

CHAPTER ONE

1. INTRODUCTION

1.1. Introduction

The medicinal value and benefits of traditional plants were recognized since ancient times (**Mohamed, 2012**).

An antimicrobial activity is substance that kills or inhibits the growth of microorganisms. Some plants have been investigated significantly for the their antimicrobial activity, and large numbers of plants products have been shown to inhibit the growth of pathogenic microorganisms (**Suleiman,2013**).

In recent years workers in medical field return back to nature particularly in the use of medical plants to treat human ailment. This trend is now supported by the recent World Health Organization (WHO), orientation of biochemically based health care (**Suleiman, 2013**).

In Africa and other developing countries, these traditional medicines derived from plants have continued to form the basis of rural medical care. This is due to the fact that this medicines are easy to get and available in cheap prices (**Mohamed, 2012**).

In Iranian traditional medicine "ITM" the use of plants in treatment of burns, dermatophytes and infectious diseases as antiseptic and anti-inflammatory is common (**Suleiman, 2013**).

According to the World Health Organization " WHO" plants are a source of compounds that have the ability to combat diseases, anti microbial, anti-viral and anti-fungal activities , this affort to any scientific research in Sudan the credibibility of the use of herbs and plants as an effective sources of both traditional and modern medicine (**Suleiman, 2013**).

Microbial infections of the vagina among pregnant women are serious problems, because they can lead to serious medical complication, such as preterm labor, amniotic fluid infection, premature rupture of the fetal membranes and low birth weight of the neonate leading to high prenatal mortality. However, proper identification and treatment will reduce the risk of preterm birth and its consequences (**Abdelaziz et al, 2014**).

On the other hands, multidrug resistant microorganisms are becoming a major challenge worldwide because of irrational use of antibiotics and the increasing population of immune-compromised individuals. In the last decades there has been steady increase in the incidence of systemic opportunistic fungal infections especially in Sub-Saharan Africa. This rise associated with Acquired Immunodeficiency Syndrome (AIDS) pandemic, prolonged antimicrobial therapy, invasive procedures and immunosuppressive therapy (**Nelson et al, 2013**).

Vaginal *Candida species* are emerging as significant opportunistic organisms that increased over the past few decades. The available

treatment of vaginal candidiasis includes azoles (fluconazole, ketoconazole, itraconazole, voriconazoles and clotrimazole) and polyene antifungals such as nystatin and amphotericin B (**Nelson *et al*, 2013**).

1.1. Rationale

Vaginal *Candida species* are emerging as significant opportunistic organisms that increased over the past few decades, particularly among pregnant women. Although conventional antifungal drugs are available include azoles groups groups (fluconazole, ketoconazole, itraconazole) and polyene nystatin and amphotericin B) in considerable quantities, increase resistance to this drugs can result in treatment failure.

Therefore, there is crucial need for the discovery of new safer, and more effective antifungal agents from traditional Sudanese plants, and to verify the claimed activity of this plants uses to treat the infectious diseases.

In Sudan many attempts were carried out to explore the role of these plants in medical field to compact infectious diseases e.g. **Suleiman (2013)** on Guava leaves and Safflower seeds extracts against wound infection bacteria, **Mohamed (2012)** on antifungal activity of *Citrullus colocynthis* on pathogenic dermatophytes, **Abd-Elnabi et al (1992)** on antimicrobial activity of *Acacia nilotic* willd, **Alashry et al (2003)** on Myrrh components, therapeutic value and uses, **Abdallah et al (2009)** on antibacterial activity of *Commiphora molmol* and *Boswellia papyrifera* against methicillin resistant *Staphylococcus aureus* (MRSA) and **Dolara et al (2000)** on local anaesthetic, antibacterial and antifungal activity of Myrrh.

1.3. Objectives

1.3.1. General objective

To detect antifungal activity of *Commiphora myrrha* against *Candida species* isolated from vaginal candidiasis among pregnant women.

1.3.2. Specific objectives

1. To isolate and identify the *Candida species* isolated from vaginal candidiasis.
2. To study antifungal activity of *Commiphora myrrha* methanol, petroleum ether and volatile oil extracts against vaginal *Candida species*.
3. To determine the minimum inhibitory concentrations (MICs) and minimum cidal concentration (MCCs) of selected plants and to compare their activity with the commonly used antifungal agents in Sudan.
4. To identify the major chemical components of the most effective extracts of the tested plants.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. The medicinal plants, and their traditional uses

Herbal medicine - also called botanical medicine or phytomedicine - refers to using a plant's seeds, berries, roots, leaves, bark, or flowers for medicinal purposes. Herbalism has a long tradition of use outside of conventional medicine. It is becoming more mainstream as improvements in analysis and quality control along with advances in clinical research show the value of herbal medicine in treating and preventing the diseases **(Steven and Ehrlich, 2011)**.

Plants had been used for medicinal purposes long before recorded history. Ancient Chinese and Egyptian papyrus writings describe medicinal uses for plants as early as 3,000 BC. Indigenous cultures (such as African and Native American) used herbs in their healing rituals, while other developed traditional medical systems (such as Ayurveda and Traditional Chinese Medicine) in which herbal therapies were used. The researchers found that people in different parts of the world tended to use the same or similar plants for the same purposes **(Steven and Ehrlich, 2011)**.

During the 8th until 11th centuries, Arab physicians upgraded the existing knowledge about herbs and their potential efficacy and safety. The greatest contribution to modern medicine were the immunology system and introduction of microbiological science **(Azaizeh et al, 2008)**.

The Middle Eastern region was covered with more than 2600 plant species of which more than 700 plants were noted for their use as medicinal herbs or botanical pesticides.

Recent ethno-pharmacological surveys revealed that 200-250 plant species till now are used in Arab traditional medicine for the treatment of various diseases **(Azaizeh et al, 2008)**.

In the early 19th century, when chemical analysis first became available, scientists began to extract and modify the active ingredients from plants. Later, chemists began making their own version of plant compounds and over time, the use of herbal medicines declined in favor of drugs. Almost one fourth of pharmaceutical drugs are derived from plants **(Steven and Ehrlich, 2011)**.

Recently, the World Health Organization (WHO) estimated that 80% of people worldwide rely on herbal medicines for some part of their primary health care. In Germany, about 600 - 700 plants based medicines are available and are prescribed by some 70% of German physicians. In the past 20 years in the United States, public dissatisfaction with the cost of prescription medications, combined with an interest in returning to natural or organic remedies has led to an increase in herbal medicine use **(Steven and Ehrlich , 2011)**.

Herbal medicine is used to treat many conditions, such as asthma, eczema, premenstrual syndrome, rheumatoid arthritis, migraine, menopausal symptoms, chronic fatigue, irritable bowel syndrome and cancer (**Steven and Ehrlich, 2011**).

Some of the traditional medicine are still included as part of the habitual treatment of various maladies for example: Myrrh shows numerous beneficial properties including anti-inflammatory, antifungal, antimicrobial, antiseptic, astringent, expectorant and its ability to relax the senses without sedating. it can also be applied topically to fungal infections of the fingernail and toenail, and diluted in a carrier oil for skin applications. Neem demonstrates antifungal, antibacterial, antiviral, antiseptic and antiparasitic. The oil of the Neem nut is one of the oldest and most popular medicinal substances in India. It is added to toothpastes, soaps, shampoos and skin care products. Olive leaf extract has strong antibacterial, antifungal, antimicrobial, antiviral, antiparasitic and antioxidant properties. It is often used as a natural defense against the onset of a cold or flu with good results, and has also been demonstrated to have blood sugar lowering effects. Olive leaf extract can be taken internally or used topically. Henna - Though most commonly used as a natural hair and skin dye, henna also contains a compound called lawsone, which demonstrates antibacterial and antifungal capabilities. Garlic - Used raw to get the medicinal effects. It can be used to treat vaginal yeast infections and fungal infections of the skin or nails (**Natrural Candida-cure website**).

The crude alcoholic extract of *Spsheeranthus indicus* flowers showed antibacterial activity was presented in alkaloidal and in no alkaloidal fraction. Four new alkalodal have been isolated by chromatographic methods (**Sheikh et al, 1986**).

2.2. Botanical ethno- pharmacological properties of myrrh gum

Different parts of selected myrrh gum were recognized as component of the traditional medicine in Sudan. They were arranged with their family, scientific and common name, distribution, botanical description, chemical constituents, antimicrobial activity and medicinal uses.

2.2.1. Myrrh gum *Commiphora myrrha*

Family: *Burseraceae*

Scientific name (s): *Commiphora myrrha* (T.Nees) Engl, *Commiphora abyssinica* (Bevg.) Engl. or, *Commiphora molmol*. Engl.

Common names: African myrrh, Arabian and Yemen myrrh, Somali myrrh, myrrh gum, myrrha, myrrhe, murr (**Blumenthal et al, 1998**).

2.2.1.1. Distribution

The *Commiphora species* native to Africa, Eastern Mediterranean countries, and South Arabia (**Evans, 1989**).

2.2.1.2. Botanical description

The *Commiphora* species that serve as source of myrrh are thorny shrubs or small trees that grow to up 3 meters high. A pale yellow-white viscous liquid exudes from natural cracks or fissures in the bark (Evans, 1989). When air dried, this exudates hardens into a reddish-brown mass that often contains white patches. These tears are for the basis of myrrh resin. Myrrh is usually collected in the summer months (Leung 1980; Evans, 1989; Michie and Cooper 1991).

2.2.1.3. Chemical constituents

Myrrh is oleo-gum resin obtained from the stem of *C.molmol* (Evans, 1989; Michie and Cooper 1991). It contains 2% to 10 % of volatile oil composed of sesquiterpenes, sterol and steroid. Water-soluble gum portion (30% to 60%) polysaccharides as well as ethanol-soluble. After undergoing hydrolysis the myrrh produce variety of sugar 9Furo sesquiterpenes are responsible of myrrh odor and are believed to exert anesthetic, antibacterial, antifungal and hypoglycemic effect (Hanus *et al*, 2005; Zhu *et al*, 2003). When oleo-gum resin is mixed with water, it form an emulsion (Al Ashry *et al*, 2003).

2.2.1.4. Antimicrobial activity of *Commiphora myrrha*

Dolara *et al*, (2000), extracted, purified and characterized 8 sesquiterpene fractions from *Commiphora molmol*. In particular, were focused on a mixture of furanodiene-6-one and methoxyfuranoguaia-9-ene-8-one, which showed antibacterial and antifungal activity against standard pathogenic strains of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*, with minimum inhibitory concentrations ranging from 0.18 to 2.8 µg/ml. These compounds also had local anesthetic activity, blocking the inward sodium current of excitable mammalian membranes.

Aqueous extract of fruit collected from central Sudan showed activity against *C.albicans*, Gram positive and Gram negative bacteria (Abdelnabi *et al*, 1992).

Methanol extract of *Pasidium guajava* leaves inhibit one or more of four tested bacteria (*S.aureus*, *E.coli*, *K.pneumoniae* and *P.aeruginosa*) , but the aqueous extract of *Carthamus tinctorius* seeds exhibited no activity against all tested bacteria and the n-hexane extract of two medicine plants also exhibited no activity against all tested bacteria (Suleiman, 2013).

An in vitro study of 2 sesquiterpenes derived from myrrh discovered antibacterial activity against *S.aureus* and *E.coli* ,and antifungal activity against *C.albicans*. Local anesthetic activity was also noted in mammalian nerve cells (Dolara *et al*, 2000).

