazole were determined. The organism appeared to be most susceptible to the azoles group; ketoconazole, itraconazole and voriconazole, with minimum inhibitory concentrations (MICs) of 0.125, 0.064 and 0.125 μg/ml, respectively. These MICs correlate with attainable serum levels. Fluconazole was the least effective azole, inhibiting the fungal growth at MIC 90 of 16.90 μg/ml. Although these MICs are high, they still meet physiologically attainable serum levels. Amphotericin-B, appeared to be less effective than ketoconazole, itraconazole and voriconazole (MIC 2 μg/ml). Interestingly, Madurella mycetomatis also proved to be susceptible to tea tree oil, with a MIC 90 of 0.25% (v/v). However, it appeared to be resistant to 5-flucytosine and artemisinin (Van de Sande et al, 2007; Ahmed and van de Sande, 2004). The azole group of antifungals is currently used in the medical treatment of eumycetoma caused by Madurella mycetomatis. Surprisingly,
although some case reports and personal experiences showed that ketoconazole and itraconazole treatment resulted in complete cure in some patients, the clinical response to both agents in most patients is often poor (Van de Sande et al, 2007; Ahmed et al, 2004). The black compound in the *Madurella mycetomatis* grain is melanin produced by the organism. It was thought to protect the fungus from the host immune system and antifungal agents; a fact that was proved experimentally (Van de Sande et al, 2007; Ahmed et al, 2004). This may explain the poor response to ketoconazole and itraconazole in clinical practice (Van de Sande et al, 2007). There are some sporadic case reports on the susceptibility of eumycetoma causative organisms to various antifungals including terbinafine, posaconazole, voriconazole, caspofungin and anidulafungin, but these need further clinical trials to establish their therapeutic potential, safety and efficacy for the treatment of eumycetoma (Badalli et al, 2009; Loo, 2006). Medical treatment for both types of mycetoma must continue until the patient is clinically, radiologically, ultrasonically and cytologically cured. Cure is considered when the skin becomes normal, the mass disappears, the sinuses heal and the organisms are eliminated from the tissue. Clinical improvement is judged by reduction in the size of the mass and healing of most of the sinuses. Radiological examination is an essential tool for follow up of patients on medical treatment. It usually shows reappearance of normal bone pattern and the disappearance of the soft-tissue mass. Absent grains cytologically with type III tissue reaction and the disappearance of the grains and cavities ultrasonically are reliable evidences for cure (Fahal, 2006; Ahmed et al, 2004; Fahal, 1997). Patients cured with medical treatment demonstrate many histological changes at the site of the lesion, and that includes the replacement of the neutrophils that usually surround the grains with both
lymphocytes and giant cells. Grains are commonly fragmented and distorted, and they become faintly stained with hematoxylin and eosin. Fibrosis is common in the mycetoma lesion and granuloma formation with type III tissue reaction is indicative of complete cure (Fahal et al., 1995).

Fig. 9: Patient with head and neck actinomyctoma before (a) and after (b) treatment (Fahal, 2004)
1.6.2.1 Some antifungal agents used in eumycetoma management:

With the exception of 5-flucytosine, the antifungal drugs in common usage are directed in some way against ergosterol, the major sterol of the fungal plasma membrane, which is analogous to cholesterol in mammalian cells. Ergosterol in the fungal membrane contributes to a variety of cellular functions. It is important for the fluidity and integrity of the membrane and for the proper function of many membrane-bound enzymes, including chitin synthetase, which is important for proper cell growth and division (Joseph-Horne and Hollomaon, 1997).

1.6.2.1.1 Polyenes:

The Polyenes are a class of antifungal drugs that target membranes containing ergosterol. These drugs, which include amphotericin-B and nystatin, are amphipathic, having both hydrophobic and hydrophilic sides. The drugs are thought to intercalate into membranes, forming a channel through which cellular components, especially potassium ions, leak and thereby destroying the proton gradient within the membrane (Vanden Boscche et al, 1994). The specificity of amphotericin-B for ergosterol-containing membranes may also be associated with phospholipid fatty acids and the ratio of sterol to phospholipids (Vanden Boscche et al, 1994). Polyenes are less likely to interact with membranes containing cholesterol. It has also been suggested that amphotericin-B causes oxidative damage to the fungal plasma membrane (Vanden Boscche et al, 1994). However, recent evidence suggests that amphotericin-B has antioxidant effect in vivo, which protects fungal cells against oxidative attack from the host (Osaka et al, 1997).
1.6.2.1.2 Ergosterol Biosynthesis Inhibitors:

Several inhibitors of the ergosterol biosynthetic pathway have been developed for use against medically important fungi, including allylamines and thiocarbamates, azoles, and morpholines. All of these drugs interact with enzymes involved in the synthesis of ergosterol from squalene, which is produced from acetate through acetyl coenzyme A, hydroxymethylglutaryl coenzyme A, and mevalonate. Ergosterol is an important sterol for fungi, since it is the predominant or “bulk” sterol in fungal plasma membranes. In addition, it has an essential “sparking” function, in which trace amounts of ergosterol are necessary for the cells to progress through the cell cycle. This sparking function is independent of the bulk sterol in the fungal membranes, since certain sterols can replace the bulk sterol of the membrane without supplying the sparking function for the cell cycle (Hitchcock, 1993). The ergosterol biosynthesis inhibitors disrupt both the bulk sterol of fungal membranes and the sparking function in the cell cycle, they include:

1.6.2.1.2.1 The allylamines:

Naftifin, terbinafine and thiocarbamates: tolnaftate and tolciclate inhibit the conversion of squalene to 2, 3-oxidosqualene by the enzyme squalene epoxidase (Favre and Ryder, 1996; Ryder, 1992; Monk et al, 1991). This enzyme is the product of the ERG1 gene, which has recently been cloned in Candida albicans (Rossener et al, 1993). This class of drugs may inhibit the epoxidase through a naphthalene moiety common to both types of drugs (Vanden and Bossche, 1991). These drugs are noncompetitive inhibitors, and cells accumulate squalene in their presence.
1.6.2.1.2.2 The azoles:

Azoles including both imidazoles (ketoconazole and miconazole) and triazoles (fluconazole, itraconazole, and voriconazole), are directed against lanosterol demethylase in the ergosterol pathway. This enzyme is a cytochrome P-450 enzyme containing a heme moiety in its active site (Hitchcock, 1991; Vanden Boscche, 1991). The azoles act through unhindered nitrogen, which binds to the iron of the heme, preventing the activation of oxygen which is necessary for the demethylation of lanosterol (Joseph-Horne and Hollomon, 1997). In addition to the unhindered nitrogen, second nitrogen in the azoles is thought to interact directly with the apoprotein of lanosterol demethylase. It is thought that the position of this second nitrogen in relation to the apoprotein may determine the specificity of different azole drugs for the enzyme (Hitchcock, 1991; Vanden Boscche, 1991). At high concentrations, the azoles may also interact directly with lipids in the membranes (Joseph-Horne and Holloman, 1997; Hitchcock et al, 1987).

Two other antifungal drug classes, the pyridines (Buthiobate and Pyrifenox) and the pyrimidines (Triarimol and Fenarimol), inhibit lanosterol demethylase and are used extensively as antifungal agents in agriculture but are not used in medicine (Marichal et al, 1997).

1.6.2.1.2.3 The morpholines:

Fenpropimorph and Amorolfine inhibit two enzymes in the ergosterol biosynthetic pathway, C-14 sterol reductase and C-8 sterol isomerase. The genes for these two enzymes, (ERG-24) and (ERG-2), have not yet been cloned from any medically important fungus, but have been cloned from Saccharomyces cerevisiae. However, little is known of the interaction of the morpholines with the two genes (ERG-24) or (ERG-2).
1.6.2.1.2.4 5-Flucytosine:

5-Flucytosine (5-FC) has an entirely distinct mode of action from the azoles. 5-FC is taken up into the cell by a cytosine permease and deaminated into 5-fluorouracil (FU) by cytosine deaminase. 5-FC is fungus specific since mammalian cells have little or no cytosine deaminase (Vanden Boscche et al, 1994). FU is eventually converted by cellular Pyrimidine-processing enzymes into 5-fluoro-dUMP (FdUMP), which is a specific inhibitor of thymidylate synthetase, an essential enzyme for DNA synthesis, and 5-fluoro-UTP (FUTP), which is incorporated into RNA, thus disrupting protein synthesis.

1.6.3 Surgery in mycetoma:

Surgery in mycetoma is indicated in early localized lesions, for diagnostic proposes, cases resistant to medical treatment or patients with massive mycetoma not responding to repeated long term medical therapy and it can be a life saving procedure. Eumycetoma lesion is well encapsulated and great care must be exercised not to rupture the capsule, which may lead to recurrence by disseminating the grains into other parts of the operative field. Actinomycetoma lesion has an ill-defined border; therefore a margin of healthy tissue should always be excised with the lesion. A bloodless operative field using a tourniquet is mandatory to identify margins of the lesion. It is advisable to flood the wound at the end of surgery with tincture of iodine for elimination of any residual fungal elements. In advanced cases of mycetoma not responding to medical treatment for a prolonged period amputation is recommended. Extensive repeated excisions of the diseased tissue, including bone, coupled with chemotherapy may be carried out several times to avoid the social consequences of amputation. In less advanced cases less mutilating surgery is advised for example, toe, mid tarsal or Syme’s amputation.
However, in many cases of inadequate surgery recurrence is inevitable. Surgery is indicated in mycetoma for localized lesions, resistance to medical treatment or for better response to medical treatment in patients with massive disease (Fahal, 2006; Fahal, 2004). The surgical options range from wide local and debunking excisions to amputations. Amputation is indicated in advanced mycetoma not responding to medical treatment with severe secondary bacterial infection, and it can be a life-saving procedure. The amputation rate ranges from 10 to 25% in most series (Fahal, 2006; Fahal, 2004). Diagnostic surgical procedures are indicated to obtain tissue biopsy for histochemical and immunohistochemical studies, and grains for microbiological identification. Local anesthesia is contraindicated as the disease extending along tissue planes is unpredictable. The postoperative recurrence rate varies from 25 to 50%, and this can be local or distant at the regional lymph nodes. This could be due to the disease biology and behavior, inadequate surgical excision due to the use of local anesthesia and lack of surgical experience and drug compliance due to financial reasons and/or lack of health education (Ahmed et al, 2007).