Hammer *et al*, (1999) investigated the antimicrobial activity of essential oil and resin extract of *commiphora myrrha* against *A cinetobacter baumanii*, *Aeromonas veronii* biogroup *sobria*, *Candida albicans*,

Enterococcus faecalis, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Serratia marcescens* and *Staphylococcus aureus*, using agar dilution method, which showed the minimum inhibitory concentration % (v/v) as follows >2.0, >2.0, >2.0, 0.25, >2.0, >2.0, >2.0, >2.0, >2.0 and 0.5 respectively), when using the broth microdilution method, and showed the minimum inhibitory concentrations (MICs) and minimum cidal concentrations (MCCs) (% v/v) in *Escherichia coli* was > 4.0 and > 0.5 respectively and in *Candida albicans* MICs was >4.0, MCCs was >4.0.

According to **Abdallah et al, (2009)** who investigated the antibacterial activity of the petroleum ether, ethyl acetate, methanol and water extracts of the oleo-gum resins of two Arabian medicinal plants, *Commiphora molmol* Engl. And *Boswellia papyrifera* Hochst. against methicillin resistant *Staphylococcus aureus* (MRSA), and showed the minimum inhibitory concentration (MIC) ranged between 31.25 and 250 µg/ml for oleo-gum resin methanol extract of *C. molmol* and of *B. papyrifera* ranged from 62.5 to 500 µg/ml, respectively.

2.2.1.5. Medicinal uses

The Arabic term "murr" means bitter and describes myrrh's taste and balsamic odor (**Michie and Cooper 1991; Al Ashry et al, 2003**). Myrrh has been used for centuries as incense (Evans, 1989), and for medicinal purposes (**Michie and Cooper, 1991**). Medicinally, it has been used as an astringent, antiseptic, antiparasitic, antitussive, emmenagogue, and antispasmodic agent. It was commonly included in mixtures used to treat worm, wounds, sepsis, skin and mouth infections, and as suppository form to treat proctitis (**Michie and Cooper, 1991**).

Myrrh has also been reported to treat gout, headache, jaundice, throat ailments, indigestion, fatigue, and paralysis (**Greene, 1993**).

Myrrh has also been used in variety of infectious diseases, including leprosy and syphilis and to treat cancers (**Al Ashry et al, 2003**).

2.3. Genus Candida

Yeast are unicellular, eukaryotic, budding cells, generally round to oval or, less often, elongate or irregular in shape. They multiply principally by blastoconidia (buds), when blastoconidia are produced one from the other, in linear fashion without separating, structure termed a pseudohyphae is formed (**Hazen and Howell 2003**).

The germ tube (GT) is a characteristic morphology observed only in *C. albicans*, confirmation of GT is available as rapid method for identifying *C. albicans* can be reliably identified in 2.5-3 hours using a germ tube test (**Collee et al, 1996**).

2.3.1. Classification of genus Candida

The heterogeneous genus *Candida* belong to the family *Candidaceae* within the ascomycetes. The genus contains approximately 200 species.

This number is not immutable .Technological advances that effect apparent taxonomic relationships will continually result in reassignments of present species and discovery of new species. Teleomorphs encompassing several genera have been demonstrated for different species of *Candida*. The teleomrphic genera include *Calvispora*, *Debaryomyces*, *Issatchenkia*, *Kluyveromyces*, *Pichia* and *Yarrowia*. (Hazen and Howell 2003).

2.3.2. Candidiasis

Are acute to chronic fungal infections involving the mouth, vagina, skin, nails, bronchi or lung, alimentary tract, blood stream, urinary tract, and, less commonly, the heart or meninges.

They are caused by *C. albicans* or other species of *Candida*, and predisposed by extremes of age, wasting and nutritional diseases, excessive moisture, pregnancy, diabetes, long-term antibiotic and steroid use, indwelling catheters, immune suppression, and AIDS (Johnson *et al*, 2002).

Candida albicans is seen as yeasts on body surfaces, and part of the normal flora of the skin, mucous membranes, and gastrointestinal tract, along with other *Candida species*. Normal colonization must be distinguished from infection, when *Candida* invades the tissues forming pseudohyphae and true hyphae (Johnson *et al*, 2002).

In the present study of Nelson *et al*, (2013), five vaginal *Candida species* were isolated and identified. These species included *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida tropicalis* and *Candida parapsilsis*.

2.3.3. Prevalence and transmission

The candidiasis are common among women of reproductive age, with high incidences during pregnancy. For unknown reasons, they are more prevelant in women in Sub-Saharan Africa than in women in developed countries. In Sudan, there is little data about vaginal infections in pregnant women .

Bacterial vaginitis (BV) has been found in 49.8% of subjects, candidiasis in 16.6% , trichomoniasis in 0.5%, gonorrhoeae in 1.8% , and *Chlamydia trachomatis* in 31.3% of Sudanese women in Omdurman Maternity Hospital in Khartoum, Sudan (Abdelaziz *et al*, 2014).

2.3.4. Clinical manifestation

The symptoms of vaginal candidiasis included itching, difficult in walking, dysuria and presence of thick adherent plaques on the vuval, vaginal or cervical epithelium (Nelson *et al*, 2013).

2.3.5. Treatment

The available treatment of vaginal candidiasis includes azoles (Fluconazole, ketoconazole, Itraconazole, Voriconazoles and

Clotrimazole) and polyene antifungals such as nystatin and amphotericin B (Nelson *et al*, 2013).

According to the protocol of ministry of health in Sudan to treat the vaginal candidiasis medicated the Itraconazole used in either creams or suppositories. In case of systemic vaginal yeast infection the doctors medicated single dose of ketoconazole. To prevent and treat vaginal discharges: the genital area must be clean and dry, yogurt with live cultures or *Lactobacillus acidophilus* tablets must be taken to prevent the yeast infection, feminine hygiene sprays, wearing extremely tight fitting pants or shorts must be avoided. Cotton under wear should be put and the blood sugar levels must be under control in case of diabetic women.

2.4. Antifungal susceptibility testing

Antimicrobial susceptibility testing methods for fungi recently have been standardized by the clinical and laboratory standards institutes (CLSI), formerly known as the National committee for clinical laboratory standards (NCCLS, 2000).

The CLSI first published methods for antifungal susceptibility testing of *Candida spp*, and *Cryptococcus neoformans* in 1997, whereas the methods For filamentous fungi were approved in 2002.

A macrodilution methods for susceptibility testing was approved first , quickly followed by a micro dilution method that is simpler and equally effective. It is important to note that only non proprietary methods are standardized by the CLSI. Thus, some methods including colorimetric or E-test methods, although shown in the studies to compare with the micro dilution methods will not be standardized by the CLSI. Although in some instances proprietary tests may be more costly. They are often simpler and more easily implemented in laboratories with little experience working with fungi .Recently, a disk diffusion methods for testing yeast has also been approved by the CLSI (NCCLS, 2004).

Several important factors have been evaluated and standardized in these published guidelines including the use of synthetic media, temperature of incubation, duration of incubation and end of point definition. Several review articles have been published, providing more details than can be discussed here (Mohamed, 2012).

2.4.1. Antifungal susceptibility testing methods

A. Broth dilution

The aim of broth and agar dilution methods determined the lowest concentration of the assayed antimicrobial agent (minimal inhibitory concentrations, MICs) which, under defined test conditions, inhibits the visible growth of the yeast colonies were being investigated. MICs values are used to determine susceptibilities of fungi to drugs and also to evaluate the activity of new antimicrobial agents. The most commonly

used method in the dilution techniques is the broth dilution technique, which described by **Rahman *et al*, (2001)**.

Broth dilution methods for antifungal can be performed by macrodilution or microdilution methods (**NCCLS, 2002**).

The macrodilution method is prepared in test tubes in 1 ml volumes. This method has been established as basis for comparing all other methods of susceptibility testing for yeast. The macro dilution method has largely been replaced by the micro dilution method that is performed in a 96-well micro dilution plate in volumes of 200 μ l per well. Both methods use a starting inoculum of $0.5-2 \times 10^3$ colony-forming units (CFU/ML) for *Candida spp* and 1×10^4 CFU/ML for *C. neoformans*. The plates are incubated at 35 °C for 48 or 72 h for *Candida spp* and *C. neoformans* respectively. The minimum inhibitory concentrations (MICs) end point differs among the antifungal agents currently approved.

The end point for amphotericin B is defined as lowest concentration resulting in no visible growth, whereas the end point for the azole antifungals is when either 80 or 50% reduction in fungal growth reduction occurs micro dilution and macro dilution methods respectively. Trailing has been describe when micro dilution methods are performed on *Candida species* to evaluate the MICs of the a zole antifungal. Trailing is reduced, but persistent growth of organism even in the presence of every high concentration of azole. This persistent growth cause confusion when reading the MIC end point for the azoles against *Candida species*. Not all *Candida* display these phenomena, and it may not be apparent at 24 h. The trailing effect can be minimized by decreasing with the pH of culture medium or the addition of dextrose and starting with a higher inoculums. The methods approved for testing common filamentous fungi include *Aspergillus species*, *Fusarium species*, *Rhizopus species*, *Pseudallescheria byodii* and *Sporothric schenckii* (**NCCLS, 2002**).

B. Colorimetric testing

Colorimetric tests have been developed for both yeast and filamentous fungi (**Espinel-Igroff *et al*, 2009**).

Others methods are spectrophotometric testing, disk diffusion and flow Cytometry (**Espinel-Igroff *et al*, 2009**).

2.4.2. Fungicidal testing

The ability of compound to kill a pathogen as opposed to simply inhibiting its growth is an apparently desirable quality, particularly in the testing of decreased immunity. Although several studies have characterized the fungicidal activity of antifungal agents. There is no standardized method for doing so.

NCCLS has published guidelines for evaluating the cidal activity of antibacterial agents (**NCCLS, 1998**).

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Study design

Descriptive - crosses sectional study.

3.2. Study area

Al-Saudi and Al-Shaikh Fadol Maternity Hospital, in Khartoum State.

3.3. Study population

All clinically suspected cases of *Candida* vaginitis arrived the unit of emergency during period of the study from March to June 2014.

3.3.1. Inclusion criteria

Pregnant women who presented with self-reported symptoms of *Candida* vaginitis .

3.3.2. Exclusion criteria

Pregnant women with bacterial vaginitis and trichomoniasis were excluded.

3.4. Sampling

Non-probability sampling.

3.4.1. Sample size

A total of ninety vaginal swab samples (n=90) were collected.

3.5. Study variables

Screen of *Candida* vaginitis (dependent variable). Etiological agents, age , trimester of pregnancy, history of abortion and Diabetes mellitus (DM) as independent variables.

3.6. Data collection

By questionnaire contain all study variables (**Appendix I**).

3.7. Ethical Clearance

Permission of this study obtained from the local authorities in the area of the study, the objectives of the study clearly and simply explained to all individuals participating in the study, verbal inform consent was obtained (**Appendix I**).

3.8. Data analysis

All collected data were analyzed using Microsoft Office Excel 2007 and SPSS (Statistical Package of Social Science) soft program version 11.5.

3.9. Experimental Work

3.9.1. Specimen collection and sampling technique

Vaginal swabs were collected from pregnant women with symptoms of vaginal candidiasis. The symptoms included itching, difficult in walking, dysuria and presence of thick plaques on vulval, vaginal or cervical epithelium.

3.9.2. Method of collection

Specimens were collected from patients by aid of sterile cotton tipped swabs with help of gynecologist. Samples collected were taken immediately -or put in Aims transport medium (ATM)- to the hospital

laboratory for *Candida species* isolation, identification and screening of antifungal activity of *Commiphora myrrha*.

3.9.3. Microscopic examination

Vaginal swab examined microscopically by normal saline wet mount and Gram's staining for the presence of budding yeast and pseudo-hyphae of *Candida species*.

3.9.3.1. Wet preparation

The swab was rolled on clean slide and a drop of sterile normal saline was added, and covered with cover slip, then examined under microscope using X10 and X40 for budding yeast and pseudo-hyphae of *Candida species* (Chessbrough , 2000).

3.9.3.2 .Gram's stain

The swab was put on clean slide to make smear air dried, then the smear was fixed with alcohol, covered with crystal violet stain for one minute. Then washed by tap water, and then covered with lugol's iodine for one minute. Iodine was washed off, and smear was decolourized with acetone-ethanol for few seconds and washed by tap water. Safranin was added for two minutes, washed off with tap water and let to air dry and microscopically examined using oil immersion objective (X100) to observe yeast cell morphology, size, Gram positive reaction and presence of pus cells, epithelial cells (Collee *et al*,1996).

3.9.4. Culture Swab

The swab was cultured on Sabouraud Dextrose Agar (SDA) with Gentamycin (50 ml SDA/ 48 mg/ml Gentamycin) and incubated at 37 °C for 24-48 hours for growth of *Candida species*.

3.9.5. Identification of *Candida species*

3.9.5.1. Gram's stain

Indirect Gram's stain was performed for yeast suspected colonies which revealed Gram positive yeast cells .

3.9.5.2. Germ tube test (GTT)

Test proves yeast germination, and its characteristic for the detection of *Candida albicans* (unless when yeast germination is not characteristic). This is rapid test for presumptive identification of *C.albicans*. Three drops of serum were put into small vitek tube by using a Pasteur pipette, a colony of yeast was touched by sterile wire loop and emulsified it in the serum. After incubation at 37 °C for 2-4 hours but no longer, then a drop of the serum was transferred to a slide covered by cover slip and examine microscopically using X40 objective. Germ tubes are appendages half the width and 3 to 4 times the length of the yeast cell from which they arise. There is no constriction between the yeast cell and the germination tube. Positive test: presence of short lateral filament (germ tube) for *C.albicans*, Negative test: yeast cell only for *C. non albicans* (Collee *et al*, 1996).

3.9.5.3. CHROM agar *Candida*

Candida chromogenic media prepared according to manufacturer (BDTM diagnostic) instruction, and the organism inoculated in the media, then incubated at 37 °C for 48 hour. After that the growth of *Candida spp* observed by the change in the colour of the colonies according to the pigment, as a result of reaction between chromogenic substrate and enzymes that secreted by different *Candida spp*, allowing organisms to be identified to the species level by their colour and colony characteristics. CHROMagar has been shown to allow differentiation of *Candida* yeast by colour and morphology (Odds and Bernaerts, 1994).

3.9.5.4. Dalmue culture plate (Corn meal agar)

By using sterile inoculating needle or loop, the appropriate yeast colony was touched and immediately scraped or cut “X” through prepared corn meal agar according to manufacturer instruction (HIMEDIA) (in the middle on one half of the agar plate, the arms of the x should be about 2 cm long. This procedure was repeated, making a duplicate” X” in the middle on the other half of the agar plate. Using sterile forceps, sterile cover slip was centered over the cross of one of the “X” patterns. Plate was inverted and incubated up to 4 days (96 hour) at 25 ± 2 °C. Plates were examined daily for the development of chlamydospores with the aid of dissecting or stage microscope. The” X” without cover slip serves as a growth control. The result seen by microscopic examination of the yeast growth under the cover slip revealed. Chlamydospore that appear as terminal double walled spheres on the pseudo hyphae indicates positive result of *C.albicans* (Collee *et al*, 1996).

3.9.5.5. Zymogram (Carbohydrate fermentation test)

Fermentative yeasts recovered from clinical specimens produce carbon dioxide and alcohol. Production of gas rather than a pH shift is indicative of fermentation. Galactose, maltose, sucrose, galactose and trehalose were used in the test. The five ml of carbohydrate (PH, 7.4) containing 1 % peptone, 1 % sugar, 0.3 % beef extract and 0.5 % NaCl, 0.2 % Andrad’s in distilled water medium was dispensed in sterilized Durham tube and 0.2 ml of saline suspension of the test organism was added and incubated at 37 °C for 10 days (Collee *et al*, 1996).

3.9.5.6. Auxanogram (Carbohydrate assimilation test)

The carbohydrate assimilation test determines the ability of a yeast isolate to use a particular carbohydrate substrate as its sole carbon in a medium. Yeasts were cultured in SDA for 24 h at 37°C, then colonies were suspended in sterile normal saline and adjusted to 0.5 McFarland standard. Basal medium I (Yeast Nitrogen Base + agar base) (YNB, Difco) was prepared and dispensed in 20 ml amount and autoclaved at 121 °C for 15 minutes. The agar medium was allowed to cool at 45 °C. A volume of 20 ml of agar medium was mixed with 1.5 mL of *Candida spp*

suspension in Falcon tubes. The suspension was placed in 15 cm Petri dishes and after solidification of the medium, discs with the 2% carbohydrates (maltose, trehalose, xylose, galactose, lactose, sucrose and glucose) were placed following a guide. The suspension was incubated in 30°C for 48 h, and observed daily. Glucose was used as positive control, since all the species of *Candida* assimilate this carbohydrate. The carbohydrate assimilation was observed with a presence of a halo of growth around each carbohydrate (Collee *et al*, 1996).

3.10. Extraction of medicinal plants

3.10.1. Collection and preparation of plant sample

The myrrh gum(*Commiphora myrrha*) samples used in this study was collected from United Kingdom of Saudia Arabia. They were authenticated by protocol of Medicinal and Aromatic Plant Research Institute (MAPRI). The dried myrrh gum sample was cleaned from dust and grass then were separately crushed to a powder form using sterilized mortar and pestle.

3.10.2. Plant extraction

3.10.2.1. Preparation of crude extract

Extraction was carried out according to method described by **Sukhdev *et al*, (2008)**: 100 g of *Commiphora myrrha* was successively extracted with petroleum ether and methanol using soxhelt extractor apparatus. Extraction carried out for about 4 hours for petroleum ether and, 8 hours for methanol. Solvents were evaporated under reduced pressure using rotary evaporator apparatus. Finally extracts allowed to air in Petri dishes till complete dryness and the yield percentages were calculated as followed: $\text{Weight of extract obtained} / \text{Weight of plant sample} \times 100$.

3.10.2.2. Distillation of volatile oils

Hundred grams of *Commiphora myrrha* was placed in 2000 ml rounded bottom capacity flask. 1000 ml of distilled water was added and the Clevenger receiver (lighter than water) (Duran West Germany) and condenser attached to the top of the flask. The system was heated at 100 °C for about 4 hours till the volume of oil above water layer at the receiver was constant. Oil was pipetted, dried over sodium sulphate anhydrous and stored in a dark container in a refrigerator till used .The yield percentages were calculated as followed: $\text{Volume of oil} / \text{weight of plant sample} \times 100$.

3.10.3. Preparation of fungal suspension

The fungal cultures were maintained on Sabouraud dextrose agar (SDA), incubated at 25 °C for 4 days. The fungal growth was harvested and washed with sterile normal saline and finally the suspension was put in 100 ml of sterile normal saline, and was stored in the refrigerator until used.

3.10.4. Quality Control

strains Prior to screening of antifungal activity of *Commiphora myrrha*, each isolate was sub-cultured at least twice on SDA for 24 hours before use. This was to obtain pure culture of each isolate. The Quality Control strain used was *Candida albicans* ATCC 7596 .

3.11. In vitro antifungal activity of Commiphora Myrrha extracts

Antifungal activity was measured *in vitro* in order to determine:

- a. The potency of an antifungal agent in solution. Sensitivity tests were also used to evaluate new antimicrobial agents by testing them against large number of organisms of known susceptibility and to compare these results with drugs already available (**Garrod *et al*, 1981**).
- b. Its concentration in body fluids or tissue
- c. The sensitivity of a given microorganism to known concentration of the drug.

3.11.1. Measurement of antifungal activity

Determination of these quantities was undertaken by one of two methods: dilution or diffusion. Using an appropriate standard test organism, these methods can be employed to estimate either the potency of antifungal in the sample or the sensitivity of the microorganism.

3.11.1.1. Diffusion methods

In the diffusion technique, the tested organisms were exposed on sabouraud dextrose agar , then put the sterile filter paper disc on the agar, which was impregnated by diffusion gradient of the chemotherapeutic drug arising from a reservoir (**Anderson,1970; Garrod *et al*, 1981**). The reservoir may be a compressed tablet (**Lund *et al*, 1951**).

3.11.1.2. Dilution methods

Dilution tests on solid media involve addition of series concentrations of *Commiphora myrrha* extracts (50, 25, 12.5 and 6.25 mg/ml) to 20 ml of Sabouraud dextrose agar 2x2 which had been melted and cooled to 45-50° C, the resultant mixtures are then poured as plates into Petri-dishes or as slants into test tubes. Standardized inocula are seeded onto the surface of the medium and MIC read after an appropriate incubation period (**Garrod *et al*, 1981**).

In these methods, it is essential to test strains of known susceptibility with each series of unknowns in order to be sure against drug deterioration, inaccuracies in dilution or variation in the medium (**Gould *et al*, 1975**). Breakpoint concentrations of antifungal drugs were used to characterize antifungal activity: the interpretive categories were susceptible, moderately susceptible (intermediate), and resistant. These concentrations were determined by considering pharmacokinetics, serum and tissue concentrations following normal doses, and the population distribution of MICs of a group of fungi for a given drug (**Gould *et al*, 1975**).

3.11.2. Determination of minimum inhibitory concentrations (MICs) by agar plate dilution method

The principle of the agar plate dilution was the inhibition of the growth on the surface of the agar by the plant extracts incorporated into the medium. Plates were prepared in the series of decreasing concentrations of the plant extraction in the following order 50, 25, 12.5, 6.25 mg/ml. The bottom of each plate was marked off into 5 segments. The tested organisms were grown in broth over night to contain 10^8 organisms per ml. Loop-full of diluted culture was spotted with a standard loop which delivers 0.001 ml on the surface of each segment and then incubated at 37 °C for 24 hours. The end point (MIC) was the least concentration of antimicrobial agent that completely inhibits the growth. Results were reported as the MIC in mg/ml.

3.11.3. Determination of minimum inhibitory concentrations (MICs) and minimum cidal concentrations (MCCs) by broth micro dilution method:

All extracts were subjected to antifungal testing and the most prominent active was further tested to find out MICs and MCCs.

3.11.3.1. Resazurin solution

Resazurin is an oxidation-reduction indicator used for evaluation of viable cells. It is a blue, non-fluorescent and non toxic dye that become pink and fluorescent when reduced to resafurin by oxidoreductase enzyme within the viable cells. The resazurin solution was prepared by dissolving 5 g of resazurin powder (Sigma-Eldrich GmbH, Germany) in 10 ml of sterile distilled water. The solution was mixed on a vortex mixer until powder was completely dissolved and the solution was homogeneous.

3.11.3.2. Micro titter plate assay:

The antifungal activity of *Commiphora myrrha* extractions were assessed by using modified version of the micro dilution technique which described by Ivanova *et al*, (2013). The antifungal assay was performed by using sterile 96-well plate, and the Minimum Inhibitory Concentrations (MICs) value was determined for the estimating the antifungal activity. All the assay were performed under aseptic conditions. Resazurin used as an indicator for the yeast growth assay, 0.5 McFarland standard used to adjust fungal concentration, and sabouraud dextrose agar was used as a medium for fungal culture.