1.7 Drug Resistance in Mycetoma:

The clinical, cellular, and molecular factors may contribute to antifungal drug resistance. While many contributing factors have been identified. There is only rudimentary information on the mechanism by which these factors contribute to overall drug resistance or the initiating events that trigger any of these factors. Thus, the study of resistance is progressing from a catalog of the basic resistance mechanisms to an understanding of the details of each mechanism and of the steps involved in the activation of these mechanisms. The ongoing developments in the understanding of resistance
should assist in the development of diagnostic strategies that identify resistant clinical isolates in patient populations, treatment strategies for resistant fungal infections, and prevention strategies that forestall the development of antifungal drug resistance. Despite all of these efforts, we are faced with the fact that fungi will continually develop new resistance mechanisms to the available antifungal drugs. In the past decade, the frequency of diagnosed fungal infections has risen sharply due to several factors, including the increase in the number of immunocompromised patients resulting mainly from the AIDS epidemic and treatments during and after organ and bone marrow transplants. Linked with the increase in fungal infections is a recent increase in the frequency with which these infections are recalcitrant to standard antifungal therapy. The factors that contribute to antifungal drug resistance can be categorized based on medical literature into three levels:

1.7.1 Clinical factors:

Clinical factors that contribute to resistance are associated with the immune status of the patient, with the pharmacology of the drugs, or with the degree or type of fungal infection. The AIDS epidemic, improved life-sustaining technologies, and aggressive anticancer therapy have contributed to today’s severely immunosuppressed patient population who survive longer in the immunocompromised state. Mucosal and systemic fungal infections are common in patients lacking intact host defenses, increasing the dependence on antifungal agents for prophylaxis and treatment. Coincident with this increased usage, resistance has been observed.
1.7.2 Cellular factors:
Cellular factors are associated with a resistant fungal strain at a cellular level; antifungal drug resistance can be the result of replacement of a susceptible strain with a more resistant strain or species or the alteration of an endogenous strain by a mutation or gene expression to a resistant phenotype.

1.7.3 Molecular factors:
Molecular factors are responsible for the resistance phenotype in the cell: The molecular mechanisms of resistance that have been identified to date in some important fungal infections includes overexpression of two types of efflux pumps, overexpression or mutation of the target enzyme, and alteration of other enzymes in the same biosynthetic pathway as the target enzyme (Theodore, 1998). In the 1990s, fungal drug resistance has become an important problem in a variety of infectious diseases including human immunodeficiency virus (HIV) infection, tuberculosis, and other microbial infections which have profound effects on human health (Theodore, 1998; Law et al, 1994; Vanden et al, 1994; Odds, 1993; Graybill, 1988). In summary the current knowledge of the clinical and cellular factors that contribute to antifungal drug resistance and focus on the molecular mechanisms of drug resistance is still a work in progress. An attempt will be made to highlight research areas that have not yet been investigated.

1.8.4 The Role of Susceptibility Testing in determination of resistance:
In the past and before the development of susceptibility testing of yeasts as outlined by the National Committee for Clinical Laboratory Standards (NCCLS), MIC determinations were inconsistent and varied in different laboratories. NCCLS document M-27, first published in 1992, has recently been revised (M27-A3) and subsequent
studies have demonstrated that the interlaboratory reproducibility of MIC determination approximates that of antibacterial testing (Rex et al, 1996). This method has set the groundwork for the development of less cumbersome methods adapted for the clinical laboratory. These methods, relying on macrodilution with or without colorimetric indicators or agar diffusion, have been shown to be reproducible and consistent with the standardized microdilution method (Pfaller et al, 1997). One complication of the NCCLS protocol is that for certain isolates, the timing of the end point determination can have a major effect (up to 128-fold) on the MIC. A recent study suggests that determining the end point after 24 hours instead of 48 hours ensures that the MICs for these isolates correlate with the *in vivo* response to azoles (Rex et al, 1997). In addition, there have been significant advances in susceptibility testing for filamentous fungi (Denning et al, 1997), as well as *in vitro* testing of amphotericin B susceptibility. Technical advances of antifungal susceptibility testing have been recently reviewed (Pfaller et al, 1997). Historically, clinical resistance has been defined as persistence or progression of an infection despite appropriate antimicrobial therapy. A successful clinical response to antimicrobial therapy typically not only depends on the susceptibility of the pathogenic organism but also relies heavily on the host immune system, drug penetration and distribution, patient compliance, and absences of a protected or persistent focus of infection e.g., a catheter or abscess. This is particularly true for fungal infections. The *in vitro* resistance of an isolate can be described as either primary or secondary. An organism that is resistant to a drug prior to exposure is described as having primary or intrinsic resistance. Secondary resistance develops in response to exposure to an antimicrobial agent. Both primary and secondary resistances to antifungal agents have
been observed. A correlation of *in vitro* susceptibility with in vivo response has been observed for mucosal candidal infections in HIV-infected patients. Many groups have noted that the clinical outcome is generally dependent on the *in vitro* susceptibility of the organism (Lacassin *et al*, 1996; Maenza *et al*, 1996; Barchiesi *et al*, 1995; Hawser *et al*, 1995; Newmann, 1994; Cameron *et al*, 1993).

Infection caused by a resistant organism does not always predict clinical failure. A clinical response to fluconazole occurred in 11 of 13 patients with oral candidiasis even though they were infected with isolates for which the MICs were 32 or 64 μg/ml (Ghannoum *et al*, 1996; Revanker *et al*, 1996). For aid in clinical interpretation of antifungal susceptibility testing, the NCCLS Subcommittee for Antifungal Susceptibility Testing recently established interpretive breakpoints for testing of fluconazole and itraconazole for *Candida* infections (Rex *et al*, 1997). The breakpoints for fluconazole MICs are as follows: <8 μg/ml, sensitive; 8 to 32 μg/ml, susceptible dose dependent; and \( \geq 64 \) μg/ml, resistant. The breakpoints for itraconazole MICs are as follows: \( \leq 0.125 \) μg/ml, sensitive; 0.25 to 0.5 μg/ml, susceptible dose dependent; and \( \geq 1.0 \) μg/ml, resistant. (Pfaller *et al*, 1997).

**1.7.4.1 The XTT – Viability assay method:**

Tetrazolium salts have been widely used as detection reagents for many years in histochemical localization studies and cell biology assays (Scudiero DA, *et al*.,1988) The second generation tetrazolium dye, XTT (sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt), can be effectively used in cell-based assays to measure cell growth, cytotoxicity, and apoptosis assay (Marshall,1999). XTT is reduced to a soluble, brightly colored orange derivative by a mix
of cellular effectors (Fig.11 and 12). The sensitivity of an XTT assay is greatly improved by the usage of an intermediate electron carrier, PMS (N-methyl dibenzopyrazine methyl sulfate). PMS helps drive XTT reduction and the formation of its formazan derivative. The XTT cell proliferation assay was first described in 1988 by Scudiero et al (Scudiero et al, 1988); as an effective method to measure cell growth and drug sensitivity in tumor cell lines. XTT is a colorless or slightly yellow compound that when reduced becomes brightly orange, (Fig.10). This color change is accomplished by breaking apart the positively-charged quaternary tetrazole ring (Bridgette, 2005). The formazan product of XTT reduction is soluble and can be used in real-time assays.

Fig.10: The reduction of XTT to form the coloured formazan derivative
Fig.11: The colorimetric reduction of XTT by cellular enzymes

XTT is thought to be excluded from entering cells by its net negative charge. Considerable evidence suggests that XTT dye reduction occurs at the cell surface facilitated by trans-plasma membrane electron transport. Mitochondrial oxidoreductases are thought to contribute substantially to the XTT response with their reductants being transferred to the plasma membrane (Fig.11). It has been proposed that XTT assays actually measure the pyridine nucleotide redox status of cells (Marshall et al, 1999; Berridge, 2005).
XTT can be used alone as a detection reaction. XTT assay results are greatly improved when an intermediate electron acceptor, such as PMS (N-methyl dibenzopyrazine methyl sulfate), is used with XTT (Figure 12). PMS is the Activation Reagent included in the XTT Cell Proliferation Assay Kit. Findings suggest that PMS mediates XTT reduction by picking up electrons at the cell surface, or at a site in the plasma membrane that is readily accessible, and forms a reactive intermediate that then reduces XTT to its highly pigmented formazan product. XTT has the following merits:

- Homogenous Assay. Easy to use with no solubilization step.
- Reproducible Accuracy, (Dye absorbance proportional to cell number).
- Non-Radioactive. Safe, no hazardous waste.
- Convenient storage. The kit is stable for 18 months when stored under refrigeration in the dark.