The first step of the assay was added 50 µl of normal saline to all 96-well. The second step was added 50 µl of crude extracts and other fraction into the first 10 rows of the plate. Serial dilutions were performed using a multichannel pipette. Tips were discarded after use, each well had 50 µl of the test material in serially descending concentrations. The first 10 rows were used for evaluation of activity of the extractions, while the

last 2 rows were used for positive and negative control. The third step was added to each well 30 µl of sabouraud dextrose broth (SDB) 3×3, then 10 µl of fungal suspension (5×10^8 CFU/ml) was added. Each plate had a set of controls: positive control (viability control) comprised of 30 µl of SDB, 10 µl of fungal suspension and 10 µl of resazurin, while the negative control (sterility control) comprised of 50 µl of SDB and 10 µl resazurin added after incubation period.

Each plate was wrapped loosely with cling film to prevent dehydration and contamination, then the plates were placed in an incubator at 37°C for 24-48 h. After incubation period 10 µl of resazurin was added to all wells. A blue colored solution indicated the growth inhibition in test wells, while pale pink to colorless indicated microbial growth or absence of inhibition. The lowest concentration at which no color change occurred was taken as the MICs.

To determined MCCs: after the first incubation period all wells were subcultured on sabouraud dextrose agar, then the plates or Petri-dishes were placed in an incubator at 37°C for 24-48 h. After incubation period the lowest concentration of plant extracts which gave sterile culture was regarded as MCCs. All the test were performed in triplicate.

3.11.3.3. MICs

The lowest concentration of the plant extract that did not permit any visible growth of the inoculated test organism in broth culture was regarded as MICs in each case.

3.11.3. 4. MCCs

The lowest concentration of plant extract which gave sterile culture after incubation period was regarded as MCCs.

3.12. Phytochemical screening

The general phytochemical screening for the active constituents was carried out for the most effective methanol extract of *Commiphora myrrha* using the Gas Chromatographic Mass Spectroscopy (GC-MS).

In gas chromatography , the mobile phase (or moving phase) was a carrier gas, usually an insert an un reactive gas such as nitrogen. The stationary phase was a microscopic layer of liquid or polymer on an insert solid support inside a piece of glass or metal tubing called a column (a homage to the fractionating column used in distillation).

The instrument used to perform gas chromatography was called Chromatograph (or Aerograph, gas separator). The gaseous compound was being analyzed interact with the walls of the column, which was coated with a stationary phase. This causes each compound to elute at a different time, known as the retention time (RT) of the compound. The comparison of retention times were what gives GC its analytical usefulness (Suleiman, 2013).

CHAPTER FOUR

4. RESULTS

In the present study (4) vaginal *Candida species* were isolated and identified. These species included *Candida albicans*, *Candida glabrata*, *Candida parapsilosis* and *Candida guilliermondii*.

4.1. Identification of yeasts

The characteristic and biochemical properties of clinical isolates of *Candida species*, are shown in table 1.

Table 1: The characteristic and biochemical properties of tested *Candida species* isolates.

Candida species	Characteristic and Biochemical properties															
				Assimilation of							Fermentation of					
	GT	MYC	UR	G	M	S	L	G ₂	R	I	G	M	S	L	G ₂	T
<i>Candida albicans</i>	+	+	-	+	+	+	-	+	-	-	+	+	-	-	+/-	+/-
<i>C.parapsilosis</i>	-	+	-	+	+	+	-	+	-	-	+	-	-	-	+/-	-
<i>C.guilliermondii</i>	-	+	-	+	+	+	-	+	+	-	+	-	(+)	-	(+)	(+)
<i>C.glabrata</i>	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	+

Key GT: germ tube/chlamydospores, MYC: pseudo-or true mycelium; ,UR: urease, G: glucose, M: maltose , S: sucrose , L: lactose, G₂: galactose, R: raffinose, I: inositol, T: trehalose, (-): negative, (+): positive, (): indicates variation, with most likely reaction given within the brackets.

4.1.1. Growth on chromogenic agar

The result of growth on chromogenic agar was as the following : the product identifies *C.albicans* by growth as light to medium green colonies, *C.tropicalis* by growth as steel blue colonies accompanied by purple pigment diffusion into surrounding agar, and *C.krusei* by growth as large, fuzzy, ross-colored colonies with white edge, we were able to

identify the following individual non albicans species: *C.glabrata* (dark pink and wet colonies), *C.krusei* (light pink and dry colonies) and we were able to differentiate it from *C.glabrata* by urease test, *C.albicans* (green and wet colonies) and other *Candida species* (white and wet colonies) ,are shown in figure 1



Fig 1: Growth of different *Candida species* on CHROM agar

4.2. Frequency of samples according to the age of pregnant women
Out of the 90 specimens, 50 were ranged in 25-34 age group, 23 were ranged in 35-44 age group, 15 were in 15-24 age group, whereas 2 were in more than 44 age group, as shown in figure 2.

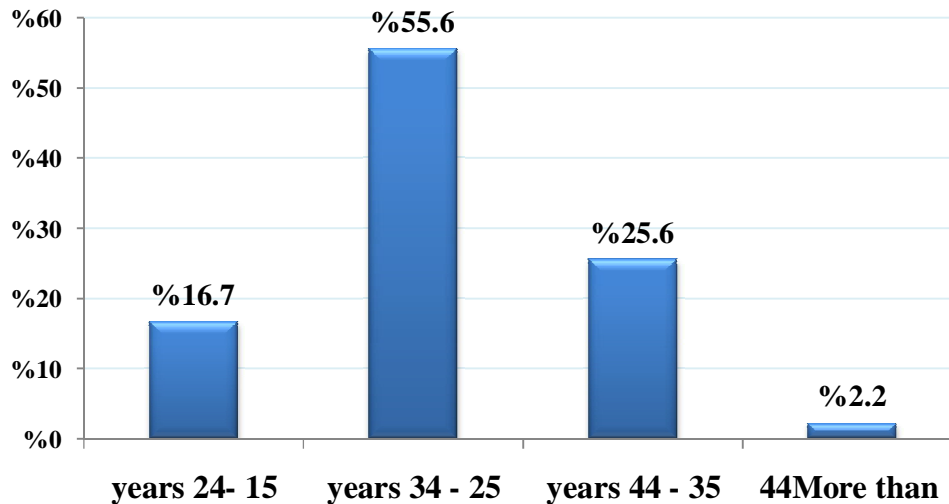


Fig 2: Frequency of samples according to the age of pregnant women

4.3. Frequency of samples according to the trimester of gestation

Out of 90 specimens, 57 (63.3%) were in the third trimester of gestation, 14(15.6%) were in the second trimester of gestation , whereas 19 (21.1%) were in the first trimester of gestation, as shown in table 2

Table 2: Frequency of samples according to the trimester of gestation

Trimester	Frequency	Percent
1 st	19	21.1%
2 nd	14	15.6%
3 rd	57	63.3%
Total	90	100.0%

4.4. The frequency and percentage of *Candida* growth

Out of the 90 investigated samples, 50 (55.6%) showed no growth, while 40 (44.4%) showed the *Candida* growth as shown in table 3.

Table 3: The frequency and percentage of *Candida* growth

Isolate	Frequency	Percent
No growth	50	55.6%
Growth	40	44.4%
Total	90	100.0%

4.5. Frequency and percentage of tested *Candida species*

As shown in table 4 and figure 3, four different *Candida species* were isolated in order to frequency. *Candida albicans* 20(50%), *C.guilliermondii* 8(20%), *C.glabrata* 6 (15%) and *C.parapsilosis* 6 (15%).

Table 4: Frequency and percentage of tested *Candida species*

Isolate	Frequency	Percent
<i>Candida glabrata</i>	6	15.0%
<i>Candida guilliermondii</i>	8	20.0%
<i>Candida albicans</i>	20	50.0%
<i>Candida parapsilosis</i>	6	15.0%
Total	40	100.0%

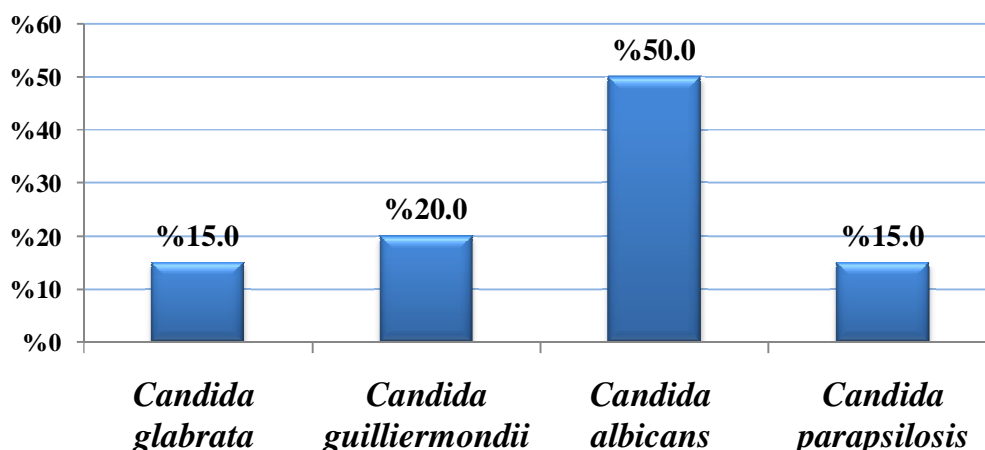


Fig 3: Frequency and percentage of tested *Candida species*

4.6. Frequency and percentage of tested *Candida species* according to the trimester of gestation

Out of the 40 isolated *Candida species*, 27/40 isolated from the third trimester of gestation, followed by 7/40 isolated from the first trimester of gestation and 6/40 isolated from second trimester of gestation. The distribution of the isolates according to the trimesters are shown in table 5. All types of isolates are evident and the majority in third trimester.

Table 5: Frequency and percentage of *Candida species* according to the trimester of gestation

Trimester	Isolate				Total
	<i>Candida glabrata</i>	<i>Candida guilliermondii</i>	<i>Candida albicans</i>	<i>Candida parapsilosis</i>	
1 st	0 (0%)	1 (14.3%)	5 (71.4%)	1 (14.3%)	7 (100.0%)
2 nd	0 (0%)	1 (16.7%)	3 (50.0%)	2 (33.3%)	6 (100.0%)
3 rd	6 (22.2%)	6 (22.2%)	12 (44.4%)	3 (11.1%)	27 (100.0%)
Total	6 (15.0%)	8 (20.0%)	20 (50.0%)	6 (15.0%)	40 (100.0%)

4.7. Frequency and percentage of tested *Candida species* according to the diabetic pregnant women

Out of the 40 isolated *Candida species*, 31/ 40 were in the non-diabetic pregnant women , while 9/ 40 were in the diabetic pregnant women, as shown in table 6. *Candida albicans* was the major isolate in both diabetic and non diabetic women.

Table 6: Frequency and percentage of tested *Candida species* according to the diabetic pregnant women

Diabetic	Isolate				Total
	<i>Candida glabrata</i>	<i>Candida guilliermondii</i>	<i>Candida albicans</i>	<i>Candida parapsilosis</i>	
No	6 (19.4%)	5 (16.1%)	14 (45.2%)	6 (19.4%)	31 (100%)
Yes	0 (0%)	3 (33.3%)	6 (66.7%)	0 (0%)	9 (100%)
Total	6 (15.0%)	8 (20.0%)	20 (50.0%)	6 (15.0%)	40 (100.0%)

4.8. Distribution of vaginal candidiasis according to the history of abortion

Distribution of vaginal candidiasis in pregnant women according to their history of abortion is shown in table 7. No significant association ($p = 0.233$) was found between history of abortion and the presence of vaginal candidiasis in the studied group, as shown in table 7.