1.7.5 Role of the melanin in the eumycetoma drug resistance:

Melanins are negatively charged, hydrophobic pigments of high molecular weight (Jacobson, 2000; Nosanchuk et al., 1999; Nosanchuk and Casadevall, 1997; White, 1958), that are composed of polymerized phenolic and/or indolic compounds (Wakamatsu and Ito, 2002; Herrero et al., 1993). Melanins are produced by organisms in all biological kingdoms, including a wide variety of pathogenic bacteria, fungi, and helminthes. Remarkably little is known about the structures of melanins, despite their abundance in the global biomass. This is due to the inability of current biochemical and biophysical techniques to provide a definitive chemical structure, because these complex polymers
are amorphous, insoluble, and not amenable to either solution or crystallographic structural studies. Consequently, our information on the structure of melanin is derived from the analysis of their degradation products and spectroscopic analysis of the melanin polymer (Da Silva et al., 2006; Gautam et al., 2005; Mednick et al., 2005; Wang et al., 1995). Characteristically, melanins are dark in color, insoluble in aqueous or organic fluids, resistant to concentrated acid, and susceptible to bleaching by oxidizing agents (Prota, 1992; Butler and Day, 1988; Nicholaus, 1964). Methods for partial chemical degradation of melanins followed by high pressure liquid chromatographic microanalysis have been developed and are useful for the characterization of specific types of melanins (Wakamatsu and Ito, 2002; Wakamatsu et al., 2002; Enochs et al., 1993). Many diverse functions have been attributed to melanins. Melanins can serve as energy transducers and affect cellular integrity (Mylonakis et al., 2002; Rosas et al., 2001; Hill, 1992). Melanin is also used for sexual display and camouflage, for instance, the coloration in black and red hair arises from melanin (Castanet and Ortonne, 1997). An example in which melanin is used for camouflage is the release of ink, a suspension of melanin particles, by the cuttlefish (Sepia officinalis) in response to danger (Nappi and Christensen, 2005; Fiore et al., 2004; Richman and Kafala, 1996; Marmaras et al., 1996). Melanins in melanocytes in skin provide protection against sunlight and are also believed to contribute to the resistance of melanoma to therapeutic radiation (Zecca et al., 2002; Zecca et al., 2001; Hill, 1991). In mammals, melanin synthesis is catalyzed by a tyrosinase (Sanches-Ferrer et al., 1995). In contrast, microbes generally synthesize melanin via various phenoloxidases, such as tyrosinases, laccases, or catacholases, and/or the polyketide synthase pathway (Rosaz and Casadevall, 1997; Zhadnova et al., 1974; Zhdanova et al.,
Melanins generated from 3, 4-dihydroxyphenyalanine (DOPA) by phenoloxidases are referred to as eumelanins, which are generally black or brown. Yellow or reddish melanins are called pheomelanins and incorporate cysteine with DOPA. Brownish melanins derived from homogentisic acid by tyrosinases are called pyomelanins, (Nonaschuk and Casadevall, 1997; Zunino and Martin, 1997; Forgarty and Tobin, 1996; Yabuuchi and Ohuya, 1972). Melanins formed from acetate via the polyketide synthase pathway are typically black or brown and are referred to as dihydroxynaphthalene melanins. Melanin synthesis has been associated with virulence for a variety of pathogenic microbes (Cunha et al, 2005), Sporothrix schenckii (Peltroche-Lacsahuanga, 2003; Rommero-Martinez et al, 2000; Feng et al, 2001). Melanin is believed to contribute to microbial virulence by reducing a pathogen's susceptibility to killing by host antimicrobial mechanisms and by influencing the host immune response to infection (Doering et al, 1999; Jacobson and Hong, 1997; Nyhus et al, 1997; Wang et al, 1996; Gan et al, 1976). Melanin and melanin synthesis pathways are potential targets for antimicrobial drug discovery. Interestingly, the drug-binding properties of both host and microbial melanins could influence the outcome of antimicrobial therapy. The capacity for melanin to bind to diverse compounds can affect the testing of antimicrobial drugs and reduce the activity of antimicrobial therapy (Mednick et al, 2005; Steenbergen and Casadevall, 2003; Steenbergen et al, 2001; Nosanchuck and Casadevall, 1997; Weng et al, 1995).

1.8 Medicinal plants; a source of new drugs:

Plants have formed the basis for traditional medicine systems in most societies and have been used for thousands of years. Hundreds of years ago there were few or no
synthetic medicines; up to 300,000 species of higher plants were the main source of drugs. During this period, when the developed world shifted largely from natural to synthetics, life expenses and population doubled, citizens began to argue that substances occurring naturally are inherently safe and more healthful than synthetic compounds (Mohammed, 2002). The WHO has estimated that approximately 80% of the world’s inhabitants rely mainly on traditional medicines for their primary health care, where plant-based systems still play a vital role in health care. In developed countries, plant-based drugs are also extremely important: currently at least 119 chemicals derived from plant species can be considered as important drugs in use (Mulholland, 2000). Digoxin, morphine, quinine, taxol, artemisinin and lapachol... etc. and /or their synthetic analogues are examples of natural products which are currently used in modern medicine (Evans, 2003; Hounhton, 2000). Moeover, medicinal and aromatic plants are considered as a major source of valuable extracts and essential oils, which are used worldwide in pharmaceuticals, food, cosmetics, perfumery, flavor and fragrance industries.

1.8.1 Plant phytoconstituents with antimicrobial activity:

Literature reports show the wide range of active secondary metabolites of plants as antimicrobial agents e.g phenolic compounds and derivatives (Corthout et al., 1994). Tanins (Sotohy et al., 1995; Sato et al., 1997; Burapadga and Bunchoo, 1995). Falvanoids (Encarnacion et al., 1994). The essential oil and /or their active constituents of many plant species proved to be responsible for their antimicrobial activity (Filamini et al, 1999; Harkenthal et al, 1999; Carson and Riley, 1995; El-alfy et al, 1975). Triterpenes that are widely occur in nature and in the view of their diverse pharmacological
properties were also shown to have antimicrobial effects (Sharma et al, 2009; Hifzur and Mohammed, 2011; Raquel, et al, 2011).

1.8. 2 Triterpenes; Plant-based therapeutic approach:

Triterpenes (Fig.12) are a large group of natural products, generally they have common origin and their structures can be considered as being derived from that of the acyclic precursor squalene – C30 – triterpene. They may be classified into two main groups, namely: the tetracyclic and pentacyclic compounds: the former ones of the steroidal type occur in plants (phytosterols), animals (zoosterols) and microorganisms (mycosterols) while the latter are mostly found in the plant kingdom (Buckingham, 1996; Buckingham, 1994). About 2500 triterpenes have been studied so far, few of them are investigated for their biological importance. However, several triterpenoids have diverse pharmacological properties including antifungal, (Hifzur and Mohammed, 2011; Pooi, et al, 2011; Zaidi and Crow, 2005), antibacterial and antimutagenic, (Hifzur and Mohammed, 2011; Molnar, et al, 2006; Lin, et al, 2003).
Fig. 12: Biosynthesis of tertracyclic and pentacyclic triterpenoids
1.8.2.1 Possible mechanism of action of triterpenes as antimicrobial agents:

It was speculated that the mode of action of triterpenes as antimicrobial agents may be due to change of microorganisms cellular membrane permeability arising from membrane lipid alteration leading to disruption, a property that had been evident with triterpenes and phytosterols (Tan, et al. 2008); to inhibit the growth of a several types of bacteria and fungi species. C24 - Sterol methyl transferase enzyme (24-SMT) catalyses the conversion of C24-Sterol acceptor to C24-alkyl phytosterol. (Fig.12). This enzyme is not synthesized in animals, making a novel target for rational drug design (Jialin and William, 2009). However, fusidic acid which was first isolated from the fungus *Fusidium coccineum* in the early 1960s, and the chemically related cephalosporin P1 (not related to cephalosporins that inhibit peptidoglycan synthesis), are lanosta-type of steroidal triterpene antibiotics (Fig. 13) and found to exert their activity against organisms by inhibition of protein synthesis by the prevention of translocation on the ribosome, (O’Neill, et al, 1997).

![Fig.13: Structures of fusidic acid (a) and cephalosporin P1 (b)](image-url)
1.8.3 Selection criteria of medicinal plants screened for antimycetomal activity:

Seven plant species based on the following criteria were selected:

(a) Native traditional herbal-healers reports (Ethnomedical uses).

(b) Presence of reported data of antifungal activity together with studies on phytochemical characteristics of the plant-species in the medical literature.

(c) Local availability and accessibility of natural plant species

(d) Cost-effectiveness of the investigated plant species

(e) Toxicity data of the plants under investigation, (edible/ non-toxic).

(f) Synergistic activity expected in different plant phytoconstituents found simultaneously in the same plant species, such as DNAmodulating agents e.g. triterpenes, flavonoids, non-protein amino-acids (Kamadam et al., 1998; Hammond, 1995)

(g) Anti-tyrosinase effect of many natural agents including: Phthalates, bioamines and amino-acids, quinones, Polyphenols. That could possibly possess the property of blocking eumelanin biosynthesis, where the tyrosinase is one of the key enzymes responsible for some types of melanin biosynthesis, a metabolite thought to be essential in eumycetoma pathogenicity and resistance, (Jacobson, 2000; Nosannchuk and Casadevall, 1997).

1.8.4 The most active plant species in the study:

1.8.4.1 Acaia nubica (Benth):

*Acaia nubica* of the family Mimosaceae, is one of the most common wild African Acacia and of widespread throughout central Sudan (Fig.14) The presence of mimosine, a non protein amino acid and related metabolites limits the potential attributes of these plants as supplements in human and animal diets, (Kamadam *et al.*, 1998; Hammond, 1995)
1.8.4.2 *Nigella sativa* L.:

*Nigella sativa* (True black cumin), which belong to the family Ranunculaceae, is an erect annual herb (Fig.15). It is cultivated in various parts of the world and is especially grown in East Mediterranean countries. In Sudan, it is mainly cultivated in northern Sudan and Darfur. Nigella seeds are employed world wide for countries as a condiment/spice in cooking. Black seed is a complex substance of large number of natural compounds most of which are volatile constituents (Fig.16). Biologically, the benefits packed into this tiny seed include its use as carminative antiasthmatic and expectorant and as potential antioxidant and immunomodulatory, antimicrobial, analgesic and antiinflammatory (Elhadi, 2005; Ali *et al*., 1999)
1.8.4.3 *Boswellia papyrifera* (Del.) Hochst:

*Boswellia papyrifera* (Del.) Hochst, of the family Burseraceae, is abundantly growing tree in dry hilly tracts of India, Arabia and East Africa (Fig.17). In Sudan it is endemic to Ingasana and Nuba mountains. It is used medicinally since ancient times. It is a source of gum resin (olibanum/frankincense). It contains essential oil, gum resin. The oleogum resin is rich in triterpenoids. Triterpenes, the main active ingredients of Boswellia, are gaining more and more importance as antituour, antimicrobial and in chronic inflamtory diseases, (Sharma, *et al.*, 2009; Dougall and Bogdan, 1958). Boswellia oleogum resin is fragrant, of brownish yellow colour and commonly used as an incense by the natives (Sharma *et al*, 2009)
1.9 Rationale of the study:

Eumycetoma caused by *Madurella mycetomatis* is treated surgically and with high doses of ketoconazole. Therapeutic responses are poor, and recurrent infections are common. Eumycetoma is an orphan infectious disease in desperate need of international recognition. The disease has a worldwide distribution in the mycetoma belt of 15° north and 30° south; but it is endemic in Gezira, central Sudan. Ketoconazole and itraconazole show varying degrees of clinical efficacy. Hopes on effective medical treatment are increasing with the introduction of broad-spectrum triazoles such as voriconazole and posaconazole. In poor endemic regions in those developing countries where these agents are required most, however, their use may be limited by a lack of essential healthcare facilities, the absence of health insurance coverage, and the high cost of the azoles. In addition, many patients in whom medical treatment was stopped without a well-validated clinical score suffered from recurrence after initial improvement.
1.10 Objectives of the study:

1.10.1 General objectives:

The aim of the study is to investigate the existence of antimycetomal activity among some local Sudanese medicinal plant species, critically selected.