Table 7: Association between vaginal candidiasis and numbers of abortions in pregnant women

Abortion	Growth of isolates		Total	P value
	No growth	Growth		
No	31 (62.0%)	19 (38.0%)	50 (100.0%)	0.223
Yes	13 (50.0%)	13 (50.0%)	26 (100.0%)	
Total	44 (57.9%)	32 (42.1%)	76 (100.0%)	

4.9. Screening for antifungal activity of *Commiphora myrrha*

In the preliminary screening for antifungal activity of *Commiphora myrrha*, belonging to *Burseraceae* family, the total number of extracts examined against the different *Candida species* (standard and clinical isolates) was three. the three extracts exhibited inhibitory effect against 4 clinical isolates of *Candida species*: *Candida albicans*, *C.parapsilosis*, *C. guilliermondii*, *C.glabrata* and standard *Candida albicans* ATCC 7596.

Table 8: Weight and yield % of extracts obtained by using different solvents

Wight of <i>Commiphora myrrha</i> gums	Petroleum ether		Methanol		Volatile oil	
	Wight of extract	yield %	Wight of extract	yield %	Wight of extract	yield %
100 g	8.53 g	8.53 %	25.472 g	25.472%	6.8 g	6.8 %

Methanol (MeOH), petroleum ether (C₆H₁₄) and volatile oil extracts of *Commiphora myrrha* showed antifungal effect against *Candida albicans*, *C.parapsilosis*, *C. guilliermondii*, *C.glabrata* and standard *Candida albicans* ATCC 7596 with the concentration 100%, are shown in table 9 and table 10 and figures 4-11.

Table 9: Antifungal activity of *Commiphora myrrha* (soxhlet extracts and volatile oil extract) against *Candida species* (standard and clinical isolates)

Family/ botanical/ Vernacular Names	Part used	Solvent Used	Microorganisms and their susceptibility to different extracts				
			<i>Candida albicans</i>	<i>Candida glabrata</i>	<i>Candida parapsilosis</i>	<i>Candida guilliermondii</i>	<i>Candida albicans</i> ATCC 7596
<i>Burseraceae</i> , <i>Commiphora myrrha</i> , Murr (Almurr alhigazi)	Resin (gum)	MeOH	S (100%)	S (100%)	S (100%)	S (100%)	S (100%)
		C ₆ H ₁₄	S (100%)	S (100%)	S (100%)	S (100%)	S (100%)
		Volatile Oil	S (100%)	S (100%)	S (100%)	S (100%)	S (100%)

Note

C₆H₁₄ (petroleum ether) , MeOH (methanol), S (sensitive).
Concentration used 100 mg/ml (10%) at 0.1 ml/disk.

Table 10: The means zone of inhibition of plant extracts (soxhlet extracts and volatile oil) against *Candida species* (standard and clinical isolates)

Solvent Used	Microorganisms and the mean zone of inhibition (in mm) according to solvents used				
	<i>Candida albicans</i>	<i>Candida glabrata</i>	<i>Candida parapsilosis</i>	<i>Candida Guilliermodii</i>	<i>Candida albicans</i> ATCC 7596
MeOH	24.5	19	21.5	24.5	20
C ₆ H ₁₄	22.5	20.5	25.5	24.5	23.5
Volatile oil	26.5	25.5	30	34.5	30.5

Note

C₆H₁₄ (petroleum ether) , MeOH (methanol), S (sensitive).

Concentration used 10 mg/ml (100%) at 0.1 ml/disk.

Interpretation of the result Means Diameter of growth Inhibition Zone (MDIZ) in (mm) average of (2) replicates.

If MDIZ

- 18 sensitive
- 14-18 intermediate
- 14 resistant

4.10. Antifungal activity of reference drugs against *Candida species* (standard and clinical isolates)

Ketoconazole was effective against *Candida albicans* and *Candida albicans* ATCC 7596 with the concentration of 100% , and non effective against *C.parapsilosis*, *C. guilliermondii*, *C.glabrata* with concentration 100%. Itraconazole exhibited activity against *Candida albicans* and *Candida albicans* ATCC 7596 with the concentration 75% and 50% respectively , and no activity against *C.parapsilosis*, *C. guilliermondii*, *C.glabrata* with concentration 100%. Fluconazole was non effective against *Candida albicans*, *C.parapsilosis*, *C. guilliermondii*, *C.glabrata* and *Candida albicans* ATCC 7596 with the concentration of 100%, shown in table 11 and table 12 and figures 18 and 19.

Table 11: Antifungal activity of reference drugs against *Candida* species (standard and clinical isolates)

Drugs Used	Microorganisms and their susceptibility to different drugs				
	<i>Candida albicans</i>	<i>Candida glabrata</i>	<i>Candida parapsilosis</i>	<i>Candida Guilliermodii</i>	<i>Candida albicans</i> ATCC 7596
Ketoconazole	S(100%)	R (100%)	R (100%)	R (100%)	S (100%)
Itraconazole	S (75%)	R (100%)	R (100%)	R (100%)	S (50%)
Fluconazole	R (100%)	R (100%)	R (100%)	R (100%)	R (100%)

Note: **R**; resistant, **S**; sensitive

Table 12: The means zone of inhibition of reference drugs against *Candida species* (standard and clinical isolates)

		Microorganisms and the mean zone of inhibition (in mm) (MDIZ) of drugs used				
Drugs Used	Conc. mg/ml	<i>Candida albicans</i>	<i>C. glabrata</i>	<i>C. parapsilosis</i>	<i>C. guilliermondii</i>	<i>Candida albicans</i> ATCC 7596
Ketoconazole	40	25	0	0	0	30
	20	23	0	0	0	28
	10	19	0	0	0	25
	5	15	0	0	0	20
Itraconazole	40	19	0	0	0	23
	20	16	0	0	0	13
	10	15	0	0	0	0
	5	0	0	0	0	0
Fluconazole	40	11	0	0	0	13
	20	0	0	0	0	0
	0	0	0	0	0	0
	0	0	0	0	0	0

4.11. The minimum inhibitory concentrations (MICs) of *Commiphora myrrha* obtained by the agar dilution method

The MICs of *Commiphora myrrha* oil and extracts obtained by the agar dilution method are shown in table 13 and figure 12. Petroleum ether and volatile oil extracts inhibited *Candida albicans*, *C.parapsilosis*, *C. guilliermondii*, *C.glabrata* and standard *Candida albicans* ATCC 7596 , at 12.5 mg/ml MICs ,while the methanol extract inhibited *Candida albicans*, *C.parapsilosis*, *C. guilliermondii*, *C.glabrata* and standard *Candida albicans* ATCC 7596 at the highest concentrations 25 mg/ml .

Table 13: The MICs of *Commiphora myrrha* oil and extracts obtained by the agar dilution method

Solvents Used	Conc.	<i>Candida albicans</i>	<i>C. glabrata</i>	<i>C. parapsilosis</i>	<i>C. guilliermondii</i>	<i>Candida albicans</i> ATCC 7596
C₆ H₁₄	50	-	-	-	-	-
	25	-	-	-	-	-
	12.5	-	-	-	-	-
	6.25	+	+	+	+	+
MeOH	50	-	-	-	-	-
	25	-	-	-	-	-
	12.5	+	+	+	+	+
	6.25	+	+	+	+	+
V.oil	50	-	-	-	-	-
	25	-	-	-	-	-
	12.5	-	-	-	-	-
	6.25	+	+	+	+	+

Note

C₆H₁₄ (petroleum ether) , MeOH (methanol), V.oil (volatile oil), (+): visible growth, (-):no visible growth.

Concentration used 100 mg/ml (10%) at 0.1 ml/disk.

Interpretation of the result

The MICs were determined as the lowest concentration of oil and soxhlet extracts which inhibiting the visible growth of each organism on the agar plate. The presence of one or two colonies was disregarded.

4.12. Minimum inhibitory concentrations (MICs) and minimum cidal concentrations (MCCs) obtained by the broth micro dilution method

Tables 14 and 15 and figures 13-17 , showed the Minimum inhibitory concentrations (MICs) and minimum cidal concentrations (MCCs) obtained by the broth microdilution method .According to the result ,the petroleum ether extract had the lowest MICs ranging from 0.781-6.25 mg/ml, followed by volatile oil extract had MICs ranging from 6.25-≥100 mg/ml. While the methanol extract had the highest MICs ranging from 12.5-≥100 mg/ml. On other hands, the MICs of ketoconazole resemble to the MICs of methanol extract (MICs ranging from 12.5-≥100 mg/ml) , followed by fluconazole had MICs ranging from 25-≥100 mg/ml, while itraconazole had highest MICs at >100 mg/ml.

The greatest difference was for *C. guilliermondii* and petroleum ether extract, where the MIC obtained by agar dilution was 12.5 mg/ml compared with the MIC by broth microdilution at 0.781 mg /ml.

Table 14: Minimum inhibitory concentrations (MICs) of *Commiphora myrrha* oil and soxhlet extracts data (mg/ml) obtained by the broth microdilution method.

Solvent, drugs and their MICs (in mg/ml)	MeOH	C ₆ H ₁₄	V.oil	Flu.	Itra.	Keto.
	MICs	MICs	MICs	MICs	MICs	MICs
<i>Candida albicans</i>	25	6.25	>100	25	100	12.5
<i>C.glabrata</i>	>100	6.25	25	>100	>100	>100
<i>C.parapsilosis</i>	12.5	6.25	6.25	>100	>100	>100
<i>C. guilliermondii</i>	>100	≤ 0.781	>100	>100	>100	>100
<i>Candida albicans</i> ATCC 7596	>100	>100	>100	>100	>100	>100

Note

C₆H₁₄ (petroleum ether) , MeOH (methanol), V.oil (volatile oil), Itra (itraconazole), Flu (fluconazole), Keto (ketoconazole), MICs (minimum inhibitory concentrations)

Concentration used 10 mg/ml (10%) at 50 µl /well

Interpretation of the result

The MICs were determined as the lowest concentration of oil and soxhlet extracts which did not permit any visible growth of each organism on the broth culture was regarded as MICs in each case.

Table 15: Minimum cidal concentrations (MCCs) of *Commiphora myrrha* volatile oil and soxhlet extracts data (mg/ml) obtained by the broth microdilution method

Solvent, drugs and their MCCs (in mg/ml)	MeOH	C ₆ H ₁₄	V.oil	Flu.	Keto.	Itra.
	MCCs	MCCs	MCCs	MCCs	MCCs	MCCs
<i>Candida albicans</i>	12.5	6.25	100	100	>100	100
<i>C.glabrata</i>	1.562	100	3.125	3.125	50	≤ 0.781
<i>C.parapsilosis</i>	50	25	3.125	>100	>100	>100
<i>C. guilliermondii</i>	50	>100	12.5	>100	>100	>100
<i>Candida albicans</i> ATCC 7596	25	6.25	12.5	12.5	≤ 0.781	≤ 0.781

Interpretation of the result

The MCCs were determined as the lowest concentration of oil and soxhlet extracts and drugs used that give sterile culture after incubation period was regarded as MCCs in each case.