1.10.2 Specific objectives:

- To conduct a general bioguided screening of the selected seven Sudanese medicinal plant species searching for their antimycetomal activity.
- To establish an *in vitro* Minimum inhibitory concentration and Minimum fungicidal concentration for these plants extracts and to use these data to determine the anti-eumycetomal efficacy.
- To select the most active plant species and to be subjected to further phytochemical investigation.
- To isolate and identify the active compound(s) from the most active antimycetomal plant species using bio-guided fractionation assays.
- *In vitro* testing of the identified compounds for their antimycetomal profile.
Chapter Two

Materials and Methods
2. Material and methods

2.1 General screening of seven selected plant extracts for antimycetomal activity:

2.1.1 Fungi used:

To determine the antifungal activity of the plant extracts *Madurella mycetomatis* clinical isolates were collected, fungus was identified; inoculum size was determined and tested.

2.1.2 The plant material:

Based on selection criteria stated previously, for this study we used the following seven plant species:

- Gum resin of *Boswellia papyrifera* of the family Burseraceae
- Root bark of *Acacia nubica* family Mimosaceae
- Seeds and fruits of *Nigella sativa* family Renunculaceae
- Flower buds of *Eugenia caryophyllus* of the family Myrtaceae
- Rhizome of *Zingiber officinalis* of the family Zingiberaceae
- Stem peel of *Cinnamomum verum* of the family Lauraceae
- Fruits of *Piper nigrum* of the family Piperaceae.

Plants samples were purchased from Wad medani local market, Gezira State, Sudan in December 2008 and January 2009, except the root bark of *Acacia nubica* peeled from wild plants, growing in the local vicinity of Wad medani town. Medicinal plants were botanically identified by the Department of Pharmacognosy, Faculty of Pharmacy, University of Gezira. Traditional ethnomedical usage, common vernacular names and plant parts used to obtain extracts are listed in Table (2).
Table 2: Ethno-botanical information on some Sudanese medicinal plants screened for their antimycetoma activity

<table>
<thead>
<tr>
<th>No</th>
<th>Botanical name and (Family)</th>
<th>Common (Vernacular name)</th>
<th>Part used</th>
<th>Ethnomedical use</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td><em>Cinnamum verum</em> (Lauraceae)</td>
<td>Cinnamon (Gerfa)</td>
<td>Stem bark</td>
<td>Spice, hypoglycemic, Antimicrobial</td>
<td>Lu et al. (2012); Burapadga and Bunchoo (1995)</td>
</tr>
<tr>
<td>3</td>
<td><em>Piper nigrum</em> (Piperaceae)</td>
<td>Blackpepper, (Filfil)</td>
<td>Fruit</td>
<td>Spice, Constipation gangrene, expectorant</td>
<td>Mehmood;Gilani (2000)</td>
</tr>
<tr>
<td>4</td>
<td><em>Boswellia papyrifera</em> (Burseraceae)</td>
<td>Olipanum/ Frankincense (Luban)</td>
<td>Gum resin</td>
<td>Anti-inflammatory, fumigant, perfume, Antimicrobial</td>
<td>Gupta et al. (1998); Sato (1997); Carson (1995)</td>
</tr>
<tr>
<td>5</td>
<td><em>Acacia nubica</em> (Leguminosae)</td>
<td><em>Acacia nubica</em> (La’aout)</td>
<td>Root bark</td>
<td>Fumigant, Antirheumatic, Antiinflammatory</td>
<td>Encarnacion (1994); Al-alfy (1975)</td>
</tr>
</tbody>
</table>
2.1.2.1 Preparation of the plant extracts:

2.1.2.1.1 Crude methanolic extracts of seven plant species:

To assess if those seven plants had any antifungal activity against *Madurella mycetomatis*

Plant extracts were prepared from dried coarsely powdered samples macerated separately in conical flasks with methanol over seven days at room temperature. Following filtration, crude methanolic extracts were dried under vacuum using Rotatory evaporator at 60°C. The extracts were dissolved separately in 70% alcohol and subjected to liquid-liquid partitioning using hexane to obtain crude methanol extract, hexane fractions and defatted methanol fractions. After solvent removal, were subjected to biological assays against fungus *Madurella mycetomatis*.

2.1.3 Biological *in vitro* susceptibility testing of the seven crude methanolic extracts against *Madurella mycetomatis*:

The whole experiment procedure was conducted at Mycetoma Research Center, Soba University Hospital and University of Khartoum, Sudan. In order to determine if the crude extracts had any inhibitory activity against clinical *Madurella mycetomatis* isolates, a broth microdilution method was performed. The procedure started with the culturing of *Madurella mycetomatis* for 10 days at 37° in RPMI-1640 medium supplemented with L-glutamine (0.3g/L) and 6 ml MOPS of 0.165M. The resulting mycelia were harvested by 5 minutes centrifugation at 2158 RPM and washed with normal saline. The mycelia were homogenized by sonicating for 20 seconds at 28 micron, using a sonicator (Mesonix-CE MODEL3510E-MT-Mexico S/Nqko40517794). The final inoculum was prepared by adjusting the homogenized fungal suspension to obtain an optical transmission of 70% at 660nm. Of this suspension 100 microliters were transferred into a microtitration plate. To
each well 2 µl of a two-fold dilution of each extract in DMSO was added. The microtitration plate was incubated for 7 days at 37°C before the endpoints were read. The MIC was determined to be the first well in which no growth was visible. Biological assays were compared to negative, positive; inoculum and solvent controls. Results are shown in (Table 8).

2.1.3.1 Reading of results:

The microtitration plates were read using visual reading. Based on that three most active plant species were selected and further subjected to fractionation using different solvents with different polarities into seven extracts designated as (Ex-1 to Ex-7).

2.2 Determination of antimycetomalous activity of the three most active plant species with various extracts (Ex-1 to Ex-7):

This part of the study was implemented at the Department of Medical Microbiology and Infectious Diseases, Erasmus University Medical Center, Rotterdam, Kingdom of the Netherlands. The aim of this part of the study was to determine the minimum inhibitory concentration (MIC) of the three most active plant species. The crude methanol extracts of gum resin of *Boswellia papyrifera* of the family Burseraceae, root bark of *Acacia nubica* family Mimosaceae, seeds and fruits of *Nigella sativa* family Ranunculaceae, were subjected to liquid liquid fractionation procedure using hexane to end with crude methanol extracts, hexane fraction extract and defatted methanol extract of each, (Table 9).
Table: 3 The seven plant extracts (Ex-1 to Ex-7); derived from the three most active plant species extract

<table>
<thead>
<tr>
<th>Extract number</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex-1</td>
<td>Crude methanol extract of <em>Boswellia papyrifera</em></td>
</tr>
<tr>
<td>Ex-2</td>
<td>Hexane fraction of <em>Boswellia papyrifera</em></td>
</tr>
<tr>
<td>Ex-3</td>
<td>Defatted methanol fraction of <em>Boswellia papyrifera</em></td>
</tr>
<tr>
<td>Ex-4</td>
<td>Hexane fraction of <em>Acacia nubica</em></td>
</tr>
<tr>
<td>Ex-5</td>
<td>Defatted methanol extract of <em>Acacia nubica</em></td>
</tr>
<tr>
<td>Ex-6</td>
<td>Hexane fraction of <em>Nigella sativa</em></td>
</tr>
<tr>
<td>Ex-7</td>
<td>Defatted methanol extract of <em>Nigella sativa</em></td>
</tr>
</tbody>
</table>

Solvents were removed, and then all seven extracts were subjected to biological testing against the selected fungus *Madurella mycetomatis*.

**2.2.1 Fungi:**

For the antifungal activity evaluation, strains from the ErasmusMC Type Culture Collection were used: *Madurella mycetomatis*. It was isolated in 1998 in the Mycetoma Research Centre, University of Khartoum and maintained in the ErasmusMC laboratories. These isolates were proven to be *Madurella mycetomatis* based on PCR-RFLP and ITS-sequencing. All fungal strains (36 strains) were maintained on Sabouraud Dextrose Agar (SDA, Oxoid, Basingstoke, UK) at 4°C and transfers were done at three-month intervals.
2.2.1.1 Culture media and inoculum size of Madurella mycetomatis strains:

Sabouraud Dextrose Agar was used for the bioautographic test. Synthetic RPMI (Sigma, St. Louis, MO, USA) medium with L-glutamine buffered to pH 7.0 with 0.165 morpholine propanesulfonic acid (MOPS, Sigma) was prepared according to the CLSI M27-A2 document (Manual of Clinical and Laboratory Standards Institute, 2002) and used for determination of the Minimal Inhibitory Concentration (MIC). Fungal cultures, freshly grown at 35ºC, and fungal suspensions were prepared and the inoculum size was adjusted to 70% optical transmission or 1.5 ± 1.0 x 10³ cfu/mL using Novaspec visible spectrophotometer.

2.2.1.2. Procedure of preparation of fungal suspension of Madurella mycetomatis:

This was made in two steps, stated as follows:

2.2.1.2.1 Transference of cultures of Madurella mycetomatis from solid to liquid media Procedure:

a. 16 falcon tubes were collected and put in relevant tube holding rack into two rows
b. 10 ml of colourless RPMI-1640+ L-glutamine +MOPS were added to each tube (the 10 ml volume is dictated by being the optimum volume when using the sonicator). L-glutamine was added as powder form, then six ml of MOPS were added (3-morpholinopropanesulfuric acid) as a biological buffer. e. Cultures were gently taken from (Sabrauds agar + Gentamicin) plates surface to release fungus from media. Fungus is put on eight falcon tubes (First row), then were sonicated for 5 seconds at maximum force, 15 ml RPMI-1640+L-Glu+MOPS were added, falcon tube were placed in a rack and put in the incubator at 37.5⁰C for 7 days.
2.2.1.2.2 Specification of the inoculum size of *Madurella mycetomatis*:

The 16 falcon tubes prepared a week ago were sonicated for 5 seconds at maximum power to get a homogenous suspension, then were topped up to 25 ml with RPMI-1640+L-glutamine+ MOPS, tubes were centrifuged with round buttom centrifugator (Hettich-Rotana NAP), velocity was adjusted to 3400 Round per Minute, temperature for 23°C, Time 5 minutes, liquid supernatant was poured-off and collected in a big flask, destroyed at waste room, 15 ml colourless RPMI-1640+L-glutamine+MOPS, was added.

The suspension in the 16 tubes is adjusted to 70% optical transmission solution using Novaspec visible spectrophotometer with aid of Semi-micro cuvettes, 1.6 ml (Greiner bio-one GmbH, Austria). The wave length was set to 660 nm, the function was set to transmission, the reference was set to RPMI as 100% optical transmission.15 µl of fungal suspension were added to each well in the flat buttom microtitre plates. Stock solution of Ketoconazole (1600 µg/ml) was prepared and stored at -80°C. The antifungal plant species extracts Ex.1 to Ex.7 were diluted according to the following schedule:

**Table 4: Dilution/ Concentration Schedule of the three most active plant species fractionated to seven extracts (Ex-1 to Ex-7)**

<table>
<thead>
<tr>
<th>Number</th>
<th>Conc.</th>
<th>Source</th>
<th>Volume</th>
<th>DMSO</th>
<th>New Concentartion</th>
<th>Final Concentartion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1600</td>
<td>Stock</td>
<td>25</td>
<td>25</td>
<td>800</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>1600</td>
<td>Stock</td>
<td>25</td>
<td>75</td>
<td>400</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>1600</td>
<td>Stock</td>
<td>25</td>
<td>175</td>
<td>200</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>200</td>
<td>3</td>
<td>25</td>
<td>25</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>200</td>
<td>3</td>
<td>25</td>
<td>75</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>200</td>
<td>3</td>
<td>25</td>
<td>175</td>
<td>25</td>
<td>0.5</td>
</tr>
<tr>
<td>7</td>
<td>25</td>
<td>6</td>
<td>25</td>
<td>25</td>
<td>12.5</td>
<td>0.25</td>
</tr>
<tr>
<td>8</td>
<td>25</td>
<td>6</td>
<td>25</td>
<td>75</td>
<td>6.25</td>
<td>0.125</td>
</tr>
<tr>
<td>9</td>
<td>25</td>
<td>6</td>
<td>25</td>
<td>175</td>
<td>3.31</td>
<td>0.060</td>
</tr>
<tr>
<td>10</td>
<td>25</td>
<td>9</td>
<td>25</td>
<td>25</td>
<td>1.56</td>
<td>0.030</td>
</tr>
</tbody>
</table>
3µL of antifungal agent was added to each of the wells. Plates were covered and taped with the yellow tape and incubated for 7 days at 37.5°C

2.2.2 Bioguided antimycetomal assays of the three most active plant species, fractionated to seven extracts, designated as (Ex-1 to Ex-7):

This part of the study was carried out at Erasmus University Medical Center- Rotterdam - Netherlands, Department of Clinical Microbiology and Infectious Diseases. In vitro susceptibilities of Madurella mycetomatis to the three most active Sudanese medicinal plants, (Boswellia papyrifera, Acacia nubica and Nigella sativa) was assessed, using the standard Modified NCCLS or CLSI method: The Viability-Based 2,3-Bis(2-Methoxy-4-Nitro-5-Sulfophenyl)-5-[(Phenylamino)Carbonyl]-2H-Tetrazolium Hydroxide (XTT) Assay.

2.2.2.1 Preparation of the XTT solution:

Menadione 0.01 mg frozen powder was weighed and put in sterile test tube, then 4 ml acetone was added to the test tube containing menadione, Sodium chloride was added in aliquots of 150, 150, 36, 264 the dissolved, Material in solution is put in aliquots of 30 ml each, then all tubes were put at (-20 °C) refrigerator ready for MIC testing.

2.2.2.2 XTT viability testing:

The seven plant extract fractions were separately tested. The protocol for susceptibility testing (broth macrodilution) was based on the NCCLS procedure for filamentous fungi (approved standard M38-A. National Committee for Clinical Laboratory Standards, 2002). Based on this initial screening, the MICs for the most active plants were also determined at the Department of Medical microbiology and infectious diseases, Erasmus
University Medical Center, Rotterdam, Netherlands using the XTT assay on 13 clinical *Madurella mycetomatis* strains. In this assay fungal cultures, were freshly grown at 37 °C in colourless RPMI-1640 medium supplemented with L-glutamine (0.3g/L) and 6 ml MOPS of 0.165 M for 10 days. The resulting mycelia were harvested by 5 minutes centrifugation at 2158 RPM and washed with normal saline. The mycelia were homogenized by sonicating for 20 seconds at 28 micron, using a sonicator (Soniprep) and fungal suspensions were prepared and the inoculum size was adjusted to 70% optical transmission using Novaspec visible spectrophotometer. To each well 150 µl of this fungal suspension was added. The crude plant extracts F1 to F7 were diluted into DMSO according to, Table (4), and 3 µl of each extract was added to the corresponding wells. All assays were performed in triplicate. Plates were covered and taped with the yellow tape and incubated for 7 days at 37°C. To facilitate endpoint reading the viable fungal mass was determined calorimetrically with XTT as the substrate as described previously (Meletiadis et al, 2001). Tubes containing final concentrations of 250 µg of XTT/ml and menadione (58 µM) were incubated for 2 h at 37°C and for another 3 h at room temperature. The tubes were then centrifuged, and the extinction coefficient of the supernatant was measured at 450 nm in a microplate reader. As a comparator agent, ketoconazole was applied in all plates. All assays were performed in triplicate.

### 2.2.2.3 Reading of the microtitre plates:

Reading was made using spectrophotometer linked to ELIZA reader and a computer software microtitre plate manager. A new file was created, each plate was separately put at right position in the ELIZA reader, an excel sheet was opened. The measured values found on the microplate – reader were filled. The positive control was taken as the values
number (1) + value number (2) + value number (3) + value number (4) and so on up to value number (8) divided by eight. The programmed excel sheet started calculating automatically

2.3. Phytochemical and Bioguided assay of *Boswellia papyrifera*:

2.3.1. Phytochemical investigation of *Boswellia papyrifera*:

Some of the crude methanol extract of *Boswellia papyrifera* was fractionated using separating funnel successively with hexane and ethyl acetate to end with exhausted methanol fraction. (Table 5)

**Table 5: Various fractions of *Boswellia papyrifera* tested for their antimycetomal activity**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-1</td>
<td>Hexane fraction</td>
</tr>
<tr>
<td>F-2</td>
<td>Crude methanol extract</td>
</tr>
<tr>
<td>F-3</td>
<td>Ethyl acetate fraction</td>
</tr>
<tr>
<td>F-4</td>
<td>Methanol fraction (exhausted)</td>
</tr>
</tbody>
</table>

2.3.2 Biological *in vitro* susceptibility testing of *Boswellia papyrifera* fractions:

Biological testing of the four fractions was carried out at Erasmus University Medical Center. The *in vitro* activities of the fractions described under 2.6 were determined with the XTT assay as described under 2.5.2.2 with only one minor modification. Instead of using 150 µl fungal inoculum and 3 µl of the compound to be tested, in this assay 100 µl fungal inoculum and 2 µl of the material to be tested was used.
2.4 Phytochemical and bioguided assay of the active fraction of *Boswellia papyrifera*:

2.4.1 Phytochemical investigation of the most active fractions (2) and (3) of *Boswellia papyrifera* by GC-MS analysis:

Based on the *in vitro* susceptibilities described under paragraph 2.6.1, Fractions 2 and 3 were further phytochemically investigated using Gas liquid Chromatography hybrid Mass spectrophotometry (GC-MS), Shimadzu GC-MS ,QP,capillary column RP,carrier gas helium at flow rate of 1.2 ml/min. Extracts were dissolved in ethyl acetate , one µl injections were made in split mode at injection temperture 280ºC. Initial temperture was 80ºC, then increased at 10ºC/min; compounds were identified by comparison of their mass spectra with those from the NIST’98 mass spectral database.