Fig 4: Activity of methanol extract of *Commiphora myrrha* against *C.albicans* isolate



Fig 5: Activity of methanol extract of *Commiphora myrrha* against standard *C.albicans* ATCC 7596



Fig 6: Activity of methanol extract of *Commiphora myrrha* against *C. glabrata* isolate



Fig 7: Activity of methanol extract of *Commiphora myrrha* against *C. guilliermondii* isolate



Fig 8: Activity of volatile oil extract of *Commiphora myrrha* against standard *C.albicans* ATCC 7596



Fig 9: Activity of volatile oil extract of *Commiphora myrrha* against *C.guilliermondii* isolate



Fig 10: Activity of petroleum ether extract of *Commiphora myrrha* against *C.albicans* isolate

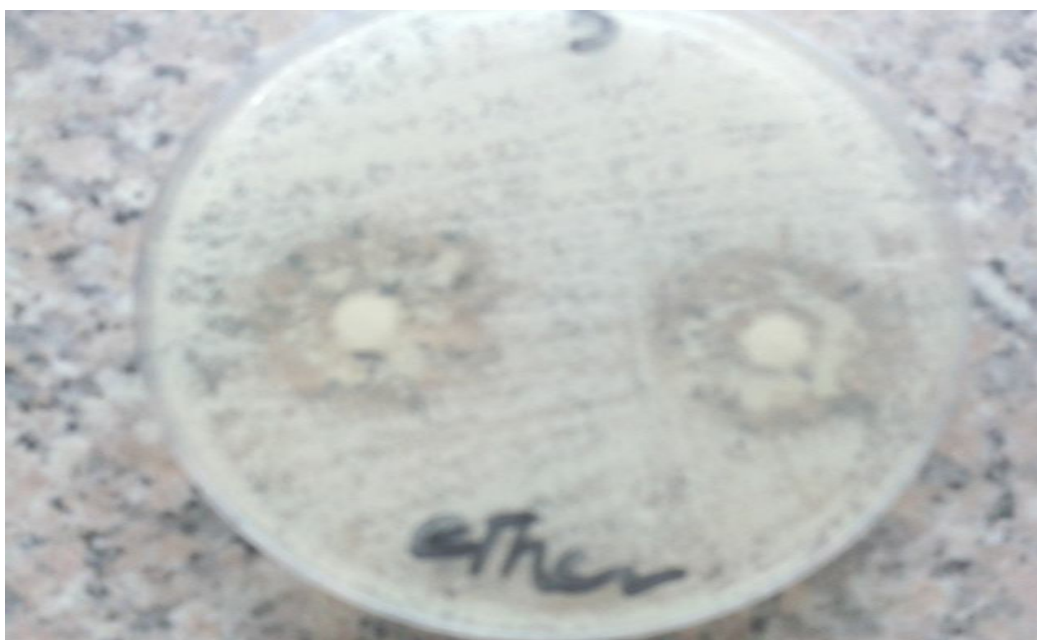


Fig 11: Activity of petroleum ether extract of *Commiphora myrrha* against standard *C.albicans* ATCC 7596

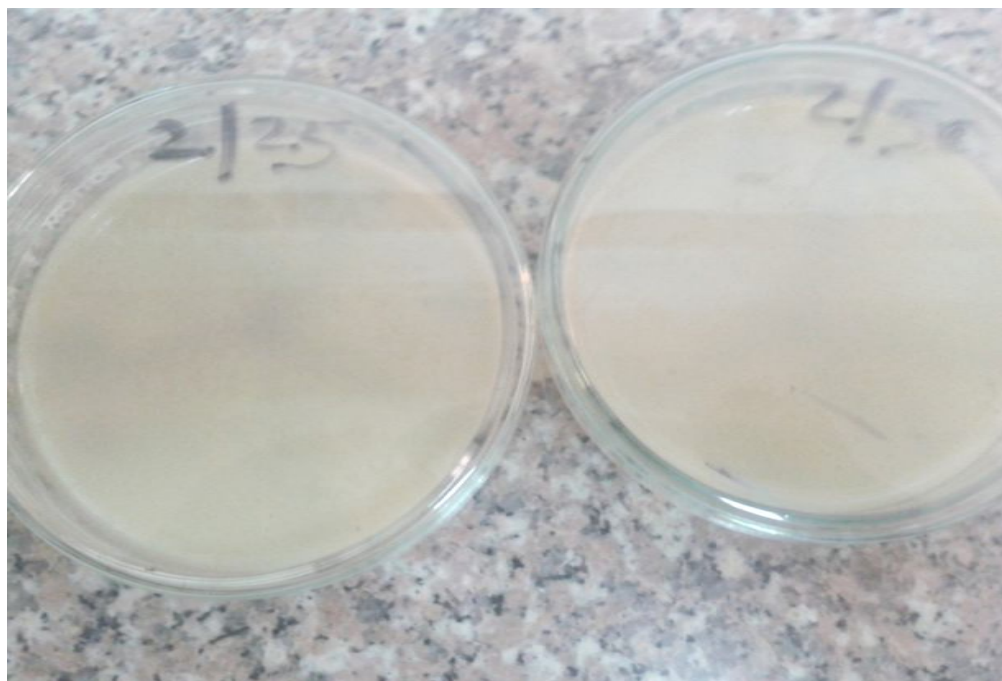


Fig 12: The minimum inhibitory concentrations (MICs) of petroleum ether extract of *Commiphora myrrha* against *Candida* species (MICs at 12.5 mg/ml) obtained by the agar dilution method

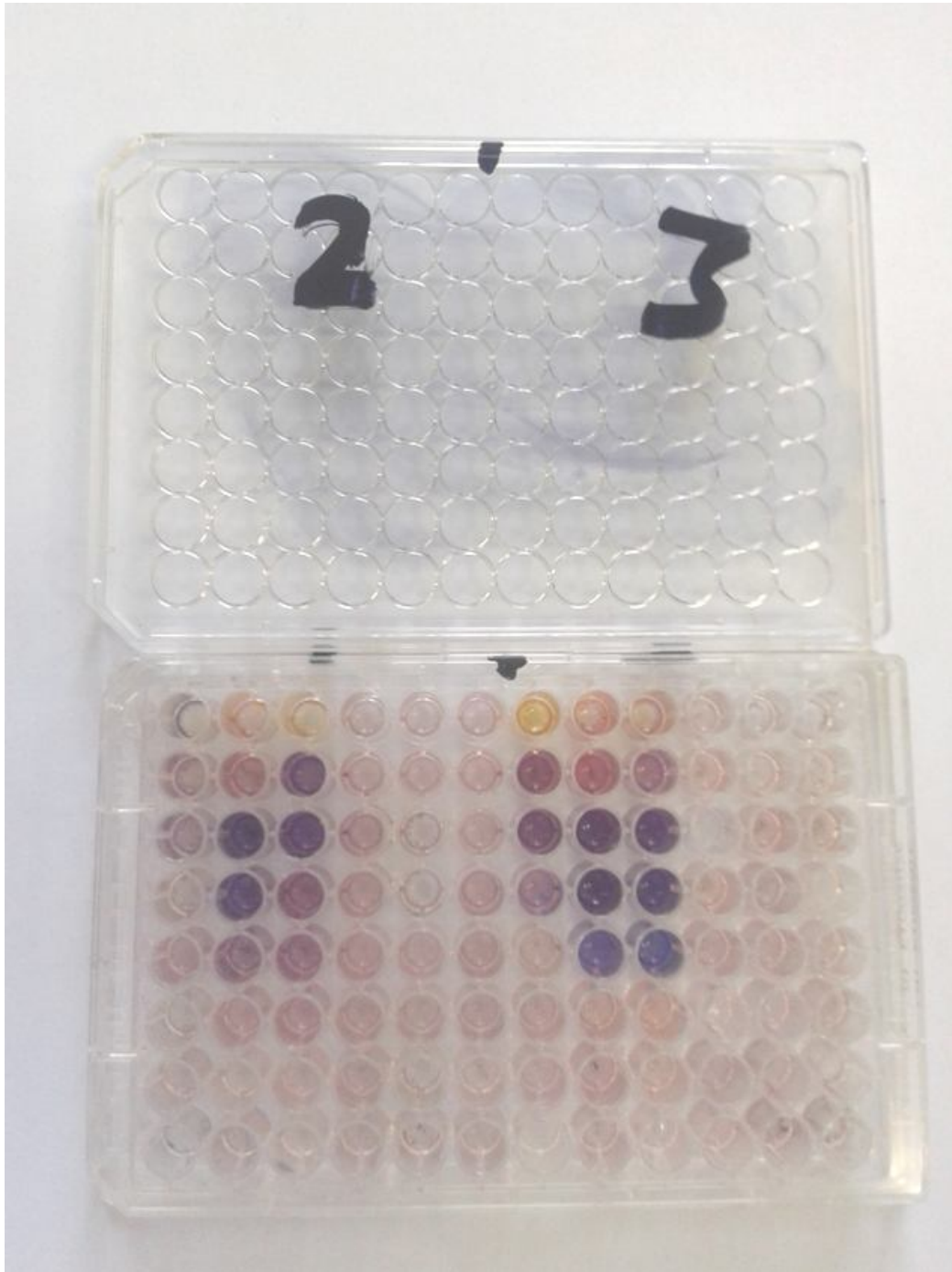


Fig 13: Minimum inhibitory concentrations (MICs) of *Commiphora myrrha* oil and soxhlet extracts against *C.galabrata* isolate(2) and *C.parapsilosis* isolate(3) obtained by the broth microdilution method

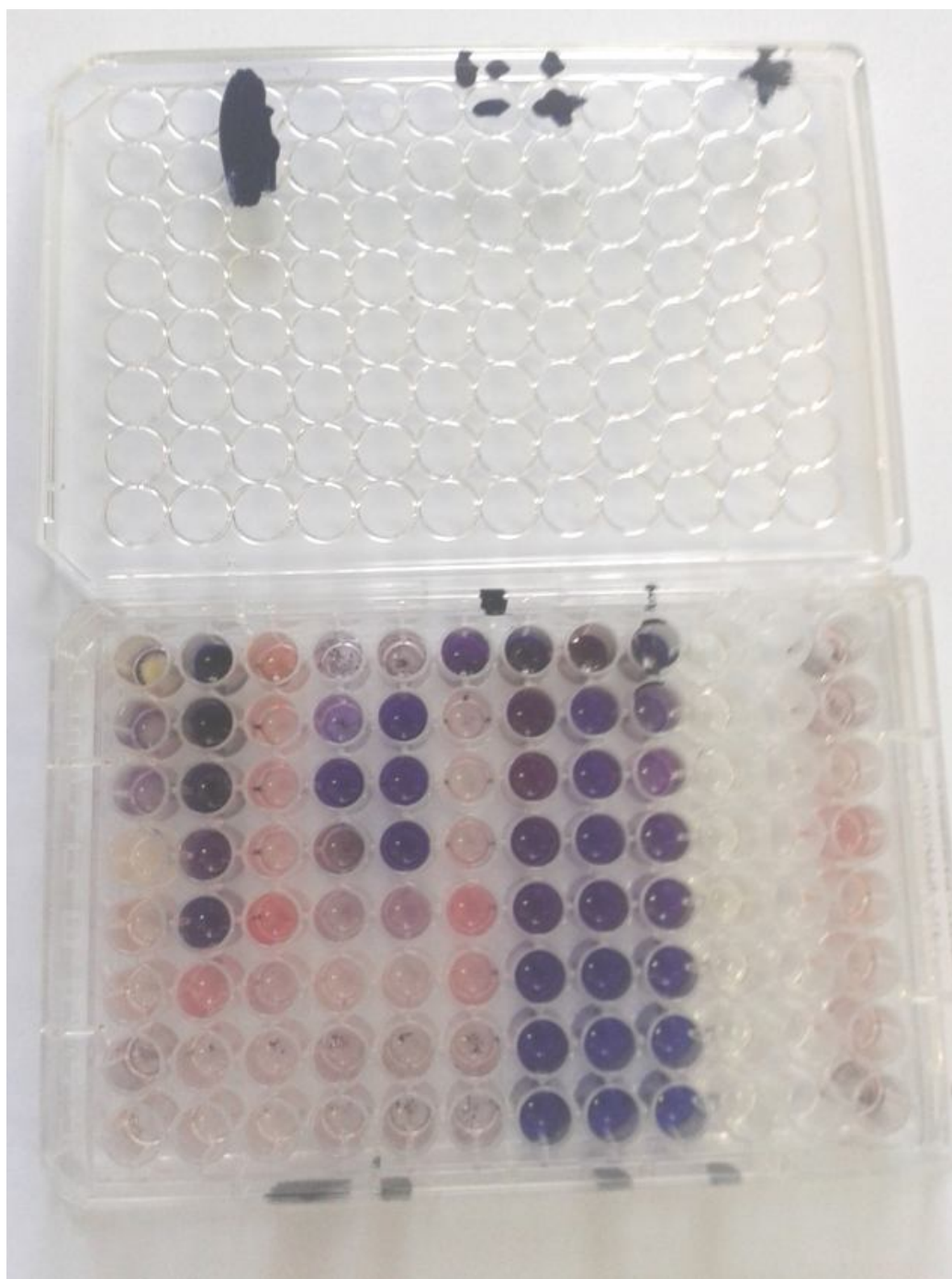


Fig 14: Minimum inhibitory concentrations (MICs) of *Commiphora myrrha* oil and soxhlet extracts against *C.albicans* isolate (1) and positive and negative control(+ve/-ve) obtained by the broth microdilution method