2.4.2 Biological investigation of the specific triterpenes phytocontituents found in *Boswellia papyrifera* active fractions against *Madurella mycetomatis*:

Biological *in vitro* testing of the four specific triterpene phytoconstituents: Beta-amyrin, Beta-amyrone, Beta-Sitosterol and Stigmatriene, was carried out at Erasmus University Medical Center. The *in vitro* activities of these triterpene against *Madurella mycetomatis*,were determined with the XTT assay as described before, with only one minor modification, instead of using 150 µl fungal inoculum and 3 µl of the compound to be tested, in this assay 100 µl fungal inoculum and 2 µl of the compound to be tested was used. Authenticated reference samples of these triterpene phytoconstituents were purchased from different sources (Table 6).
Table 6: Sources of various specific phytoconstituents authenticated samples found in Boswellia papyrifera active fractions

<table>
<thead>
<tr>
<th>No.</th>
<th>Phytoconstituent</th>
<th>Source of the phytoconstituents</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Beta-amyrin</td>
<td>Fluka</td>
</tr>
<tr>
<td>2</td>
<td>Amarone</td>
<td>Sigma-aldrich</td>
</tr>
<tr>
<td>3</td>
<td>Sitosterol</td>
<td>Carbbosynth</td>
</tr>
<tr>
<td>4</td>
<td>Stigmatriene</td>
<td>Sigma-aldrich</td>
</tr>
</tbody>
</table>

The extinction was read in a microplate reader software linked to enzyme linked Immunosorbent assay (ELISA) at 450 nm, results were saved in an excell sheet, data was introduced into standard software excel program to determine MICs, MICs were determined as MIC-50 values. The MIC-50 is an important parameter for determination of the potency and efficacy of novel antifungal agents, it is defined as the minimum concentration of antifungal required to kill or inhibit the growth of 50% of the fungal population.
Table 7: Diagrammatic representation of the Microdilution plate, showing sites allocated for each compound, dilution/concentration, positive, negative and growth controls

<table>
<thead>
<tr>
<th>Number</th>
<th>Compound</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Beta-myrin</td>
<td>A</td>
<td>128</td>
<td>64</td>
<td>32</td>
<td>16</td>
<td>8</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>0,5</td>
<td>0,25</td>
<td>0,125</td>
</tr>
<tr>
<td>2</td>
<td>Amyrone</td>
<td>B</td>
<td>64</td>
<td>32</td>
<td>16</td>
<td>8</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>0,5</td>
<td>0,25</td>
<td>0,125</td>
<td>0,0625</td>
</tr>
<tr>
<td>3</td>
<td>Sitosterol</td>
<td>C</td>
<td>64</td>
<td>32</td>
<td>16</td>
<td>8</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>0,5</td>
<td>0,25</td>
<td>0,125</td>
<td>0,0625</td>
</tr>
<tr>
<td>4</td>
<td>Stigmatriene</td>
<td>D</td>
<td>64</td>
<td>32</td>
<td>16</td>
<td>8</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>0,5</td>
<td>0,25</td>
<td>0,125</td>
<td>0,0625</td>
</tr>
<tr>
<td>5</td>
<td>Ketoconazole</td>
<td>F</td>
<td>16</td>
<td>8</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>0,5</td>
<td>0,25</td>
<td>0,125</td>
<td>0,0625</td>
<td>0,03125</td>
<td>0,016</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G</td>
<td>Only DMSO</td>
<td>1:1 DMSO:EtOH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>Only EtOH</td>
<td>1:2 DMSO:EtOH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
From figure (7) diagram shows the experimental set up of the plate which was crucial in the experimentation process. It is clearly indicated that:

- Row A was allocated for β-amyrin
- Row B for amyrone
- Row C for Sitosterol
- Row D for Stigmatriene
- Row F for Ketoconazole positive control.
- Column 1 to 11 shows the sequential, serial dilution of each of the six drugs under investigation
- Column 12 was being allocated for Negative control, containing only the solvent of each compound, without any drug
- Row G: column 1, 2 and 3 containing only DMSO, was being allocated for growth control of Ketoconazole.
- Row G: column 4, 5 and 6 containing only ethyl alcohol, was being allocated for Growth control of Stigmasterol
- Row H: column 1, 2, and 3 containing DMSO:EtOH, 1:1 ratio, was being allocated for β-amyrin, amyrone and stigmatriene
- Row H: column 4, 5 and 6 containing DMSO:EtOH, 1:3 ratio, was being allocated for Sitosterol.
Chapter Three

Results and Discussion
3. Results and Discussion

3.1 General screening for detection of anti-mycetomal activity of seven crude methanol plant extracts:

An *in vitro* susceptibility assay that performed in the test tubes for the determination of the antifungal activity of the methanol extracts of seven plant species against a clinical isolate of *Madurella mycetomatis* showed that all seven plant extracts were able to inhibit *Madurella mycetomatis* growth at a concentration of 50µg/ml or less as detected visually. The visual finding of these tubes after ten days incubation were so encouraging to conduct a research using more standardized antifungal susceptibility testing techniques. The microdilution test that made in triplicate and the visual reading of the plates and as demonstrated on Tables (8) showing activity to the entire seven plant species methanol extracts at a low concentration of 50µg/ml or less. Out of these seven species three were found to possess more reliable activity of as low as 0.781µg/ml. These three ranked in sequential manner according to activity as follows: *Boswellia papyrifera*, *Acacia nubica* and *Nigella sativa* (Fig.18-a, 18-b).

The inhibition of mycetoma growth produced by methanol extracts of the tested plants is really an important property, that for the first time to be disclosed. Results obtained also give a good chance and useful guide for further phytochemical processes to identify the active fraction(s) and/or compound(s).
Table 8: Antimycetomal activity of methanol extracts of seven plant species using microtitration plate visual reading

<table>
<thead>
<tr>
<th>Column</th>
<th>Methanol Extract</th>
<th>1 100 µg/ml</th>
<th>2 50 µg/ml</th>
<th>3 25 µg/ml</th>
<th>4 12.5 µg/ml</th>
<th>5 6.25 µg/ml</th>
<th>6 3.125 µg/ml</th>
<th>7 1.56 µg/ml</th>
<th>8 0.781 µg/ml</th>
<th>9 +ve C</th>
<th>10 -ve C</th>
<th>11 Inn. C</th>
<th>12 Sol. C</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><em>Eugenia caryophillus</em></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td><em>Cinnamum verum</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td><em>Piper nigrum</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td><em>Boswellia papyrifera</em></td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E</td>
<td><em>Acacia nubica</em></td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F</td>
<td><em>Zingiber officinalis</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G</td>
<td><em>Nigella sativa</em></td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

N.B.: (-) = No effect or activity on the growth of the fungus; (+) = Moderate activity; (++) = Excellent activity
Fig. 18.a: Mycetoma inhibition by the methanol extracts of seven plant species using microtitration technique

Fig. 18 b: Mycetoma inhibition by the methanol extracts of seven plant species using microtitration technique
3.2. Determination of minimum inhibitory concentration (MIC) for three most active plant species:

The in vitro testing of plant extracts (Ex-1 to Ex-7), prepared by liquid –liquid extraction with hexane obtained from the original methanol extracts of the three most active plant species (Table 3) showed a considerable outcome of results when tested against thirteen strains Madurella mycetomitis that made in triplicate (Table 9). The microplate manager reading using the viability-based (XTT) assay method and after introduction of data into excel sheet software program, all extract exhibited a potential anti-mycetoma effects. Furthermore all proved to possess MIC-50 of 1- 4 µg/ml (Table 10). When MIC values was calculated (Table 11), MIC values affirmed the ability of Boswellia papyrifera to inhibit the growth of Madurella mycetomatis and supersedes the others in activity. Moreover, similar results (Fig.19 - 21) were obtained when the biological tests were performed on Madurella mycetomatis strain (55), that known to contain a characteristic pigment (Ahmed et al, 2004).

Altogether, the considerable MICs demonstrated by the three plants provided a therapeutic potential that is not yet being utilized in the treatment of mycetoma infection.
Table 9: Median MIC values for the seven plant extracts of the three most active plants against 13 *Madurella mycetomatis* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Times tested</th>
<th>Ketoconazole Ref. value</th>
<th>Ketoconazole</th>
<th>Ex1</th>
<th>Ex 2</th>
<th>Ex 3</th>
<th>Ex 4</th>
<th>Ex 5</th>
<th>Ex 6</th>
<th>Ex 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>mm13</td>
<td>3</td>
<td>0.25</td>
<td>0.25</td>
<td>4</td>
<td>4</td>
<td>0.5</td>
<td>4</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>mm14</td>
<td>3</td>
<td>0.064</td>
<td>0.032</td>
<td>1</td>
<td>16</td>
<td>1</td>
<td>16</td>
<td>128</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>mm22</td>
<td>3</td>
<td>0.064</td>
<td>0.032</td>
<td>4</td>
<td>32</td>
<td>8</td>
<td>0.25</td>
<td>128</td>
<td>128</td>
<td>128</td>
</tr>
<tr>
<td>mm25</td>
<td>3</td>
<td>0.064</td>
<td>0.125</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>???</td>
<td>???</td>
</tr>
<tr>
<td>mm30</td>
<td>3</td>
<td>0.032</td>
<td>0.032</td>
<td>1</td>
<td>1</td>
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<td>4</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>mm31</td>
<td>3</td>
<td>0.064</td>
<td>0.125</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>128</td>
<td>8</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>mm35</td>
<td>3</td>
<td>1</td>
<td>0.25</td>
<td>1</td>
<td>0.25</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>0.25</td>
</tr>
<tr>
<td>mm39</td>
<td>3</td>
<td>0.032</td>
<td>0.032</td>
<td>2</td>
<td>0.5</td>
<td>2</td>
<td>128</td>
<td>0.5</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>mm41</td>
<td>2</td>
<td>0.125</td>
<td>0.06</td>
<td>0.5</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.5</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>mm43</td>
<td>3</td>
<td>0.032</td>
<td>0.125</td>
<td>4</td>
<td>128</td>
<td>0.5</td>
<td>16</td>
<td>16</td>
<td>64</td>
<td>32</td>
</tr>
<tr>
<td>mm45</td>
<td>3</td>
<td>0.25</td>
<td>0.25</td>
<td>128</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>mm49</td>
<td>3</td>
<td>0.032</td>
<td>0.125</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>mm54</td>
<td>3</td>
<td>0.032</td>
<td>0.125</td>
<td>128</td>
<td>8</td>
<td>128</td>
<td>0.5</td>
<td>128</td>
<td>1</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Table 10: Calculated MIC-50 of seven plants extracts against 13 *Madurella mycetomatis* strains

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Extract</th>
<th>MIC-50</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ketoconazole reference value</td>
<td>0.125 µg/ml</td>
<td>0.032-0.25</td>
</tr>
<tr>
<td>Ex-1</td>
<td><em>Boswellia papyrifera</em> Crude methanol extract</td>
<td>1 µg/ml</td>
<td>0.5 – 128</td>
</tr>
<tr>
<td>Ex-2</td>
<td><em>Boswellia papyrifera</em> Hexane fraction</td>
<td>1 µg/ml</td>
<td>0.25 – 128</td>
</tr>
<tr>
<td>Ex-3</td>
<td><em>Boswellia papyrifera</em> Methanol fraction</td>
<td>2 µg/ml</td>
<td>0.25 – 128</td>
</tr>
<tr>
<td>Ex-4</td>
<td><em>Acacia nubica</em> Hexane soluble fraction</td>
<td>2 µg/ml</td>
<td>0.25 – 128</td>
</tr>
<tr>
<td>Ex-5</td>
<td><em>Acacia nubica</em> Defatted methanol extract</td>
<td>4 µg/ml</td>
<td>0.5 – 128</td>
</tr>
<tr>
<td>Ex-6</td>
<td><em>Nigella sativa</em> Hexane soluble fraction</td>
<td>2 µg/ml</td>
<td>0.25-128</td>
</tr>
<tr>
<td>Ex-7</td>
<td><em>Nigella sativa</em> Defatted methanol extract</td>
<td>4 µg/ml</td>
<td>0.25 – 128</td>
</tr>
</tbody>
</table>
Fig. 19: Results of XTT-plate, of the seven extracts of the three plants

Fig. 20: The activity of three active plants with seven extracts on *Madurella mycetomatis* strain-55
3.3 Antimycetomal biological testing of the active *Boswellia papyrifera* fractions:

Fractions (Table 5) from the crude active methanolic extract of *Boswellia papyrifera* obtained by liquid-liquid successive extraction and on biological testing conducted against *Madurella mycetomatis* strain (55) the activity was found to be resided in the crude methanol extract (8µg/ml), as well as the ethyl acetate soluble fraction. The other two fractions showed a reasonable fungistatic activity based on the gradual inhibition of the fungus seen on the plate (Table 11 and Fig.21). Recently, the essential oil and methanol extract of *Boswellia papyrifera*, showed bactriocidal against some bacterial species and fungicidal activity against *Candida albicans* and Aspergillus species (Fatouma *et al.*, 2012)

**Table 11: MIC values of *Boswellia papyrifera* fractions**

<table>
<thead>
<tr>
<th>Row</th>
<th>Fraction</th>
<th>Content</th>
<th>MIC-50</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>F-1</td>
<td>Hexane soluble fraction</td>
<td>128 µg/ml</td>
</tr>
<tr>
<td>B</td>
<td>F-2</td>
<td>Crude methanol extract</td>
<td>8 µg/ml</td>
</tr>
<tr>
<td>C</td>
<td>F-3</td>
<td>Ethyl acetate soluble fraction</td>
<td>8 µg/ml</td>
</tr>
<tr>
<td>D</td>
<td>F-4</td>
<td>Methanol soluble fraction(exhausted)</td>
<td>128 µg/ml</td>
</tr>
<tr>
<td>E</td>
<td>F-1</td>
<td>Hexane soluble fraction</td>
<td>128 µg/ml</td>
</tr>
<tr>
<td>E</td>
<td>F-2</td>
<td>Crude methanol extract</td>
<td>8 µg/ml</td>
</tr>
<tr>
<td>F</td>
<td>F-3</td>
<td>Ethyl acetate soluble fraction</td>
<td>8 µg/ml</td>
</tr>
<tr>
<td>G</td>
<td>F-4</td>
<td>Methanol soluble fraction</td>
<td>128 µg/ml</td>
</tr>
</tbody>
</table>
Fig.21: Inhibition of the fungus Madurella mycetomatis strain (55), by various polarity-based fractions of Boswellia papyrifera

Then experiment was repeated on the same plate on a sequential manner. Results were identical (Table11) and (Figure21) above.

3.4 Phytochemical investigation of the active fractions of Boswellia papyrifera:

Two extracts of crude methanol extract (F-2) and ethyl acetate soluble fraction (F-3) of Boswellia papyrifera were subjected to further phytochemical isolation and identification procedure using GC-MS analysis for the actual specific ingredients therein and thought to be responsible for the antimycetoma activity. The triterpenoidal types of compounds and the considerable amount in both extracts were identified as: Beta-amyrin, Beta-amyrone, Beta-sitosterol, and Stigmatriene (Table 12).

It is of interest that results of GC-MS analysis of Boswellia gum extracts demonstrate a high level of content of triterpenes. The presence of triterpenes together with mono and
diterpenes has been shown in Boswellia (Sharma et al, 2009). However, tetracyclic steroids, stigmasterene and sitosterol were found to occur as dominating triterpenes in the investigated Sudanese Boswellia. The results from GC-MS analysis indicate that Boswellia gum could serve as a source of triterpenes.
Table 12: GC-MS analysis presenting triterpenes identified by GC/MS in *Boswellia papyrifera* extracts

<table>
<thead>
<tr>
<th>Compound name and Synonyms</th>
<th>Type of extract</th>
<th>Retention Time</th>
<th>% Area</th>
<th>Diagnostic MS data (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Base Peak</td>
</tr>
<tr>
<td>Beta-amyrone (Olean-12-en-β-3-one)</td>
<td>Crude methanol extract (F2) and Ethyl acetate fraction (F3)</td>
<td>20.614</td>
<td>18.24 (F2) 12.46 (F3)</td>
<td>218</td>
</tr>
<tr>
<td>Stigmatriene Stigmasta(5,7,22-trien-β-3-ol)</td>
<td>Crude methanol extract (F2) and Ethyl acetate fraction (F3)</td>
<td>20.757</td>
<td>0.48 (F2) 72.41 (F3)</td>
<td>392</td>
</tr>
<tr>
<td>Beta-sitosterol (Stigmast(5-en-β-3-ol)</td>
<td>Ethyl acetate fraction (F3)</td>
<td>21.752</td>
<td>2.53</td>
<td>129</td>
</tr>
<tr>
<td>Beta-amyrin (Olean-12-en-β-3-ol)</td>
<td>Crude methanol extract (F2)</td>
<td>22.09</td>
<td>20.17</td>
<td>218</td>
</tr>
</tbody>
</table>
Fig. 22: Chemical structure of triterpenes identified in *Boswellia papyrifera* extracts
Fig. 23: Fragmentation pattern of triterpenes identified by GC–MS in *Boswellia papyrifera* extracts
3.5. Biological testing of the triterpene phytoconstituents of *Boswellia papyrifera*

**active fractions:**

Biological testing of triterpenes found in *Boswellia papyrifera* when tested for their antimycetomal activity on 12 strains of *Madurella mycetomatis*, results on Excel software (Table 13) revealed that: β-amyrin, amyrone, Sitosterol and stigmatriene exhibited varying antimycetomal activity compared to Ketoconazole. On calculation of the MIC-50 (Table 14), results showed that: Stigmatriene possessed the best activity compared to others, indicated by the lowest MIC-50 value achieved by the compound (32µg/ml). This when compared to results shown in (Table 11 and 12), where F2 and F3, exhibited MIC-50 value as lower as eight. It gave an evidence for the hypothesis that some synergistic activity was exerted by other triterpenoid phytoconstituents found in the fractions.

Studies on cultural and medical importance of Boswellia gum resin provided an arsenal of bio-active molecules of various chemical natures and with considerable therapeutic potentials, mainly in the treatment of inflammation, cancerous diseases, wound healing and for their antimicrobial activity (Moussaif and Mechoulam, 2009). It thus seems that the triterpenes reported in this study play a significant role in the effects that Boswellia gum resin exerts in inhibiting the growth of mycetoma.
Table 13: Overview of the inhibitory effects of triterpene phytoconstituents in *Boswellia papyrifera* extract on 12 strains of *Madurella mycetomatis*

<table>
<thead>
<tr>
<th>1. beta-amyrin</th>
<th>2. amyrone</th>
<th>3. Sitosterol</th>
<th>4. stigmatriene</th>
<th>6. ketoconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>#1</td>
<td>#2</td>
<td>#3 Median</td>
<td>#1</td>
</tr>
<tr>
<td>mm13</td>
<td>&gt; 128</td>
<td>&gt; 128</td>
<td>&gt; 128</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>mm14</td>
<td>&gt; 128</td>
<td>32</td>
<td>&gt; 128</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>Mm22</td>
<td>&gt; 128</td>
<td>&gt; 128</td>
<td>&gt; 128</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>Mm25</td>
<td>&gt; 128</td>
<td>0.5</td>
<td>2</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>Mm30</td>
<td>&gt; 128</td>
<td>&gt; 128</td>
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</tr>
<tr>
<td>Mm35</td>
<td>&gt; 128</td>
<td>&gt; 128</td>
<td>&gt; 128</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>Mm39</td>
<td>&gt; 128</td>
<td>&gt; 128</td>
<td>&gt; 128</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>Mm41</td>
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<td>0.5</td>
<td>0.5</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>Mm45</td>
<td>&gt; 128</td>
<td>0.5</td>
<td>0.5</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>Mm54</td>
<td>0.5</td>
<td>&gt; 128</td>
<td>&gt; 128</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>Mm55</td>
<td>2</td>
<td>2</td>
<td>&gt; 128</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>MIC-50</td>
<td>&gt; 128</td>
<td>&gt; 64</td>
<td>&gt; 64</td>
<td>&gt; 64</td>
</tr>
</tbody>
</table>

MIC-50: Minimum Inhibitory Concentration of 50%
N.B.: # = Reading number
Table 14: MICs achieved by the triterpenes found in *Boswellia Papyrifera* extracts on different *Madurella mycetomatis* strains (n=12) tested