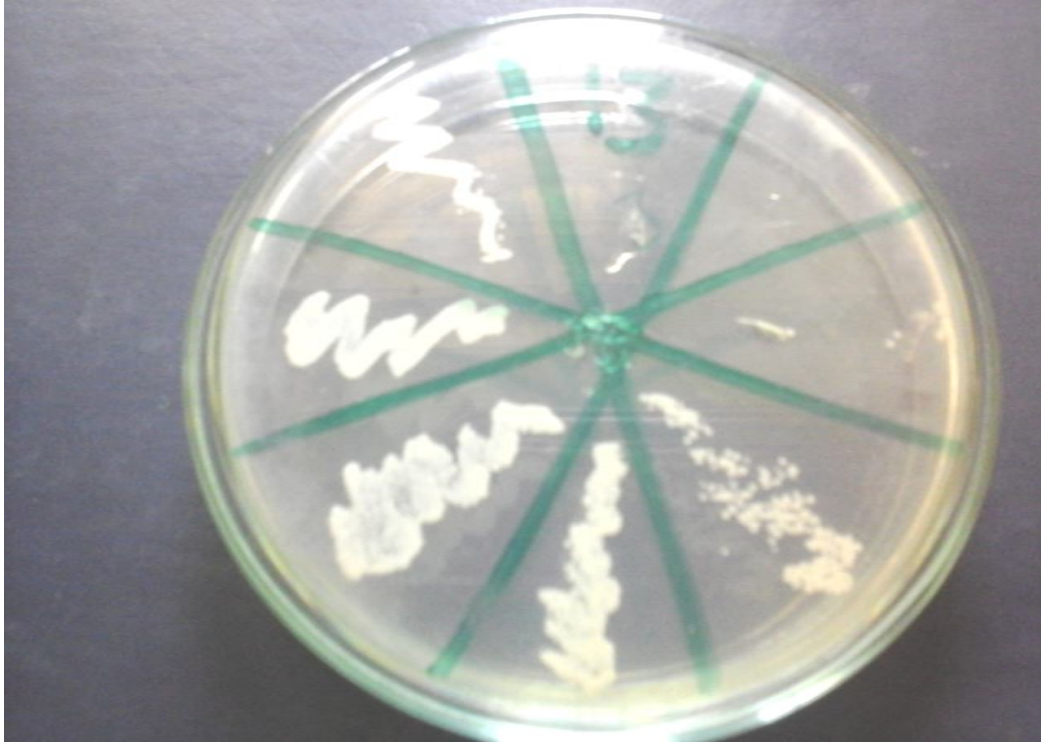


Fig 15: Minimum cidal concentrations (MCCs) of volatile oil extract of *Commiphora myrrha* oil against *C.albicans* isolate obtained by the broth microdilution method



Fig 16: Control positive (viability control) of broth micro-dilution method (micro-titre plate assay)

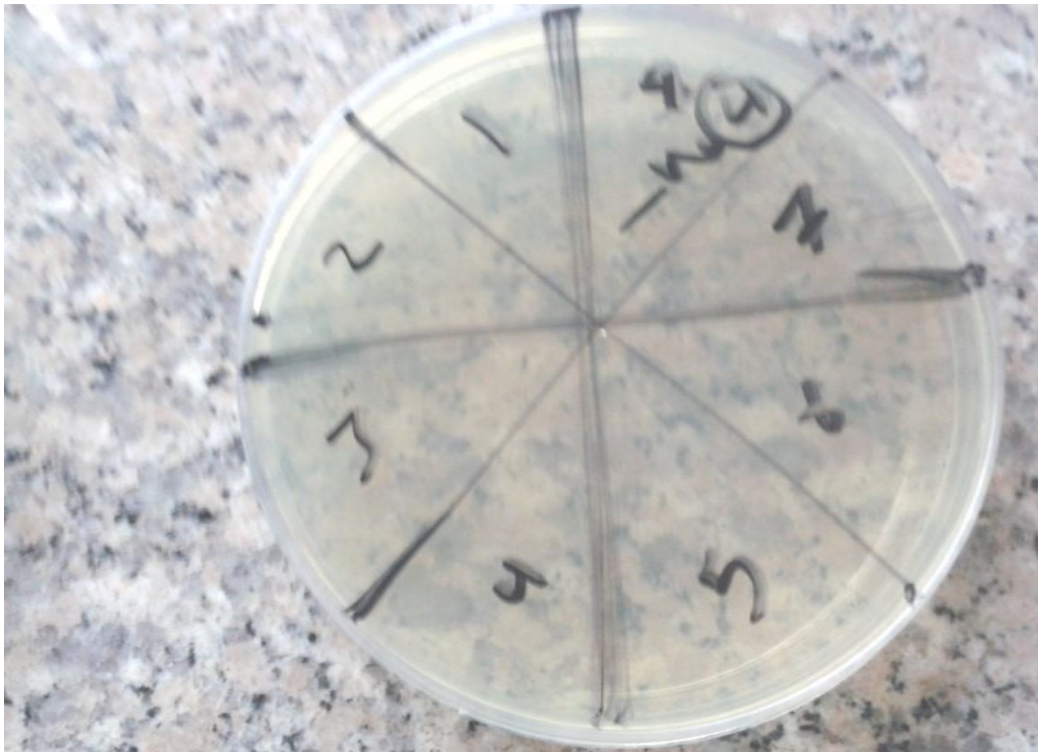


Fig 17: Control negative (sterility control) of broth micro-dilution method (micro-titre plate assay)



Fig 18 : Activity of Itraconazole against *Candida albicans* isolate



Fig 19: Activity of Itraconazole against standard *Candida albicans* ATCC 7596

4.13. Gas chromatography results

Table 16 and figure 20 revealed that *Commiphora myrrha* (methanol extract) contain 10 phytochemical components. The active ingredients was not evident in this study.

Table 16: Gas chromatography analysis of *Commiphora myrrha*

Peak	Retention time	Area	Area %	Name
1	24.217	4087126	4.01	Cyclohexane,1-ethenyl-1-methyl-2
2	25.217	1968961	1.93	Germacrene B (CAS)
3	26.906	27976124	27.42	Benzofuran
4	28.409	1613949	1.58	1,5-Cyclodecadiene
5	30.050	32828572	32.18	2-(2-Nitro-1-p-toly-ethyl)-cyclohexanone
6	30.202	11954833	11.72	2-Dimethylene bicyclohexyl-3
7	31.040	2182018	2.14	4-Trimethyl-3-(1-methyl ethenyl)
8	31.380	2480868	2.43	Azuleno [4,5-6] furan-2
9	32.035	13024688	12.77	4-(2,6,6-Trimethylcyclohexa-1,3-dienyl)pent-3-en-2-ol
10	35.502	3895966	3.82	Acetic acid 6-(1-hydroxymethyl-vinyl)-4

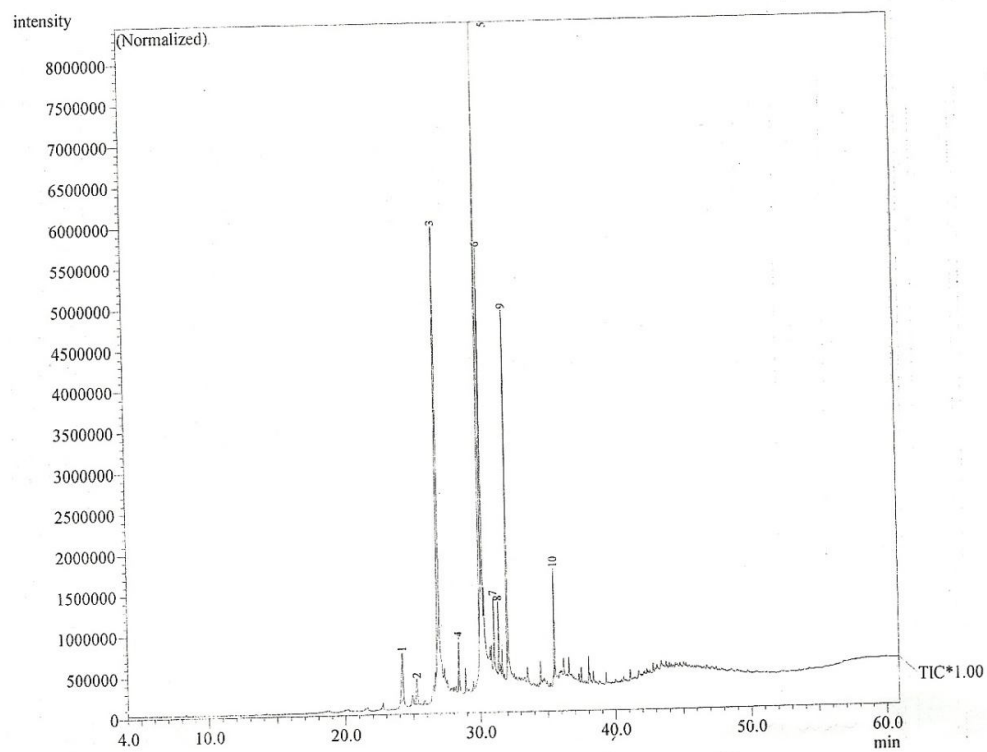


Fig 20: The phytochemical components found in *Commiphora myrrha*

CHAPTER FIVE

5.DISCUSION

5.1. Discussion

The traditional use of plants as medicines provide the basis for indicating which essential oils and plant extracts may be useful for specific medical conditions. Historically, many plant oils and extracts, such as tea tree, myrrh and clove, have been used as topical antiseptics, or have been reported to have antimicrobial properties. It is important to investigate scientifically those plants which have been used in traditional medicines as potential sources of novel antimicrobial compounds. Also, the resurgence of interest in natural therapies and increasing consumer demand for effective, safe, natural products means that quantitative data on plant oils and extracts are required (Hammer *et al*, 1999).

In this study the volatile oil, petroleum ether and methanol extracts of the oleo-gum resins *Commiphora myrrha* gave high antifungal activity against the standard and clinical isolates of *Candida species*: *Candida albicans*, *C.parapsilosis*, *C. guilliermondii*, *C.glabrata* and *Candida albicans* ATCC 7596. This result is in agreement with report of **Dolara *et al* (2000)** and **Hammer *et al* (1999)**.

The Means Diameter of growth Inhibition Zone (MDIZ) of microorganism isolates were increases with the increase in drugs concentration. This result is in agreement with report of **Suleiman (2013)**.

Petroleum ether extract of *Commiphora myrrha* exhibited higher activity against the standard and clinical isolates of *Candida species*, but Fluconazole gave no activity against them. This result is in disagreement with report of **Nelson *et al* (2013)**.