<table>
<thead>
<tr>
<th>Strain</th>
<th>B-amyrin</th>
<th>Amyrone</th>
<th>Sitosterol</th>
<th>Stigmatriene</th>
<th>Ketoconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mm 13</td>
<td>&gt; 128</td>
<td>&gt; 64</td>
<td>&gt; 64</td>
<td>&gt; 64</td>
<td>1</td>
</tr>
<tr>
<td>Mm 14</td>
<td>&gt; 128</td>
<td>&gt; 64</td>
<td>&gt; 64</td>
<td>0.125</td>
<td>0.016</td>
</tr>
<tr>
<td>Mm 22</td>
<td>&gt; 128</td>
<td>&gt; 64</td>
<td>&gt; 64</td>
<td>64</td>
<td>0.25</td>
</tr>
<tr>
<td>Mm 25</td>
<td>2</td>
<td>&gt; 64</td>
<td>&gt; 64</td>
<td>0.125</td>
<td>0.063</td>
</tr>
<tr>
<td>Mm 30</td>
<td>&gt; 128</td>
<td>0.25</td>
<td>&gt; 64</td>
<td>&gt; 64</td>
<td>0.016</td>
</tr>
<tr>
<td>Mm 31</td>
<td>&gt; 128</td>
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<td>32</td>
<td>0.031</td>
</tr>
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<td>Mm 35</td>
<td>&gt; 128</td>
<td>&gt; 64</td>
<td>&gt; 64</td>
<td>&gt; 64</td>
<td>1</td>
</tr>
<tr>
<td>Mm 39</td>
<td>&gt; 128</td>
<td>&gt; 64</td>
<td>&gt; 64</td>
<td>32</td>
<td>1</td>
</tr>
<tr>
<td>Mm 41</td>
<td>&gt; 128</td>
<td>0.25</td>
<td>&gt; 64</td>
<td>64</td>
<td>1</td>
</tr>
<tr>
<td>Mm 45</td>
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<td>1</td>
<td>0.031</td>
</tr>
<tr>
<td>Mm 54</td>
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<td>&gt; 64</td>
<td>0.125</td>
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</tr>
<tr>
<td>Mm 55</td>
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<td>&gt; 64</td>
<td>0.125</td>
<td>&gt; 64</td>
<td>1</td>
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<tr>
<td>Median</td>
<td>&gt; 128</td>
<td>&gt; 64</td>
<td>&gt; 64</td>
<td>32</td>
<td>0.031</td>
</tr>
</tbody>
</table>

For stigmatriene MIC-50, was calculated as 32µg/ml; on the other hand an important finding about stigmatriene was that, it managed to inhibit three out of twelve strains at a concentration of 0.125µg/ml (Table 15).
Table 15: Frequency of eumycetoma strains inhibited by each component of *Boswellia papyrifera* active triterpenes

<table>
<thead>
<tr>
<th>MIC</th>
<th>β-amyrin</th>
<th>Amyrone</th>
<th>Sitosterol</th>
<th>Stigmasterene</th>
<th>Ketoconazole</th>
</tr>
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<tr>
<td>0.015625</td>
<td>0</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
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<td>1</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>1</td>
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<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>0.5</td>
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</tr>
<tr>
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<td>64</td>
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<td>0</td>
<td>2</td>
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</tr>
<tr>
<td>128</td>
<td>0</td>
<td>8</td>
<td>10</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>256</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Sitosterol showed inhibitory effect for only one strain at this concentration. At concentrations lower than 0.125 µg/ml none of the triterpenoid phytoconstituents was showing antimycetoma activity. The overall picture of activity profile indicates that the activity of these triterpenes is concentration dependent.
Fig.24: Frequency of *Madurella mycetomatis* strains inhibited by beta-amyrin at various concentrations

β-amyrin at concentration of 0.5 µg/ml, succeeded to inhibit two out of the 12 trains, again at the concentration of 2µg/ml two strains were inhibited, but most of the strains 8 were inhibited at a concentration of equal or more than 256 µg/ml.

Fig.25: Frequency of *Madurella mycetomatis* strains inhibited by beta-amyrone at various concentrations
β- amyrone: At concentration of 0.25µg/ml manage to inhibit three out twelve strains and another strain was inhibited at 64µg/ml and the majority of strains were inhibited at a concentration of 128µg/ml. Natural triterpenes found in flowering plants of pentacyclic type or those of steroidal moiety (tetracyclic) are reported to inhibit the growth of several types of bacteria and fungi, Lupeol (Hifzur and Mohammed,2011) β–amyrin, oleanolic acid (Jabeen ,et al.,2011) have been shown to inhibit the growth of variety of fungal species.

Fig.26: Frequency of Madurella mycetomatis strains inhibited by Sitosterol at various concentrations

Sitosterol: showed antimycetoma inhibitory activity of one strain at a concentration as low as 0.125 µg/ml and another at a concentration of 1µg/ml, ten strains were killed by the compound at a concentration of 128µg/ml. Sitosterol is known to be used in many topical pharmaceutical preparations as active antimicrobial agent. (Al-meshaan et al, 2008).
Sitosterol from methanol extract of aerial parts of *Senecio lyratus* has been shown to exhibit antifungal and antibacterial activity (Kiprono, *et al.*, 2000). It was also found that purified β-sitosterol (20µg/ml) has antimicrobial activity almost equivalent to the standard (Amit, *et al.*, 2012).

**Fig. 27: Frequency of *Madurella mycetomatis* strains inhibited by Stigmatriene at various concentrations**

**Stigmatriene:** Three strains of *Madurella mycetomatis* were inhibited at a concentration of 0.125µg/ml, one strain at a concentration of 1µg/ml and four of the tested strains were inhibited at a concentration less than /or equal to 64 µg/ml. The highest concentration of the compound (128µg/ml) found to kill the remaining four strains. As many studies revealed the usefulness of phytosterols in control of diseases caused by pathogenic fungal species, remarkably active antimycetomal steroids stigmatriene and Sitosterol could possibly mimic certain steroidal like compounds, particularly the well-known antibiotic fusidic acid which exerts its antimicrobial activity by inhibition of protein synthesis (O’Neill *et al.*, 2012).
Fig. 28: Frequency of *Madurella mycetomatis* strains inhibited by Ketoconazole at various concentrations

For **Ketoconazole**: The positive control, it succeeded to inhibit 3 strains of *Madurella mycetomatis* at a concentration of 0.01625µg/ml and all strains were inhibited at a concentration less than or equal to 1 µg/ml. The *in vitro* sensitivity of Ketoconazole is to be looked for in light of the drug resistance imposed by the endogenous ability of the fungus to produce DHN-melanin leading to obligatory increment of dose to achieve acceptable tissue levels with the consequent serious toxic effects of Ketoconazole which is comprising the anti-oestrogen effect and erectile dysfunction, effect of the drug on the mood causing depression and psychosis as well as the hepatotoxicity. These facts highlights the importance of research and drug discovery and development to find new chemical or biomarkers to treat *Madurella mycetomatis*.

It is worth noting that, the tested groups of triterpenoid compounds represent a therapeutic potential in the treatment of mycetoma infection, by virtue of the
characteristic ability of the four compounds to inhibit 1 to 3 *Madurella mycetomatis* strains, at a concentration as low as 0.5µg/ml (Table 15).
Chapter Four

Conclusion and Recommendation
4. Conclusion and Recommendation

4.1 Conclusion:

4.1.1 Summary of findings:

- Screening of seven Sudanese medicinal plants for antimycetomal activity revealed that, all plant extracts were able to inhibit *Madurella mycetomatis* growth at various concentrations, a property that for the first time to be disclosed.

- *Boswellia papyrifera* is the most potential anti-eumycetomal agent.

- Two medicinal plants: *Acacia nubica* and *Nigella sativa* were found to be equal, but next in activity to *Boswellia papyrifera*.

- When *Boswellia papyrifera* was fractionated based on polarity; the crude methanol and the ethyl acetate soluble fraction were found to contain the active part of *Boswellia papyrifera* in comparison to the other two fractions.

- GC-MS hybrid analysis of the active fractions of *Boswellia papyrifera* detected for the first time five phytoconstituents, triterpenoid in nature: Beta-amyrin, Beta-amyrene, Sitosterol and Stigmatriene.

- All identified triterpenes in *Boswellia papyrifera* were found to exhibit considerable varying degree of antimycetomal activity.

- Stigmatriene was found to be the best antimycetomal agent, compared to the other four triterpenoid phytoconstituents; when biologically tested against various strains of *Madurella mycetomatis*.

- Stigmatriene, with the aid of hybrid GC-MS, data sheet was found to be the major component of *Boswellia papyrifera* active fraction, constituting an area percentage of 72.41%.
• Active fractions of *Boswellia papyrifera* were found to show activity at low MIC-50 equal to 8µg/ml. This validates the synergistic activity exerted by other triterpenoid phytoconstituents in the mother extract.

• Stigmatriene killed three mycetoma strains at low concentration as 0.125µg/ml

• Sitosterol managed to kill 1/12 *Madurella mycetomatis* strains at a concentration of 0.125µg/ml, where Sitosterol is known in medical literature for its’ ability to destabilize the fungal cell membrane.

4.1.2 Final conclusion:

• *Boswellia papyrifera* is found to contain four triterpenes as active phytoconstituents. These phytoconstituents namely Beta-amyrin, Beta-amyrone, Beta-sitosterol and Stigmatriene exert their activity in synergistic manner and the highest activity was found to be residing in the Stigmatreine, the major triterpene therein.

• The identified triterpenoids represent an important class of new drugs that have potential for clinical use for prevention and treatment of mycetoma and other fungal ailments.

4.2 Recommendations:

• The sole use and/or synergized combination of either of these triterpenes or their parent natural products extracts with conventional antifungal agents will represent an attractive prospect for the development of new management strategies for mycetoma and should be investigated further *in vitro* models. However, more research will need to be made before this will be clinically practical.
• *Acacia nubica* and *Nigella sativa* are to be considered for further investigation as antimycetoma drugs, in light of their proven potential activity and with reference to the special merits of the two, where; both are locally accessible as wild and/or widely cultivated in Sudan.

• The possible use of *Boswellia* gum or *Acacia nubica* crude extracts could be incorporated in *Nigella* oil as topical preparation for their antimycetomal activity alone or combined to conventional systemic antifungal agents for synergy. Moreover, Local administration of pure active triterpene isolates, though the microfiltration and/or transdermal patches techniques, especially those triterpenes of tetracyclic nature: β-sitosterol and stigmatriene are worthy possible trials. However further studies are needed to evaluate their efficacy and pharmacokinetic properties in the treatment of eumycetoma.

• Melanin pigments produced by fungi have been linked to virulence in some human and phytopathogenic fungi. Hence inhibitors of the secreted tyrosinase of mycetoma targeting its virulence might turn out to be particularly rewarding.

• Natural products rich in antityrosinase molecules that inhibit melanin biosynthesis in *Madurella mycetomatis*, could have a major protective and curative role in management of mycetoma, and should be further investigated.
Chapter Five

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