Itraconazole and Ketoconazole gave higher activity against *Candida albicans* and no activity against *C.parapsilosis*, *C. guilliermondii* and *C.glabrata*. This result is in agreement with report of **Nelson *et al* (2013)**, but observed higher activity of Itraconazole and Ketoconazole against *C.parapsilosis* and *C.glabrata*.

The methanol extract of *Commiphora myrrha* inhibited *Candida albicans* isolate less than 5 mg/ml of ketoconazole , Itraconazole, and higher than 40 mg/ml of Fluconazole , so the methanol extract of *Commiphora myrrha* more potent than these antifungal drugs. This study is in agreement with report of **Omer *et al* (1999)** but they observed antifungal activity against *Candida albicans* isolated from gazelles held.

The methanol extract of *Commiphora myrrha* inhibited standard *Candida albicans* ATCC 7596 less than 5 mg/ml of ketoconazole and less than 40 mg/ml of Itraconazole. The volatile oil extract of *Commiphora myrrha* inhibits the standard *Candida albicans* ATCC 7596 less than 5 mg/ml of

ketoconazole , less than 40 mg/ml of Itraconazole, and higher than 40 mg/ml of Fluconazole.

The volatile oil extract of *Commiphora myrrha* inhibits *Candida albicans* isolate less than 5 mg/ml of ketoconazole and Itraconazole, and higher than 40 mg/ml of Fluconazole, while inhibits *C.parapsilosis*, *C. guilliermondii* and *C.glabrata* higher than 40 mg/ml of Itraconazole, ketoconazole and Fluconazole , so the volatile oil extract of *Commiphora myrrha* more potent than these antifungal drugs.

In this study the petroleum ether and methanol extracts of the oleo-gum resins *Commiphora myrrha* gave high activity against the standard and 4 clinical isolates of *Candida species*: *Candida albicans*, *C.parapsilosis*, *C. guilliermondii*, *C.glabrata* and *Candida albicans* ATCC 7596. This study is in agreement with report of **Hammer et al (1999)**, **Omer et al (1999)** and **Dolara et al (2000)** but they observed antifungal activity against *Candida albicans* only.

The volatile oil extract of the *Commiphora myrrha* exhibited high activity against the standard and 4 clinical isolates of *Candida species*: *Candida albicans*, *C.parapsilosis*, *C. guilliermondii*, *C.glabrata* and *Candida albicans* ATCC 7596. This study is in agreement with report of **Hammer et al (1999)**.

In similar study, **Dolara et al (2000)** reported that the 8 sesquiterpene fractions from *Commiphora myrrha* exhibited high activity against *Candida albicans*. In the World many attempts were carried out of explore the role of the *Commiphora myrrha* in medical field to compact infectious diseases e.g. **Alashry et al (2003)** on Myrrh components, therapeutic value and uses, **Abdallah et al (2009)** on antibacterial activity of *Commiphora molmol* and *Boswellia papyrifera* against methicillin resistant *Staphylococcus aureus* (MRSA), **Omer et al (1999)** on antimicrobial Activity of *Commiphora myrrha* against some bacteria and *Candida albicans* Isolated from gazelles at King Khalid wildlife research centre, **Zhu et al (2003)** on isolation and characterization of several aromatic sesquiterpenes from *commiphora myrrha* and **Wanner et al (2010)** on chemical composition and antibacterial activity of selected essential oils and some of their main compounds. Comparison of the data obtained in this study with previously published results is problematic. Because firstly, the composition of plant oils and extracts are known to vary according to local climatic and environmental conditions . Furthermore, some oils with the same common name may be derived from different plant species (**Hammer et al ,1999**).

Secondly, the method used to assess antimicrobial activity, and the choice of test organism(s), vary between publications (**Ivanova et al, (2013)**; (**Hammer et al, 1999**).

A method frequently used to screen plant extracts and reference drugs for antimicrobial activity is the agar disc diffusion technique. The usefulness of this method is limited to the generation of preliminary, qualitative data only, as the hydrophobic nature of most essential oils and plant extracts prevents the uniform diffusion of these substances through the agar medium. Agar and broth dilution methods are also commonly used.

The results obtained by each of these methods may differ as many factors vary between assays. These include differences in microbial growth, exposure of micro-organisms to plant oil, the solubility of oil or oil components, and the use and quantity of an emulsifier. These and other elements may account for the large differences in MICs obtained by the agar and broth dilution methods in this study. *In vivo* studies may be required to confirm the validity of some of the results obtained (**Hammer et al,1999**).

The results of gas chromatography exhibited 10 compounds of *Commiphora myrrha* and also these compounds were identified qualitatively by the Retention time, and quantitatively by the area under the curve. The active compound which inhibited the growth of fungi may be one or more of these 10 compounds, so further study must be performed to identify the compounds and the active ingredients.

5.2. Conclusion

- The volatile oil, methanol and petroleum ether extracts of *Commiphora myrrha* had high activity against tested *Candida species*.
- *Commiphora myrrha* had high activity, which justify their traditional uses as antiseptic for treatment of skin and mouth infections.
- *Commiphora myrrha* extracts was more effective than antifungal drugs combating the pathogenic *Candida species* studied.
- The efficiency of the antifungal activity of the extracts was found to increase by increasing the concentrations.

5.3. Recommendations

1. Based on this study and result, it is recommended that to isolate and identify the active ingredient in the compounds extracts responsible for the antifungal activity.
2. Determination of the minimum inhibitory concentrations (MICs) and minimum cidal concentrations (MCCs) for the active ingredients on each fungi including those in the study.
3. Determination of the toxicity of the active ingredients.
4. More research is required for verify these results.

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Appendix 1:
Sudan University of Science and Technology
College of Graduate studies

**Antifungal Activity of *Commiphora myrrha* (Murr) against *Candida*
species Isolated from Vaginal Candidiasis in pregnant women**
Questioner

Age:

Trimester of gestation: 1st () 2nd () 3rd ()

D.M No() yes ()

History of abortion No() yes ()

Medication description

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.....
.....
.....
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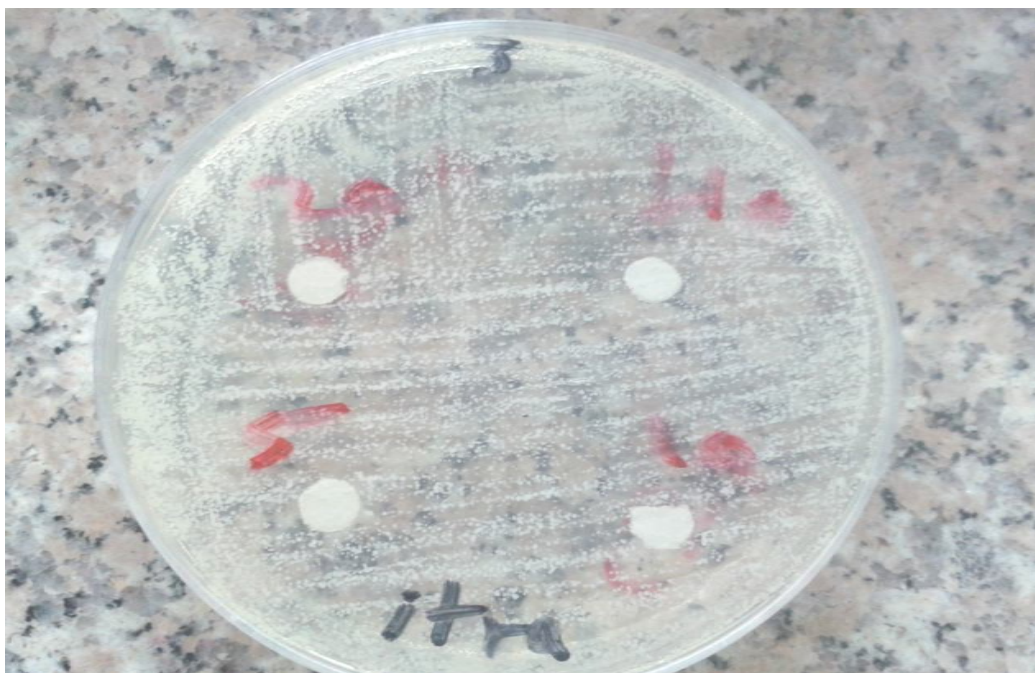
Antifungal Activity of *Commiphora myrrha* (Murr) against *Candida species* Isolated from Vaginal Candidiasis in pregnant women in Khartoum State

Submitted by: Safaa Omer Eltybe Mohammed

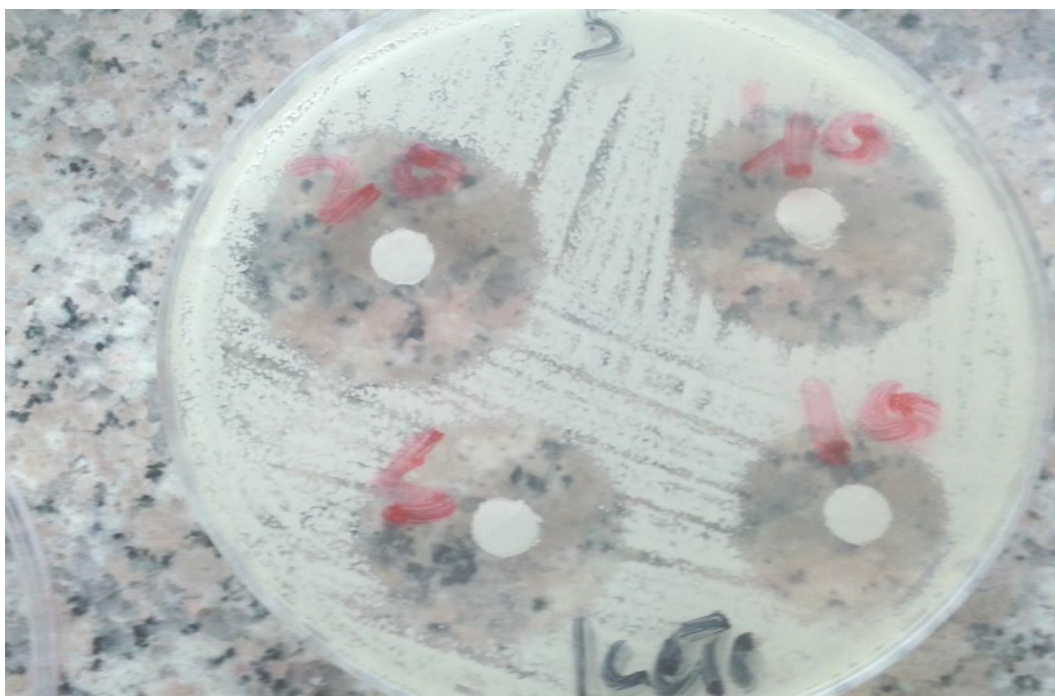
اقرار بالموافقة علي المشاركة في البحث

انا
بعد ان تم لي شرح طبيعة الدراسة واعطيت فرصة كافية للاستفسار عنها وقد تم الاجابة علي كل
اسئلتي بصورة كافية وفهمت ان لي الحق في التوقف عن هذه الدراسة في اي وقت من غير ان
افقد الحق في الرعاية الطبية او أي حقوق اخري ، اوافق طوعية علي المشاركة في هذه الدراسة.
أسم المريض.....
التوقيع..... التاريخ.....
شاهد أول
التوقيع..... التاريخ.....
شاهد ثاني
التوقيع..... التاريخ.....

Appendix 2:



Activity of Itraconazole against *Candida guilliermondii* isolate



Activity of Ketoconazole against standard *Candida albicans* ATCC
7596



Activity of Ketoconazole against *Candida parapsilosis* isolate



Activity of Ketoconazole against *Candida albicans* isolate



Activity of Fluconazole against standard *Candida albicans* ATCC 7596



Activity of Fluconazole against *Candida albicans* isolate